Ph.D. Thesis

Sapna K

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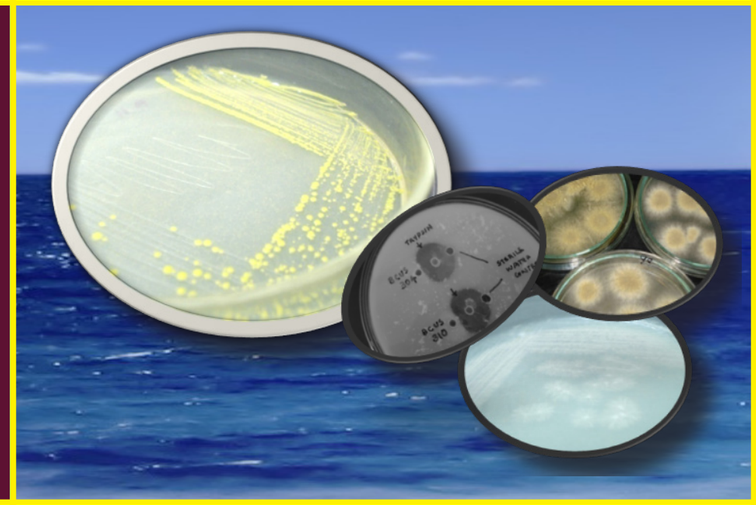
Isolation, purification, characterization and application of proteinaceous protease inhibitor from marine bacterium *Pseudomonas mendocina* BTMW 301



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March 2013

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ISOLATION, PURIFICATION, CHARACTERIZATION AND APPLICATION OF PROTEINACEOUS PROTEASE INHIBITOR FROM MARINE BACTERIUM *PSEUDOMONAS MENDOCINA* BTMW 301

Thesis submitted to the
Cochin University of Science and Technology
Under the Faculty of Science
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for the degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

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This is to certify that the research work presented in the thesis entitled **"Isolation, purification, characterization and application of proteinaceous protease inhibitor from marine bacterium *Pseudomonas mendocina* BTMW 301"** is based on the original research work carried out by **Mrs. Sapna K,** under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology in partial fulfilment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any other degree.

Thenchippalam
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This is to certify that the research work presented in the thesis entitled **"Isolation, purification, characterization and application of proteinaceous protease inhibitor from marine bacterium *Pseudomonas mendocina* BTMW 301"** is based on the original research work carried out by Mrs. Sapna K under the guidance and supervision of Dr. Elyas K K and my co-guidance at the Department of Biotechnology, Cochin University of Science and Technology in partial fulfilment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any degree.

Cochin–22
19.03.2013

SARITA G BHAT

DECLARATION

I hereby declare that the thesis entitled "**Isolation, purification, characterization and application of proteinaceous protease inhibitor from marine bacterium *Pseudomonas mendocina* BTMW 301"** 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.

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19.03.2013

Dedicated to my
Achan and *Amma*

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SAPNA K

LIST OF ABBREVIATIONS

%	-	Percentage
°C	-	Degree Celsius
µg	-	microgram
µL	-	microlitre
µM	-	micromole
nM	-	nanomole
A_{280}	-	Absorbance at 280 nm
BLAST	-	Basic Local Alignment Search Tool
bp	-	base pair
AIDS	-	Acquired immunodeficiency syndrome
Arg	-	Arginine
Asn	-	Asparagine
Asp	-	Aspartate
ATP	-	Adenosine tri phosphate
BAPNA	-	α-*N*-benzoyl-DL-arginine-*p*-nitroanilide
BSA	-	Bovine serum albumin
C18	-	Octadecyl bonded Silica
cfu	-	Colony forming unit
cm	-	Centimetre
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
MALDI	-	Matrix Assisted Laser Desorption Ionization
(M_r)	-	Relative molecular weight
mg	-	milligram

min	-	minutes
MIC	-	Minimum inhibitory concentration
mL	-	millilitre
mm	-	millimetre
NCBI	-	National Center for Biotechnology Information
OD	-	Optical density
PAGE	-	Polyacrylamide gel electrophoresis
PCR	-	Polymerase chain reaction
PEG	-	Polyethylene glycol
pI	-	Isoelectric point
PI	-	Protease inhibitor
PMSF	-	Phenyl methyl sulphonyl fluoride
RNA	-	Ribonucleic acid
rpm	-	Revolutions per minute
RP	-	Reverse phase
RT	-	Room temperature
SDS	-	Sodium dodecyl sulphate
Ser	-	Serine
Sl. No.	-	Serial number
Smf	-	Submerged fermentation
sp.	-	Species
TCA	-	Trichloro acetic acid
TE	-	Tris-EDTA
TEMED	-	N-N-N'-N'-Tetramethyl ethylene diamine

Thr	-	Threonine
TPCK	-	*N*- tosyl-L-phenylalanyl chloromethyl ketone
u	-	Protease inhibitor activity expressed for caseinolytic assay
U	-	Protease inhibitor activity expressed for BAPNA assay
UV-VIS	-	Ultraviolet-Visible
V_{max}	-	Maximal velocity
v/v	-	Volume/volume
w/v	-	Weight/volume
w/w	-	Weight/weight

CONTENTS

Chapter 1

INTRODUCTION

Enzyme inhibitors are agents that combine with an enzyme in such a manner as to slow down or prevent the catalytic action of the enzyme. Enzyme inhibitors are important as therapeutic agents, as regulators in controlling the enzymic processes in living organisms, and as useful agents in the study of enzyme structures and reaction mechanisms (Bode and Huber, 1992; Cyran, 2002; Imada, 2005; Robert, 2005). Protease enzyme inhibitors exercise control of unwanted proteolysis and play an essential role in physiological and pathological regulation. Applications of protease inhibitors are intimately connected to the proteases they inhibit. To understand the inhibitors, understanding of proteases and the modes of regulation of their proteolytic activity is important.

Proteolytic enzymes or proteases are the single class of enzymes, which occupy a pivotal position with respect to their applications in both physiological and commercial fields (Poldermans, 1990). They are protein degrading enzymes that catalyze the cleavage of peptide bonds in proteins and perform essential metabolic and regulatory functions in many biological processes. These functions extend from the cellular level to the organ and organism level to produce a cascade of systems such as homeostasis and inflammation, and complex processes at all levels of physiology and pathophysiology. They are involved in various processes including fertilization, digestion, tissue morphogenesis and remodelling, angiogenesis, neurogenesis, ovulation, wound repair, stem cell mobilization, homeostasis, blood coagulation, inflammation, immunity, autophagy, senescence, immune and endocrine functions and also in many pathological processes like cardio pulmonary disease emphysema, pancreatitis, rheumatic disease, cancer, AIDS, as well as other bacterial, viral and parasitic diseases (Darby and Smith, 1990; Demuth, 1990; Johnson and Pellecchia, 2006; Klemm et al., 1991; Koivunen et al., 1991; Lasson, 1984; Lopez-Otin and Bond, 2008; Nilsson, 1987;

Sabotic and Kos, 2012; Tetley, 1993; Turk, 2006; Utermann, 1989; Willoughby et al., 1991).

Proteases are ubiquitous, present in a wide diversity of sources such as plants, animals and microorganisms. Among the six major groups of proteases, serine proteases have been studied in great detail in numerous physiological systems (Kraut, 1977; Neurath, 1989). Proteases are one of the uppermost value commercial enzymes. These enzymes find applications in detergents, leather processes, food processing, silk gumming, pharmaceuticals, bioremediation, biosynthesis and biotransformation (Bhaskar et al., 2007; Gupta et al., 2002) and are important tools in studying the structure of proteins and polypeptides (Bhosale et al., 1995).

The major cause of food spoilage is microbial proteases (Chandrasekaran, 1985). Thorough understanding of the growth and activity of spoilage microflora in seafood as well as any other foods is crucial for the development of effective preservation techniques and subsequent reduction of losses due to spoilage. The use of an adequate amount of natural protease inhibitors could be an effective way to extend the shelf life of many proteinaceous seafoods such as salted fish products. Microbial proteases have been recognized as virulence factors in a variety of diseases caused by microorganisms. These enzymes have also been responsible to degrade proteins that function in host defense *in vivo* (Sakata et al., 1993). In the light of rapidly spreading antibiotic resistance, bacterial proteases are promising targets for the design of novel antibiotics. Serine proteases are important pathogenesis factors in bacteria like *Treponema denticola* involved in dental diseases (Sabotic and Kos, 2012).

Proteases are potentially hazardous to their proteinaceous environment and their activities need to be kept strictly under control. Any system that encompasses normal and abnormal bodily functions in such a way must have effective regulatory, counterparts, important amongst which are the interactions of the enzymes with inhibitor proteins. Specific inhibition of these proteases can be

used as a strategy for drug designing. In medicine, protease inhibitors can be used as diagnostic or therapeutic agents for viral, bacterial, fungal and parasitic diseases as well as for treating cancer and immunological, neurodegenerative and cardiovascular diseases. Some diseases may be subjected to treatment with the inhibitors administered as drugs, with synthetic inhibitors that take over their function, or with the natural inhibitors made available by gene therapy. Gene therapy to introduce inhibitors is under consideration (Grant and Mackie, 1977; Hamilton et al., 2001). There are a number of inherited diseases that are attributable to abnormalities in protease inhibitors. These include forms of emphysema, epilepsy, hereditary angioneurotic oedema and Netherton syndrome (Bitoun et al., 2002; Lehesjoki, 2003; Lomas et al., 2002; Ritchie, 2003).

Moreover, protease inhibitors are indispensable in protein purification procedures to thwart undesired proteolysis during heterologous expression or protein extraction. Protease inhibitors are also important tools for simple and effective purification of proteases, using affinity chromatography. They can be involved in crop protection against plant pathogens and herbivorous pests (Sabotic and Kos, 2012). Search for novel protease inhibitors with potential protective function is very important in crop protection for the development of environmentally friendly pest and pathogen management strategies. In agriculture, genetically modified crop plants expressing inhibitors of the digestive enzymes of their insect pests are already under study (Samac and Smigocki, 2003 ; Telang et al., 2003)

Although there are numerous protease inhibitors isolated and studied from plants (Bijina et al., 2011a; Joshi et al., 1998; Lorito et al., 1994; Ryan, 1990) there are only a small number of reports on proteinaceous inhibitors from microbial sources (Anderson et al., 2009; Stoeva and Efferth, 2008; Zeng et al., 1988). Microorganisms represent an efficient and low-cost source of protease inhibitors due to their rapid growth, limited space required for cultivation and ready accessibility for genetic manipulation (Pandhare et al., 2002). Nearly 50,000

natural products have been discovered from microorganisms. Over 10,000 of these are reported to have biological activity and over 100 microbial products are in use today as antibiotics, antitumour agents, and agrochemicals (Filippis et al., 2002). Protease inhibitors of microbial origin have been studied and already proven useful in many different applications (Rawlings, 2010; Rawlings and Barrett, 2011). The search for novel types of inhibitors from natural sources such as fungi and microbes, is important for identifying new lead compounds (Sabotic and Kos, 2012).

The marine environment is characterized by high salinity and low concentrations of organic matter. The existence of marine microorganisms was first reported in the late 19th century, and they were found to be metabolically and physiologically different from terrestrial microorganisms. Their microbial growth and metabolic products differ significantly from those of terrestrial microorganisms (Bitoun et al., 2002). The marine environment contains over 80% of world's plant and animal species (McCarthy and Pomponi, 2004). In recent years, many bioactive compounds have been extracted from various marine organisms (Donia and Hamann, 2003; Haefner, 2003). Thermo-stable proteases, lipases, esterases, starch and xylan degrading enzymes have been actively sought and in many cases are found in bacterial and archaeal hyperthermophilic marine microorganisms (Bitoun et al., 2002). The search for new metabolites from marine organisms has resulted in the isolation of more or less 10,000 metabolites (Fuesetani and Fuesetani, 2000), many of which are endowed with pharmacodynamic properties. Apart from the fact that the biodiversity in the marine environment far exceeds that of the terrestrial environment, research to exploit marine natural products is still in its infancy. In addition, there are numerous reports that disclose the physiological and functional similarity of marine organisms to that of terrestrial ones (Halvey et al., 1990; Salisbury, 1971; Wolf et al., 1978). So the products from them will be more compatible to our body systems to use as biopreservatives and as pharmaceutical agents.

Several naturally occurring inhibitors, such as the anticoagulant hirudin, are being used as the basis of engineered proteins for injection in their own right (Filippis et al., 2002). In agriculture, genetically modified crop plants expressing inhibitors of the digestive enzymes of their insect pests are already under study (Filippis et al., 2002; Hunneke et al., 2000). In spite of the advances in computer – assisted drug design, in molecular biology and gene therapy there is a pressing need for new drugs to counteract medical problems such as drug-resistant pathogens and multi-drug resistant cancers or to combat Alzheimers disease and the Human Immunodeficiency Virus (HIV) (Borowitzka, 1995; Schaeffer and Krylov, 2000; Witvrow and Clercq, 1997).

Certain marine microbial extracts have been shown to inhibit binding at brain muscarinic receptors or to activate protein kinase C, both sites which may be linked to the etiology of AD. Moreover, the continual emergence of new natural products with desired biological activities promise well for the utility of natural products. The great apprehension today in all industries is to develop the ability to find new products for use and to accelerate the speed with which newer ones are discovered and developed. This will only be successfully achieved if the procedures for target revelation and lead compound identification and optimization are accomplished.

Unrelenting research into natural sources will continue to deliver newer and more promising products with novel mechanisms of action that suits for specific applications and even with higher degrees of efficiency. While plants have been the commonly searching sources for new natural products; many other sources are now starting to be explored, as well as the marine environment. Detailed study of new marine microbial proteinaceous inhibitors will provide the basis for future research. So an attempt has made to screen effective inhibitor for trypsin from marine environment.

OBJECTIVES OF THE PRESENT STUDY

Protein protease inhibitors constitute a very important mechanism for regulating protease activity. The marine environment representing approximately half of the global biodiversity estimated between 3 and 500 million different species offer an enormous resource for novel compounds. To date, many bioactive compounds with pharmaco dynamic properties have been isolated from marine organisms. Among them, protease inhibitors have drawn the attention recently due to their key role in pharmaceutical, agricultural and industrial fields. The serine proteases are being recognized as important factors in the control of multiple pathways associated with many physiological as well as pathological processes. Hence they are often targets for therapeutic interventions.

Regardless of the reports available on the scope for utilizing plants and other terrestrial organisms as useful source for deriving protease inhibitors, marine microorganisms have not been explored as potential source. With the anticipation that the abundant microbial floras inhabiting the 70% of the Earth's surface covered by the ocean waters which remain relatively unexplored could produce industrially and pharmacologically important protease inhibitor, an attempt was made to screen marine microorganisms for protease inhibitors and select a potential candidate for possible application.

Thus, the primary objectives of the present study included

1. Screening of marine bacteria, actinomycetes and fungi for serine protease inhibitor
2. Optimization of bioprocess towards indigenous production of the protease inhibitor
3. Purification of protease inhibitor
4. Characterization and property studies of protease inhibitor
5. Evaluation of protease inhibitor for various applications

Chapter 2

REVIEW OF LITERATURE

2.1 Proteases

Proteases are considered mainly as "enzymes of digestion". They are one of the prevalent and most diverse families of enzymes known and are present in all living organisms, including viruses, bacteria, archaea, protists, fungi, plants and animals. Proteases constitute one of the largest groups of functional proteins, with more than 560 members actually described (Barrett et al., 1998). They are linked to every aspect of organismal function and play a critical role in many physiological and pathological processes such as protein catabolism, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, blood coagulation, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins, and transport of secretory proteins across membranes (Chambers and Laurent, 2001).

Proteolytic enzymes are essential for the survival of all kinds of organisms, and are encoded by approximately 2% of all genes (Barrett et al., 2001). Proteases encompass a broad range of hydrolytic enzymes that are found across nature which catalyse the cleavage of targeted protein substrates. All promote the hydrolysis of peptide bonds by nucleophilic attack, but there are variations in their catalytic mode of action which forms the basis of their classification. 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. Proteases are also classified according to their catalytic type into aspartic, cysteine, glutamic, serine and threonine peptidases, based on

the functional amino acid residue present at the active site. But for metallopeptidases, the catalytic activity depends on the presence of a divalent metal ion bound within the active site (Sabotic and Kos, 2012).

Peptidases are classified further into families, according to their sequence similarity, and into clans, according to their structural similarity in the MEROPS database (http://merops.sanger.ac.uk/). There are 226 peptidase families assigned in the MEROPS database (Release 9.5, July 2011) and based on the structural data, divided in to 57 clans (Barrett et al., 2001; Rawlings, 2010). Originally thought to be simply involved in the non-discriminate degradation of unwanted proteins, the fact that the human genome has revealed at least 500 protease genes is indicative of the complexity of their biological roles (Rawlings et al., 2006). Proteases catalyze the addition of water across amide (and ester) bonds to influence cleavage using a reaction involving nucleophilic attack on the carbonyl carbon of the scissile bond. The exact mechanisms of cleavage and the active site substituents vary broadly among different protease subtypes. Cleavage of peptide bonds can be general, which leads to the degradation of the entire protein substrate into their constituent amino acids, or it can be specific, leading to selective protein cleavage for post-translational modification and processing. Aspartic, glutamic and metalloproteases exploit a coordinated water molecule to destabilise the peptide bond of substrates, whereas cysteine, serine and threonine protease classes use these respective amino acids in their active sites as nucleophiles (Lopez-Otin and Bond, 2008).

Serine peptidases form the most abundant class comprising about 1/3 of the total proteases, being recognized as important factors in the control of multiple pathways associated with coagulation, fibrinolysis, connective tissue turnover, homeostasis, fertilization, complement activation and inflammatory reactions (Ana et al., 2010), followed by metallo-, cysteine, aspartic and threonine peptidases. In eukaryotic organisms there has been an explosive growth of the number of peptidase families observed, there being 100 peptidases in bacterial genomes and

half as many in archaeal genomes and from 400 to 700 peptidase genes in plant and mammal genomes. In addition, there is a remarkable difference between the compositions of eubacterial and eukaryotic degradomes (the complete set of proteases present in an organism). There are 16 peptidase families that comprise the core of the nearly ubiquitous peptidase families present in all living forms. Additional 34 peptidase families are widely distributed in eukaryotic organisms, while another 10 are unique to higher metazoan organisms, performing mainly limited proteolysis in extracellular environments (Page and Cera, 2008; Rawlings et al., 2010).

In addition to the MEROPS database, information on proteases can be found in several other online databases, including the Degradome database (http://degradome.uniovi.es/) (Quesada et al., 2009) and the Proteolysis Map (PMAP) (http://www.proteolysis.org/) that comprises five different databases (CutDB, PathwayDB, ProteaseDB, SubstrateDB and ProfileDB) (Igarashi et al., 2009). 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).

2.2 Protease inhibitors

The presence of proteases in all living organisms signifies their role in essential metabolic and regulatory functions in various biological processes. Uncontrolled proteolytic pathways have been clearly linked to diseases. Some proteases are the key virulence factors in many pathogenic bacteria, parasites and viruses (Lopez-Otin and Bond, 2008; Turk, 2006). Specific and selective inhibition of proteases can be a powerful strategy for preventing pathogenesis. Proteolytic enzymes have a long history of application in various biotechnological industries (Kumar and Takagi, 1999; Rao et al., 1998; Sabotic and Kos, 2012). But uncontrolled proteases can be hazardous to the system and must be regulated both in time and place. Proteases in biological systems are regulated by diverse

mechanisms. Inactivation of proteases can be achieved by degradation or by binding with inhibitor molecules. Interaction with protease inhibitors constitutes a very important mechanism of protease regulation (Lopez-Otin and Bond, 2008; Rawlings et al., 2010) and among them protein protease inhibitors constitute a very important control mechanism over proteases. The inhibitor can bind at the active site by mimicking the structure of the tetrahedral intermediates in the enzyme-catalyzed reaction (Bode and Huber, 2000). Serine protease inhibitors are the largest and most important superfamily of protease inhibitors which act as suicide substrates by covalently binding to their target leading to inactivation. They act as modulators, performing key roles in regulating the activities of numerous serine and cysteine proteases (Gettins, 2002). The study of enzyme inhibitors give important information on the method and pathway of enzyme catalysis, the nature of the active site functional group, the specificity of the enzyme to the substrate and the contribution of certain functional group in maintaining the active site conformation of the enzyme molecule.

The specific inhibition of proteases by their inhibitors can be used as a strategy for drug design for the prevention of propagation of the causative agents of many dreadful diseases like malaria, cancer and AIDS (Johnson and Pellecchia, 2006). Excessive proteolysis plays a significant role in cancer and in cardiovascular, inflammatory, neurodegenerative, bacterial, viral and parasitic diseases. Due to the evident relevance of protease inhibitors, they have been studied extensively with the intent to develop therapeutic drugs (Drag et al., 2010; Haq et al., 2010; Turk, 2006). Thus, the studies on protease inhibitors, the valuable regulators of proteases are very important. Better understanding of the enzymes' specificity for the substrate and inhibitor binding enables a more rational design of potent inhibitors, suitable for a particular enzyme.

2.3 Sources of protease inhibitors

Protease inhibitors, especially serine protease inhibitors are one of the most abundant classes of proteins in eukaryotes widely distributed in plants, animals and microorganisms as well as archea (Silverman et al., 2010; Umezawa, 1982). Protein inhibitors of mammalian serine proteases have been purified from a number of plant and animal sources (Laskowski and Kato, 1980; Lorand, 1976).

2.3.1 Microorganisms as the source of protease inhibitors

Protease inhibitors of microbial origin have already found many different applications (Rawlings and Barrett, 2011; Sabotic and Kos, 2012). The number and diversity of proteases found in microorganisms (Rao et al., 1998) and higher fungi (Sabotič et al., 2007b) make them an important source of novel protease inhibitors with unique features. A review has identified bacterial proteinases as targets for development of "second-generation" antibiotics (Travis and Potempa, 2000). Even though a plethora of low molecular weight non-protein inhibitors of various proteases from microorganisms have been reported, (Imada et al., 1985a) there are only a few reports of proteinaceous protease inhibitors. The microorganisms of prokaryotic domains archaea and bacteria and of the kingdom of fungi constitute important sources of protease inhibitors. Extracellular proteolytic enzymes hydrolyze organic nitrogen compounds in the medium and are thought to be harmful to cells. The production of inhibitors of the proteolytic enzymes by microorganisms has probably evolved as a mechanism to provide cell protection. Specific inhibitors of microbial origin have been used as useful tools in biochemical analysis of biological functions and diseases. Microbial protease inhibitors are versatile in their structures and mechanisms of inhibition in ways that differ from those of other sources. They have therefore found countless applications in the fields of medicine, agriculture and biotechnology (Sabotic and Kos, 2012).

Majority of the extracellular protein protease inhibitors produced by microorganims are from the genus *Streptomyces.* The widely distributed and well-characterized proteinaceous inhibitors from *Streptomyces* are the inhibitors of bacterial serine alkaline protease, subtilisin (Kourteva and Boteva, 1989 ; Sato and Murao, 1973). Besides the subtilisin inhibitors there are reports of other related inhibitors of trypsin and other serine proteases from *Streptomyces.* A potent plasmin inhibitor, plasminostreptin has been studied from *S. antiplasminolyticus* (Sugino et al., 1978). Two naturally occurring abundantly produced trypsin inhibitors have been purified from *S. lividans* and *S. longisporus* (Strickler et al., 1992*)*. A novel double-headed proteinaceous inhibitor of serine and metalloproteases has been reported from a *Streptomyces* sp. (Hiraga et al., 2000). Kexstatin, a proteinaceous Kex 2 proteinase and subtilisin inhibitor was purified from the culture supernatent of *Streptomyces platensis* (Oda et al., 1996). A *Streptomyces* sp., which produces an alkaline protease inhibitor (API) exhibiting antifungal activity has been isolated from soil (Pandhare et al., 2002; Vernekar et al., 1999). *Streptomyces lactacystinaeus* has been reported to obstruct replication of several viruses, including influenza virus, herpes simplex virus type 1, paramyxovirus and rhabdoviruses, as well as cytomegalovirus (Kaspari and Bogner, 2009).

Ecotin, a serine protease inhibitor found in the periplasm of *E. coli,* is a competitive inhibitor that strongly inhibits trypsin, chymotrypsin and elastase (Chung et al., 1983; Eggers et al., 2004; Yang et al., 1998). A number of pathogenic Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens* or *Erwinia chrysanthemi* seem to be able to protect themselves against their own proteases by producing periplasmic protease inhibitors such as the protease inhibitor ecotin, which has orthologous sequences widely distributed in the bacterial kingdom (Eggers et al., 2004). *Erwinia chrysanthemi*, a phytopathogenic bacterium, produces a protease inhibitor which is a low-molecular-weight, heat-stable protein. In addition to its action on the three

E. chrysanthemi extracellular proteases A, B and C, it also strongly inhibits the 50 kD extracellular protease of *Serratia marcescens* (Letoffe et al., 1989). A broad spectrum protease inhibitor was isolated from the entemopathogenic bacterium *Photorhabdus luminescens* (Wee et al., 2000). A novel serine protease inhibitor gene designated as *Spi1C* was cloned via the sequenced-based screening of a metagenomic library from uncultured marine microorganisms (Cheng-Jian Jiang et al., 2011). Two serine protease inhibitor genes were identified as encoding proteins from *Clostridium thermocellum* which act as protectors or regulators of external proteases (Schwarz et al., 2006). Different role for serine protease inhibitors in bacteria was proposed for *Bacillus brevis* and *Prevotella intermedia*, where they are thought to protect endogenous proteins against proteolysis (Grenier, 1994; Shiga et al., 1992; Shiga et al., 1995). 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). A few inhibitors of the cytomegalovirus protease have been described from bacterial (*Streptomyces*) and fungal (*Cytonaema*) origins (Anderson et al., 2009; Stoeva and Efferth, 2008). Kinetic analysis, expression pattern and production of a recombinant fungal protease inhibitor in tasar silkworm *Antheraea mylitta* were carried out (Roy et al., 2012).

Microorganisms represent an efficient and inexpensive source of protease inhibitors due to their rapid growth, limited space required for cultivation and ready accessibility for genetic manipulation (Pandhare et al., 2002). It was reported that entomopathogenic nematode symbiotic bacterium is a valuable resource of protease inhibitors which can be engineered into plants for insect pest management (Zeng et al., 2012). The advantage of using microbial and fungal protease inhibitors is that many of them display unique inhibitory profiles and resistance to proteolytic cleavage, as well as high thermal and broad pH range stability, with the latter being very convenient since harsh conditions may be used

for immobilization (Rawlings, 2010; Rawlings and Barrett, 2011; Sabotic and Kos, 2012).

Marine microorganisms, with their unique nature differ very much in many aspects from their terrestrial counterparts and are known to produce diverse spectra of novel useful substances including protease inhibitors (Imada, 2004; Kanaori et al., 2005; Rawlings and Barrett, 2011). The potential for marine microbes to become valuable sources of serine protease inhibitors and other industrial enzymes is also proven (Mayer and Lehmann, 2000; Yooseph et al., 2010). It has been reported that among sea organisms, sponge was the most potential producer of bioactive agents including enzyme inhibitor components (Lee et al., 2001). Sponge-associated bacteria are also produce bioactive components (Webster et al., 2001). Bacterial and cyanobacterial symbions of sponge, especially *Aplysina aerophobia*, could reach up to 40% of total sponge biomass (Ahn et al., 2003). It was reported that a bacterium designed as isolate 6A3 (identified as *Chromohalobacter* sp.) isolated from sponge *X. testudinaria* produced protease inhibitor against protease produced by *P. aeruginosa* (Wahyudi et al., 2010). It was also showed that *Pseudomonas sagamiensis,* the marine bacterium produced protease inhibitor (Kobayashi et al., 2003).

2.3.2 Plants as the source of protease inhibitors

Plant protease inhibitors are generally small proteins or peptides that occur in storage tissues, such as tubers and seeds and also in the aerial parts of plants (Macedo et al., 2003; Valueva and Mosolov, 2004). There are numerous protease inhibitors isolated and studied from plants (Bijina et al., 2011a; Green and Ryan, 1972; Joshi et al., 1998; Lorito et al., 1994; Ryan, 1990). Of these, the serine PIs are the most studied and have been isolated from various Leguminosae seeds (Macedo et al., 2002; Macedo and Xavier-Filho, 1992; Mello et al., 2002; Oliva et al., 2000; Souza et al., 1995). Legume seeds contain various PIs classified as Kunitz-type, Bowman–Birk-type, potato I, potato II, squash, cereal superfamily

and thaumatin-like and Ragi A1 inhibitors (Richardson, 1991). Plant protease ihibitors prevent proteolysis in the insect gut which leads to poor nutrient uptake, retarded development and, eventually, death by starvation (Gatehouse et al., 1999).

Plant protease inhibitors have received special attention because of their potential applications in agriculture as bioinsecticide, nematicidal, acaricidal, antifungal and antibacterial agents. In biomedical ground they are remarkable candidates in the production of therapeutic agents. Plant protease inhibitors are usually regulators of endogenous proteinases and also function as plant defense agents blocking the insect and microbial proteinases (Kim et al., 2009). 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 (Kim et al., 2005).

Plant protease inhibitors occur naturally in a wide range of plants as a part of their natural defence system against herbivores or phytophagous insects where the inhibitors impair protein digestion (Broadway and Duffey, 1986; Ryan, 1990). In some cases moulting and non-digestive enzyme regulation could also be affected (Faktor and Raviv, 1997). The pea and soybean trypsin-chymotrypsin inhibitors (PsTI-2,SbBBI) belonging to the Bowman–Birk family (Rahbe´ et al., 2003b) and the mustard-type trypsin-chymotrypsin variant Chy8 (Ceci et al., 2003) induced significant mortality and growth inhibition on the pea aphid *Acyrthosiphon pisum*. The phytocystatin oryzacystatin I (OCI) isolated from rice seeds (Abe et al., 1987) significantly reduced adult weight and fecundity of the aphid *M. persicae* (Rahbe´ et al., 2003a).

A novel protease inhibitor, designated mungoin, with both antifungal and antibacterial activities, was isolated from mung bean (*Phaseolus mungo*) seeds (Wanga et al., 2006). Effects of plant protease inhibitors, oryzacystatin I and soybean Bowman–Birk inhibitor, on the aphid *Macrosiphum euphorbiae*

(Homoptera, Aphididae) and its parasitoid *Aphelinus abdominalis* (Hymenoptera, Aphelinidae) was studied (Azzouz et al., 2005).

The defence against pathogens in plants involves the activation or repression of different signalling pathways leading to the over expression of target genes with defence properties. Protease inhibitors are one of the main groups of proteins induced after plant pathogen exposition. Various plant protease inhibitors with significant defensive role have been isolated from several plant species. Differential *in vitro* and *in vivo* effect of cysteine and serine protease inhibitors from barley on phytopathogenic microorganisms were analysed (Carrillo et al., 2011a). Abundant accumulation of serpins in seeds and their presence in phloem sap suggest additional functions in plant defense by irreversible inhibition of digestive proteases from pests or pathogens (Robert et al., 2012). The use of recombinant protease inhibitors to protect plants has emerged as an interesting strategy for insect pest control using genetic engineering (Lawrence and Koundal, 2002; Reeck et al., 1997; Whetstone and Hammock, 2007). Expression of a nematode symbiotic bacterium-derived protease inhibitor protein in tobacco, enhanced tolerance against *Myzus persica* (Zhang et al., 2012). It was found that the Arabidopsis extracellular Unusual serine Protease Inhibitor (UPI) functions in resistance to necrotrophic fungi (*Botrytis cinerea* and *Alternaria brassicicola)* and herbivorous insect, *Trichoplusia ni* (Laluk and Mengiste, 2011). *In silico* characterization and expression analysis of the multigene family encoding the Bowman–Birk protease inhibitor (BBI) in soybean, identified 11 potential BBI genes in the soybean genome (Barros et al., 2012). Studies were conducted on physical organization of mixed protease inhibitor gene clusters, coordinated expression and association with resistance to late blight at the StKI locus on potato chromosome III and found that protease inhibitors (PIs) play a role in plant defence against pests and pathogens (Odeny et al., 2010).

Protease inhibitors from potato (Bryant et al., 1976; Melville and Ryan, 1972; Pearce et al., 1982; Richardson, 1977; Ryan, 1973), sweet potato (Sugiura et

al., 1973), *Alocasia macrorhiza* and *Colocasia antiquorum* (Sumathi and Pattabiraman, 1979), arrow root tuber (Rao et al., 1983), chick pea (Smirnoff et al*.,* 1976), flax (*Linum usitatissimum* L.) (Kubis et al., 2001) and *Fagopyrum tataricum Seeds* (Tartary buckwheat) (Ruan et al., 2011) have been studied. Effects of the medicinal plant *Mucuna pruriens* protease inhibitors on *Echis carinatus* venom were studied (Onyekwere et al., 2012). Potato protease inhibitors were found to inhibit food intake and increase circulating cholecystokinin levels by a trypsin-dependent mechanism (Komarnytsky et al., 2010). Coexpression of potato type I and II proteinase inhibitors in cotton plants showed that the inhibitors are produced by solanaceous plants as a defense mechanism against insects, give protection against insect damage in the field (Dunse et al., 2010). Interaction of recombinant CanPIs, protease inhibitor of *Capsicum annuum* (common name: hot pepper; Solanaceae) with *Helicoverpa armigera* gut proteases reveals their processing patterns, stability and efficiency which signify isoform complexity in plant protease inhibitors and insect proteases (Mishra et al., 2010). The effect of protease inhibitors derived from potato was formulated in a minidrink, and its effect on appetite, food intake and plasma cholecystokinin levels in humans etc. were studied (Peters et al., 2011).

2.3.3 Protease inhibitors from animals

Mammalian defence protease inhibitors belong to two classes: the active-site inhibitors, represented by superfamilies of serpins and cystatins; and the α2-macroglobulins. The members of the former group inactivate enzymes by binding to the active site, the latter act as molecular traps for the proteases (Władyka and Pustelny, 2008). A novel protease inhibitor in *Bombyx mori* is involved in defense against *Beauveria bassiana* (Li et al., 2012). The primary structure of a new Kunitz-type protease inhibitor InhVJ from the sea anemone *Heteractis crispa* was determined. InhVJ amino acid sequence was shown to share high sequence identity (up to 98%) with the other known Kunitz-type sea anemones sequences

(Gladkikh et al., 2012). Cysteine protease inhibitors namely sialostatins L and L2 have been demonstrated in the saliva of tick *Ixodes scapularis*, in which they play essential roles in transmitting the pathogenic spirochete *Borellia burgdoferi* (Kotsyfakis et al., 2010).

A 30.5 kDa cysteine protease inhibitor (Eel-CPI-1) with lectin activity was isolated from the epidermis of the eel which was shown to bind strongly to both lactose and carboxymethylated papain-affinity gels (Saitoh et al., 2005).

Protease inhibitor (FPI-F) of 6000 Da in the haemolymph of the silkworm, *Bombyx mori* was purified which inhibit fungal proteases and subtilisin (Eguchi et al., 1993). A Kunitz-type protease inhibitor (Gm KTPI) was characterized from the hemolymph of *Galleria mellonella* larvae immunized with *Escherichia coli* which was capable of inhibiting only the trypsin-like activity of the larval midgut extracts. Gm KTPI induced the activation of extracellular signal-regulated kinase (ERK) in the fat bodies and integument cells, and this kinase is known to perform a central role in cell proliferation signaling. It was suggested that Gm KTPI might be responsible for the protection of other tissues against proteolytic attack by trypsin-like protease(s) from larval midgut during metamorphosis, and might play a role in the proliferation of cells in the fat body and integument (Lee et al., 2010). A 10.4 kDa inhibitor of *Aspergillus oryzae* fungal protease was purified to homogeneity (AmFPI-1) from the hemolymph of fifth instar larvae of Indian tasar silkworm, *Antheraea mylitta.* After cDNA cloning and sequence comparison, it was clear that the sequence exhibits similarity to several *Bombyx mori* ESTs and in particular to N-terminal amino acid sequence of an inducible serine protease inhibitor (ISPI-1) from *Galleria mellonella,* indicating its relatedness to ISPI-1 of *G. mellonella*. The presence of this protease inhibitor in the hemolymph may play an important role as a natural defense system against invading microorganisms (Shrivastava and Ghosh, 2003).

Spermiogenesis is a series of poorly understood morphological, physiological and biochemical processes that occur during the transition of immotile spermatids into motile, fertilization-competent spermatozoa. A serpin (serine protease inhibitor) family protein (As_SRP-1) secreted from spermatids during nematode *Ascaris suum* spermiogenesis (also called sperm activation) facilitated sperm motility acquisition and also inhibited in trans, the activation of surrounding spermatids by inhibiting vas deferens-derived As_TRY-5, a trypsin-like serine protease necessary for sperm activation (Zhao et al., 2012).

A Kunitz-type serine protease inhibitor was identified from adult *Ancylostoma ceylanicum* RNA by using a PCR-based approach. The inhibitor plays a role in parasite survival and the pathogenesis of hookworm anemia (Milstone et al., 2000). The recombinant protein (AceKI) inhibits the pancreatic enzymes chymotrypsin, pancreatic elastase, and trypsin *in vitro*. The native AceKI protein was also purified from adult hookworm excretory-secretory (ES) products, which strongly suggests that it has a role in the biology of the adult hookworm (Chu et al., 2004). A number of bioactive molecules from adult *Ancylostoma caninum* hookworms were isolated, including a family of anticoagulant serine protease inhibitors (Cappello et al., 1995; Stassens et al., 1996). The translated amino acid sequence of the *Ancylostoma ceylanicum* Kunitz type inhibitor1 (AceKI-1) cDNA demonstrates homology to members of the Kunitz type family of serine protease inhibitors (Jespers et al., 1995) and a chymotrypsin inhibitor from the silkworm *Bombyx mori* (Sasaki and Kobayashi, 1984). A protease inhibitor of the Kunitz Family from skin secretions of the tomato frog, *Dyscophus guineti* (Microhylidae) was identified and it was demonstrated that selective evolutionary pressure acted to conserve those domains in the molecule (corresponding to positions 12–18 and 34–39) that interact with trypsin. The broad spectrum antimicrobial activity of the inhibitor was described which hypothesize that the synthesis of a proteinase inhibitor in the skin of the tomato frog may be a

component of an alternative strategy of this animal to defend it self against microorganisms (Conlon and Kim, 2000).

In plants, the apoplast (intercellular fluid) forms a protease-rich environment that is colonized by many pathogens, including *P. infestans* and the fungus *Cladosporium fulvum*.The oomycete *Phytophthora infestans* causes late blight, a ravaging disease of potato and tomato. An extracellular protease inhibitor, EPI1, from *P. infestans* was characterized which contains two domains with significant similarity to the Kazal family of serine protease inhibitors. Database searches suggested that Kazal-like proteins are mainly restricted to animals and apicomplexan parasites but appear to be widespread and diverse in the oomycetes. Inhibition of tomato proteases by EPI1 could form a novel type of defense-counterdefense mechanism between plants and microbial pathogens. In addition, this study pointed to a common virulence strategy between the oomycete plant pathogen *P. infestans* and several mammalian parasites, such as the apicomplexan *Toxoplasma gondii* (Tian et al., 2004). Parasitic eukaryotes often face inhospitable environments in their hosts. For example, parasites that colonize or transit through the mammalian digestive tract must adapt to the diverse and abundant array of proteases secreted in the gastric juices (Dubey, 1998; Milstone et al., 2000; Morris et al., 2002). The apicomplexan obligate parasite *Toxoplasma gondii* secretes TgPI-1 and TgPI-2, four-domain serine protease inhibitors of the Kazal family (Lindh et al., 2001; Morris et al., 2002; Pszenny et al., 2000).

Lymnaea trypsin inhibitor (LTI) was purified and characterized from *Lymnaea albumen* gland extracts. Comparison of the LTI sequence with other known serine protease inhibitors indicates that LTI is a member of the bovine pancreatic trypsin inhibitor family. Abundant amounts of intact LTI are packaged in egg masses. The presence of a trypsin inhibitor in the perivitelline fluid compartment of the egg mass may minimize digestion of peptides and proteins in the perivitelline fluid that are important for the development of the embryo (Nagle et al., 2001).

2.4 Classification of protease inhibitors

Protease inhibitors can be classified according to the source organism (microbial, fungal, plant, animal), according to their structure (primary and three-dimensional), or according to their inhibitory profile (broadrange, specific) and reaction mechanism (competitive, non-competitive, uncompetitive as well as reversible or irreversible). They are commonly classified according to the class of protease they inhibit (aspartic, cysteine or serine protease inhibitors).

Protease inhibitors are grouped broadly in to two;

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

While protein inhibitors can gain potency through the burial of a large surface area and specificity through contacts with specific exosites, small-molecule inhibitors primarily gain potency through interactions with the catalytic machinery of the enzyme, and specificity through interactions with the substrate binding sites. While there are several examples of successful small-molecule protease inhibitors in the clinic, selectivity and potency can be significant challenges when targeting particular protease family members.

2.4.1 Small molecule inhibitors

Small molecule inhibitors (SMIs) include naturally occurring compounds such as pepstatin, bestatin and amastatin, as well as synthetic inhibitors generated in a laboratory. So it is difficult to provide any of natural classification, unlike the peptidases and protein inhibitors and a new series of identifiers has been created. Accordingly each SMI is assigned an identifier consisting of an initial J followed by a five digit number. For example, pepstatin is J00095 and ethylene diamine tetraacetic acid is J00149 (Rawlings and Barrett, 2011).

SMIs are inhibitors that are not proteins, including peptides and synthetic inhibitors that are generally of microbial origin and are low molecular weight

peptides of unusual structures (Umezawa, 1982). Many of them have been synthesized in the laboratory; however, those that occur naturally have been isolated from bacteria and fungi (Rawlings, 2010). Small molecule inhibitors which have proved useful include reversible transition state mimics such as peptide aldehydes and boronates, and irreversible reagents such as peptidyl chloromethanes and sulfonyl fluoride derivatives (Powers and Harper, 1986). These include many substances that are laboratory reagents used in the characterization of peptidases, and others that are compounds that are inhibitors of peptidases known to be important in diseases, such as retropepsin of the HIV virus (Kempf et al., 1998) thrombin (Gustafsson et al., 1998) which can cause thrombosis; dipeptidyl-peptidase IV (Feng et al., 2007; Hughes et al., 1999; Kim et al., 2005), which is implicated in type 2 diabetes; γ-secretase (Imbimbo, 2008) which is implicated in Alzheimer's disease; renin (Wood et al., 2003) and angiotensin-converting enzyme (Sybertz et al., 1987), which control blood pressure; and peptidases from the malarial parasite *Plasmodium* (Andrews et al., 2006).

Among the small-molecule inhibitors of bacterial and fungal origin, peptidyl aldehydes such as leupeptin and antipain, hexapeptide pepstatin and epoxysuccinyl peptide E-64 and their analogues have been studied as anticancer agents. 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. They were designed on the basis of the X-ray crystal structures of individual cathepsins, and the most studied were cathepsin B specific inhibitors CA-074 and CA-030, cathepsin L specific inhibitors CLIK-148 and CLIK-195, and cathepsin X specific inhibitor AMS-36. Cathepsin S specific inhibitor CLIK-060 was designed on the basis of the structure of leupeptin and antipain

(Katunuma, 2011). Antitumour activity was exhibited particularly by CA-074, a specific inhibitor of the cysteine protease cathepsin B (Johansson et al., 2000), which appears to be crucial for tumour cell invasion (Lah et al., 2006). Better cell permeability was demonstrated for ethyl ester E-64 and the methyl ester of CA-074, which are also highly soluble and effective for prolonged periods (Frlan and Gobec, 2006).

A few examples of utilization of microbial small-molecule inhibitors as antinutritional agents are available, e.g. aminopeptidase inhibitors of actinomycetes amastatin and bestatin against the red flour beetle (*Tribolium castaneum*) (Oppert et al., 2011), aspartic protease inhibitor pepstatin A from actinomycetes against the cowpea bruchid (*Callosobruchus maculatus*) (Amirhusin et al., 2007), the serine and cysteine protease inhibitor leupeptin from actinomycetes against western corn rootworm (*Diabrotica virgifera*) (Kim and Mullin, 2003) and cysteine protease inhibitor E-64 from *Aspergillus japonicus* against Colorado potato beetle (*Leptinotarsa decemlineata*) (Bolter and Green, 1997).

For secreted recombinant proteins, small-molecule inhibitors can be added to the culture medium to inhibit the predominant secreted proteolytic activity of the host organism that is often of the serine and aspartic catalytic type (Sabotic et al., 2012). Allosteric small-molecule inhibitors could be useful against many proteases by, for example, binding to protease exosites and preventing protein substrate binding or recognition. A recent breakthrough in allosteric protease inhibitor design has been achieved with the development of the first allosteric caspase inhibitors: 5-fluoro-1*H*-indole-2-carboxylic aci (2-mercapto-ethyl)-amide (FICA) and 2-(2,4-dichlorophenoxy)- *N*-(2-mercapto-ethyl)-acetamide (DICA). These were shown to bind to a cysteine residue in the vicinity of the active site cleft of caspases 3 and 7, respectively, locking the specificity loops of the protease into a zymogen-like conformation, thereby abolishing enzymatic activity (Hardy et al., 2004).

Some bacteria synthesize peptides and derivatives of peptides that are efficient peptidase inhibitors, often ones that affect peptidases from different families and different catalytic types. The best known of these is leupeptin (N-acetyl-L-leucyl-L-leucyl-D,L-argininaldehyde), which was originally isolated from *Streptomyces exfoliates* and inhibits a wide range of serine, cysteine and threonine-type peptidases, including trypsin, PACE4 calpain, clostripain and the trypsin-like activity of the proteasome (Aoyagi et al., 1969; Kembhavi et al., 1991; Kurinov and Harrison, 1996; Mains et al., 1997; Moldoveanu et al., 2004; Savory et al., 1993). Other small molecule inhibitors produced by actinomycetes include bestatin and amastatin (inhibitors of aminopeptidases) and tyrostatin, which inhibits sedolisin of family S53 (Aoyagi et al., 1978; Oda et al., 1989; Umezawa et al., 1976).

2.4.2 Proteinaceous inhibitors

Protein inhibitors of proteases are ubiquitous and have been isolated from numerous plants, animals and microorganisms (Birk, 1987; Leo et al., 2002). Naturally occurring proteinaceous inhibitors are of immense interest as templates for the modification of natural control mechanisms and as a source of basic design principles.

Formerly, protease inhibitors were grouped according to the kind of protease inhibited. Then, they were classified as cysteine, serine, aspartic, and metalloprotease inhibitors (Laskowski and Kato, 1980). With the exception of the plasma macroglobulins, which inhibit proteases of all classes (Barrett, 1981), individual protein inhibitors inhibit only proteases belonging to a single mechanistic class. Of these inhibitors, the most extensively studied are the inhibitors of serine proteases. Protein inhibitors of aspartic proteases are relatively uncommon and are found in only a few specialized locations (Bennet et al., 2000). Few of the examples include a 17 kDa inhibitor of pepsin and cathepsin E from the parasite *Ascaris lumbicoides* (Kageyama, 1998), proteins from potato (Kreft et

al., 1997), and a pluoripotent inhibitor from sea anemone of cysteine protease as well as cathepsin D (Lenarcic and Turk, 1999). There is a report of an 8 kDa polypeptide inhibitor of the vacuolar aspartic protease (protease A or saccharopepsin) from yeast (Saheki et al., 1972).

It was evident that peptidase inhibitors could best be classified in their homologous families (Laskowski and Kato, 1980), but the sequence information then available allowed only about a dozen families to be recognized and they are now classified in function of their sequence similarities and three dimensional structures (Rawlings, 2010). Hundreds of protein inhibitors of peptidases are now known and they are the subjects of thousands of research communications. A detailed classification of protein protease inhibitors based on their evolutionary relationship is available in the MEROPS database (http://merops.sanger.ac.uk/inhibitors/) which follows a hierarchy similar to that for proteases. PIs have been grouped into families and subfamilies and into different clans on the basis of sequence relationship and the relationship of protein folds of the inhibitory domains or units. An 'inhibitor unit' was defined as the segment of the amino acid sequence containing a single reactive site (or bait region, for a trapping inhibitor) after removal of any parts that are known not to be directly involved in the inhibitory activity. A protein that contained only a single inhibitor unit was termed a simple inhibitor, and one that contained multiple inhibitor units was termed a compound inhibitor (Rawlings et al., 2004).

The classification of protein peptidase inhibitors is continually being revised by MEROPS database and currently inhibitors are grouped into 71 families based on comparisons of protein sequences which include 17451 inhibitor sequences. Their molecular weight and mechanism of inhibition varies from inhibitor to inhibitor. These families can be further grouped into 39 clans based on comparisons of tertiary structure. The family and clan of some protein peptidase inhibitors are depicted in Table 2.1. 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 2.1 Families and clans of proteinaceous inhibitors

Family or subfamily	Clan	Inhibitor and unit name (source)	Reference
I1	IA	Ovomucoid unit 3 (*Meleagris gallopavo*)	(Laskowski and Kato, 1980; Lu et al., 2001)
I2	IB	Aprotinin (*Bos taurus*)	(Ascenzi et al., 2003; Beierlein et al., 2005)
I3	IC	Soybean trypsin inhibitor (*Glycine max*)	(Laskowski and Kato, 1980), (Oliveira et al., 2001)
I4	ID	α_1-proteinase inhibitor (*Homo sapiens*)	(Huntington et al., 2000), (Al-Khunaizi et al., 2002)
I5	IA	Ascidian trypsin inhibitor (*Halocynthia rorefzi*)	(Kumazaki et al., 1994)
I6	IJ	Ragi seed trypsin/ α-amylase inhibitor (*Eleusine coracana*)	(Hojima et al., 1980)
I7	IE	Trypsin inhibitor MCTI-1 (*Momordica charantia*)	(Wieczorek et al., 1985)
I8	IA	Nematode anticoagulant inhibitor (*Ascaris suum*)	(Bernard and Peanasky, 1993), (Griesch et al., 2000)
I9	JC	Protease B inhibitor (*Saccharomyces cerevisiae)*	(Kojima et al., 1999)
I11	IN	Ecotin (*Escherichia coli*)	(Chung et al., 1983)
I12	IF	Bowman-Birk plant trypsin inhibitor (*Glycine max*) unit 1	(Odani and Ikenaka, 1973), (Hatano et al., 1996)
I13	IG	Eglin C (*Hirudo medicinalis*)	(Heinz et al., 1991)
I14	IM	Hirudin (*Hirudo medicinalis*)	(Bode and Huber, 1992)
I15	IM	Antistasin unit 1 (*Haementeria officinalis*)	(Rester et al., 1999)

I16	IY	Subtilisin inhibitor (*Streptomyces albogriseolus*)	(Mitsui et al., 1979), (Taguchi et al., 1998)
I17	IP	Mucus proteinase inhibitor unit 2 (*Homo sapiens*)	(Tsunemi et al., 1993)
I18	JD	Mustard trypsin inhibitor (*Sinapis alba*)	(Menegatti et al., 1992)
I19	IW	Proteinase inhibitor LCMI I (*Locusta migratoria*)	(Eguchi et al., 1994)
I20	JO	Proteinase inhibitor II (*Solanum tuberosum*)	(Barrette-Ng et al., 2003)
I25	IH	Ovocystatin (*Gallus gallus*)	(Bode et al., 1988), (Alvarez-Fernandez et al., 1999)
I27	II	Calpastatin unit 1 (*Homo sapiens*)	(Todd et al., 2003)
I29	JF	Cytotoxic T-lymphocyte antigen	(Guay et al., 2000)
I31	IX	Equistatin (*Actinia equina*)	(Strukelj et al., 2000)
I32	IV	BIRC-5 protein (*Homo sapiens*)	(Riedl et al., 2001)
I33	IR	Ascaris pepsin inhibitor PI-3 (*Ascaris suum*)	(Ng et al., 2000)
I34	JA	Saccharopepsin inhibitor (*Saccharomyces cerevisiae*)	(Phylip et al., 2001)
I35	IT	Timp-1 (*Homo sapiens*)	(Gomis-Ruth et al., 1997), (Lee et al., 2003)
I36	IU	Streptomyces metalloproteinase inhibitor (*Streptomyces nigrescens*)	(Hiraga et al., 1999)
I37	IE	Potato carboxy peptidase inhibitor (*Solanum tuberosum*)	(Bode and Huber, 1992)
I38	IK	Metalloproteinase inhibitor (*Erwinia chrysanthemi*)	(Feltzer et al., 2003)
I39	IL	α_2-macroglobulin (*Homo sapiens*)	(Barrett, 1981)
I40		Bombyx subtilisin inhibitor (*Bombyx mori*)	(Pham et al., 1996)

I42	JL	Chagasin (*Leishmania major*)	(Monteiro et al., 2001)
I43	JM	Oprin (*Didelphis marsupialis*)	(Neves-Ferreira et al., 2002)
I44	JJ	Carboxypeptidase A inhibitor (*Ascaris suum*)	(Homandberg et al., 1989)
I46	IS	Leech carboxypeptidase inhibitor (*Hirudo medicinalis*)	(Reverter et al., 2000)
I47	IH	Latexin (*Homo sapiens*)	(Normant et al., 1995)
I48	IC	Clitocypin (*Lepista nebularis*)	(Brzin et al., 2000)
I49		ProSAAS (*Homo sapiens*)	(Basak et al., 2001)
I50	IQ	Baculovirus p35 caspase inhibitor (*Spodoptera litura* nucleopolyhedrovirus)	(Xu et al., 2003)
I51	JE	Carboxypeptidase Y inhibitor (*Saccharomyces cerevisiae*)	(Bruun et al., 1998)
I52	IB	Tick anticoagulant peptide (*Ornithodorus moubata*)	(Charles et al., 2000)
I57	IK	Staphostatin B (*Staphylococcus aureus*)	(Rzychon et al., 2003)
I58	IK	Staphostatin A (*Staphylococcus aureus*)	(Rzychon et al., 2003)
I59	IZ	Triabin (*Triatoma pallidipennis*)	(Fuentes-Prior et al., 1997)
I63	JB	Pro-eosinophil major basic protein (*Homo sapiens*)	(Glerup et al., 2005)
I67	IF	Bromein (*Ananas comosus*)	(Sawano et al., 2005)
I68	JK	Tick carboxypeptidase inhibitor (*Rhipicephalus bursa*)	(Arolas et al., 2005)
I73	JN	Veronica trypsin inhibitor (*Veronica hederifolia*)	(Conners et al., 2007)
I83	JH	AmFPI-1 (*Antheraea mylitta*)	(Roy et al., 2009)
I85	IC	Macrocypin 1 (*Macrolepiota procera*)	(Sabotic et al., 2009)

- **Distribution of families amongst organisms**

Over 634 protein peptidase inhibitor species are distributed throughout the organisms from viruses to animals. Of the 71 families, 27 include members of microbial and fungal origin (Tables 2.2). Of these, seven families include members of exclusively bacterial origin (I10, I16, I36, I38, I57, I58, I69), and five families include members of exclusively fungal origin (I34, I48, I66, I79, I85) (Rawlings, 2010).

Table 2.2 Families of proteinaceous inhibitors of microbial and fungal origin (Rawlings and Barrett, 2011)

Family	Common name	Families of peptidases inhibited
I1	Kazal	M10, S1A, S1D, S8A, S9A
I2	Kunitz-BPTI	S1A, S7
I4	Serpin	C1A, C14A, S1A, S7, S8A, S8B
I9	YIB	S8A
I10	Marinostatin	S1A, S8A
I11	Ecotin	S1A
I16	SSI	M4, M7, S1A, S8A, S8B
I31	Thyropin	A1A, C1A, M10A
I32	IAP	C14A
I34	IA3	A1A
I36	SMI	M4
I38	Aprin	M10B
139		A1A, A2A, C1A, C2A, C11, M4, M10A,M10B, M12A, M12B, S1A, S1B, S8A
I42	Chagasin	C1A
I43	Oprin	M12B
I48	Clitocypin	C1A, C13
I51	IC	S1A, S10
I57	Staphostatin B	C47
I58	Staphostatin A	C47
I63		M43B, S1A

I66	Cnispin	S1A
I69		C10
I75	CIII	M41
178		S1A, S8A
179	AVR2	C1A
I85	Macrocypin	C1A, C13, S1A
187	HflKC	M41

2.4.3 Classification according to the physiological outcome or relevance of the inhibition

The physiological inhibition of proteases depends on temporal and spatial co-localization of the protease and its inhibitor, their relative concentrations and binding kinetics; this leads to two major categories of physiological inhibitors (Bode and Huber, 2000)

i) Emergency type inhibitors

These are characterized by a large excess concentration of inhibitor, rapid binding and no co-localization with proteases. The major function is to block any protease activity in an inappropriate compartment. Eg: cystatins (Turk et al., 2002).

ii) Regulatory type inhibitors

They are often co-localized with proteases and are responsible for the fine regulation of protease activity. Regulatory type inhibitors can be subdivided into the following categories:

- **Threshold inhibitors:** low concentration and rapid binding, their major function is to neutralize accidental protease activation. Eg: inhibitors of apoptosis (Deveraux et al., 1997).
- **Buffer-type inhibitors:** weak binders, these temporarily block proteases to prevent inappropriate activity and can be easily displaced from the complex. Eg: propeptides of cathepsins (Turk et al., 2000).

- **Delay-type inhibitors:** often irreversible, slow binders that enable protease activity for a limited amount of time. Eg: antithrombin (Olson et al., 2002).
- **Pro-inhibitors:** synthesized as inactive, and require proteolytic processing to become active. Eg: invariant chain p41 fragment resulting from the proteolytic processing of major histocompatibility class II molecules (Bevec et al., 1996).

2.5 Mechanism of action of protease inhibitors

There are two general mechanisms of protease inhibition, namely, irreversible "trapping" reactions and reversible tight-binding reactions. Trapping reactions work only on endopeptidases and are the result of a conformational change of the inhibitor triggered by cleavage of an internal peptide bond by the host protease (Fig. 2.1). Only three families utilize a trapping mechanism: I4 (serpins), I39 (α2-macroglobulin) and I50 (viral caspase inhibitors). Four different mechanisms for protease inhibition have been described so far (Bode and Huber, 2000).

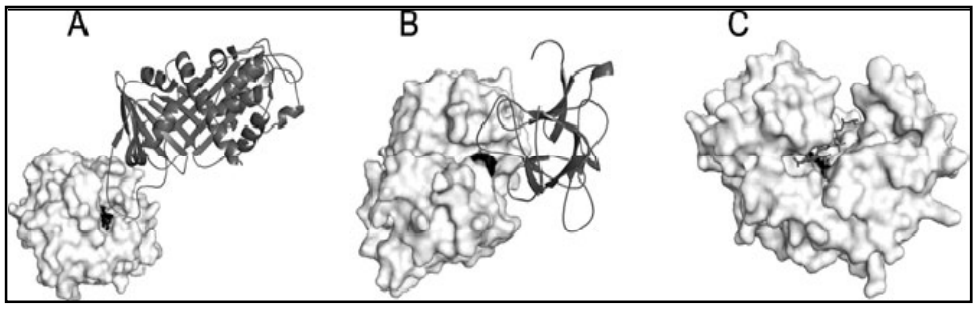

Fig. 2.1 Examples of protease inhibitors utilizing irreversible "trapping" reaction (A) and reversible tight-binding reactions (B and C). Proteases are shown in light grey, their active site residues in black and inhibitors in dark grey. **A**. Serine protease trypsin in complex with serpin (family I39) (PDB ID 1K9O). The

protease cleaves the reactive centre loop of serpin, which triggers a conformational change in the inhibitor and trapping of the protease in an inactive covalent complex. **B**. Cysteineprotease cathepsin V in complex with clitocypin (family I48) (PDB ID 3H6S). The inhibitor binds to the protease active site cleft and obstructs access of substrate. **C**. Aspartic protease plasmepsin IV in complex with the small-molecule inhibitor pepstatin A (PDB ID 1LS5). The inhibitor binds in the active site of the protease (Sabotic and Kos, 2012).

2.5.1 Competitive inhibition

The vast majority of protease inhibitors are competitive. Most protease inhibitors bind a critical portion of the enzyme in the active site in a substrate-like manner (Fig 2.2). Related proteases often show a high degree of homology in the active site, substrate-like binding often leads to inhibitors that can potently inhibit more than one target protease. The most thoroughly studied mechanism of protein protease inhibitors is that of the standard (or canonical, or Laskowski) mechanism inhibitors of serine proteases (Laskowski and Kato, 1980). Canonical inhibitors (lock-and-key) bind to their targets in a substrate-like manner to form an almost Michaelis-type complex, a principle used by serine protease inhibitors (Silverman et al., 2001). These inhibitors include the Kazal, Kunitz, and Bowman-Birk family of inhibitors and bind in a lock-and-key fashion. Standard-mechanism inhibitors insert into the active site of the protease a reactive loop that is complementary to the substrate specificity of the target protease and binds in an extended β-sheet with the enzyme in a substrate-like manner (Figure 2.2 A, B). While bound to the protease, the “scissile bond” of standard-mechanism inhibitors is hydrolyzed very slowly, but products are not released, and the amide bond can be relegated (Zakharova et al., 2009). Reversible tight-binding inhibition is widespread, in which the inhibitor has a peptide bond that is cleaved by the peptidase active site in a substrate-like manner. The inhibitor is only slowly released due to the conformational stability of the stabilized loop that can mimic a substrate. This

mechanism has been conclusively demonstrated only for inhibitors of serine proteases. Other reversible tight-binding protease inhibitors physically block the protease active site by high-affinity binding to sites on either side of the active site.

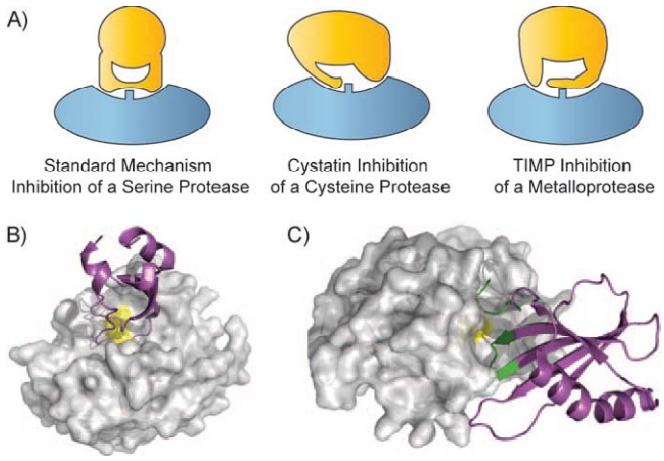

Fig 2.2 Competitive, active-site inhibitors of proteases. **A.** Inhibitors bind in the active site, but not in a substrate-like manner. Peptide extensions bind in specificity subsites, and can interact with the catalytic residues (rectangle). **B.** Crystal structures of a serine protease (matriptase/MT-SP1, PDB ID: 1EAW) in complex with the standard-mechanism inhibitor aprotinin, and **C.** the cystatin stefin A in complex with a cysteine protease (cathepsin H, PDB ID: 1NB5). Both inhibitors bind in the active-site groove of their targets (Farady and Craik, 2010).

The standard mechanism is used by many structurally disparate protein scaffolds to create potent inhibitors. However, the majority of standard-mechanism protease inhibitors tend to have relatively broad specificity within subclasses of serine proteases. For example, bovine pancreatic trypsin inhibitor (BPTI) efficiently inhibits almost all trypsin-fold serine proteases with P1-Arg specificity with sub-nanomolar potency, and can also potently inhibit

chymotrypsin (Phe P1 specificity) with a *K*i of 10 nm (Castro and Anderson, 1996). Binding of an inhibitor to the active site can also be irreversible, when an electrophilic reactive group of the inhibitor forms a covalent bond with an amino acid residue in the enzyme active site.

The majority of protease inhibitors bind in and blocks access to the active site of their target protease, but do not bind in a strictly substrate-like manner. Instead they interact with the protease subsites and catalytic residues in a noncatalytically competent manner. This differentiates them from standard mechanism inhibitors; however, like standard-mechanism inhibitors, they get most of their potency from interactions within the protease active site, and tend to potently inhibit many related proteases. The cystatins, a superfamily of proteins that inhibit papainlike cysteine proteases, are a classic example of these inhibitors (Fig 2.2 A, C). The cystatins insert into the V-shaped active site of a cysteine protease a wedge-like face of the inhibitor that consists of the protein N terminus and two hairpin loops. The N-terminal residues bind in the S3–S1 pockets in a substratelike manner, but the peptide then turns away from the catalytic residues and out of the active site. The two hairpin loops bind to the prime side of the active site, which provides the majority of the binding energy for the interaction. Thus, both the prime and nonprime sides of the active site are occupied, but no interactions are actually made with the catalytic machinery of the enzyme (Bode and Huber, 2000). The four human tissue inhibitors of metalloproteases (TIMPs) are responsible for the inhibition of dozens of extracellular metalloproteases (Fig 2.2 A). They bind to their target enzymes in a two-step mechanism similar to that of the cystatins. While the N-terminal residues of cystatins bind to the nonprime side of cysteine proteases, TIMPs N termini bind in the P1-P3' pockets of the protease, coordinate the catalytic Zn^{2+} ion, and exclude a catalytic water molecule from the active site. Meanwhile a second loop of the TIMP binds both in the P3 and P2 pockets, and binds to the N terminus of the matrix metalloprotease (MMP). Despite the similarities in mechanistic architecture between TIMPs and cystatins

(hairpin loops and N-terminal residues in substrate binding pockets), TIMPs interfere with the catalytic machinery of MMPs by chelating the catalytic Zn^{2+} (Brew et al., 2000).

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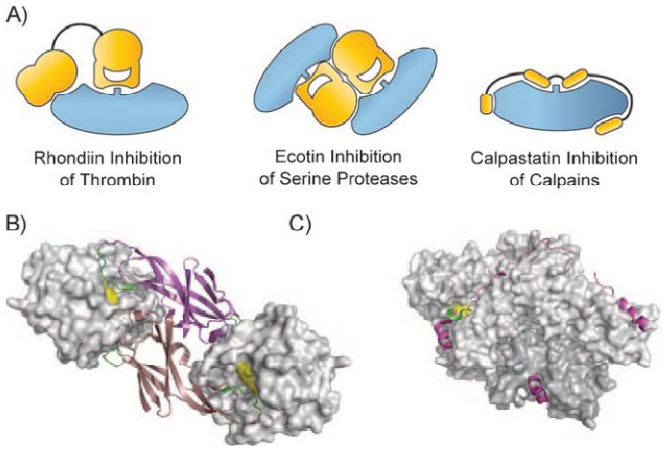


Fig 2.3. Inhibitors that take advantage of exosite binding. (**A**) Most exosite inhibitors are competitive inhibitors that prevent substrate binding at the active site. In the case of ecotin (bound to trypsin, PDB ID: 1EZU), the exosites provide binding energy and allow for broad specificity (**B**), while calpastatin (**C**) gains binding energy and specificity by forming critical interactions across the calpain protease surface (PDB ID: 3BOW) (Farady and Craik, 2010)

2.5.2 Competitive inhibition with exosite binding

A number of protease inhibitors are competitive, and bind in the protease active site, but also have secondary binding sites outside the active site that are critical to inhibition. Exosite-binding inhibitors directly block the active site by binding adjacent to it and covering it partially in a substrate-like manner. This principle was observed initially with cystatins and cysteine cathepsins (Stubbs et al., 1990). Exosite binding provides two major benefits; it increases the surface

area of the protein protein interaction, leading to a greater affinity, and it can have a significant effect on the specificity of the inhibitor. Rhodniin, a thrombin inhibitor from the assassin bug *Rhodnius prolixus* has two Kazal-type inhibitory domains, a common standard-mechanism serine protease inhibitor domain (Fig 2.3A). While the N-terminal domain binds and inhibits through the standard mechanism, the second Kazal-type domain has evolved to bind to exosite I on thrombin. The inhibitor gains both potency and specificity from exosite binding. In contrast, the *E. coli* serine protease inhibitor ecotin uses its exosites to provide binding energy and to actually broaden the inhibitor promiscuity to protect the bacteria from diverse host proteases (Fig 2.3 A, B). Ecotin is a dimeric protein that inhibits trypsin-fold serine proteases, regardless of primary specificity. It inhibits using a standard mechanism at its primary binding site, but also has a secondary binding site. The effect of the secondary binding site on affinity was found to be inversely proportional to the strength of binding at the primary site. The exosite actually makes the inhibitor less specific, or more capable of inhibiting a broad range of proteases, and allows one bacterial inhibitor to protect against a number of host proteases (Eggers et al., 2001).

The recent structures of the calcium-dependent protease calpain 2 in complex with the inhibitory domain CAST4 of calpastatin (Fig 2.3 A, C) reveal another unique use of binding sites outside the active site (Moldoveanu et al., 2008). Free calpastatin is intrinsically unstructured. Upon binding to calpain, the polypeptide forms three helices, strung across the surface of the enzyme, and binds in the protease active site to act as a competitive inhibitor. Incredibly, CAST4 buries on calpain, approximately three times the surface area that standard-mechanism protease inhibitors (Scheidig et al., 1997). The majority of competitive protease inhibitors do not show much induced fit upon binding, and thus do not require a lot of buried surface area. In contrast, CAST4 uses this large amount of buried surface area outside the protease active site to compensate for the entropic penalty of ordering the inhibitor upon binding, and still allows for a

*K*i in the low-nanomolar range. The use of exosites to increase the surface area of a protease inhibitor, and allow for structural arrangements to take place upon binding, is a theme that has been extremely useful in the design of novel protein protease inhibitors. Inhibitors might also bind in a quasi-substrate-like manner that is a combination of the canonical and exosite-binding mechanisms (for example, as with tissue inhibitor of metalloproteinases) (Gomis-Ruth et al., 1997). There are also some inhibitors that block the exosites, to which substrate binding occurs in addition to the active site in some proteases (Christeller, 2005; Rawlings et al., 2004; Rawlings, 2010).

2.5.3 Allosteric inhibition

Such as X-linked inhibitor of apoptosis protein, which inhibits caspase prevent dimerization by binding in the interdomain region away from the active site (Shiozaki, 2003). With the exception of allosteric inhibitors all other endogenous protein inhibitors are mostly competitive.

2.5.4 Irreversible inhibition

Some protease inhibitors use proteolytic activation by an enzyme to covalently modify and thereby inhibit it. They are called suicide substrates. This sort of activity-dependent inhibition is powerful and fundamentally different from the competitive mechanisms. The inhibitor acts as a substrate, then utilizes the enzymes' catalytic machinery to trap and inhibit the enzyme. The inhibitor α-2-macroglobin (α2M) and its relatives are responsible for clearing excess proteases from plasma.

The serpins are a family of inhibitors that covalently and irreversibly inhibit primarily serine proteases (Gettins, 2002). Serpins have a large reactive center loop (RCL) that is presented to a protease for proteolytic processing. Upon productive cleavage of the RCL, the N-terminal half of the RCL, still attached to

the protease as an acylenzyme intermediate is inserted into a β-sheet in the body of the inhibitor. The resulting free-energy change is enough to translocate the protease (still covalently attached to the RCL) to the distal side of the inhibitor, and the resulting steric collisions completely deform the protease active site, thus leaving completely inactive. The serpin inhibitory mechanism is completely irreversible (Fig 2.4). Because of the drastic nature and irreversibility of this mechanism, serpins function as protease scavengers, protecting cells and tissues from unwanted proteolytic activity. These types of inhibitors, which take advantage of the catalytic activity of a protease to trap and inhibit the enzyme, are effective and powerful inhibitors that are responsible for protecting the organism from aberrant proteolytic activity from a wide range of proteases. Thus, they tend to be relatively nonspecific.

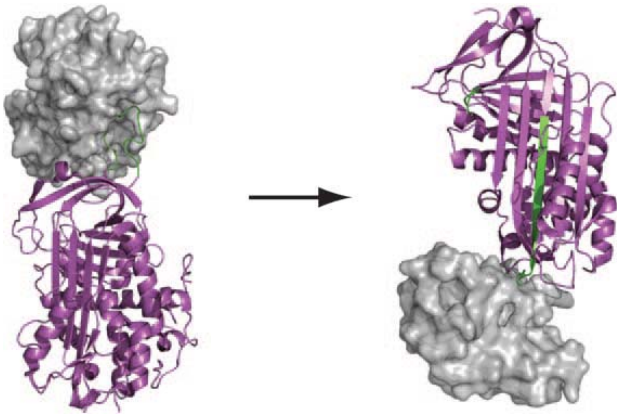


Fig 2.4 Serpins inhibit serine proteases by binding a reactive center loop in the active site, forming a covalent complex with the enzyme, undergoing a large conformational change, and irreversibly distorting the active site of the protease (PDB IDs: 2GD4 and 1EZX) (Farady and Craik, 2010).

2.6 Protease inhibitor purification methods

Protease inhibitors are ubiquitous and are among the most intensively studied proteins. Purification to homogeneity of inhibitors is a necessary and critical step in order to define their structural characteristics and binding

specificity to the proteases. Isolate these from all other proteins that are present in the same biological source is a difficult task, since the protease inhibitors have a large molecular diversity. So the process of purification includes a combination of separation strategies, such as: extraction, fractionation by precipitation and chromatographic techniques addressing different properties. The type and amount of technique will depend on the nature and characteristics of the molecule of interest, as well as the degree of purity desired in the final product.

Once inhibitory activity is detected in a crude sample, it is important to perform an initial simple fractionation of the inhibitor activity: eg. ammonium sulphate, organic solvent and polyethylene glycol fractionation. Protease inhibitors are 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). This is to determine whether the specific activity of the inhibitor increases after such fractionation. Methods like SDS-PAGE, Isoelectric focusing, MALDI-TOF etc. are used for confirming homogeneity. Protease inhibitor with antipathogenic and anti-proliferative activities from mung bean has been purified by ammonium sulphate precipitation, Cation-exchange chromatography on CM-Sephadex C-50 column, perfusion chromatography on a BioCAD 700E work station and C18 capillary reverse-phase high-performance liquid chromatography (Wanga et al., 2006). To establish effective purification of the inhibitor sample it is important to determine the specific activity of the inhibitor sample in each step of purifications (Hideaki and Guy, 2001). The homogeneity is confirmed by methods like SDS-PAGE, Isoelectric focusing and MALDI-TOF. Depending on the type of protein-resin interactions, there are four types of chromatographic techniques. These techniques are ion exchange, affinity, size exclusion and hydrophobic chromatography. Though all four chromatographic techniques are used in the purification process, affinity and ion exchange chromatography are by far the widely used techniques in the biopharmaceutical industry and in academia.

Purification procedures like ion exchange chromatography, gel filtration chromatography, high performance liquid chromatography, reversed-phase high-performance liquid chromatography and affinity chromatography are used for purifying protease inhibitor from the crude extract. Ion exchange chromatography is based on the principle of charge-charge interaction, wherein similar charges repel and opposite charges attract. The protein migration in an ion exchange column is dependent on the interaction between the protein net charge and the charge of resin. The net charge of the protein is primarily dependent on the protein's isoelectric point (pI). In ion exchange chromatography, the whole protein surface with its charge moieties modulates the protein binding process. The matrices for ion exchange chromatography contain ionizable functional groups such as diethyl amino ethyl (DEAE) and carboxy methyl (CM), which gets associated with the charged protein molecule, thereby adsorbing the protein to the matrices. The adsorbed protein molecule is eluted by a gradient change in the pH or ionic strength of the eluting buffer or solution (Arindam et al., 2006; Shrivastava and Ghosh, 2003).

Another very rapid and selective method for peptide and protein purification is reversed-phase high performance liquid chromatography on porous, microparticulate, chemically bonded alkylsilicas (Hearn, 1982). The most commonly used reverse phase HPLC column includes, octadecylsilane column (Shoji et al., 1999), Vyda C18 HPLC column (Cesar et al., 2004), Vydac 218 TP 1022 C-18 (Sivakumar et al., 2005) and μ-Bondapak C18 column (Ligia et al., 2003). Among the chromatographic techniques, the affinity chromatography, by which specific biological properties can be exploited, stands out for its high purification capacity. For example, affinity chromatography on columns containing immobilized enzymes provides an efficient and rapid process of isolation and purification of protease inhibitors from different sources. This procedure provides advantages as high enrichment of inhibitor fraction, reduction of purification steps due to high binding specificity of the protein immobilized on

a chromatographic matrix and purification of different protease inhibitors in the same fraction by differential elution of material retained on chromatographic matrix.

Affinity chromatography is a very efficient technique capable of purifying proteins based on reversible interactions between a protein and a specific ligand coupled to a chromatographic matrix. The affinity chromatography is usually used as one of the last steps in the purification process of protease inhibitors. The binding property of the protease inhibitors with enzymes has been exploited for the affinity purification of inhibitors from various sources. Nevertheless, due to their specific and reversible binding capacity to the enzymes (without undergoing chemical change), protease inhibitor purification can be greatly enhanced by the use of affinity chromatography techniques, where the binding agent are particular proteases (Araújo et al., 2005; Gomes et al., 2005). The common affinity ligands used for the protease inhibitor purification includes, S-carboxymethyl-papain-Sepharose (Gounaris et al., 1984), trypsin-Sepharose (Alinda et al., 2003), chymotrypsin-Sepharose (Gennis and Cantor, 1976) and trypsin-agarose (Sivakumar et al., 2005). Trypsin inhibitors from wild-type soybean (Glycine soya) (WBTI) and domesticated soybean (*Glycine max*) (SBTI) are purified using prepared chitosan resin-trypsin as filler on the affinity chromatography column (Zhang et al., 2009). Serine protease inhibitor from the tissue-penetrating Nematode *Anisakis simplex* was purified using C3 reverse-phase HPLC chromatographic procedure and streptavidin-agarose affinity chromatography (Morris and Sakanari, 1994). Serine protease inhibitor (AmPI) purified from larval hemolymph of tasar silkworm *Antheraea mylitta* using trypsin-sepharose CL-6B affinity column (Rai et al., 2010). A heat-stable serine protease inhibitor (Potide-G) from the tubers of new potato variety ‘‘Golden Valley’’ through extraction of the water-soluble fraction, dialysis, ultrafiltration and DEAE-cellulose and C18 reversed-phase high performance liquid chromatography (Mi-Hyun et al., 2006).

Affinity chromatography on Affi-gel blue gel is used for the purification of a 20-kDa Kunitz-type trypsin inhibitor from *Gymnocladus chinensis* (Yunnan bean) seeds (Zhu et al., 2011). 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). A Kunitz-type trypsin inhibitor with high stability from *Spinacia oleracea* L. seeds is purified by Sepharose 4B-trypsin affinity chromatography (Kang et al., 2009). Purification of the inhibitors from skin extract of Atlantic salmon (*Salmo salar* L.) was carried out in four chromatographic steps: papain-affinity chromatography on papain-Sepharose column, gel filtration on a Superdex 200 HR10/30 (Pharmacia Biotech) column, anion exchange chromatography on a Mono Q HR 5/5 (Pharmacia Biotech) column and reverse phase Chromatography on a 0.46 X 4 cm TSK TMS 250 column (C1, TosoHaas Corporation, Japan) (YloÈnen et al., 1999).

2.7 Characterization of protease inhibitors

Protease inhibitors play various important roles in living systems. They can perform important functions due to their unique structures (Pandhare et al., 2002; Sabotic and Kos, 2012). The characters of proteinaceous protease inhibitors isolated from diverse sources were thoroughly studied based on their physiological roles and accordingly the possible use of inhibitors can be determined. Therefore, characterizing protease inhibitors by understanding the relationship between protein structure and its function is crucial for determining the scope and application. The determination of the physicochemical parameters characterizing the structural stability of the inhibitors is essential to select effective and stable inhibitors under a large variety of environmental conditions. Moreover, the knowledge of their structural features is fundamental to understand the inhibitor-enzyme interactions and allow novel approaches in the use of synthetic inhibitors aiming for drug design.

2.7.1 pH and temperature stability

Protease inhibitors constitute one of the most important classs of proteins that has got applications in various fields. Higher thermal stability is one of the crucial properties for their biotechnological applications. A novel thermostable protein inhibitor of trypsin and subtilisin, called BN, was isolated from the seeds of *Brassica nigra* (Genov et al., 1997). The pH and temperature stability of the alkaline protease inhibitors and the influence of various additives on their thermostability were investigated as a prelude to their exploitation as biocontrol agents (Pandhare et al., 2002). *Serratia marcescens* produced very small amounts of a thermostable inhibitor protein (SmaPI) that shows an inhibitory activity against extracellular 50 kDa metalloprotease (Kim et al., 1995). The pH and temperature stability of the inhibitor can be attributed to the presence of disulfide linkages (Vernekar et al., 1999). 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). There is a correlation between the conformational stability and thermostability of serine protease inhibitors (Kwon et al., 1994). Probably the extreme conditions of pH and temperature might have totally distorted the structure of the inhibitor such that they no longer bind with the enzymes or with their substrates. Temperature, ionic strength, pH and other physicochemical factors can influence protein–protein interactions (Veselovsky et al., 2002).

Thermostable variants of serpins may be of practical use because heat-induced deactivation of serpins was shown to be a concentration-dependent aggregation process (Kwon et al., 1994; Lomas et al., 1993). Temperature and pH are important physical variables in enzyme-catalyzed reactions. The majority of the protease inhibitors exhibiting anti-feedent properties reported so far, are active against the neutral serine proteases such as trypsin and chymotrypsin (Ryan,

1990). The alkaline protease inhibitor from *Streptomyces* sp. was found to be stable over a wide pH and temperature ranges. Therefore, one can envision the direct application of it as a biocontrol agent for the protection of plants against phytopathogenic fungi by encapsulation for surface application or can be sprayed directly (Vernekar et al., 1999). 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. The thermostability of protease inhibitor signifies its role as a stablizer for many commercially important proteases, which has a major role in detergent industries to withstand higher temperature (Pandhare et al., 2002).

High pH-stability and thermostability was shown by preparations of new low molecular weight protein inhibitors of serine proteinases from buckwheat *Fagopyrum esculentum* seeds (Tsybina et al., 2004). The pH stability dictates the affinity of the inhibitor with different digestive proteases of the pest and insects, mainly neutral proteases like trypsin and chymotrypsin implies the role of inhibitors as biopesticides (Joshi et al., 1999). Protease inhibitor (PISC-2002) isolated from culture supernatants of *Streptomyces chromofuscus* was found to be pH tolerant and thermostable due to the presence of proline and a high content of hydrophobic amino acids (Angelova et al., 2006).

2.7.2 Effect of metal ions on inhibitor

The presence of metal ions is necessary for the activity of some protease inhibitors as they have a major role in maintaining the structural integrity of protease inhibitor. The structural stability of a protein is enhanced by divalent metal ions. This is because the metal ions serve to attain the critical conformation that is needed for biological activity of the protein. Cysteine protease inhibitor (CPI) from pearl millet needs the Zn^{2+} for the protease inhibitory and antifungal activity of the protein, indicating a key role for this metal ion in these activities.

Analysis of metal chelated CPI using CD spectroscopy indicated a significant change in the secondary structure (Joshi et al., 1999).

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). 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). 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 et al., 1983).

2.7.3 Effect of oxidizing agents on inhibitor

When proteins are exposed to oxidising agents such as hydrogen peroxide (H_2O_2), periodate, dimethyl sulfoxide, chloramine-T, *N*-chlorosuccinamide and to oxidants released by neutrophils (*e.g.* superoxide, hydroxyl radical), they are subjected for oxidation (Brot and Weissbach, 1983; Vogt, 1995). Usually a decline in the biological activity of the protein is observed by the oxidation of methionine residues. The effect of methionine oxidation of α1-protease inhibitor (α1-PI) was thoroughly studied. There are eight methionine residues and upon oxidation of one of these residues (Met358), a complete loss of inhibitory activity of α1-PI was observed toward its primary biological target, elastase (Johnson and Travis, 1979). The specificity pattern of chymotrypsin has changed significantly upon oxidation of methionine to its sulfoxide (Weiner et al., 1966). Studis on α2-macroglobulin

revealed that the loss of activity of the protein on oxidation is due to the 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 oxidising agents DMSO and H_2O_2 (Bijina et al., 2011a).

2.7.4 Effect of reducing agents on inhibitor

In many proteins the covalent link of cysteine residues by disulfide bonds is important in maintaining a stable conformational stability for their biological activity. So conformational destabilization of the protein is occured by the removal of the disulfide bonds link of cysteine residues by reduction or substitution for another amino acid residue (Kawamura et al., 2008; Zavodszky et al., 2001). This covalent linkage 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). There was no effect on protease inhibitors isolated from *Peltophorum dubium* and *Erythrina caffra*, a Kunitz type trypsin inhibitor when lower concentration of dithiothreitol (DTT) was used (Lehle et al., 1996; Macedo et al., 2003). Unfolding of mutant leech carboxypeptidase inhibitor (LCI) was resulted when reduced with dithiothreitol (DTT) (Arolas et al., 2009).

2.7.5 Effect of detergents on inhibitor

Three distinct modes of interaction of detergents with proteins has been proposed (a) association with specific binding sites of native proteins; (b) cooperative association between protein and a large number of detergent molecules without major conformational change; (c) cooperative association with conformational changes in the protein in which most of the hydrophobic residues are presumably exposed for association with the detergent (Reynolds and Tanford, 1974). Proteases are added as key ingredients in detergents, which accounts for approximately 25% of the total worldwide sales of enzymes and all detergent proteases currently used in the market are serine proteases (Rao et al., 1998). So

studying the effect of detergents on inhibitor is significant when it is used along with their corresponding proteases.

Detergents (cationic, anionic, zwitterionic and non-ionic) are used for solubilizing proteins from lipid membranes. Moreover, proteinase inhibitors and detergents are routinely used together in cell lysis buffers to inhibit unwanted proteolysis and facilitate membrane protein solubilization in protein purification procedures. Conformation of *C. cajan* inhibitor changed in the presence of SDS and deoxycholate (DOC), in the presence of anionic bile salt, deoxycholate resulting in the loss of inhibitory activity (Cardamone et al., 1994). 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 unspecifically to the protein surface, which usually lead to protein unfolding (Mogensen et al., 2005). Triton X-100, Tween 20 and Tween 80 are nonionic polyoxyethylene detergents and the major interaction with proteins are hydrophobic (Salameh and Wiegel, 2010). The effects of SDS and Tween 80 on thermal stability to the alkaline protease inhibitors (API-I and API-II) from actinomycetes were studied. SDS provided 60% protection only to API-I and Tween-80 conferred 45% and 30% protection to API-I and API-II, respectively (Pandhare et al., 2002).

The inhibitory activity of protease inhibitor isolated from *Moringa oleifera* showed an increase in the inhibitory activity in the presence SDS (Bijina et al., 2011a). Small amounts of nonionic or zwitterionic detergents are used in crystallizing integral membrane proteins (Garavito et al., 1983; Michel, 1982; Michel and Oesterhelt, 1980). These detergents help to inhibit micelle formation and permit crystallization of proteins (Gilliland and Davies, 1984). The three dimensional conformation of soybean Kunitz inhibitor has been changed due to the action of SDS (Jirgensons, 1973). The property of detergents to mimic the native, hydrophobic environment of the phospholipid bilayer is explored for the

application in protein chemistry and in biotechnological processes (Israelachvili, 1991; Jones and Chapman, 1995; Tanford, 1980).

2.7.6 Effect of chemical modifiers on inhibitor

Chemical modification offer a very useful approach for giving proteins some new and desired characteristics related to stability and catalytic activity. In chemical modification, a chemical reagent binds covalently to specific amino acid side chains of a protein to produce changes in the biological property of a protein (Jonossen and Svendson, 1982; Yang et al., 1998). Chemical modification is an important tool for studying structure–function relationship of biologically active proteins. This can be done without knowing the protein structure (Gote et al., 2007). Chemical modification is an alternative approach to genetic modification for studying the structure-function-stability relationship of proteins and for modifying their activity-stability properties reynold (Kaspari and Bogner, 2009). It is widely used as a tool for studying role of individual amino acids, their participation in the maintenance of the native conformation and for their stabilization (He et al., 2000; Torchilin et al., 1979). The involvement and role of amino acids in the dimerization of Bowman-Birk type of protease inhibitors from the seeds of Horsegram **(***Dolichos biflorus***)** was investigated by chemical modification (Kumar et al., 2004).

Chemical modification is an effective approach to obtain inhibitors highly active against proteinases of the pests. This approach is exemplified by the new form of oryzacystatin I, a protease inhibitor which differs from the original inhibitor by the absence of one amino acid residue, Asp86 and is highly active against proteinases of the pest, *Caenorhabditis elegans* and toxic to the parasitic nematode *Globodera pallida* (Urwin et al., 1995). Chemical modification of soybean cystatin scN and tomato (*Lycopersicum esculentum* L.) multicystatin reveals that substitution of individual amino acid residues in the N-terminal portion of one of multicystatin domains may considerably affect its ability to

inhibit diverse proteinases (Kiggundu et al., 2006; Koiwa et al., 2001). In the majority of specific serine protease inhibitors, the inhibitory action is localized to a specific reactive site situated within a loop closed by a disulphide bridge (Mosolov and Valueva, 2005). It was reported that chemical modification of Pearl millet cysteine protease inhibitor provided evidence for the presence of two distinct sites responsible for antifungal and antifeedant activities (Joshi et al., 1998).

Amino acids are usually modified with following chemicals: serine with Phenyl methyl sulphonyl fluoride (PMSF), aspartic acid with *N*-Ethyl-5-phenylisoxazolium-39-sulfonic acid (Woodward's reagent K, WRK), arginine with phenylglyoxal, histidine with Diethylpyrocarbonate (DEPC), tryptophan with *N*-bromosuccinanmide (NBS) and cysteine with *N*-ethylmaleimide. A trypsin inhibitor (PDTI) from *Peltophorum dubium* seeds was inactivated by lysine and arginine modifying reagents, trinitrobenzene-sulfonic acid (TNBS) and 1,2-cyclohexanedione (CHD), respectively (Ligia et al., 2003). Modification of cysteine, lysine, tyrosine and carboxylic acids had no effect on the Pearl millet cysteine protease inhibitor (CPI) activity, whereas modification of arginine and histidine resulted in the activation of CPI activity (Joshi et al., 1999). The inhibitory properties of a serine proteinase inhibitor from the fruiting body of the basidiomycete, *Lentinus edodes* was studied by chemical modification. On modification of arginine residues with cyclohexane-1,2-dione, the inhibitor activity was completely abolished while modification of three lysine residues out of the eight with sodium 2,4,6-trinitrobenzene-1-sulfonate did not significantly affect the activity (Odani et al., 1999).

Peltophorum dubium protease inhibitor, a Kunitz type serine protease inhibitor, was inactivated by lysine and arginine modification with tri-nitrobenzene-sulfonic acid and 1, 2- 1 cyclohexanedione respectively (Macedo et al., 2003). Chemical modification of the carboxylic and amine groups of a bifunctional inhibitor (ATBI) from an extremophilic *Bacillus* sp. using *N*-Ethyl-5-

phenylisoxazolium-39-sulfonic acid (Woodward's reagent K, WRK) a carboxyl group modifier, and 2,4,6-trinitrobenzenesulfonic acid (TNBS) an amine group modifier of lysine resulted in the loss of inhibitory activity against xylanase and aspartic protease and also in the loss of antifungal property, suggesting the involvement of amino acids containing ionizable side chains (Lys, Asp, and Glu) in the mechanistic pathway (Dash et al., 2001b). Dimerization of protease inhibitors from the seeds of Horsegram **(***Dolichos biflorus***)** was investigated by chemical modification of arginine (using 1,2-cyclohexanedione) lysine (using citraconic anhydride) and histidine (using diethylpyrocarbonate, DEPC) residues (Kumar et al., 2004).

2.7.7 Fluorescence binding studies

The fluorescence property of a folded protein is due to the presence of aromatic amino acid residues. Tryptophan, tyrosine and phenylalanine are the three aromatic aminoacids which may contribute to the intrinsic fluorescence of a protein. The measure of the intrinsic flourescence of a folded protein is the total contribution of individual aromatic aminoacids present in it. For the understanding of the folding and binding events in proteins, fluorescence spectroscopy is used in which the fluorescence property is made use of. This is an extremely sensitive technique. Protein fluorescence is generally excited at 280 nm or at longer wavelengths, usually at 295 nm. Fluorescence spectroscopy can give information about the solvent accessibility of the chromophores (tryptophan, tyrosine, and phenylalanine). Therefore, the fluorescence spectrum is sensitive to local changes in the tertiary structure of a protein (Pace et al., 1989).

The conformational stability of potato cysteine protease inhibitor (PCPI) was investigated by fluorescence spectroscopy. The PCPI isoforms investigated have a highly similar structure at both the secondary and the tertiary level. Changes in the tertiary structure of PCPI during heating were also monitored by measuring the changes in fluorescence intensity as a function of temperature

(Pouvreau et al., 2005). In a study the monomeric form of an originally dimeric serine proteinase inhibitor, ecotin, was compared with that of the iosoforms fluorescent spectra (Gfibor and Grfif, 1996). The structural and functional studies on ecotin and its interactions with different serine proteases have been investigated by fluorescence titration (Seymour et al., 1994) which revealed the remarkable features of this protein in inhibiting a strikingly large subset of the chymotrypsin family of serine proteases (Mcgrath et al., 1999).

The role of the dimer interface on ecotin's unique broad specificities was investigated by monitoring the monomer dimer dissociation constant (Kd) (Seymour et al., 1994) by measuring the fluorescence (Yang et al., 1998). Detergent-induced structural changes of proteins were analyzed efficaciously by fluorescence emission as the fluorescence signal undergoes changes, reflective of changes in protein conformation (Haq and Khan, 2005). The value of static fluorescence spectra of the three isoforms from *Acacia plumosa* Lowe seeds measured after excitation at 280 and 295 nm, were the same and the λ_{max} emission value at 341 nm (Lopes et al., 2009) 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).

The intrinsic fluorescence spectrum of pearl millet cysteine protease inhibitor (CPI) was found to be typical of a protein devoid of tryptophan residues. The interaction of CPI modified for arginine or histidine with papain resulted in an enhancement of CPI activity accompanied by a slight decrease in fluorescence intensity. In contrast, modification of serine resulted in inhibition of CPI activity with a concomitant increase of 20% in the fluorescence intensity when complexed by the enzyme. This implies the involvement of enzyme-based tryptophan in the formation of a biologically active enzyme-nhibitor complex (Joshi et al., 1998). The tryptophan free variant α1-antitrypsin, an inhibitor of thrombin upon formation of the serpin proteinase complex the emission maximum of 340 nm of thrombin which blue shifts to 346 nm, concomitant with a 40% increase in

intensity, indicative of substantial conformational change within the proteinase (Tew and Bottomley, 2001). The binding studies on inhibition of the thermostable xylanase (Xyl I) from a *Thermomonospora* sp. by a bifunctional inhibitor ATBI, isolated from an extremophilic *Bacillus* sp. were carried out. The binding of ATBI resulted in a concentration-dependent quenching of the fluorescence. The steady-state kinetics revealed a two-step inhibition mechanism, and the conformational modes observed during the binding of inhibitor to the enzyme were conveniently monitored by fluorescence analysis. Fluorescence spectroscopy plays a very important role in the determination of kinetic constants of enzyme inhibition (Dash et al., 2001b).

2.7.8 Kinetics of inhibition

Enzyme inhibition studies are conducted to evaluate the occurrence and extent of protein-drug interactions. To characterize an inhibitor, one needs to understand the nature of inhibition process (i.e., competitive, noncompetitive, or uncompetitive) and the inhibition constant (*K*i). The inhibitor constant, *K*i, indicates the efficacy of an inhibitor to its target enzyme. A given inhibitor may inhibit a number of proteases, and potency of an inhibitor is often described as the molar concentration of the inhibitor that gives 50% inhibition of the target enzyme activity (IC_{50}) or just the percentage inhibition of the enzymatic activity of a fixed concentration of the inhibitor (Hideaki and Guy, 2001). Determination of the kinetic parameters of the inhibition of proteases will provide insight into the mechanism of the interaction between the enzyme and the inhibitor.

A broad spectrum kunitz type serine protease inhibitor (rAceKI-1) secreted by the hookworm *Ancylostoma ceylanicum* shows a dose-response behavior suggesting the competitive nature with the protease substrates. Kinetic assays showed that rAceKI-1 is a “tight binding” (Williams and Morrison, 1979) inhibitor of chymotrypsin, pancreatic elastase, neutrophil elastase, and trypsin (Aaron et al., 2000). A full-length cDNA, encoding a Bowman-Birk protease

inhibitor (BBI) from lentil immature seeds found to inhibit trypsin and chymotrypsin, with *K*i values at the nanomolar level (Caccialupi et al., 2010). This is comparable to those previously reported for trypsin/chymotrypsin BBI extracted from lentil seeds (Caccialupi et al., 2010; Ragg et al., 2006).

Ecotin, a serine protease inhibitor found in the periplasm of *Escherichia coli,* has been characterized as a potent reversible tight-binding inhibitor of the human contact activation proteases factor XIIa (FXIIa) and plasma kallikrein, having *K*i values of 89 pM and 163 pM, respectively. Ecotin also inhibited human leukocyte elastase (HLE) with high affinity (*K*i = 55 pM) (Ulmer et al., 1995). The serine protease inhibitor protein obtained from a gene *Spi1C* from a metagenomic library of uncultured marine microorganisms exhibited inhibitory activity against α-chymotrypsin and trypsin with *K*i values of around 1.79×10^{-8} and 1.52×10^{-8} M, respectively (Cheng-Jian Jiang et al., 2011).

The *K*i of a serine protease inhibitor named bicolin from the venom of *Vespa bicolor Fabricius* toward trypsin was 5.5×10^{-7} M and no inhibition was detected to elastase and α-chymotrypsin, respectivley (Yang et al., 2009). A serine protease inhibitor (Bungaruskuni) from *Bungarus fasciatus* venom had a *K*i of around 6.1×10^{-6} M to chymotrypsin (Lu et al., 2008). Protease inhibitor (PISC-2002) from culture supernatants of *Streptomyces chromofuscus* inhibited subtilisin, proteinase K, trypsin and proteinase of *S. albovinaceus* strongly, with *K*i of micromolar range and weakly inhibited pepsin (Angelova et al., 2006).

A cDNA encoding a precursor of equistatin, a potent cysteine and aspartic proteinase inhibitor, was isolated from the sea anemone *Actinia equine* and the mature protein region as well as those coding forn each of the domains were expressed in the periplasmic space of *Escherichia coli*. It was found that the whole recombinant equistatin and its first domain, but not the second and third domains, inhibited the cysteine proteinase papain (*K*i 0.60 nM) (Trukelj et al., 2000). A bifunctional inhibitor (ATBI) from an extremophilic *Bacillus* sp. exhibiting an activity against phytopathogenic fungi, inhibited xylanase and aspartic protease

competitively, with *K*i values 1.75 and 3.25 mM, respectively. The double reciprocal plots of reaction velocity versus substrate concentration obtained for the enzymes demonstrated steady-state kinetic behavior and a competitive mode of inhibition (Dash et al., 2001a). The recombinant protease inhibitor from *Clitocybe nebularis* found to inhibit trypsin with a *K*i value of 3.1 nM (Avanzo et al., 2009).

An extracellular protease inhibitor, EPI1, from *P. infestans* was characterized and the inhibitory constant (*K*i) for subtilisin was determined at 2.77 +/- 1.07 nM (Tian et al., 2004). The kinetic characteristics, of interaction between hK6 (human Kallikrein) and soybean (BBI), protease inhibitor and antitumor agent , were investigated. The hK6 were found to bind soybean in two reversible steps. The *K*i of the first step binding was 13 nM and *K*i of the second binding step was 1.6 nM. The results suggested that the interaction mechanism between hK6 and soybean was like that of trypsin with this inhibitor but with rather lower inhibitory constants values (Mellati and Diamendis, 2004). The molar ratio of proteases to protease inhibitors determined by stoichiometry. Recombinant inhibitory protein cnispin (rCnp) from basidiomycete, inhibitor from *Leucaena leucocephala* LlTI and *Dimorphandra mollis*, DmTI showed 1:1 stoichiometry (Macedoz et al., 2000; Oliva et al., 2000). Serine proteinase inhibitor from the seeds of leguminous plant *Archidendron ellipticum* (AeTI), inhibited trypsin with a stoichiometric ratio of 1:1, but lacked similar stoichiometry against chymotrypsin (Bhattacharyya et al., 2006).

2.8 Application studies of protease inhibitors

The highly specific nature of the inhibitor-protease interaction makes it a valuable tool in medicine, agriculture and food technology. The digestive systems of crop pests, human immune deficiency virus proteinase, and fish muscle proteases are some examples of targets for study.

2.8.1 Protease inhibitors as defense tools for protection

Present methods of crop protection depend primarily on the use of chemical pesticides. The exclusive use of chemical pesticides not only results in rapid build-up of resistance to such compounds, but their non-selectivity affects the balance between pests and natural predators, and is generally in favour of pests (Metcalf, 1986). To limit the harmful effects of these 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 emerged as an interesting strategy for insect pest control (Lawrence and Koundal, 2002; Reeck et al., 1997). Protease inhibitors have been involved in plant defence due to the implication of extracellular proteases from microorganisms in pathogenesis and the digestive role of proteases in herbivorous pests. In plants protease inhibitors can be counted among the defensive mechanisms displayed against phytophagous insects and microorganisms (Fan and Wu, 2005). The defensive capacities of PIs 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 (Leo et al., 2002). Protease inhibitors (PIs) are one of the prime candidates with highly proven inhibitory activity against insect pests and also known to improve the nutritional quality of food (Lawrence and Koundal, 2002). The inhibitory activities of several Kunitz Type protease inhibitors against larval midgut proteases from bertha armyworm (BAW; *Mamestra configurata*) and forest tent caterpillar (FTC; *Malacosoma disstria*), pests of populus and crucifers, respectively were demonstrated, in which the direct evidence of the role of the poplar Kunitz Type protease inhibitors in the inducible defense of poplar against insect herbivores was revealed (Major and Constabel, 2008).

Protease inhibitors as defence proteins is supported by their specificity against enzymes from insect pests (Alfonso et al., 1997; Franco et al., 2002;

Franco et al., 2000). Insecticidal effects of serine and cysteine protease inhibitors have been studied by diet incorporation assays or by *in vitro* inhibition studies. They induce delayed growth and development, reduced fecundity and sometimes increased mortality (Annadana et al., 2002; Edmonds et al., 1996; Gatehouse et al., 1999 ; Oppert et al., 2003; Ortego et al., 1998). The implication of trypsin and chymotrypsin inhibitors on fungal and bacterial growth inhibition has been previously reported (Giudici et al., 2000; Kim et al., 2005; Lorito et al., 1994). In addition to enzymes of the digestive tract of insects, the plant inhibitors of proteinases may affect proteinases of phytopathogenic microorganisms. It was shown in 1976 that trypsin and chymotrypsin inhibitors from diverse plants are capable of suppressing the activity of proteinases excreted by the fungus *Fusarium solani* (Mosolov et al., 1976). Similar results were obtained subsequently in studies of the effects of other proteinase inhibitors of plant origin on the activity of enzymes and the growth and development of phytopathogenic fungi (Dunaevskii et al., 2005; Gvozdeva et al., 2006; Ievleva et al., 2006; Valueva and Mosolov, 2004). 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. OCI significantly reduced nymphal survival of the potato aphid *M. euphorbiae* and prevented aphids from reproducing (Azzouz et al., 2005).

Transgenic plants expressing a proteinase inhibitor have enhanced levels of insect resistance. The protease inhibitor genes have been used for the construction of transgenic crop plants to be incorporated in integrated pest management programs (Haq et al., 2004; Lawrence and Koundal, 2002). Several serine and cysteine protease inhibitors have been expressed in transgenic plants to enhance their resistance against Lepidoptera (Falco and Silva-Filho, 2003; Hilder et al., 1987; Leo et al., 2001) and Coleoptera (Alfonso-Rubi et al., 2003; Lecardonnel et al., 1999). Numerous transgenic plants expressing entomotoxic proteins of various origins have thus been engineered (Carlini and Grossi-de-Sa,

2002; Gatehouse and Gatehouse, 1998; Jouanin et al., 1998; Ranjekar et al., 2003). The *in vitro* antifungal capability of cystatins, protease inhibitors from barley (HvCPI-1 to HvCPI-13) against three important phytopathogenic fungi, *Magnaporthe grisea, Plectosphaerella cucumerina* and *F. oxysporum*, and two plant pathogen bacteria, *Dickeya dadantii* and *Pseudomonas syringae* was studied (Carrillo et al., 2011a). Arabidopsis and maize plants have been transformed with the HvCPI-6 cystatin and their partial resistance against acari and aphids were characterized (Carrillo et al., 2011b; Carrillo et al., 2011c). Protease inhibitor from maize seed was able to inhibit spore germination and mycelial growth of nine different plant pathogen fungi (Chen et al., 1999). In barley trypsin inhibitor gene, Itr1,was specifically expressed and the purified protein BTI-CMe has been shown to be active *in vitro* against insect trypsin proteases (Alfonso et al., 1997) and transgenic rice, wheat and tobacco plants expressing this protein, BTI-CMe were tested against the performance of several herbivorous pests showing a negative impact on their performance (Alfonso-Rubi et al., 2003; Altpteter et al., 1999; Lara et al., 2000).

Sugarcane productivity is challenged by a wide array of biotic and abiotic stresses. The sugarcane borer *Diatraea saccharalis* (Lepidoptera: Crambidae) is an insect pest causing stalk damage to sugarcane (*Saccharum officinarum* L.) plants which results 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 genome conferred partial resistance to the sugarcane borer *D. saccharalis* as the growth of neonate larvae of *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). Insect hemolymph protease inhibitors have been suggested to serve many biological functions such as defense against invading pathogens. A low molecular mass protease inhibitor called AmPI has been successfully purified from the

hemolymph of tasar silkworm, *Antheraea mylitta.* Studies on the distribution pattern of AmPI indicate the involvement of AmPI in defense against pathogens and pests by either preventing the digestion of cuticle proteins by inhibiting the proteases of entomopathogenic fungi or by regulating the protease mediated phenoloxidase activation (Rai et al., 2010). A Streptomyces alkaline protease inhibitor (API), exhibiting antifungal activity against several phytopathogenic fungi, has been reported (Vernekar et al., 1999; Vernekar et al., 2001). An antifungal peptide ATBI from an extremophilic *Bacillus* sp. was isolated and its inhibitory activity against xylanase and aspartic protease and the correlation of these enzymatic activities to growth inhibition of plant pathogenic fungi was evaluated. ATBI was found to be active against a relatively broad spectrum of filamentous fungi, and its IC_{50}s indicated an exceptionally high potency (Dash et al., 2001a).

2.8.2 Protease inhibitors in food processing and preservation

In food technology, proteases are pervasive, perplexing, persistent and pernicious for protein and in these cases; inhibitors of proteases can be desirable. In surimi production, proteinase inhibitors are deliberately added as a means of circumventing the problem of reduced gel-forming ability that occurs as a result of the action of endogenous myosin-degrading proteinases. Microbial food spoilage is an area of global concern as it has been estimated that as much as 25% of all food produced is lost post-harvest owing to microbial activity (Baird-Parker, 2003). Postmortem muscle softening in fish, molluscs and crustaceans, and blackspot development in crustaceans are serious problems in seafood storage and processing. This softening or mushiness is mainly caused by muscle proteinases, mostly cathepsins (Garcia-Carreiio, 1996). The use of an adequate amount of natural protease inhibitors is an effective way to extend the shelf life of many types of seafood such as salted fish products. This is because the inhibitors can retard the aging and other deteriorative processes like protein degradation caused

by the action of endogenous and exogenous proteases, during the food processing and preservation (Reppond and Babbitt, 1993).

2.8.3 Protease inhibitors as therapeutic agents

Protease inhibitors are of great importance in medicine as proteolysis have significant role in almost every biological processes. Unregulated proteolysis affects physiological processes like cell-cycle progression, cell proliferation and cell death, DNA replication, tissue remodeling, haemostasis (coagulation), wound healing and immune response. Loss of proteolytic control plays significant role in cancer and in cardiovascular, inflammatory, neurodegenerative, bacterial, viral and parasitic diseases (Christopher and Clifford, 2010). As a result, proteases are often targets for therapeutic interventions (Murphy, 2008; Polanowski et al., 2003; Turk, 2006).

Due to the evident relevance of protease inhibitors, they have been studied extensively with the intent to develop therapeutic drugs (Drag et al., 2010; Haq et al., 2010; Turk, 2006). Among compounds able to selectively abrogate serine protease actions, the serine protease inhibitors are able to form less active or fully inactive complexes with their cognate enzymes and therefore they can be used either as drugs by themselves or may serve as templates for the design and development of highly specific drugs (Leung et al., 2000). Aprotinin, a potent inhibitor of plasmin, trypsin and kallikrein and has been used for antifibrinolytic therapy (Waxler and Rabito., 2003). Therapeutic intervention using protease inhibitors include angiotensin converting enzyme (ACE) inhibitors for the treatment of hypertension (Abbenante and Fairlie, 2005), HIV aspartyl protease inhibitors to prevent development of AIDS (Collier et al., 1996; Gulick et al., 1997) and application of proteasome inhibitors in the treatment of multiple myeloma (Orlowski, 2004).

Several proteasome inhibitors (e.g. lactacystin from *Streptomyces lactacystinaeus*) have been reported to obstruct replication of several viruses,

including influenza virus, herpes simplex virus type1, paramyxovirus and rhabdoviruses, as well as cytomegalovirus (Kaspari and Bogner, 2009). 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). A few inhibitors of the cytomegalovirus protease have been described from bacterial (Streptomyces) and fungal (Cytonaema) origins (Anderson et al., 2009; Stoeva and Efferth, 2008). Protease inhibitor isolated from *Streptomyces chromofuscus* was with efficient antiviral activity against influenza virus A/Rostock/34 (H7N7). This is because serine endoproteases are essential for the proteolytic cleavage of influenza virus haemagglutinin that unravels viral infectivity (Angelova et al., 2006). The reproduction of human immuno- deficiency virus type1 (Clercq, 2001), cytomegalovirus (Shu et al., 1997) and influenza virus (Lozitsky et al., 2002) was efficiently inhibited by protease inhibitor produced by *Streptomyces* species. The serine proteases NS3 and NS2 of flaviviruses are targets for antiviral drug development against hepatitis C virus and dengue virus and several protease inhibitors are in different phases of clinical evaluation to combat these diseases (Anderson et al., 2009).

Proteases are important virulence factors of many pathogenic bacteria. In the light of rapidly spreading antibiotic resistance, bacterial proteases are promising targets for the design of novel antibiotics. Omptins, proteases found in several Gram-negative bacteria, inhibited partially by serine protease inhibitors has been reported (Hritonenko and Stathopoulos, 2007; Seron et al., 2010; Yun et al., 2009). Inhibitors of thermolysin family proteases are of bacterial origin, including those isolated from Streptomyces, the small-molecule inhibitor phosphoramidon and protein inhibitor SMPI (Streptomyces metalloproteinase inhibitor) of family I36. Parasitic proteases constitute one of the very important druggable targets since they are key virulence factors due to their essential roles in cell metabolism and interaction with the host (McKerrow et al., 2008; Renslo and McKerrow, 2006; Zucca and Savoia, 2011). The ability of tumour cells to invade

extracellular barriers and to metastasize to distant sites is associated with the activity of proteases (Kos and Lah, 1998). 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.

2.9 *Pseudomonas mendocina*

The genus *Pseudomonas* is the most heterogenous and ecologically significant group of known bacteria. They include Gram-negative motile aerobic rods that are wide-spread throughout nature and characterized by elevated metabolic versatility, with a complex enzymatic system. They have primary role in the use as biocontrol agents in seawater and are therefore of great interest. The nutritional requirements of *Pseudomonas* sp. are very simple, and the genus is found in natural habitats like soil, fresh water, marine environments etc., but it has also been isolated from clinical instruments, aseptic solutions, cosmetics and medical products (Franzetti and Scarpellini, 2007).

Several species of the genus *Pseudomonas* are encountered in most natural environments, from freshwater to hypersaline habitats (Holt et al., 1994). They are known to tolerate a salt concentration up to1.2 M and the mechanism of osmoadaptation was due to the presence of glucosylglycerol. In response to osmotic stress, this species accumulated a number of compatible solutes, the intracellular levels of which depended on both the osmolarity and the ionic composition of the growth medium (Pocard Jean-Alain et al., 1994; Tripathy et al., 2006). They are intensively studied because of their primary importance in medicine, phytopathology, food spoilage, biological control, and environmental research. Traits of fluorescent *Pseudomonas* sp. involved in suppression of plant root pathogens.

Species *P. mendocina* have a particularly high potential for bioremediation of contaminated soils and waters because they can detoxify

recalcitrant pollutants such as organomercurials, pesticides, and halogenated aromatic compounds found in crude oil and petroleum (Meer et al., 1992). *P. mendocina* shares phenotypic characteristics with *Pseudomonas stutzen* and members of the fluorescent pseudomonad group (particularly *Pseudomonas aeruginosa*). Lipase of *P. mendocina* 3121-1 was shown to hydrolyze a broad spectrum of substrates (Vida˙ et al., 2004). But, so far there are no reports available on protease inhibitors from *P. mendocina.*

Chapter 3

MATERIALS AND METHODS

3.1 SCREENING OF MICROORGANISMS FOR PROTEASE INHIBITOR PRODUCTION

3.1.1 Microorganisms

Marine microorganisms were isolated from the sediment and sea water of coastal areas of Cochin, Kerala. Microorganisms; actinomycetes, bacteria and fungi were isolated on suitable media by pour plating of serially diluted samples. After incubation, the isolated colonies were picked and purified by single colony/mycelium plating technique. The isolated cultures were maintained in suitable media slants at 4^{o}C and were sub cultured periodically for further use.

3.1.2 Media

3.1.2.1 Medium for bacteria

Zobell Marine Broth 2216 (HiMedia) was used in 4% level (w/v) concentration for the isolation of bacteria for the present study. The isolates were maintained on the slants at 4^{o}C.

3.1.2.2 Medium for fungi

Mycological Broth (HiMedia) containing 2% NaCl was used for the isolation of fungi. The isolates were maintained on the slants at 4^{o}C.

3.1.2.3 Actinomycetes isolation medium

Composition

Starch	2.5 g
Glucose	1 g
Yeast extract	0.2 g

$CaCO_3$	0.3 g
NaCl	2 g
Trace salt solution*	1 mL
Water	100 mL

Contents were dissolved and autoclaved (pH 7.5)

***Trace salt solution**

$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0.5 g
$CuSO_4 .5H_2O$	0.5 g
$ZnSO_4 .7H_2O$	0.5 g
$MnCl_2.4H_2O$	0.5 g
Water	100 mL

3.1.2.4 Skimmed milk agar plates

Composition

Agar	0.8% (w/v)
Skimmed milk powder	1% (w/v)

Skimmed milk powder was dissolved in distilled water, agar was added and sterilized. The media was poured in to sterile Petri plates and allowed for solidification. The plates were kept at 4^oC for further use.

3.1.3 Screening

Screening of microbial isolates for the selection of potential trypsin protease inhibitor producing strain was carried out in two steps. Primary screening, employing plate assay as detailed under section 3.1.3.2. Positive cultures were then subjected to secondary screening by caseinolytic broth assay method as described under section 3.2.1.1.

3.1.3.1 Crude inhibitor preparation

Crude inhibitor samples were prepared from bacteria, fungi and actinomycetes as described under section 3.1.3.1.1, 3.1.3.1.2 and 3.1.3.1.3.

3.1.3.1.1 Bacteria

A loopful of cells from the freshly grown stock culture slants were transferred to a 10 mL medium (3.1.2.1) and incubated at room temperature (28 ± 2^{o}C) for 18 h with constant shaking at 150 rpm on a rotary shaker (Orbitek, Scigenics India). After incubation, this pre culture was transferred to a 40 mL medium in 250 mL Erlenmeyer flask. The flask was incubated at room temperature (28 ± 2^{o}C) with constant shaking at 150 rpm on a rotary shaker. After 48 h incubation the culture broth was subjected to centrifugation (Sigma, Germany) at 6000 rpm for 5 min. The supernatant was carefully transferred to a fresh tube. This cell free culture supernatant was used as crude inhibitor sample.

3.1.3.1.2 Fungi

The inoculum was prepared from a freshly raised 8 days old culture slants. The slants were surface washed using 0.1% Tween 80 prepared in sterile physiological saline for the preparation of spore suspension. One millilitre spore suspension (22 x 10^{8} cfu/ml) was used as inoculum. Fungi were grown at room temperature (28 ± 2^{o}C) under shaking (150 rpm) for 120 h in 250 mL Erlenmeyer flasks containing 50 mL of medium (3.1.2.2). After incubation the culture filtrate was separated by centrifugation at 6000 rpm for 5 min. This cell free culture supernatants was used as crude inhibitor for screening experiments.

3.1.3.1.3 Actinomycetes

The actinomycetes isolates were grown in the medium (3.1.2.3) for 96 h at 28°C on a rotary shaker at 200 rpm. The cells were separated by centrifugation. The cell free supernatants were collected and used as crude inhibitor preparation for screening.

3.1.3.2 Primary screening-Plate assay

The plate assay for the detection of protease inhibitor was performed in skimmed milk agar plate (3.1.2.4) with wells made in the center and on the periphery at a distance of 1 cm from the central well. Twenty microlitre of 0.5 mg/mL trypsin (SRL, India) was added in the central well. A suitable dilution of prepared crude culture supernatant (3.1.3.1.1) was added in one of the peripheral wells and sterile distilled water in the other to serve as a negative control. The plate was incubated at 37°C. The inhibition of hydrolysis of casein by trypsin was indicated by the absence of clearance zone around the well containing the inhibitor.

3.1.3.3 Secondary screening-caseinolytic broth assay

The crude inhibitor preparations (3.1.3.1.1, 3.1.3.1.2 and 3.1.3.1.3) were used to assay protease inhibitor activity, protein content and specific activity as described under section 3.2.1.1, 3.2.2 and 3.2.3 respectively.

3.2 ANALYTICAL METHODS

3.2.1 Protease inhibitor assay

Presence of protease inhibitor in the extract of *P. mendocina* 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.

3.2.1.1 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 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.

3.2.1.2 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.

3.2.2 Protein estimation

Protein content was estimated using bovine serum albumin (BSA) as the standard (Bradford, 1976) 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^{o}C.
c. Measured the absorbance at 595 nm in a UV-Visible spectrophotometer (Shimadzu, Japan).

3.2.3 Specific activity

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

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

or

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

3.3 FINAL SCREENING

Bacteria that are positive for secondary screening were selected and subjected to final screening by ammonium sulphate precipitation. For this crude inhibitor samples were precipitated with ammonium sulphate as described under section 3.3.1.

3.3.1 Ammonium sulphate precipitation

Ammonium sulphate precipitation of the prepared sample was done (Englard and Seifter, 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.

3.3.2 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.

3.3.2.1 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).

3.3.2.2 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 (3.3.2.1) (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 3.2.1.1, 3.2.2 and 3.2.3 respectively.

3.3.3 Selection of potential strain

A single protease inhibitor producing strain (bacterial isolate, BTMW 301) was selected based on the results obtained from the final screening and was used for further studies.

3.4 IDENTIFICATION OF THE SELECTED BACTERIUM BTMW 301

Molecular identification of the selected bacterium BTMW 301 was performed by PCR amplification of 16S rDNA sequences. This was carried out using two degenerate primers. PCR amplification product was subjected to sequencing, followed by homology analysis.

3.4.1 Template preparation for PCR

DNA extraction was performed employing phenol-chloroform method (Ausubel et al., 1995).

a. Transferred 2 mL bacterial overnight culture in LB medium (HiMedia, Mumbai, India) into a micro centrifuge tube.
b. Centrifuged at 8000 rpm for 10 min. and decanted the supernatant completely.
c. Resuspended the pellet in 875 mL of Tris-EDTA (TE) buffer and added 5 µL Proteinase K and 100 µL 10% sodium dodecyl sulphate (SDS) to it.
d. The mixture was incubated in a water bath at 37^{o}C for 1 h.
e. Added equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and mixed properly by inverting the tube until a white precipitate was seen.
f. Centrifuged at 8000 rpm for 10 min.
g. With the help of a wide mouth-cut-tip carefully collected the upper aqueous layer into a fresh tube, taking care not to carry over any phenol while pipetting.
h. Repeated the process twice and added equal volume of chloroform.

i. Centrifuged for 5 min. at 8000 rpm.
j. Collected the upper layer into a fresh tube and added 0.1 volume sodium acetate and double volume isopropyl alcohol.
k. Spun at 8000 rpm for 10 min and decanted the supernatant.
l. To the pellet, added 1 mL 70% ethanol and spun.
m. Decanted the alcohol and kept the pellet for air in a covered tray.
n. The pellet containing the isolated DNA was then dissolved in 1 mL TE buffer 10 mM Tris (pH 7.8) and stored at -20^{o}C for further studies.

3.4.2 Primer sequence

Primer	**Sequence (5'-→3')**	Reference
16SF	AGTTTGATCCTGGCTCA	(Shivaji et al., 2000)
16SR	ACGGCTACCTTGTTACGACTT	(Reddy et al., 2000; Reddy et al., 2002) a,b

3.4.3 Polymerase Chain Reaction (PCR)

PCR was performed using the genomic DNA (~100 ng/mL) as template and 16S rDNA specific primers as detailed above.

3.4.4 PCR Mix composition (20 µL)

10X PCR buffer	2 µL
2.5mM dNTPs	2 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
*Taq*DNA polymerase	1U (0.2 µL)
Template DNA	0.5 µL
$MgCl_2$	1.2 µL
Sterile Milli Q water to make final volume to	20 µL

3.4.5 PCR conditions

Annealing - 56^{o}C -30 sec.

Extension - 72^{o}C -2 min.

PCR was performed in a thermal cycler (BioRad, USA).

3.4.6 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. 1 kb 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).

3.4.7 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., 1990).

3.4.8 Multiple sequence alignment and phylogenetic tree construction

All the nucleotide sequences were converted into FASTA format. Multiple sequence alignment for the assembled nucleotide sequences was done by using the Clustal X program (Thompson et al., 1997) in BIOEDIT software (Hall, 1999). Aligned sequences were imported into an MEGA5: Molecular

Evolutionary Genetics Analysis (MEGA) software version 5.0 (Tamura et al., 2007) software for further analysis. The ends of the alignment were trimmed to obtain equal lengths for all sequences and the aligned sequences were converted into MEGA format for carrying out phylogenetic analysis. The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) using nucleotide based TN84 evolutionary model for estimating genetic distances based on synonymous and non synonymous nucleotide substitutions. Statistical support for branching was estimated using 1000 bootstrap steps.

3.5 BIOCHEMICAL AND MORPHOLOGICAL CHARACTERIZATION OF *P. MENDOCINA* BTMW 301

Various biochemical tests were carried out according to Bergey's Manual of Systematic Bacteriology (Palleroni et al., 1984) in order to analyse the characteristics of the selected bacterium. The colony morphology was determined based on the external appearance of the colony.

3.6 TIME COURSE STUDY AND GROWTH PROFILE

Time course of protease inhibitor production was studied in Zobell marine broth medium (3.1.2.1) using shake flask cultures. Optimum incubation time for maximum protease inhibitor production was determined by incubating the inoculated media for a total of 120 h and the samples were drawn and analysed at different time intervals for protease inhibitor production and assayed for protease inhibitory activity, protein content and specific activity as described under section 3.2.1.1, 3.2.2 and 3.2.3 respectively.

The growth of selected bacterium was studied for 120 h. Aliquots were sampled at different intervals to analyze the cell growth by checking OD at 600 nm in a spectrophotometer. All the experiments were carried out in triplicates. From the results obtained, the growth profile of the isolate was prepared by plotting a graph of OD at 600 nm against incubation time.

3.7 PROTEASE INHIBITOR PRODUCTION BY *P. MENDOCINA* BTMW 301: OPTIMIZATION OF BIOPROCESS VARIABLES BY "ONE-FACTOR-AT-A-TIME" METHOD

Various physico-chemical and bioprocess parameters influencing protease inhibitor production by *P. mendocina* under submerged fermentation were optimized towards maximal protease inhibitor production in the production medium. The strategy adopted for the optimization was to evaluate individually the effect of different parameters ("one-variable-at-a-time" method) on protease inhibitor production under submerged fermentation using minimal salt media as basal medium.

The parameters optimized included carbon sources, additional sodium chloride concentration, incubation temperature, initial pH of medium, inoculum concentration and additional nitrogen sources. All the experiments were carried out in triplicates. The results are an average of triplicate experiments and standard deviation was determined using Excel 2007 (Microsoft Corporation, Redmond, USA).

3.7.1 Minimal salt medium

Minimal salt medium was used as the basal medium for optimization studies. The contents were mixed thoroughly autoclaved at 121^{o}C for 30 min and cooled to room temperature before inoculation.

Minimal media with the composition mentioned below.

Na_2HPO_4	0.6 g
KH_2PO_4	0.3 g
NaCl	0.1 g
NH_4Cl	0.1 g
$MgSO_4.7H_2O$ (1 M)	0.2 mL

$CaCl_2$. $2H_2O$ (0.1 M)	0.1 mL
Distilled water	100 mL
pH	7

The various components of the media were added to distilled water taken in a 500 mL Erlenmeyer flask, and autoclaved.

3.7.2 Inoculum preparation

For the preparation of inoculum, a loopful of cells from the freshly grown culture (Zobell marine agar slant) of *P. mendocina* was transferred to a 5 mL sterile Zobell Marine Broth and incubated at room temperature (28 ± 2°C) at 150 rpm on a rotary shaker. After 18 h of incubation, this pre culture was transferred to a 40 mL Zobell marine broth in a 250 mL Erlenmeyer flask and incubated at room temperature (28 ± 2°C) at 150 rpm on a rotary shaker for 18 h. Cells were harvested by centrifugation at 6000 rpm for 5 min at 4°C. The cell pellet was resuspended in 10 mL of 0.8% physiological saline. This culture suspension (OD_{600} = 1) was used as primary inoculum for production medium.

3.7.3 Inoculation, incubation and recovery of the protease inhibitor

The minimal salt medium (3.7.1) was inoculated with 1% (v/v) of bacterial inoculum as prepared as mentioned under 3.8.2 and incubated on a rotary shaker at 28 ± 2°C at 150 rpm. After incubation (48 h arbitrarily taken) the inhibitor was recovered from the production broth by centrifugation at 10,000 rpm at 4°c for 15 min. The clear supernatant obtained after centrifugation was used as the crude inhibitor. This was assayed for inhibitory activity and protein content as mentioned under section 3.2.1.1, 3.2.2 and 3.2.3 respectively.

3.7.4 Carbon source optimization

Different carbon sources viz. lactose, maltose, sucrose, glycerol, mannitol, galactose, glucose, fructose, sorbitol and xylose at 25 mM concentration and starch (1%) were tested. The carbon sources sterilized separately and added to the production medium. Medium preparation, inoculation, incubation and inhibitor recovery were performed as detailed under section 3.7.1, 3.7.2 and 3.7.3.Culture supernatants were analyzed for protease inhibitor production and assayed for protease inhibitory activity, protein content and specific activity as described under section 3.2.1.1, 3.2.2 and 3.2.3 respectively.

3.7.5 Optimization of additional NaCl concentration

Optimal sodium chloride for maximum protease inhibitor was evaluated by adding different sodium chloride concentrations (1%, 2%, 3%, 4% and 5%) in addition to the NaCl in the minimal medium. Twenty five millimolar glucose was used as carbon source. Medium preparation, inoculation, incubation and inhibitor recovery were performed as detailed under section 3.7.1, 3.7.2 and 3.7.3.Culture supernatants were analyzed for protease inhibitor production and assayed for protease inhibitory activity, protein content and specific activity as described under section 3.2.1.1, 3.2.2 and 3.2.3 respectively.

3.7.6 Optimization of incubation temperature

For the optimization of different incubation temperatures the protease inhibitor production was evaluated by incubating the inoculated minimal media at 25^{o}C, 30^{o}C, 37^{o}C, 45^{o}C and 50^{o}C. Twenty five millimolar glucose was used as carbon source with 3% additional NaCl. Medium preparation, inoculation, incubation and inhibitor recovery were performed as detailed under section 3.7.1, 3.7.2 and 3.7.3.Culture supernatants were analyzed for protease inhibitor production and assayed for protease inhibitory activity, protein content and specific activity as described under section 3.2.1.1, 3.2.2 and 3.2.3 respectively.

3.7.7 Optimization of inoculum concentration

Optimal inoculum concentration that supports maximum protease inhibitor production in *P. mendocina* was evaluated using different concentrations of initial inoculum (2%, 4%, 6%, 8% and 10%) prepared as mentioned in section 3.7.2. Twenty five millimolar glucose was used as carbon source with 3% additional NaCl and incubated at 30^{o}C. The medium preparation, inoculation and inhibitor recovery were performed as detailed under section 3.7.1 and 3.7.3. Culture supernatants were analyzed for protease inhibitor production and assayed for protease inhibitory activity, protein content and specific activity as described under section 3.2.1.1, 3.2.2 and 3.2.3 respectively.

3.7.8 Nitrogen source optimization

Various nitrogen sources (1%, w/v) were incorporated in minimal medium with 25 mM glucose, 3% NaCl at pH 7. 4% inoculum was used for the study. Incubated on a rotary shaker at 30^{0} C. Effect of complex organic nitrogen source on protease inhibitor production was studied using malt extract, beef extract, yeast extract, peptone, casein, tryptone and tryptone soya broth (TSB) were added individually.

Samples were analyzed after 48 h incubation and the studies were carried out at a media pH 7, 150 rpm and at 30^{o}C incubation temperature with 25 mM glucose as carbon source and 3 % additional NaCl in minimal medium. The medium preparation, inoculation and inhibitor recovery were performed as detailed under section 3.7.1, 3.7.2 and 3.7.3. Culture supernatants were analyzed for protease inhibitor production and assayed for protease inhibitory activity, protein content and specific activity as described under section 3.2.1.1, 3.2.2 and 3.2.3 respectively.

3.8 PROTEASE INHIBITOR PURIFICATION

Proteinaceous protease inhibitor isolated from *P. mendocina* was purified employing standard protein purification methods which included ammonium sulphate precipitation, followed by dialysis, DEAE sepharose ion exchange chromatography and Trypsin sepharose affinity chromatography. The purity was further confirmed by reverse phase HPLC. All purification steps were carried out at 4°C unless otherwise mentioned.

3.8.1 Ammonium sulphate precipitation

Ammonium sulphate precipitation was done (Englard and Seifter, 1990) as described earlier under section 3.3.1.

3.8.2 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 3.3.2.

3.8.3 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.

3.8.3.1 Purification using DEAE sepharose column

DEAE sepharose was carefully packed in Bio-Rad econo column (42.5 X 2.5 cm) without any air bubble and the column was equilibrated with five column volumes of 0.01 M phosphate buffer pH 7.5. Twenty five millilitre of dialyzed sample, prepared as described in section 3.5.2, with a protein content of 120 mg

was applied to the pre equilibrated DEAE sepharose 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 1.5 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 mentioned under section 3.5.2. The dialyzed fractions were concentrated using amicon UF-30 kDa membrane (Millipore, USA) and assayed for protease inhibitory activity, protein content and specific activity as described under section 3.2.1.1, 3.2.2 and 3.2.3 respectively.

3.8.4 Affinity chromatography

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

3.8.4.1 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.

3.8.4.2 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).

3.8.4.3 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 0.01 M HCl. The bound peak was immediately dialyzed against 0.01 M phosphate buffer pH 7.5 and concentrated using amicon UF-30 kDa membrane. Protease inhibitory activity, protein content and specific activity found as described under section 3.2.1.1, 3.2.2 and 3.2.3 respectively. The yield and fold of purifications were calculated.

3.8.5 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.

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

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

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

3.8.6 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.

3.9 CHARACTERIZATION OF PROTEASE INHIBITOR

Purified inhibitor was further subjected to characterization for their biophysical as well as biochemical 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.

3.9.1 Electrophoretic methods

The active fractions of protease inhibitor from *P. mendocina*, 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 Non-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

Samples were diluted with sample buffer in 1:2 ratios and heated at 95^{o}C for 4 min.

8. 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.

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

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

11. Protein staining solution

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

12. Destaining solution

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

Silver staining

Silver staining of the gel after electrophoresis was performed (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.

13. Protein Markers for SDS-PAGE

Prestained protein molecular weight marker (Fermentas, India) was used for non-reductive SDS-PAGE analysis.

Components		**MW (M_r) in Da**
β-galactosidase	-	118,000
Bovine serum albumin	-	90,000
Ovalbumin	-	50,000
Carbonic anhydrase	-	36,000
β-lactoglobulin	-	27,000
Lysozyme	-	20,000

a. Low range molecular weight marker mix of Bio-Rad was used for the reductive SDS-PAGE. 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	-	31,000
Trypsin inhibitor	-	21,500
α-Lactalbumin	-	14,400

3.9.1.1 Native polyacrylamide gel electrophoresis

3.9.1.1.1 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.

3.9.1.2 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.

3.9.2 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).

3.9.3 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.

3.9.3.1 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.

3.9.3.2 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).

3.9.3.3 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 coomassie stained after electrophoresis.

3.9.4 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.

3.9.5 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°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 3.2.2.

3.9.6 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 3.2.2.

3.9.7 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 3.2.2. 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.

3.9.8 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.

3.9.9 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.

3.9.10 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 3.2.2.

3.9.11 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 3.2.2.

3.9.12 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 3.2.2.

3.9.13 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 3.4.2.

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).

3.9.14 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 3.2.2.

3.9.15 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.

3.9.16 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.

3.9.17 Specificity of protease inhibitor

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

3.9.17.1 Assay of elastase, proteinase K and subtilisin 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.

3.9.17.2 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.

3.9.18 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 11,567). 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.

3.9.19 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).

3.10 APPLICATION STUDIES

3.10.1 Protease inhibitor as seafood preservative

The inhibitory activity of *P. mendocina* protease inhibitor towards the spoilage microbial flora isolated from the shrimp *Peneaus monodon* was evaluated. To assess the effect of inhibitor on protein degradation of shrimp during preservation under different storage conditions like room temperature, 4°C and –20°C for different duration was analysed. For this, head was removed from the peeled shrimp. Five gram shrimp biomass was weighed out and taken in a sterile container, sealed and kept at different storage condition as control. For the test experiment, the same weight of samples were taken in the same conditions as that of control and incubated with in a sterile container, having 5 mL of (0.1 mg/mL) purified protease inhibitor prepared as mentioned under section 3.9.4 at each temperature for 8 h, 24 h and 168 h respectively.

After incubation, the samples were taken and extracted by homogenizing in sterile distilled water using a mortar and pestle under sterile conditions and kept in a rotary shaker for 30 min at 150 rpm. One milliliter of extract was taken under sterile condition, and serially diluted the sample in physiological saline. The total microbial population of each sample was analyzed by pour plating on Skim milk agar (HiMedia) plates. In the same way, the experiments were repeated with the

control also. The colony forming units are enumerated in both cases and a comparative strategy was made to analyse the efficacy of the inhibitor to prevent microbial growth.

The spoilage bacteria from *P. monodon* were isolated, purified and checked for the production of protease enzyme on Skim milk agar (HiMedia) plates. The positive cultures were characteristic of clearing zone around the colonies due to casein hydrolysis. They were picked and grown in Nutrient Broth (HiMedia) with 1% casein. To isolate spoilage protease enzyme from each bacterium, the culture broth was transferred in to centrifuge tube and subjected to centrifugation at 6000 rpm, at 4^{o}C in a rotary shaker. The resulted cell free culture supernatants were taken as crude proteolytic enzymes and stored at -20^{o}C which were used for further inhibition studies. The purified inhibitor was evaluated for its ability to inhibit the isolated spoilage proteases as described under section 3.2.1.1.

The complete protein of the samples were extracted using 5% NaCl in 0.02 M sodium bicarbonate according to (Chandrasekaran, 1985) and the cell pellet were removed by centrifugation at 10,000 rpm for 15 min at 4°C. The clear supernatant was assayed for total protein content as described in section 3.2.2.

3.10.2 Protease inhibitor as antibacterial agent

Inhibition of growth of bacteria by *P. mendocina* protease inhibitor was studied using agar well diffusion assay (Tagg and McGiven, 1971). The bacterial strains *Escherichia coli* (NCIM 5051) *Pseudomonas aeruginosa* (NCIM 2863), *Pseudomonas solanicerum* (NCIM 5103), *Pseudomonas fluorescens* (NCIM 2099), *Pseudomonas syringae* (NCIM 5102), *Pseudomonas putida* (NCIM 2650), *Salmonella typhimurium* (NCIM 2501), *Salmonella abony* (NCIM 2257), *Klebsiella pneumoniae* (NCIM 2957), *Proteus vulgaris* (NCIM 2027), *Clostridium perfringens* (NCIM 2677), *Staphylococcus aureus* (NCIM 2127), *Bacillus cereus* (NCIM 2155), *Bacillus circulans* (NCIM 2107), *Bacillus coagulans* (NCIM 2030), *Bacillus macerans* (NCIM 2131), and *Bacillus*

pumilus (NCIM 2189) were purchased from National Collection of Industrial Microorganisms (NCIM), NCL Pune, India and used as test bacteria.

Test bacteria freshly grown in nutrient broth (HiMedia) for 12 h were used for the study. Antibacterial analysis was performed in Petri plates containing Mueller-Hinton agar (HiMedia) in which each of the test bacterium was swab-inoculated. Well was cut aseptically in the test bacterial plate and 20 µL (5 mg/mL) of purified protease inhibitor was added in it. Plates were incubated for 24 h at 37°C and observed for the clearance zones of inhibited bacterial growth. Minimum inhibitory concentration (MIC) was determined by adding varying concentrations (70 µg, 75 µg, 80 µg, 85 µg, 90 µg, 95 µg and 100 µg) of inhibitor preparations in the wells made in the test bacterial plate, and observed for the clearance of inhibited bacterial growth.

Chapter 4

RESULTS

4.1 ISOLATION OF MICROORGANISMS FOR PROTEASE INHIBITOR ACTIVITY

Bacteria, fungi and actinomycetes isolated from different locations of Cochin backwaters, Kerala were screened for their protease inhibitory activity. Three hundred and eighty isolates of bacteria, 70 isolates of fungi and 150 isolates of actinomycetes were subjected to primary screening.

4.2 PRIMARY SCREENING –Caseinolytic plate assay

Results presented in Fig 4.1 shows the inhibition of casein hydrolysis by trypsin by the microbial culture extracts in skimmed milk agar plate. Four bacterial isolates were recognized as positive for protease inhibitor production and they were subjected to secondary screening.

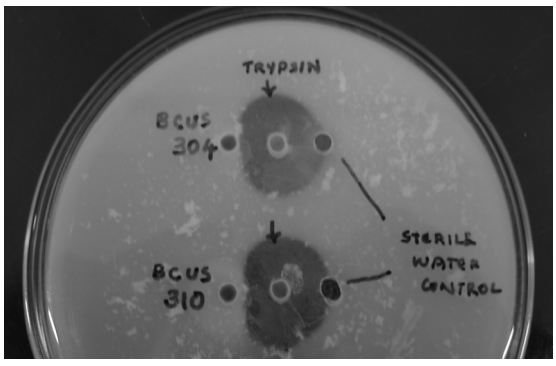


Fig 4.1 Caseinolytic plate assay: The zone of inhibition of the hydrolysis of casein was observed. Central well: trypsin 20 µL (0.5 mg/mL); Peripheral wells: (left) culture filtrate and (right) sterile distilled water as control.

4.3 SECONDARY SCREENING- Caseinolytic broth assay

Secondary screening by caseinolytic broth assay was done with culture supernatants of bacteria shortlisted after primary screening. Results of cultures which showed considerable inhibitory activity are presented in Table 4.1

Table 4.1 Secondary screening of bacteria for protease inhibitor

Serial No	Bacterial culture	Inhibitory activity (u)
1	BTMW 116	50
2	BTMW 301	41
3	BTSM 304	63.8
4	BTMW 310	41

4.4 SELECTION OF POTENTIAL SOURCE AND ISOLATION OF PROTEASE INHIBITOR

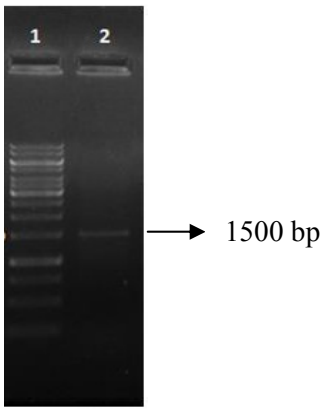
Bacterial isolates BTMW 116, BTMW 301, BTSM 304 and BTMW 310 which showed considerable protease inhibitory activity were subjected to further screening. Crude culture supernatants prepared from these bacteria were subjected to partial purification by ammonium sulphate fractionation. The precipitates obtained after each fractionation were dissolved in minimum quantity of phosphate buffer (0.01 M, pH 7.5) and evaluated for inhibitory activity by caseinolytic broth assay. The results are shown in Table 4.2. Among them the fraction obtained after 0-30% saturation of ammonium sulphate of the culture supernatant from bacterium BTMW 301 showed maximal protease inhibition (50 u) and specific activity (5 u/mg) compared to others and hence was selected as the source for the isolation of protease inhibitor. Whereas, BTSM 304 and BTMW 310 ammonium sulphate precipitates showed no protease inhibitory activity.

Table 4.2 Protease inhibition of ammonium sulphate precipitated active fractions of selected bacterial culture supernatants

Sl No.	Bacterial extract	Saturation of ammonium sulphate (%)	Inhibitory activity (caseinolytic) (u)	Specific inhibitory activity (u/mg protein)
1	BTMW 116	30-60	4	0.778
2	BTMW 301	0-30	50	5
3	BTMW 301	30-60	2	0.024

4.5 IDENTIFICATION OF THE SELECTED BACTERIAL STRAIN BTMW 301

The molecular identification of the bacterial strain BTMW 301 was done by ribotyping using partial gene sequences of 16S rRNA gene. A portion of the 16S rRNA gene (~1500 bp) (Fig 4.2) was amplified from the genomic DNA and the amplicon was subjected to sequencing followed by homology search analysis.



1 2

Fig. 4.2a

5’GTCGAGCGGATGAGAGGAGCTTGCTCCTTGATTTAGCGGCGGACGGGTGAGTAAT
GCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTATACCGC
ATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCC
TAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAAC
TGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCC
GCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCA
GTAAGTTAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTTC
GTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCG
TAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTG
GGAACCGCATCCAAAACTGGCGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTTCC
TGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCCAGTGGCCGAAGGCGA
CCACCTGGACTGATTCTGACACTGATGTGCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACAATGTCAACTAGCCGTTGGGTTCCTTGAG
AACTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGTAAG
GTCAAAACTCAAATGAATTGACCGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGA
GATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTA
CCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTAC
AATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACC
GATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGT
AATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCTTCGGGGGGAC
GGTTACCACGGAGT-3’

Fig. 4.2b

Fig 4.2 a) PCR amplicon of 16S rDNA segment.
Lanes 1. 1 kb ladder 2. Amplicon.
b) DNA sequence of amplicon.

4.5.1 Phylogenetic tree construction

The sequence showed 96% similarity (NCBI Accession No: GU139342) with already available sequences of *Pseudomonas mendocina* in the GenBank. The homology analysis was established using BLAST software. The phylogenetic tree was constructed using the dendrogram method implemented in CLUSTAL X (Fig 4.3)

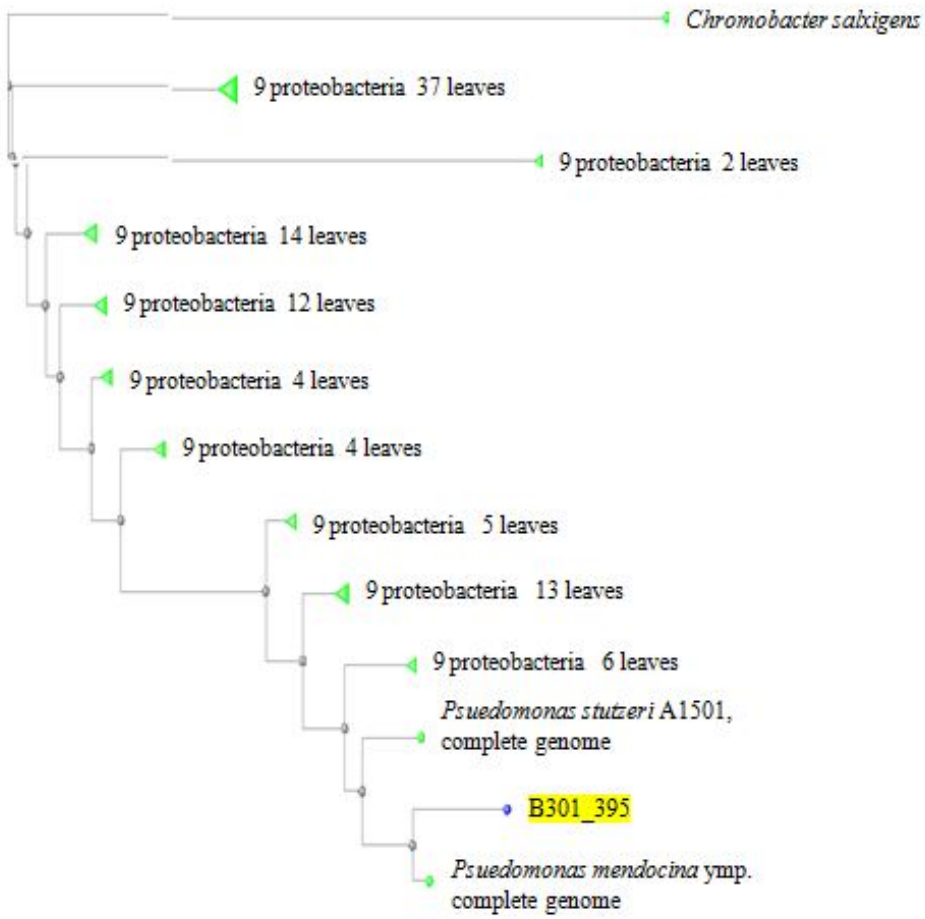


Fig 4.3 Dendrogram representing the relationship of the culture with reported *Pseudomonas mendocina.*

4.5.2 BIOCHEMICAL AND MORPHOLOGICAL CHARACTERIZATION OF *P. MENDOCINA* BTMW 301

Colony morphology of the bacterial strain was analysed and the results are given in Table 4.3. The results presented in Table 4.4 are biochemical tests catalase, urease, modified oxidation-fermentation, indole, methyl red, Voges Proskauer, Simmons' citrate utilisation and Gram's staining performed for the identification of the culture. The strain has been deposited at Microbial Culture Collection unit of National Centre for Cell Science (NCCS), DBT, Govt. of India, Pune, with the accession number MCC2069.

Table 4.3 Morphological characteristics of *P. mendocina* BTMW 301

Colony morphology	Result
Configuration	Circular
Margin	Entire
Elevation	Raised
Surface	Smooth
Pigment	Creamy orange
Opacity	Opaque
Cell Shape	Rod
Gram's reaction	Negative

Table 4.4 Biochemical characteristics of *P. mendocina* BTMW 301

Biochemical test	Result
Catalase	Negative
Urease	Negative
Modified oxidation fermentation	Positive
Indole	Negative
Methyl Red	Positive
Voges Proskauer	Negative
Simmons' citrate utilisation	Positive

4.6 Time profile for the production of inhibitor

P. mendocina was grown in the Zobell marine broth 2216 (HiMedia) and course of production of inhibitor as a function of time was monitored. The data depicted in Fig 4.4 indicated that although the production of inhibitor commenced at 4 h of incubation, a considerable level of activity (14.3 u) was recorded only after 24 h of incubation. The activity curve recorded steep rise during the period 24-48 h and maximal protease inhibitor activity (44 u) was recorded at 48 h of incubation. Later, a gradual decrease in the inhibitor production was noticed and came to negligible level after 120 h, whereas the OD at 600 nm which indicated the growth of bacteria was found to reach maximum after 20 h and the culture was found to remain in the stationary phase of growth. Thus it was inferred that maximal protease inhibitor production takes place during early stationary phase of growth, which however declined rapidly later.

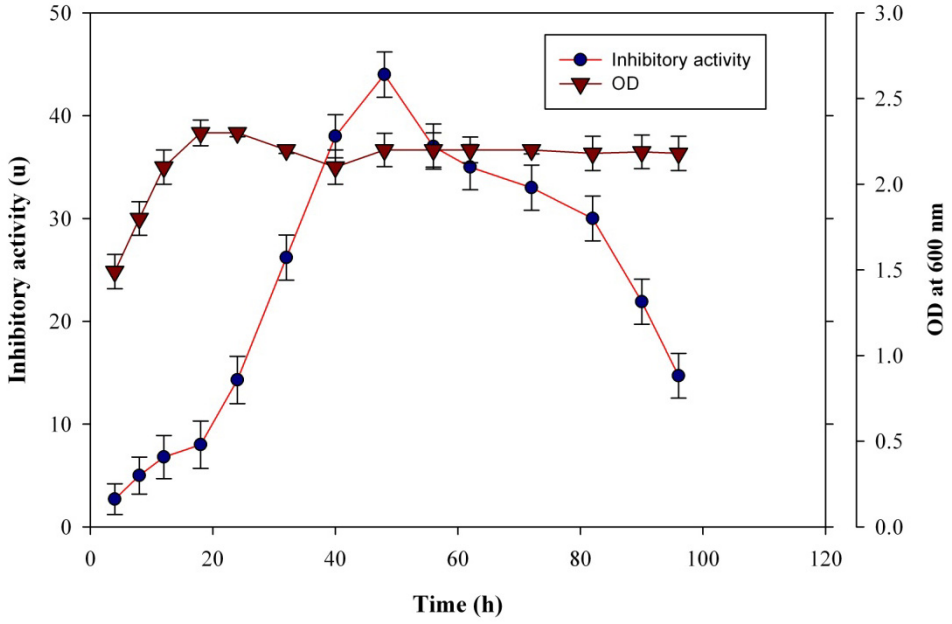


Fig 4.4 Time course of protease inhibitor production by *P. mendocina* BTMW 301.

4.7 SUBMERGED FERMENTATION (SMF): PRODUCTION OPTIMIZATION OF PROTEASE INHIBITOR BY *P. MENDOCINA* BTMW 301

Protease inhibitor production by *P. mendocina* BTMW 301 under submerged fermentation was studied using minimal medium. Various parameters that influence protease inhibitor production were optimized towards maximal production.

4.7.1 Carbon source

Eleven different carbon sources were used individually with minimal medium in order to select the best carbon source that supports maximal protease inhibitor production. The results presented in Fig 4.5 shows that among the different sugars and polysaccharides tested, maximum inhibitory activity was recorded with 25 mM glucose (19 u) followed by fructose (13.6 u). Lactose, maltose, sucrose, mannitol and starch did not support protease inhibitor production in minimal media where as 25 mM galactose (5.2 u), xylose, (3.3 u), glycerol (3.19 u) and sorbitol (2 u) showed very less activity compared to glucose. Hence, 25 mM glucose was selected as carbon source for further optimization studies.

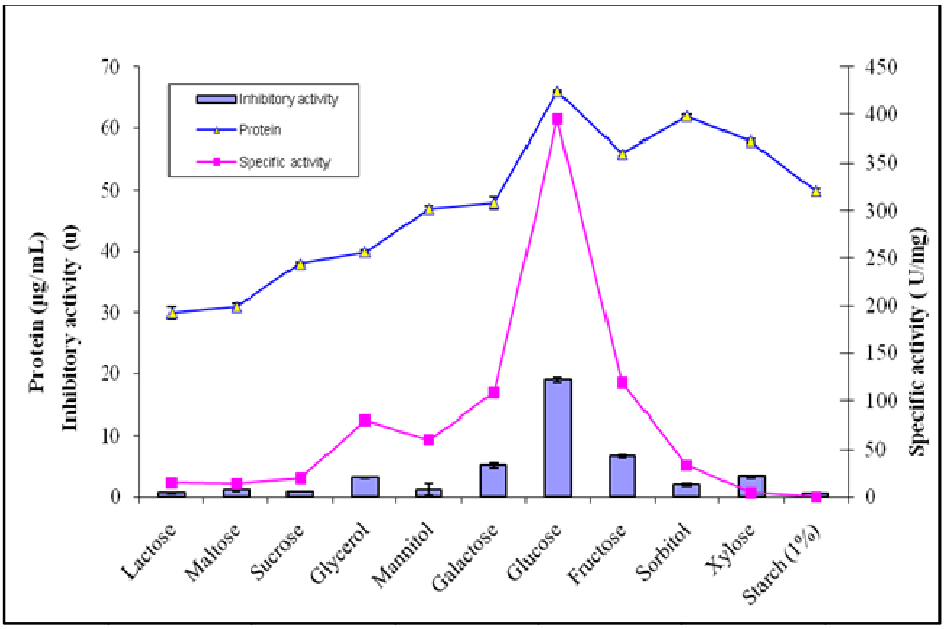


Fig 4.5 Effect of different carbon sources on protease inhibitor production by *P. mendocina* BTMW 301 in minimal medium. Optimization studies conducted in minimal medium with different carbon sources at 25 mM concentration except starch (1%) and incubated at room temperature (RT, 28 ± 2) at 150 rpm.

4.7.2 Additional NaCl concentration

Results presented in Fig 4.6 suggest that *P. mendocina* BTMW 301 is a halotolerant bacterium and among the various NaCl concentrations tested, maximum protease inhibitor production (47 u) was found at 3% of NaCl concentration followed by 2% (37.57 u), 4% (28 u) , 5% (27.5 u) and 1% of NaCl concentrations. Nevertheless, inhibitor production was recorded at considerable level at a wide range of NaCl concentrations.

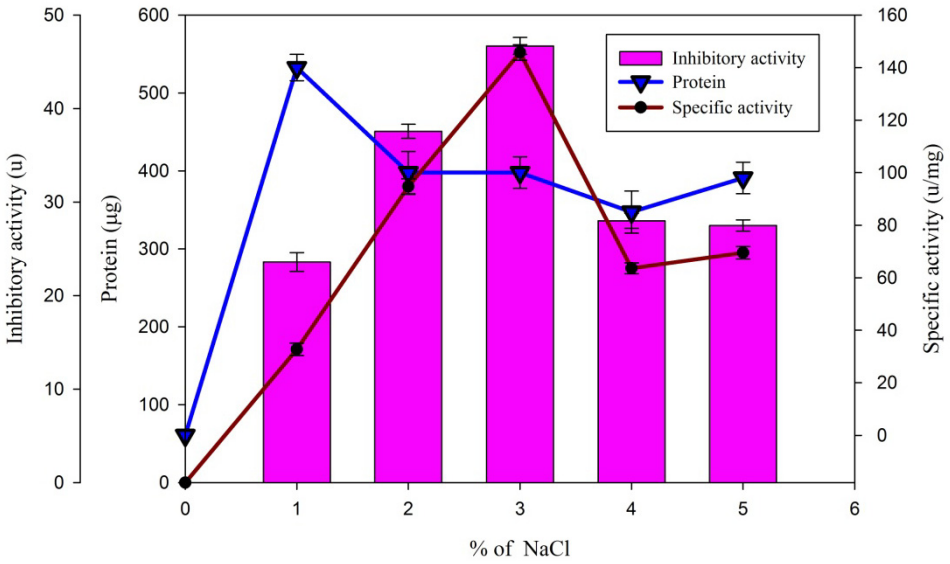


Fig 4.6 Effect of NaCl on protease inhibitor production in minimal medium. Optimization studies conducted in minimal medium by *P. mendocina* BTMW 301 with 25 mM glucose, incubated at RT, 150 rpm and with different concentrations of NaCl.

4.7.3 Incubation temperature

From the results presented in Fig 4.7 it is evident that *P. mendocina* grows well and produced protease inhibitor in minimal salt medium with 25 mM glucose as carbon source, 3% NaCl, 150 rpm and at all the temperatures studied. However maximum inhibitor production was observed at 30^{O}C (47.2 u) followed by 37^{o}C (40 u). Whereas the specific activities of protease inhibitor was found to be maximum at 25^{O}C which declined along with increase in incubation temperature.

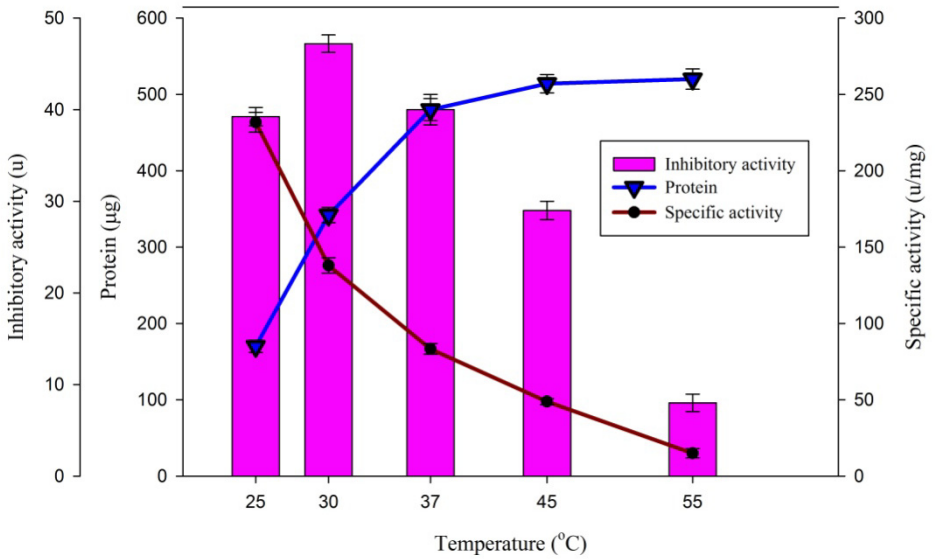


Fig 4.7 Effect of incubation temperature on protease inhibitor production in minimal medium. Temperature optimization studies conducted in minimal medium with 25 mM glucose, at 150 rpm, 3% NaCl, incubated at different temperatures at 48 h of incubation.

4.7.4 pH

Results presented in Fig 4.8 indicated that the bacterium *P. mendocina* BTMW 301 is capable of producing protease inhibitor over broad pH range from pH 3 (1.67 u) to pH 10 (33 u) even though the inhibitory activity varied considerably. There was an increase in inhibitory activity along with increase in pH from pH 3 to 7, reaching a peak value at pH 7 (38.5 u). When pH was raised above pH 7 a sudden decline in activity was observed at pH 8 (21.43 u) followed by an increase at pH 9 (32.05 u) and 10 (33 u). The trend observed for specific activity was identical to protease inhibitory activity. The protein levels recorded for different pH did not show any correlation with protease inhibitory activities in the initial pH ranges, from pH 3 to pH 6, but shows a similar trend with protease inhibitory activity up to pH 9.

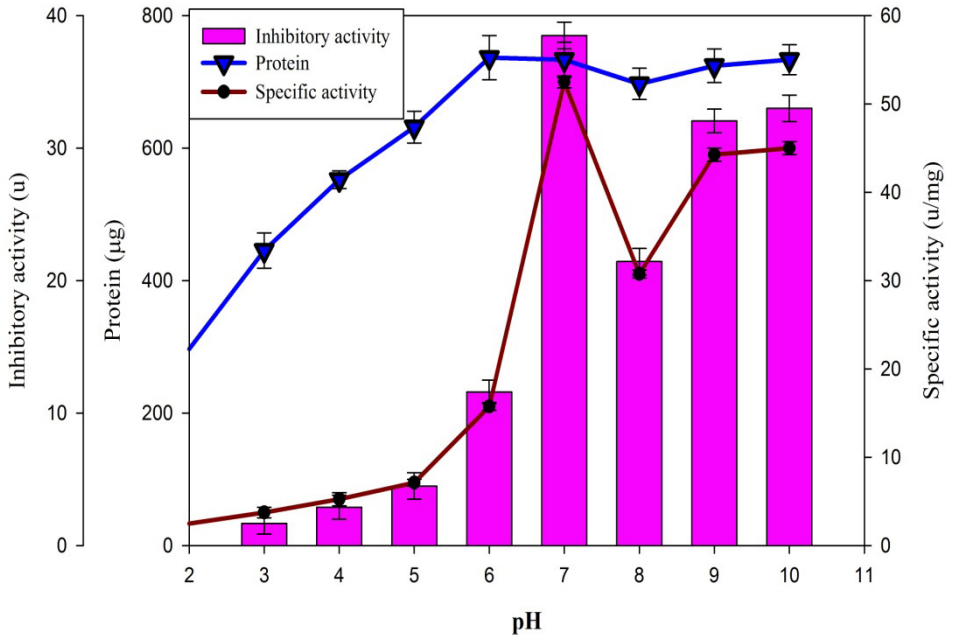


Fig 4.8 Effect of media pH on protease inhibitor production in minimal medium. pH optimization studies conducted in minimal medium with 25 mM glucose, at 150 rpm, 3% NaCl, at RT, incubated at different pH for 48 h.

4.7.5 Inoculum concentration

Data presented in Fig 4.9 showed the effect of inoculum concentration on protease inhibitor production. Four percent inoculum was found to be optimal for maximal inhibitor production (69 u) and further increase in inoculum concentration resulted in a gradual decrease in inhibitory activity. Whereas maximal specific activity of protease inhibitor was recorded with 6% inoculum while protein level did not show direct relation.

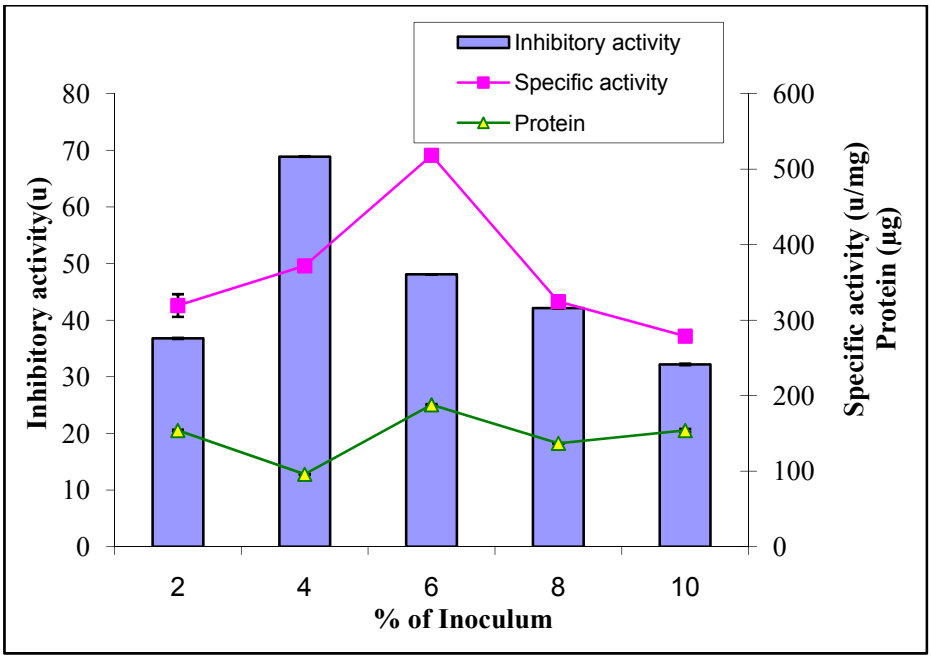


Fig 4.9 Effect of inoculum concentration on protease inhibitor production in minimal medium. Optimization of inoculum concentration conducted in minimal medium with 25 mM glucose, at 150 rpm, 3% NaCl, at pH 7 with different inoculum concentrations and for 48 h.

4.7.6 Effect of nitrogen sources

Among the nitrogen sources tested 1% malt extract showed highest inhibitory activity (97 u) followed by 1% yeast extract (59.8 u) (Fig 4.10). Other nitrogen sources did not have much effect on protease inhibitor production. The protein level was maximum when 1% peptone was used in the medium while both protease inhibitor activity and specific activity were very low. The specific activity recorded an identical trend with protease inhibitory activity whereas protein level did not show correlation.

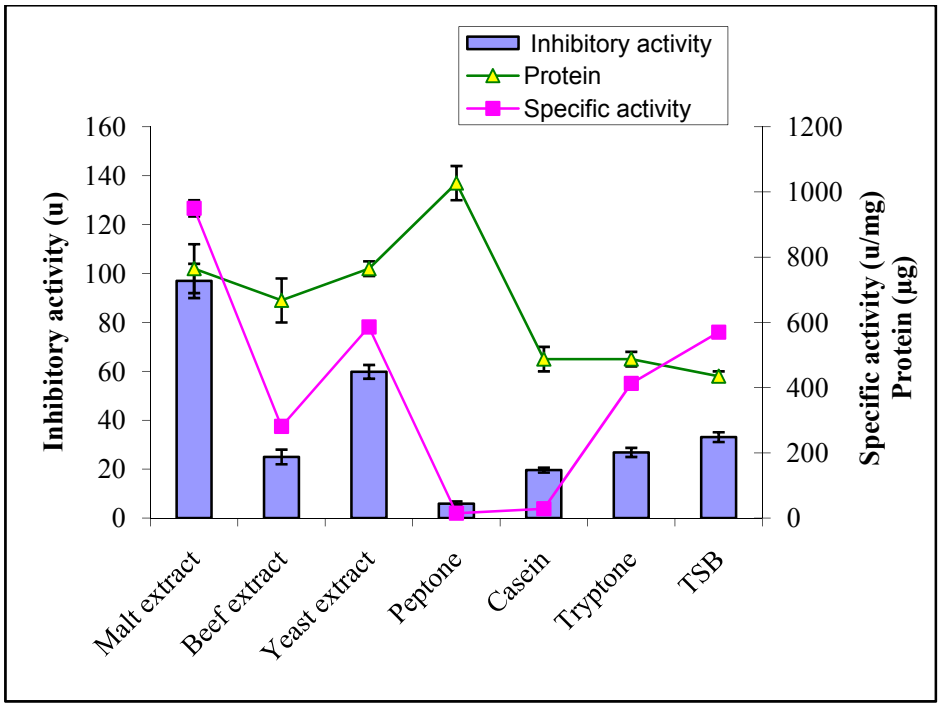


Fig 4.10 Effect of different nitrogen sources on protease inhibitor production in minimal medium. Nitrogen source optimization studies conducted in minimal medium with 25 mM glucose, at 150 rpm, 3% NaCl, at pH 7, with 4% inoculum with different nitrogen sources added separately and for 48 h.

4.8 PURIFICATION OF PROTEASE INHIBITOR

The inhibitory protein was purified to homogeneity employing standard protein purification methods, including ammonium sulphate precipitation, ion exchange chromatography using DEAE Sepharose, and trypsin affinity chromatography. The yield and fold of purification of protease inhibitor obtained in each step of purification is summarized in Table 4.5. As the first step towards the purification of protease inhibitor, the crude culture supernatant was subjected to ammonium sulphate fractionation, and concentration of ammonium sulphate required for complete precipitation of inhibitor was standardized. It was found that 0- 30% saturation of ammonium sulphate could precipitate the protease inhibitor

protein compared to others. The fold of purification of protease inhibitor obtained for ammonium sulphate precipitation, ion exchange chromatography and trypsin affinity chromatography were 3.257, 4.769 and 106.71 respectively.

Table 4.5 Yield and fold of purification[a]

Sample	Volume (mL)	Total protein (mg)	Total activity (u)	Specific activity (u/mg)	Yield of protein (%)	Yield of activity (%)	Fold of purification
Crude	1000	13000	40000	3.07	100*	100*	1*
Ammonium sulphate precipitation (0-30%)	24	120	1200	10	0.923	3	3.257
Ion-exchange chromatography (DEAE)	0.25	7	102.5	14.64	0.0538	0.256	4.769
Trypsin affinity chromatography	0.2	0.3	98.3	327.6	0.002	0.002	106.71

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

* Arbitrary taken values for crude extract.

4.8.1 Ion exchange chromatography

The dialysate obtained after ammonium sulphate saturation (0-30% fraction) was subjected to ion exchange chromatography using DEAE sepharose. 20 mL (100 mg) of ammonium sulphate (0- 30%) sample was loaded to DEAE Sepharose Column (42.5 X 2.5 cm). The elution profile of protease inhibitor depicted in Fig 4.11 shows 3 major peaks. Peak fractions were pooled and concentrated using Amicon 30 kDa membrane. Inhibitory activity was shown by the peak IV with 4.769 fold of purification.

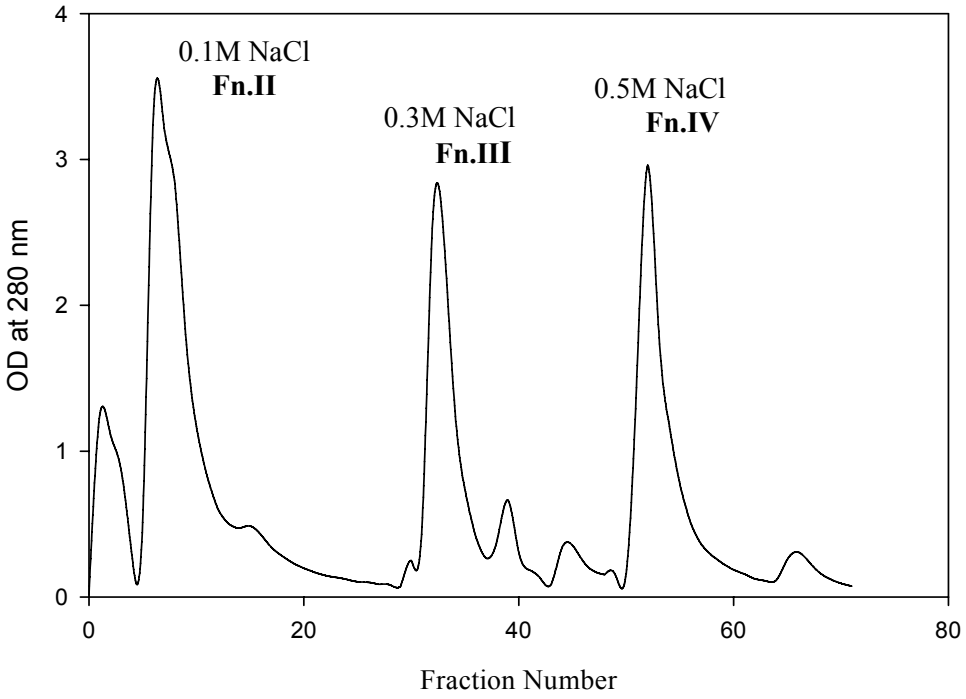


Fig. 4.11 Elution profile of protease inhibitor from DEAE Sepharose anion exchange chromatography column (42.5 X 2.5 cm) using NaCl gradient.

4.8.2 Affinity chromatography

Active ion exchange chromatographic fraction (Peak IV) that showed 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 106.71 fold of purification with a specific inhibitory activity of 327.6 units/mg protein (Fig 4.12).

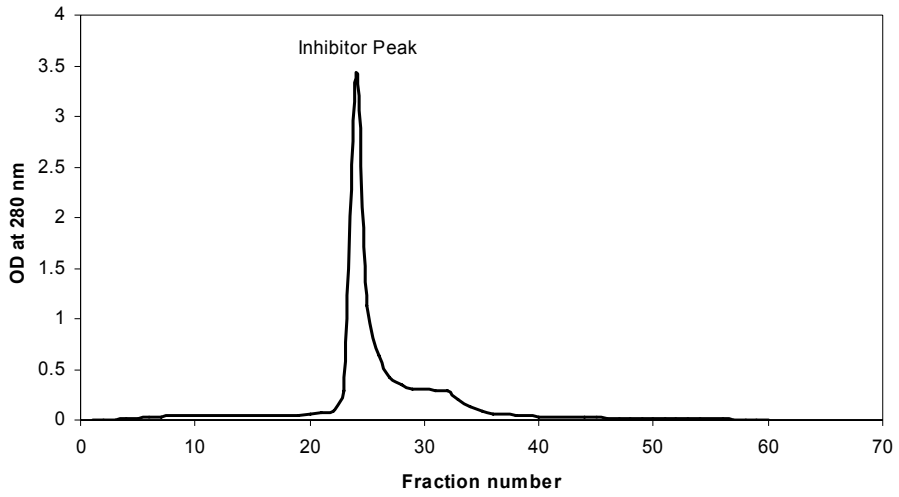


Fig. 4.12 Elution profile of inhibitor from trypsin affinity chromatography column (15 X 1 cm). Fraction No: 20 to 38 showed active bound proteins eluted with 0 .01M HCl in 0.5 M NaCl

4.8.3 Native Polyacrylamide Gel Electrophoresis

The purified inhibitor obtained after affinity chromatography was analysed through native ployacrylamide gel electrophoresis. The fraction with protease inhibitory activity was visualized as a single protein band on the gel confirming their purity and homogeneity (Fig 4.13).

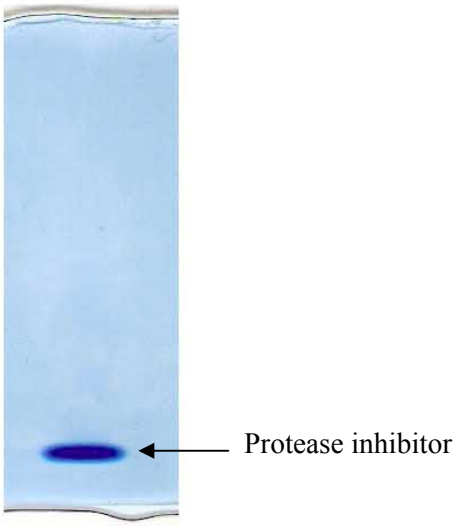


Fig. 4.13 Native PAGE analysis of purified protease inhibitor

4.8.4 HPLC profile of the inhibitor

Affinity purified active fraction (20 µL, 0.2 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. 4.14).

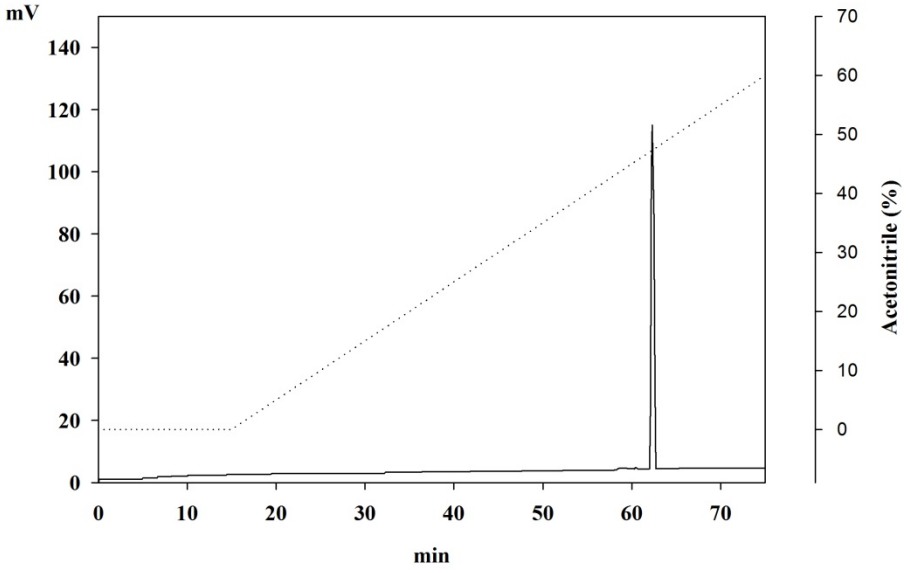
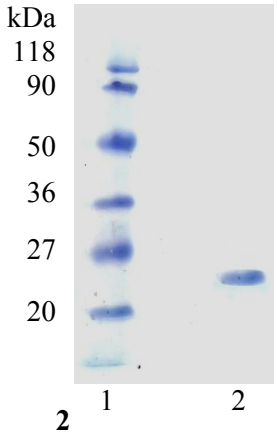


Fig. 4.14 Reverse phase HPLC profile of the purified inhibitor

4.9 CHARACTERIZATION OF PROTEASE INHIBITOR

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

SDS-PAGE was performed with the purified protease inhibitor under non-reducing and reducing conditions. The gel patterns depicted in the Fig 4.15(a) and Fig 4.15 (b) indicated that the purified protease inhibitor was resolved as single polypeptide band with a molecular weight of 22,000 Da, testifying the single polypeptide nature.



2

Fig 4.15 a

1. Marker
2. Purified protease inhibitor

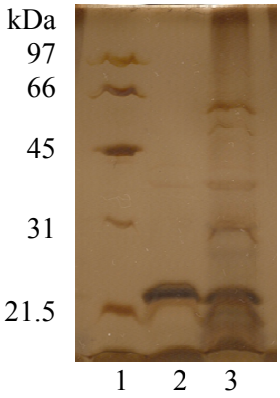


Fig 4.15 b

1. Marker
2. Purified protease inhibitor
3. Ion exchange purified active inhibitor fraction

Fig 4.15 SDS PAGE analysis of purified sample

(a) Non-reductive, **(b)** Reductive

4.9.2 Molecular mass determination by MALDI-TOF

The intact molecular mass of the inhibitor was determined by MALDI TOF/TOF. The purified inhibitor sample was desalted with ZipTip -C18 and MALDI TOF/TOF was carried out. The single peak indicating a molecular weight of 11567 Da (Fig 4.16) was recorded.

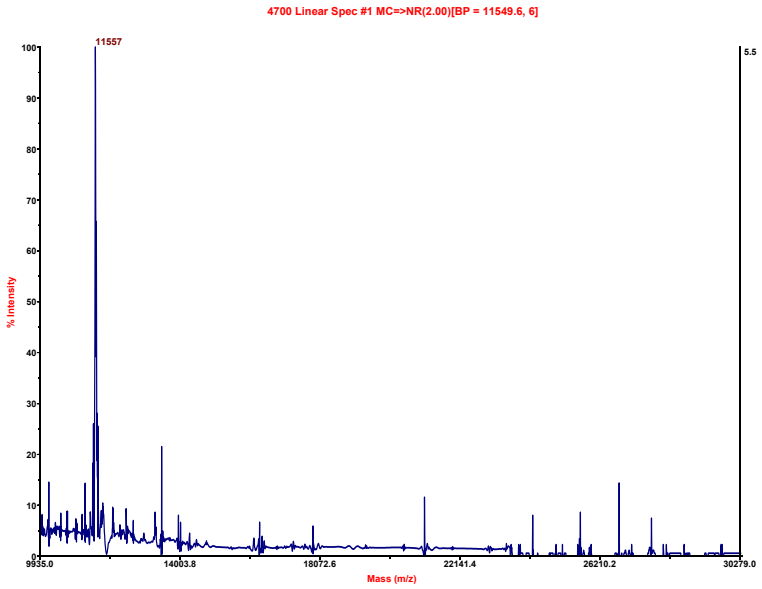


Fig 4.16 Mass of the inhibitor by MALDI TOF/TOF

4.9.3 Isoelectric focusing and 2D electrophoresis

Isoelectric point (pI) of the purified inhibitor protein was found to be 3.8, using an immobilized pH gradient (IPG) strip of pH 3-10 (Fig 4.17 a). Another strip was subjected to 2D electrophoresis to ensure the homogeneity of the protein. The presence of multiple proteins with same pI was tested out with 2D

electrophoresis. The result illustrated in Fig 4.17 (b) showed single band after coomassie staining.

Fig 4.17 a

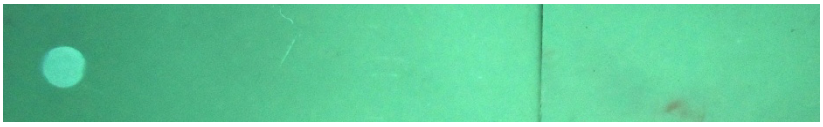
Fig 4.17 b

Fig 4.17 (a) Isoelectric focusing on pre-cast IPG strip. The protein band was visible on Coomassie stained IPG strip and resolved at pI of 3.8 **(b)** 2D gel of the protease inhibitor

4.9.4 Analysis of protease inhibitor by Dot-blot method

The dot-blot analysis of protease inhibitor activity was performed on X-ray film. The clearing zone formed due to the hydrolysis of gelatin by trypsin in

the presence and absence of protease inhibitor was analysed. The presence of inhibitor was confirmed by comparing the clearing zone formed due to gelatin hydrolysis. While a clear zone was formed due to gelatin hydrolysis by trypsin, there was a reduction or absence in clearing with the trypsin incubated with protease inhibitor. The result depicted in Fig. 4.18 indicated that the inhibitor blocked the gelatin hydrolysis by trypsin similar to that of the control protease inhibitor Soya bean trypsin inhibitor (SBTI).



1 **2** **3**

Fig 4. 18 Dot-blot analysis of protease inhibitor on X-ray film. 1. Trypsin control 2. Trypsin with Soyabean trypsin inhibitor (SBTI) as positive control 3. Trypsin with purified inhibitor.

4.9.5 Effect of temperature

The stability of protease inhibitor was assessed by pre incubation for 60 min at wide range of temperatures varying between 4 to 100^{o}C. The result depicted in Fig. 4.19 indicated that the inhibitor is considerably stable on pre incubation up to 90^{o}C. As the pre incubation temperature increases, the inhibitor recorded a gradual decrease in activity and a considerable loss of activity was evidenced after pre incubation at 60^{o}C. At 100^{o}C the inhibitor was completely inactive. Maximal activity of the protease inhibitor was recorded when pre incubated at 4^{o}C.

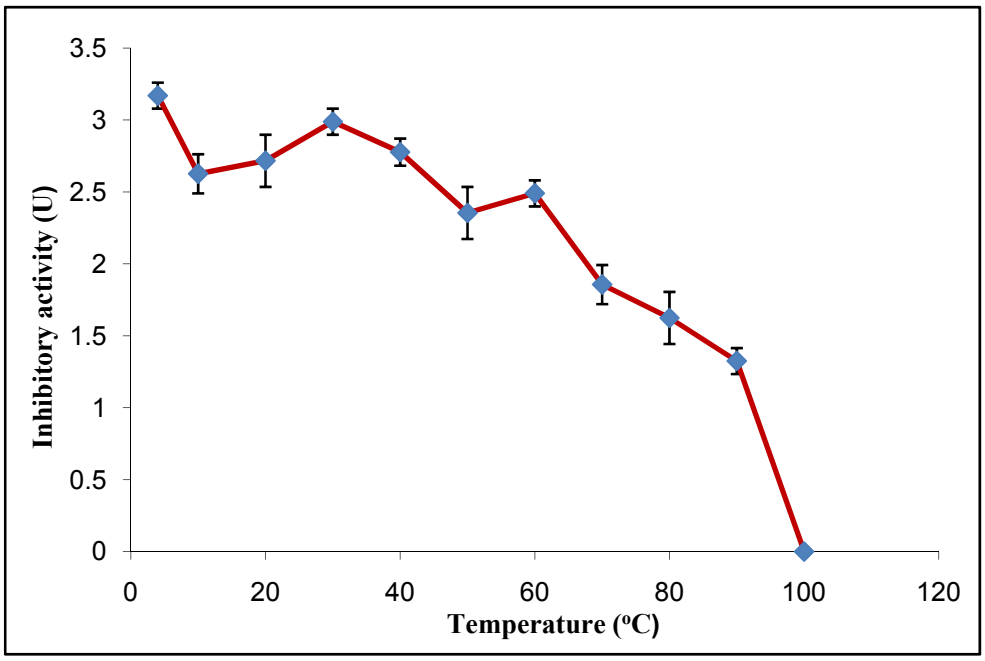


Fig. 4.19 Effect of temperature on activity of the inhibitor

4.9.6 Determination of stability of protease inhibitor at different pH

Stability studies carried out in different buffer systems for a period of 24 h demonstrated that the protease inhibitor had stability over a wide range of pH. From the results illustrated in Fig 4.20, it was evident that the activity of the inhibitor gradually increased from pH 2 and reached its maximum at pH 7 and after that gradually decreased up to pH 10. At the acidic (pH 3) and alkaline (pH 11.0) conditions the inhibitory activities were sharply declined. Further at high alkaline and high acidic conditions the protease inhibitor was found to unstable.

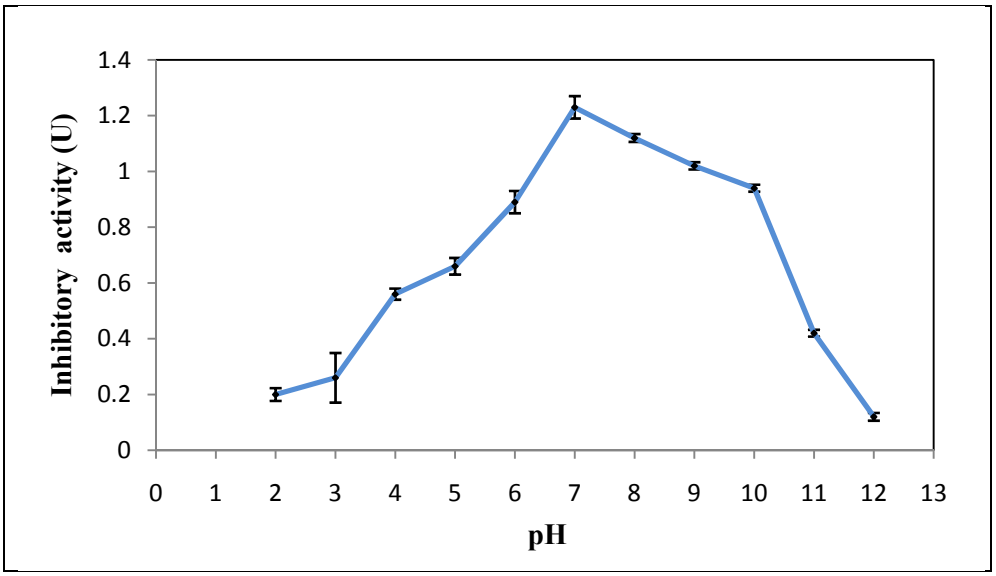


Fig. 4.20 Effect of pH on inhibitory activity of protease inhibitor

4.9.7 Effect of metal ions

The results depicted in the Fig 4.21 evidence the effect of metal ions such as Mo^{6+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Ca^{2+}, Al^{3+}, Na^{+}, Ni^{2+} and Ba^{2+} on the activities of protease inhibitor. Addition of magnesium sulphate which supplies divalent Mg^{2+} ions at a concentration of 10 mM and calcium chloride which supplies divalent Ca^{2+} ions at a concentration of 1 mM enhanced the protease inhibitory activity. Mg^{2+} (10 mM) ions enhanced the activity up to 6 U and Ca^{2+} (1 mM) ions up to 3.7 U compared to that of control (2.3 U). But Mg^{2+} at 1 mM concentration, and Ca^{2+} ions at 10 mM concentration, resulted in the decrease of inhibitory activity. Presence of Mo^{6+}, Mn^{2+}, Fe^{3+}, Ba^{2+}, Ni^{2+}, $Cd^{2+}$and Al^{3+} did not support protease inhibitory activity when compared to control and instead had a negative effect. Na^{+} and Ba^{2+} at 1 mM concentration, had no considerable effect on protease inhibitory activity but at 10 mM blocked the inhibitory activity and had a diminishing effect.

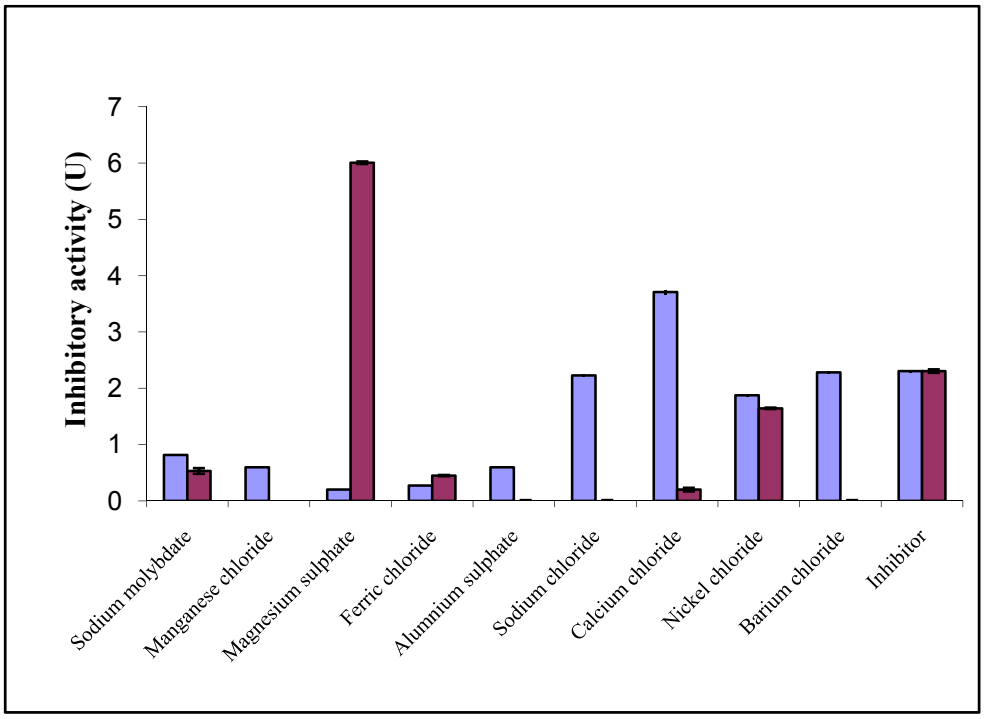


Fig. 4.21 Effect of metal ions on protease inhibitory activity

4.9.8 Metal chelation of protease inhibitor using EDTA

The results of ICP-AES analysis depicted in Table 4.6 indicated the presence of divalent cations in protease inhibitor. The atomic emission spectrum showed the presence of Ca^{2+} and Mg^{2+} in the protease inhibitor. Protease inhibitor dialyzed against deionised water was observed to contain calcium and magnesium at 40.65 and 42.46 ppm respectively, while inhibitor dialyzed against EDTA showed a negligible reduction in the metal ion concentration.

Table 4.6 Metal ion concentration of inhibitor

Sample No	Sample Name	Elements measured (ppm)	
		Ca	Mg
1	MilliQ dialysed	40.65	42.46
2	EDTA treated	32.94	22.15

4.9.9 Effect of detergents

The results presented in Fig. 4.22 testified the stability of inhibitor in the presence of detergents. Further, it was observed that the anionic detergent SDS and cationic detergent CTAB at a concentration of 1% completely inactivated the inhibitor or reduced the inhibitory activity significantly. Whereas, the nonionic detergents Triton X and Tween 80 showed a slight decrease in the protease inhibitory activity and Tween 20 lowered the inhibitory activity to half, compared to the control.

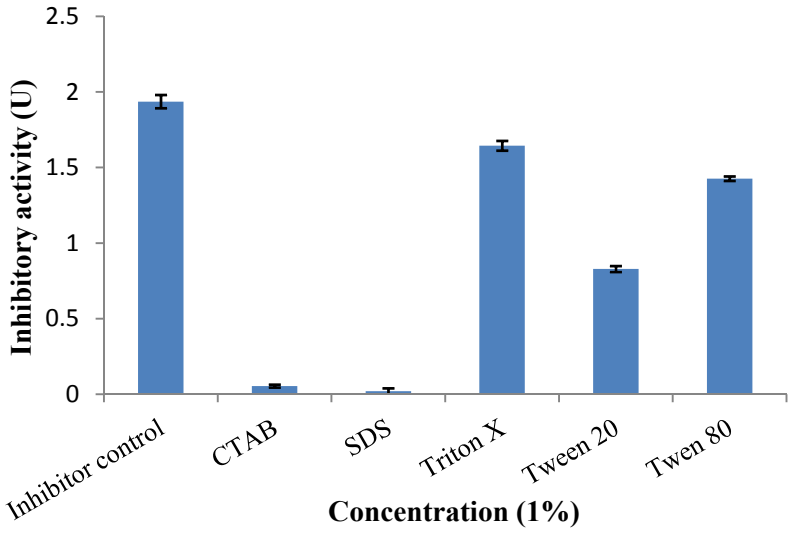


Fig 4.22 Effect of detergents on protease inhibitory activity

4.9.10 Effect of oxidizing agents

The results presented in the Fig. 4.23 suggests that the oxidizing agents H_2O_2 (Fig 4.23 a) and DMSO **(**Fig 4.23 b) have a negative effect on protease inhibitory activity. The oxidation of protease inhibitor by H_2O_2 did not influence much on the activity at 0.2% to 1% concentrations, since the activity slightly decreased along with increase in concentrations and later at the concentration above 1% there was a sudden loss of activity and complete inactivation occurs at a concentration above 1.2% .

The protease inhibitory activity was found to be decreased gradually along with increase in concentration from 1% to 5% of DMSO and complete inactivation was noticed at 6% of DMSO.

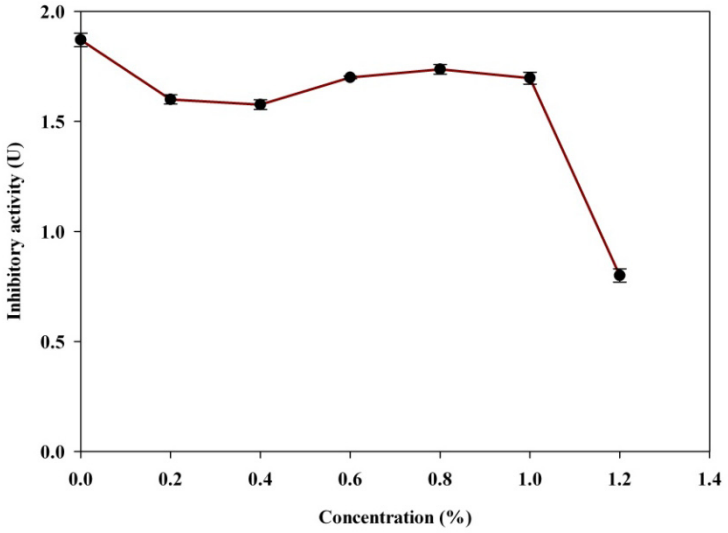


Fig 4.23 a

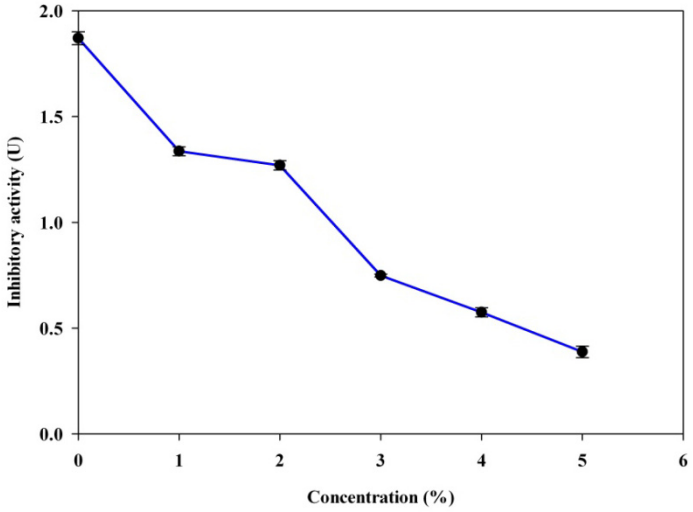


Fig 4.23 b

Fig 4.23 Effect of oxidizing agents on protease inhibitor.

(**a)** In the presence of H_2O_2. (**b)** In the presence of DMSO

4.9.11 Effect of reducing agents

The results depicted in Fig. 4.24 demonstrated the effect of reducing agents dithiothreitol and β-mercaptoethanol on protease inhibitory activity. From the results it was inferred that the activity was slightly increased by dithiothreitol up to a concentration of 30 mM, and at concentration above that led to a considerable inactivation of the inhibitor. In the case of β-mercaptoethanol inactivation was observed at a concentration of 50 µM and the effect was steady up to a concentration of 250 mM. However, at concentration above 250 mM, there was a sharp decline in the inhibitory activity, and a complete inactivation was noticed at 350 mM of dithiothreitol.

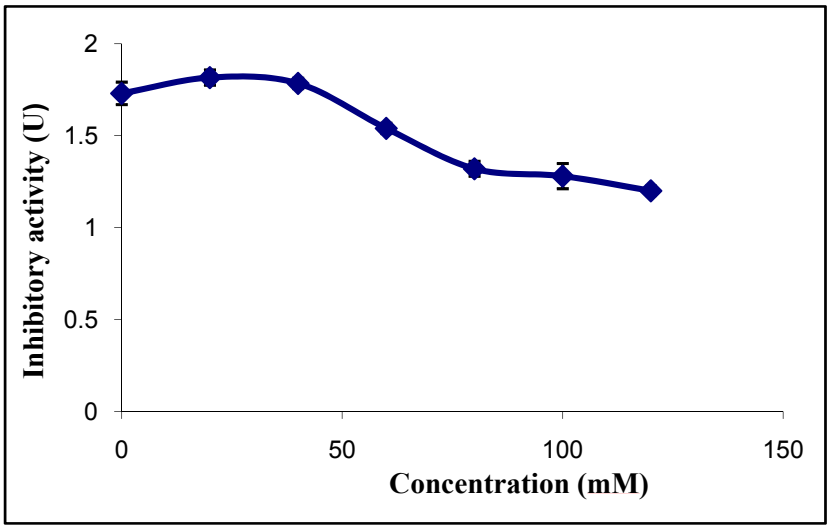


Fig 4.24 a

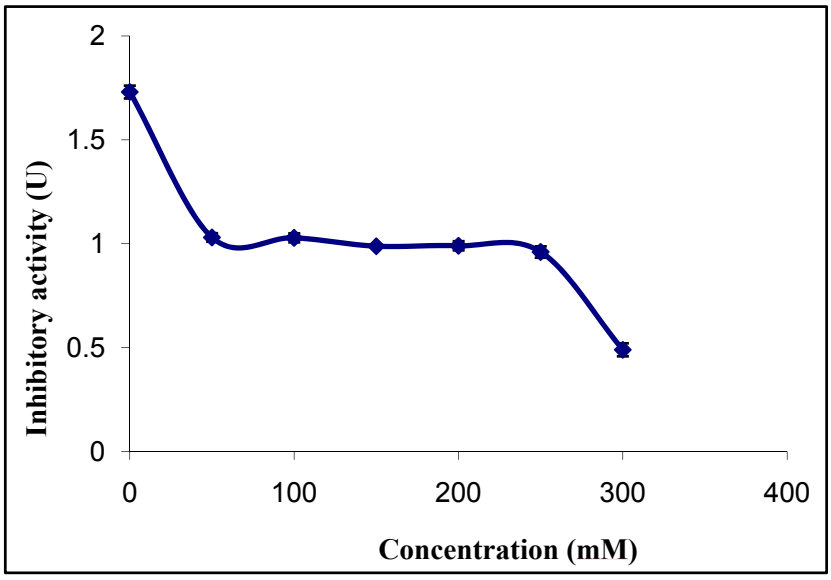


Fig 4.24 b

Fig 4.24 Effect of reducing agents on protease inhibitor

(a) Effect of dithiothreitol **(b)** Effect of β-mercaptoethanol

4.9.12 Chemical modifications of amino acids in protease inhibitor

Data depicted in Fig. 4.25 demonstrated the modification of individual amino acids using specific chemical modifiers and their effect on protease inhibitory activity. Among the three chemical modifiers used, PMSF at lesser concentrations reduced the protease inhibitory activity while a positive effect was demonstrated when used at a concentration above 2 mM. Whereas *N*-Ethylmaleimide and DEPC inactivated the protease inhibitory activity. Inhibitory activity of the PMSF modified inhibitor was reduced to 1.385 and 1.51 U/mL respectively at 1 mM and 2 mM concentration of the inhibitor compared to the control (1.74 U/mL). Further increase in concentration of PMSF resulted in an enhancement of inhibitory activity of 1.936 U/ml and 1.97 U/ml respectively at 3 mM and 4 mM. Modification of cysteine by *N*-Ethylmaleimide resulted in a rapid reduction in the inhibitory activity form 1.74 U/mL to 1.263 U/mL at 10 mM concentration. But further reduction in activity was not observed when the concentration was increased to 30 mM. Modification of histidine amino acid residue of the inhibitor with DEPC had no effect on protease inhibitory activity. The protease inhibitor was modified with 5-25 mM concentration of DEPC and the results of the study indicated that the inhibitory activity was not affected by the modifier.

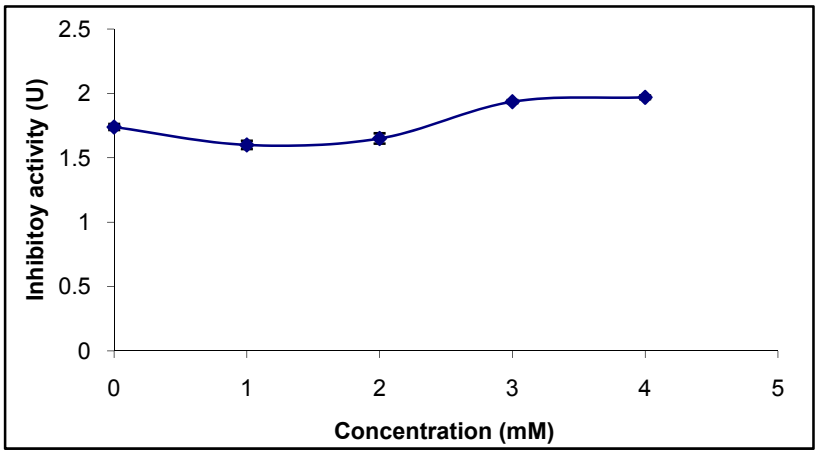


Fig 25 a

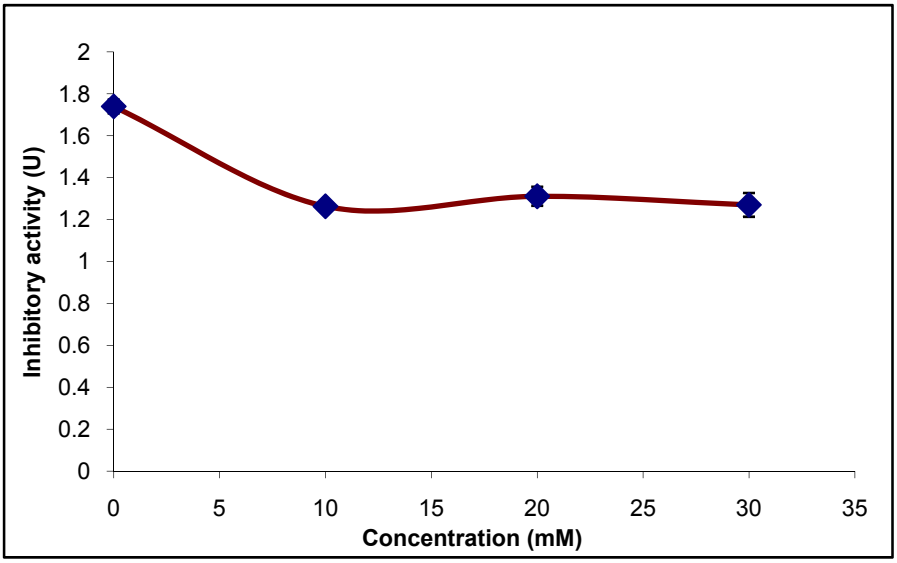


Fig 4.25 b

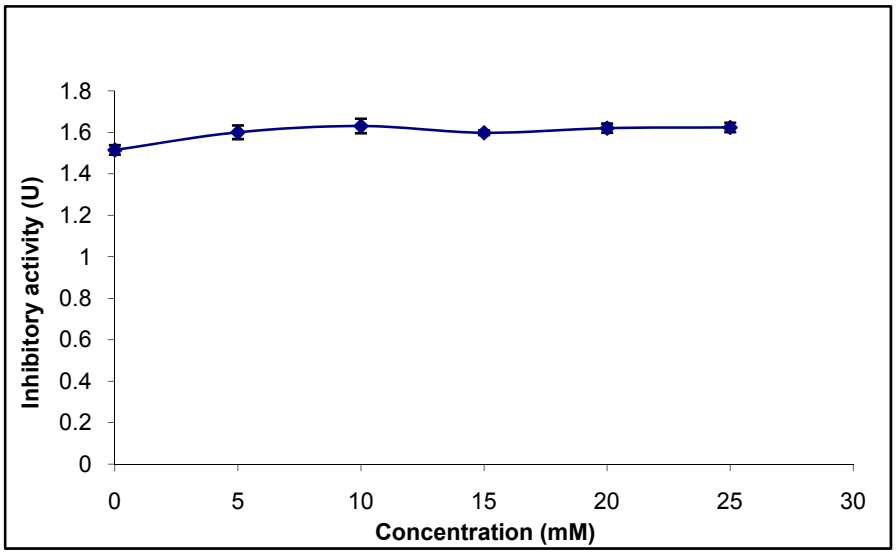


Fig 4.25 c

Fig 4.25 Effect of chemical modification on protease inhibitory activity

(a) Effect of PMSF **(b)** Effect of N-Ethylmaleimid **(c)** Effect of DEPC.

4.9.13 Effect of acid treatment on protease inhibitor

Acid treatment on protease inhibitor did not show any effect up to concentration of 0.04 M HCl. Above that a sudden decrease in the activity of protease inhibitor along with increase in the concentration was recorded and a complete inactivation of the inhibitor was noticed at 0.08 M HCl (Fig. 4.26).

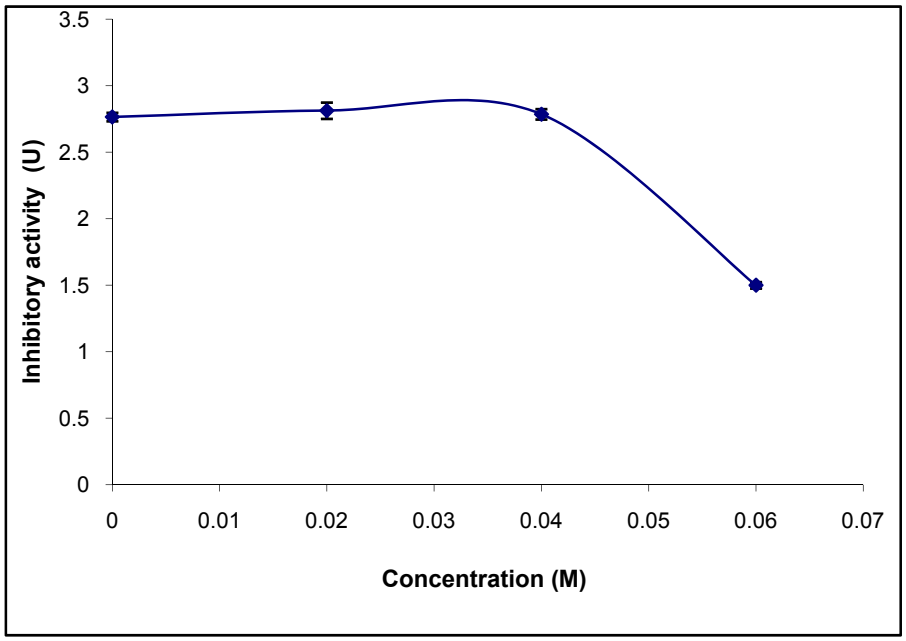


Fig 4.26 Effect of increasing concentration of HCl on the activity of protease inhibitor

4.9.14 Binding studies of inhibitor using flourimetry

Results presented in the Fig. 4.27 shows the binding of inhibitor with trypsin. The emission spectrum at 278 nm of the complex of trypsin with protease inhibitor showed complete loss in fluorescence intensity at 350 nm compared with trypsin alone, where the emission spectrum at an excitation wavelength of 278 nm disclosed a major peak demonstrating the intrinsic fluorescence of tryptophan amino acid residue. The result indicated that the strong binding of inhibitor caused the quenching of tryptophan fluorescence of trypsin.

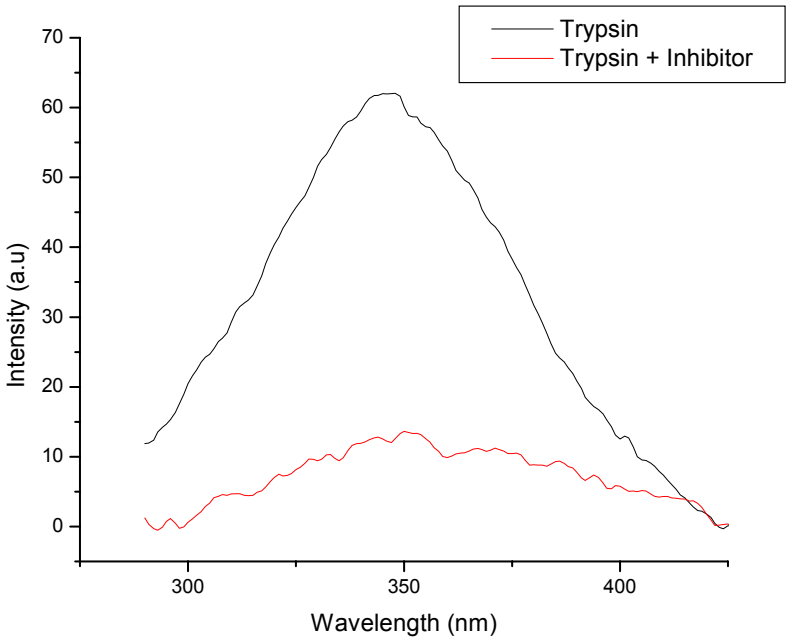


Fig 4.27 Fluorescence analysis of trypsin and trypsin-inhibitor complex

4.9.15 Peptide mass fingerprinting

Peptide mass fingerprint produced by MALDI-TOF-TOF of protease inhibitor was evaluated with the Mascot sequence matching software in Swiss-Prot database, did not match any of the inhibitors, but identified peptide sequence homology (31% sequence coverage) to Glycine cleavage system H protein of *P. mendocina* with matched sequence in bold underlined is as follows.

1 MSNIPADLRY AASHEWARLE ADGSVTVGIS DHAQEALGDV VFIELPEVGK
51 QLDAGQEAGV VESVKAASDI YAPVGGEVIA INEALVDSPE SVNSDPYGSW
101 FFKLKPSDAS ELDKLLDASA YQAAADADA

4.9.16 Specificity of the inhibitor

The evaluation of inhibitory activity of *P. mendocina* protease inhibitor with different serine proteases indicated high specificity towards trypsin. The inhibitor did not show inhibitory activity towards elastase, proteinase K, subtilisin and chymotrysin.

4.9.17 Stoichiometry of protease-protease inhibitor interaction

The data obtained for the studies carried out on protease-protease inhibitor interaction is illustrated in Fig. 4.28. 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 11.67 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 0.48 nM.

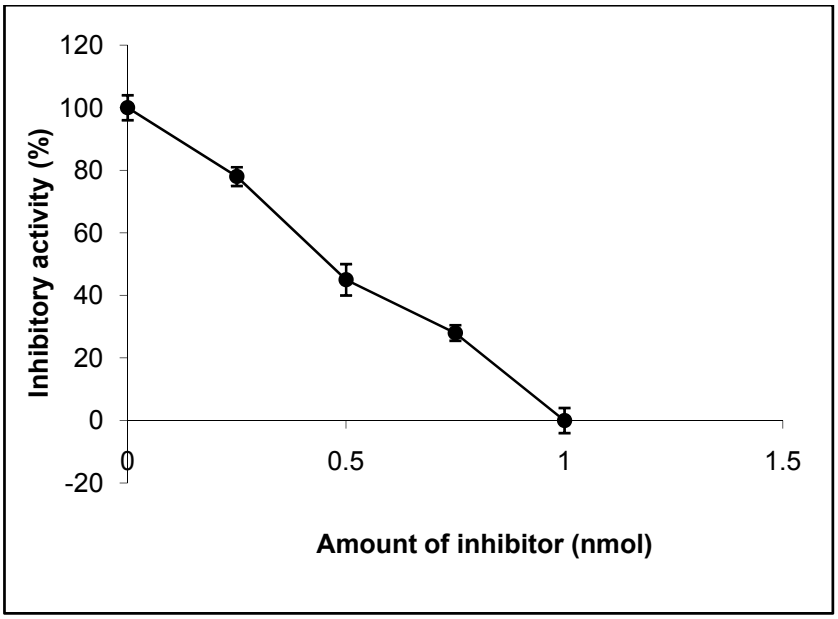


Fig 4.28 Stoichiometry of protease-protease inhibitor interaction

4.9.18 Kinetic studies of inhibition

From the primary plots obtained it was inferred that *V*max did not udergo change while *K*m changed alongwith change in the inhibitor concentration suggesting the competitive nature of the inhibitor. From the data presented in the Fig. 4.29 for the secondary plot of the inhibition kinetics, it was inferred that identical concentration of trypsin (1 nM) preincubated with different concentrations of inhibitor (0.13, 0.325 and 0.65 nM) yielded different slopes for plots 1/*v* versus 1/[s] for six different [s] values. Further 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 3.46 x 10^{-10} M under the assay conditions.

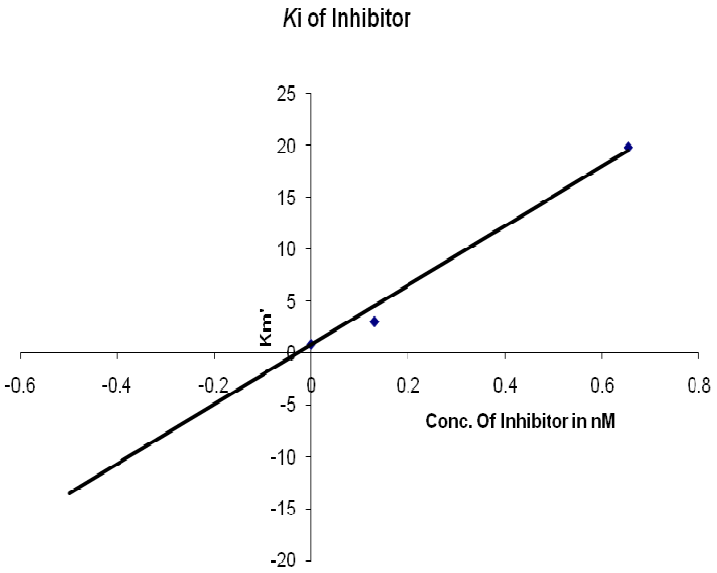


Fig 4.29 Secondary plot of the inhibitor

4.10 APPLICATION STUDIES

4.10.1 Protease inhibitor as seafood preservative

The potential of protease inhibitor as preservative for sea food preservation was assessed using shrimp, *Peneaus monodon.* Viable microbial flora naturally present on shrimp was isolated and assessed for their ability to produce protease enzyme, the major enzyme responsible for food spoilage. Among one hundred and ten bacterial isolates 98 bacteria were found to produce enormous quantities of protease enzyme in skimmed milk agar plates (Fig 4.30). The inhibitor was evaluated for its activity against these spoilage proteases by caseinolytic assay. The data obtained showed that the protease inhibitor was active against 61 of spoilage bacterial proteases (Table 4.6).

Table 4.6 Activity of *P. mendocina* protease inhibitor towards spoilage bacterial proteases

Serial number	Strain designation	Inhibitory activity (u)	Serial number	Strain designation	Inhibitory activity (u)
1	SPBt 1	42.2	25	SPBt 30	63.4
2	SPBt 3	51.4	26	SPBt 31	13
3	SPBt 4	28.6	27	SPBt 32	21
4	SPBt 6	64.29	28	SPBt 33	18.23
5	SPBt 7	29.87	29	SPBt 34	31
6	SPBt 8	19	30	SPBt 36	42.25
7	SPBt 9	11	31	SPBt 37	56
8	SPBt 10	13.09	32	SPBt 38	27
9	SPBt 11	30.9	33	SPBt 41	17.3
10	SPBt 12	33.7	34	SPBt 43	16.31
11	SPBt 13	21.44	35	SPBt 44	22.56
12	SPBt 14	50.47	36	SPBt 45	31.33
13	SPBt 15	27.7	37	SPBt 46	14
14	SPBt 17	33	38	SPBt 47	50.11
15	SPBt 18	41.3	39	SPBt 49	18
16	SPBt 19	55	40	SPBt 51	34.9
17	SPBt 21	45.6	41	SPBt 53	47
18	SPBt 22	41.2	42	SPBt 54	19.3
19	SPBt 23	28	43	SPBt 55	38.1
20	SPBt 25	39	44	SPBt 56	54
21	SPBt 26	14.06	45	SPBt 59	26.07
22	SPBt 27	28	46	SPBt 60	27.33
23	SPBt 28	51	47	SPBt 63	14.56
24	SPBt 29	45	48	SPBt 67	13.96

Serial number	Strain designation	Inhibitory activity (u)	Serial number	Strain designation	Inhibitory activity (u)
49	SPBt 69	21.5	62	SPBt 94	41.44
50	SPBt 70	36	63	SPBt 95	36.78
51	SPBt 71	33.45	64	SPBt 97	38.44
52	SPBt 73	29.4	65	SPBt 98	18.63
53	SPBt 74	44.89	66	SPBt 99	28.92
54	SPBt 77	9.41	67	SPBt 100	36.24
55	SPBt 78	20.6	68	SPBt 101	44
56	SPBt 79	24	69	SPBt 103	25.36
57	SPBt 85	46.32	70	SPBt 104	18
58	SPBt 86	59	71	SPBt 105	19.3
59	SPBt 87	18.7	72	SPBt 106	47.1
60	SPBt 90	19.35	73	SPBt 108	48
61	SPBt 93	49	74	SPBt 110	23.43

Shrimps were treated with protease inhibitor and the studies were carried out by incubating the samples at different storage temperatures. It was found that protease inhibitor influenced the total viable microbial count on the sample. The results showed that there was a considerable decrease in microbial population in the samples treated with protease inhibitor, compared to the untreated samples (Fig 4.31).

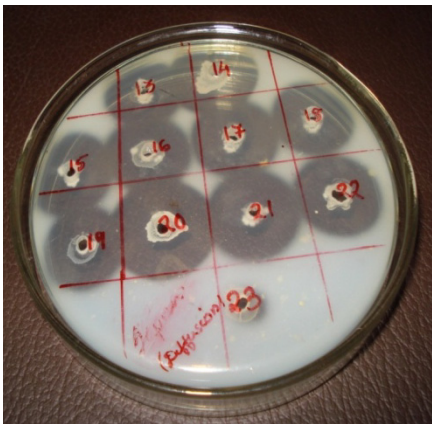
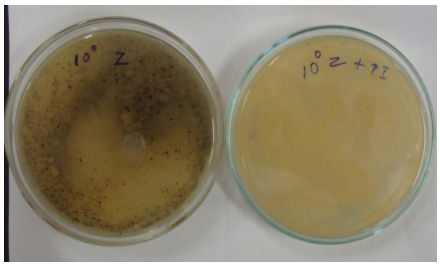


Fig 4.30 Evaluation of the spoilage microbial flora for protease production.



Untreated Treated with protease inhibitor

Fig 4.31a

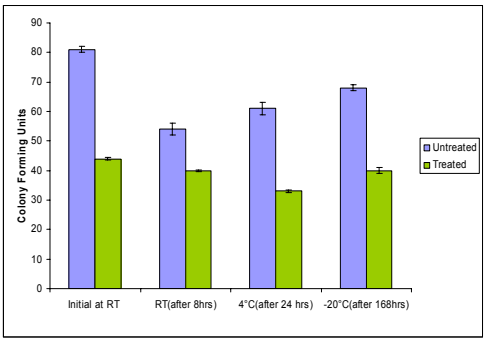


Fig 4.31b

Fig 4.31 Comparison of the microbial flora of the protease inhibitor treated and untreated *Peneaus monodon.* (a) Plates showing the difference in microbial load. **(b)** Evaluation of microbial load at different storage temperatures.

The total protein of both control and test samples were estimated and compared to evaluate the protein degradation. The protein content of both

inhibitor treated and untreated samples are depicted in Table 4.7. From the data obtained, it is evident that there is a considerable reduction in protein degradation of the treated sample compared to control at room temperature after 8 hours of incubation. When the samples were incubated at lower temperature for longer time it was noted that the protein degradation is negligible in the case of inhibitor treated samples compared with untreated samples.

Table 4.7 Effect of Protease Inhibitor on the protein degradation of *Peneaus monodon*

Sample	**Protein content (mg/mL)**			
	Initial 0 h	28± 2°C after 8 h	4°C after 24 h	-20°C after 168 h
Untreated *Peneaus monodon* peeled	14.03	9.2	11.5	11.83
Treated *Peneaus monodon* peeled	14.12	13.22	13.73	13.86

4.10.2 Protease inhibitor as antibacterial agent

Purified inhibitor preparation was checked for its ability to inhibit bacterial growth by growth inhibition method. The inhibitory activity was evaluated against 17 different standard bacterial cultures. Among them the inhibition towards *Bacillus cereus* was evident from the result obtained for growth inhibition studies (Fig 4.32). The minimum inhibitory concentration (MIC) of the inhibitor was found to be 80 μg/well.

Fig 4.32 Growth inhibition of *Bacillus cereus* using protease inhibitor from *P. mendocina* BTMW 301

Chapter 5

DISCUSSION

5.1 SCREENING OF MICROORGANISMS FOR PROTEASE INHIBITOR PRODUCTION AND PROCESS OPTIMIZATION

The microorganisms are recognized as important sources of protease inhibitors which are valuable in the fields of medicine, agriculture and biotechnology. The protease inhibitors of microbial origin are found to be versatile in their structure and mode of inhibition that vary from those of other sources. Although surplus of low molecular weight non-protein protease inhibitors from microorganisms have been reported, there is a dearth of reports on proteinaceous protease inhibitors. The search for new metabolites from marine organisms has resulted in the isolation of more or less 10,000 metabolites (Fuesetani and Fuesetani, 2000) many of which are gifted with pharmacodynamic properties. The existence of marine microorganisms was reported earlier, and they were found to be metabolically and physiologically dissimilar from terrestrial microorganisms. Marine microorganisms have potential as important new sources of enzyme inhibitors and consequently a detailed study of new marine microbial inhibitors will provide the basis for future research (Imada, 2004).

In the present study, bacteria, actinomycetes and fungi isolated from different locations of Cochin backwaters, Kerala were screened for protease inhibitors towards identification of new potential strain as source for protease inhibitor production. The primary screening of protease inhibitor on skimmed milk agar plates was carried out and the shortlisted cultures were subjected to caseinolytic liquid assay. The isolates with considerable inhibitory activity were shortlisted and further screened after partial purification using ammonium sulphate precipitation. The precipitate was dissolved and dialysed against phosphate buffer of pH 7.5. Protease inhibitor from *Moringa oleifera* (Bijina et al., 2011b) and

Streptomyces sp. (Pandhare et al., 2002) were resuspended and dialysed in phosphate buffer. The optimum activity of trypsin protease is at pH 7.5. So the pH of the phosphate buffer was selected as 7.5.

Among the four bacterial isolates shortlisted, bacterium BTMW 301 was selected as the potential source since it recorded the highest activity compared to others. This strain was identified as *Pseudomonas mendocina* by 16S ribotyping since it showed very close similarity with already existing 16S sequences of *P. mendocina* in the NCBI database. Species of the genus *Pseudomonas* are metabolically very versatile and are encountered in most natural environments, from freshwater to hypersaline habitats (Holt et al., 1994). *P. mendocina* is a salt tolerant bacterium (Palleroni et al., 1984) with high potential for bioremediation of contaminated soils and waters (Meer et al., 1992). Although there are reports available on protease inhibitor produced by marine *Pseudoalteromonas sagamiensis* (Takeshi et al., 2003), so far no protease inhibitor has been reported to be produced by *P. mendocina.* In this context the present investigation indicates the potential of *P. mendocina* as a dependable source of protease inhibitor.

Evaluation of time course of production of protease inhibitor in Zobell marine broth indicated that maximal production was at 48 h of incubation, i.e. during the commencement of stationary phase. Process optimization studies performed towards maximal production of protease inhibitor using minimal medium evidenced that protease inhibitor production was influenced by incubation temperature, media pH, NaCl concentration, carbon sources, nitrogen sources and inoculum concentration. A previous study on the production of protease inhibitor by an actinomycetes sp. showed that the production of inhibitor depends upon nitrogen and carbon sources (Pandhare et al., 2002). Among the different carbon sources studied, 25 mM glucose induced maximum production of protease inhibitor. Study conducted on the effect of additional NaCl concentrations on protease inhibitor production with the selected carbon source showed that the

production was maximum at 3% NaCl. In fact *P. mendocina* was reported as salt tolerant bacterium (Pocard et al., 1994). The tolerance to higher NaCl concentration is an added advantage when the protease inhibitor finds application as food preservative, as the protease inhibitor produced by this hyper salt tolerant strain is likely to serve a preservative function in salt preserved foods as well as for sea foods.

Temperature is a significant factor that has to be regulated. Hence temperature optimization studies for maximal production of protease inhibitor was done and it was observed that maximal production occurred at 30^{o}C, which is ambient temperature and hence an advantage considering the production economy. *P. mendocina* is capable of producing protease inhibitor over a broad range of pH from pH 3 to 10 although the production was very less in acidic pH conditions compared to neutral and alkaline pH ranges. Varying concentrations of inoculum were added to the production media to analyse the effect of inoculum concentration on the production of protease inhibitor by *P. mendocina.* The protease inhibitor production was found to be maximum when 4% inoculum was used. Above that concentration decline in inhibitory activity along with increase in inoculum concentration was noticed. For 10% of inoculum, the production of inhibitor was lesser than that produced for 2% inoculum. This decrease may be attributed to the over populated culture and nutrient limitation in the media. Among the nitrogen sources tested, the maximal protease inhibitor production was recorded when malt extract was used in the production media.

5.2 PURIFICATION AND CHARACTERIZATION OF PROTEASE INHIBITOR

Protease inhibitor obtained from *P. mendocina* was purified by ammonium sulphate precipitation, followed by DEAE sepharose ion exchange chromatography and trypsin affinity chromatography. *P. mendocina* protease inhibitor was precipitated efficiently with 0-30% saturation of ammonium

sulphate. The inhibitor eluted out from trypsin affinity column yielded a single protein fraction with maximum protease inhibitory activity. The homogeneity of this fraction was further proved by reverse phase high pressure liquid chromatography and single band was obtained after polyacrylamide gel electrophoresis, reconfirming its homogeneity. From the results obtained it is evident that the fold and yield of protein can be enhanced by repetitive purifications using combinations of several advanced purification techniques. Purification of protease inhibitors from skin extract of Atlantic salmon (*Salmo salar* L.) was carried out in four chromatographic steps: papain-affinity chromatography, gel filtration, anion-exchange chromatography and reversed phase chromatography (Anne et al., 1999) and protease inhibitor from potato tubers (*Solanum tuberosum*) was purified through extraction of the water-soluble fraction, dialysis, ultra filtration, DEAE-cellulose and C18 reversed-phase high performance liquid chromatography (Mi-Hyun et al., 2006).

Protease inhibitor purification can be significantly improved by the use of affinity chromatography techniques, where the binding agents are particular proteases (Araújo et al., 2005). Trypsin affinity chromatography was found to augment the fold of purification of trypsin inhibitors (TI) from wild-type soybean (*Glycine soya*) (WBTI) and domesticated soybean (*Glycine max*) (SBTI) using chitosan resin-trypsin on the affinity chromatography column (Zhang et al., 2009). A trypsin inhibitor was purified from *Sapindus saponaria* seeds (SSTI) using a trypsin-Sepharose column, where the inhibitor was eluted with 0.01 M HCl (Macedo et al., 2011). Trypsin agarose affinity chromatography was used for the isolation of a protein from *Helianthus annuus* flowers (Giudici et al., 2000). The purity of inhibitor was further confirmed by reverse phase HPLC. Homogeneity of the proteinase inhibitor from the marine annelid *Hermodice carunculata* was confirmed by reversed phase high performance liquid chromatography (RP-HPLC) on C-18 column (Isel et al., 2004).

The purity and homogeneity of the affinity chromatography purified inhibitor was proved by single band obtained in both Native-PAGE and SDS-PAGE. The SDS-PAGE analysis of the purified proteinaceous protease inhibitor molecule evidenced the single polypeptide nature of the inhibitor. An apparent molecular weight of 22 kDa was obtained in SDS-PAGE, both under non-reducing and reducing conditions. Whereas the intact molecular mass by MALDI-TOF analysis indicated a value of 11.567 kDa. It was considered that the MALDI-TOF analysis is a more precise method for molecular weight determination with a mass accuracy of 0.05-0.1% (Jany et al., 1986). The occurrence of a high molecular weight protein form along with protease inhibitor (PISC-2002) of 11.264 kDa isolated from *Streptomyces chromofuscus* is explained as a thin band of molecular mass 25 kDa. This band was observed which corresponded to a dimer of the inhibitor. This band did not disappear when electrophoresis was performed in the presence of β-mercaptoethanol (Angelova et al., 2006). The molecular mass of the present inhibitor is different from protease inhibitor (ecotin) isolated from Gram-negative bacteria *E. coli,* which is a homodimer of 16 kDa subunits (Maurizi, 1992). A 12 kDa protease inhibitor was isolated and studied from the entemopathogenic bacterium *Photorhabdus luminescens* (Wee et al., 2000). Marinostatin is a low molecular weight peptide inhibitor and monastatin is a novel glycoprotein with a molecular weight of 20 kDa. Both were isolated from marine bacterium *Alteromonas* sp. (Imada et al., 1986a ; Imada et al., 1985a). A serine protease inhibitor was obtained from a metagenomic library from uncultured marine microorganisms with a predicted molecular mass of about 28.7 kDa (Cheng-Jian Jiang et al., 2011). The serine protease inhibitors isolated from silk worms (pacifastin family) have molecular weight of 4 kDa and Kunitz inhibitors are in the range of 18-26 kDa (Clynen et al., 2005). 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).

Most preferred criteria for distinguishing proteins are the isoelectric point (pI). Protease inhibitors exhibit a range of pI from acidic to alkaline with respect to the source and type of inhibitor. In the present study isoelectric point of the inhibitory protein resolved on the IPG strip was 3.8, whereas isoelectric point of the protease inhibitor isolated from *Manduca sexta* is 5.4 (Wang and Jiang, 2004) and *Streptomyces chromofuscus* is 7.4 (Angelova et al., 2006). Kunitz type inhibitors generally show a pI of acidic nature. Three isoforms of protease inhibitors, ApTIA, ApTIB and ApTIC from *Acacia plumose* resolved on isoelectric focusing gel had isoelectric points of an acidic nature: 5.05, 5.25 and 5.55, respectively (Laber et al., 1989; Lopes et al., 2009). Serine protease inhibitors from the basidiomycete *Clitocybe nebularis* CnSPIs, have isoelectric points 4.8 and 5.2 (Avanzo et al., 2009). The isoelectric point of protease inhibitor isolated from the seeds of pearl millet (*Pennisetum glaucum*) was 9.8 indicating its basic nature (Joshi et al., 1998). Serine protease inhibitors (ISPI-1, 2, 3) purified from larval hemolymph of greater wax moth larvae, *Galleria mellonella* had isoelectric points ranging between 7.2 and 8.3 (Andreas et al., 2000).

Protease inhibitory activity was confirmed on X-ray film by Dot-blot analysis. The results indicated that the protease inhibitor blocked the hydrolysis of gelatin on X-ray film by inhibiting trypsin, in the same way as that of the control protease inhibitor soya bean trypsin inhibitor (SBTI). Thermal stability increases the efficiency of proteins and is one of the essential features for their commercial exploitation (Pandhare et al., 2002). In this respect thermal inactivation studies of protease inhibitor isolated from *P. mendocina* was carried out by evaluating its activity and stability at wide range of temperatures varying between 4^{o}C to 100^{o}C. Results demonstrated considerable stability of the inhibitor up to 90^{o}C although maximal stability of the protease inhibitor was observed at 4^{o}C. However a progressive decline in activity was noted after pre-incubation at temperatures above 40°C. Considerable decrease in activity was observed at 60°C to 90°C for 1 h and a complete loss of activity at 100°C. From these observations it is evident

that the protease inhibitor has high intrinsic stability in its native state, which imparts a high degree of thermal stability. The protease inhibitor of bacterium *Photorhabdus luminescens* was inactivated at temperatures above 50^0 C (Wee et al., 2000). Kinetic studies reveal the competitive nature of the inhibitor and shows high specificity to trypsin. Protease inhibitors API-I, API-II and API-III were isolated from three different actinomycetes strains showed different properties in their molecular nature as well as pH and temperature stabilities (Pandhare et al., 2002). *Streptomyces chromofuscus* protease inhibitor (PISC-2002) was stable over pH 2–10 and at temperatures 80 °C/30 min, mainly due to the presence of proline and of a high content of hydrophobic amino acids (Angelova et al., 2006). Proteases of the larger grain borer *Prostephanus truncatus* (Coleoptera: Bostrichidae) was inhibited by an 8.7 kDa protease inhibitor isolated from chia seeds (*Hyptis suaveolens* L.). This inhibitor was found to be stable over wide range of temperatures, from 4^oC-95^oC (Aguirre et al., 2004). Buckwheat *Fagopyrum esculentum* was found to be a rich source of low molecular weight protein inhibitors of serine proteinases which possessed high pH-stability in the pH range 2-12 and thermo stability (Tsybina et al., 2004).

pH stability studies were conducted using different buffer systems. The result showed that the protease inhibitor was stable over a broad range of pH showing considerable protease inhibitor activity over a range of 4-11. The inhibitor showed maximum stability at pH 7, similar to that of the inhibitor produced from the clone obtained from marine metagenome (Cheng-Jian Jiang et al., 2011), the enteropathogenic bacterium *Photorhabdus luminescens* (Wee et al., 2000) and API-III from soil actinomycete (Pandhare et al., 2002). At high alkaline (pH 11-12) and high acidic conditions (pH 2-3) the protease inhibitor was not stable. Although the *P. mendocina* inhibitor was found to be stable in the pH range of 4-11, the activity was decreased when pre incubated at pH levels above pH 7. The protease inhibitor showed sharp decline in stability at pH conditions above pH 10 as the activity reduced steeply at this range.

Intramolecular disulfide bridges are assumed to be responsible for the functional stability of the inhibitor at different temperature, pH, and in the presence of reducing agents (Oliveira et al., 2007). It is presumed that the denaturation of protein inhibitors which happen under strong acidic or alkaline conditions causes the loss of activity partially or completely. In order to control insect pests and pathogens, protease inhibitors of appropriate pH stability and activity are used as antifeedents to target their gut proteases. Lepedopteran and dipteran larvae are voracious plant feeders and have alkaline guts. They depend upon serine proteases like trypsin and chymotrypsin for digestion. But cysteine proteases are predominant in Hemiptera, Coleoptera and Thysanoptera (Sabotič and Kos, 2012). Most inhibitors in the Kunitz family are acidic and some are very sensitive to acidic pH and stable in the alkaline pH (Mello et al., 2002). The stability of *P. mendocina* protease inhibitor in a wide range of pH suggests its use as biopesticides, which can with stand highly alkaline conditions of insects gut flora. The high thermal and pH stabilities of *P. mendocina* inhibitor demonstrated 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.

Metal ions such as Mo^{6+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Ca^{2+}, Al^{3+}, Na^{+}, $Ni^{2+}$and Ba^{2+} were studied to know their influence on the activity of protease inhibitor from *P. mendocina*. Addition of 1 mM Mg^{2+} and 10 mM Ca^{2+} enhanced the protease inhibitory activity. Inductively coupled atomic emission spectroscopy (ICP-AES) analysis of the metal ion concentration of both native and demetallized protein demonstrated the presence of Mg^{2+} and Ca^{2+}. The role 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 was studied (Schirmeister and Peric, 2000).

Detergents act as surfactants as they lower the surface tension of water and mimic the native, hydrophobic environment of the phospholipid bilayer *in vivo*. The ability of detergents to solubilise the protein from lipid membranes and other protein bound membranes is made use in protein extraction processes. To prevent proteolysis, protease inhibitors along with detergents are normally employed in cell lysis buffers. This procedure facilitates membrane protein solubilization in protein purification processes. Nonionic detergents do not interact extensively with the protein surface and they are generally considered as mild detergents. The ionic detergents like SDS, generally bind non-specifically to the protein surface, leading protein unfolding (Mogensen et al., 2005). In the present study, *P. mendocina* protease inhibitor was completely inactivated or reduced the inhibitory activity significantly in the presence of anionic detergent SDS and cationic detergent CTAB at a concentration of 1%. While, the nonionic detergents Triton X and Tween 80 showed a slight decrease in the protease inhibitory activity and Tween 20 lowered the inhibitory activity to half, compared to the control. This indicated that the anionic detergent SDS and cationic detergent CTAB, at 1%, reduced the inhibitory activities significantly probably due to unfavorable electrostatic interactions that might have caused unfolding and/or disrupt trypsin binding. The interaction of mild detergents with hydrophobic amino acids of the inhibitor probably might have changed its conformation in such a way that the binding with trypsin was not disturbed. So only a slight reduction n activity was noticed.

Detergent sensitivity of Soybean Kunitz inhibitor was due to the absence of hydrogen bonded α-helical or β-structure. It was concluded that the inhibitor is stabilized mainly by hydrophobic interactions with the loop and bend structure which forms major conformation in this protein (Jirgensons, 1973). Whereas either sodium dodecyl sulfate (SDS) or sodium deoxycholate (DOC) had no effect on *Cajanus cajan* inhibitor. No significant conformational changes were observed but the inhibitory activity of the PI was decreased significantly in the presence of

DOC. Loss of inhibitory activity without a concomitant loss or change in structure indicated that certain reactive site amino acids are required for inhibiting enzyme activity (Haq and Khan, 2005).

Studies on the effect of oxidizing agents, H_2O_2 and DMSO indicated that they have a negative effect on protease inhibitory activity. A decrease in inhibitory activity with the increase in concentration of oxidizing agents was observed. Lower concentration of H_2O_2 and DMSO did not influence the inhibitory activity much, but above 1% there was a fall in activity in both cases. An abrupt loss of protease inhibitory activity was noticed at a concentration above 1% in the case of H_2O_2 where as a gradual decrease was observed with increase in concentration from 1% to 5% of DMSO. The complete inactivation was noticed when 1.4% H_2O_2 was used, but 6% DMSO was needed to cause total loss of inhibitory activity. It is clear that the oxidation of protease inhibitor by H_2O_2 was stronger than that caused by DMSO. Oxidation inactivation of the inhibitor evidenced the presence of methionine residue at the reactive site of the inhibitor isolated from *P. mendocina.*

Significant reduction in activity was observed in the case of protease inhibitor isolated from *M. oleifera.* The activity was declined along with an increase in the concentration (from 1% to 5%) of oxidizing agents DMSO and H_2O_2 (Bijina et al., 2011a). Oxidation of one of the eight methionine residues of α1-protease inhibitor (α1-PI) (Met358) resulted in complete loss of inhibitory activity of α1-PI toward its primary biological target, elastase (Johnson and Travis, 1979).

Influence of reducing agents on the activity of protease inhibitor depicts that the concentration of above 40 µM dithiothreitol resulted in significant inactivation of the inhibitor. In the case of β-mercaptoethanol reduction in inhibitory activity was observed at a concentration of 50 µM and at concentration higher than 250 µM, there was a sharp decline in the inhibitory activity. Complete inactivation was noticed at 350 µM of dithiothreitol. The intra molecular

disulphide bridges are presumably responsible for the functional stability of Kunitz type protease inhibitor in the presence of reducing agents. Dithiothretol at a concentration of 1 mM had no effect on the activity or stability of *Peltophorum dubium* protease inhibitor (Ligia et al., 2003). A Kunitz type trypsin inhibitor from *Erythrina caffra* retained its inhibitory activity after reduction with dithiothretol (Lehle et al., 1996). Leech carboxypeptidase inhibitor (LCI) was found to have a compact domain with a five-stranded β-sheet and a short α-helix stabilized with four disulfide bonds. The importance of disulfide bonds in LCI for both correct folding and achievement of a functional structure was reported (Arolas et al., 2009). Whereas, lower concentration of dithiothreitol (DTT) had no effect on protease inhibitors isolated from *Peltophorum dubium* and *Erythrina caffra*, (Lehle et al., 1996; Macedo et al., 2003).

Data obtained for the effect of chemical modifiers on the activity of *P. mendocina* protease inhibitor demonstrated that modification of amino acid serine influenced the inhibitory activity since the PMSF modified inhibitor was less active at 1 mM and 2 mM concentrations. Further increase in concentration of PMSF resulted in an enhancement of inhibitory activity. The modification of cysteine with *N*-Ethylmaleimide led to a drastic reduction in the activity even at smaller concentrations. The decline in the inhibitory activity was evident at 10 mM concentration of *N*-Ethylmaleimide. In contrast, the modification of histidine with DEPC had no effect on the activity of the inhibitor. The results indicated the occurrence of serine and cysteine in the reactive site of the inhibitor.

Protease inhibitors are modified by reactive site modification using specific chemicals proved to be a significant tool in specific protein–protein interactions. A covalent binding of chemical reagents with amino acid side chains causes changes in the properties/activity without knowing the protein structure. Lentinus proteinase inhibitor, purified from the fruiting bodies of the edible mushroom, *Lentinus edodes*, was observed to suggested involve one or more arginine residues in the inhibition of trypsin (Odani et al., 1999). Participation of

an arginine residue in amylase inhibitor activity in the case of barley has been described (Abe et al., 1993). Primary sites interacting with the target proteases called as Kunitz-domains, (and determining their protease-specificity) are short segment having 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 reported to be lost after modification of cysteine, arginine or aspartic/glutamic acid residues, where CPI activity was selectively enhanced by modification of histidine or arginine residues (Joshi et al., 1999).

Acid treatment on protease inhibitor did not affect up to 0.04 M concentration of HCl. But further increase in concentration of HCl resulted in a gradual decrease in inhibitory activity and a complete loss of activity was recorded at 0.08 M HCl.

Fluorescence binding studies of the inhibitor isolated from *P. mendocina* with trypsin showed a major peak at 350 nm in the emission spectrum of trypsin demonstrating the intrinsic fluorescence of tryptophan amino acid residue. On comparison with the emission spectrum of the complex of trypsin it was noted that binding of inhibitor resulted in the quenching of tryptophan fluorescence in trypsin. The enhancement in the tryptophan fluorescence of papain observed at 343 nm indicated that on demetallization, the inhibitor was not able to bind with the active site of the enzyme (Joshi et al., 1999). Fluorescence intensity studies of pearl millet cysteine protease inhibitor revealed that removal of a Zn^{2+} atom from the inhibitor resulted in the loss of anti-fungal and protease inhibitor activity with concomitant decline in the fluorescence intensity. This is an indication of quenching of tyrosine fluorescence upon the binding of the metal ion.

Data obtained after peptide mass fingerprinting using Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) of protease inhibitor isolated from *P. mendocina* was analysed with the MASCOT search tool in Swiss-Prot database. But none of the characteristic features of serine protease inhibitors

was found in the peptide similarity search, even though it is a potent inhibitor of trypsin. It is possible that the inhibitory edge of serine protease inhibitors could be reproduced by others. But relatively high similarity (31%) restricted to two regions of the *P. mendocina* inhibitor for glycine cleavage system H Protein of *P. mendocina.* The majority of the peptide sequence data showed similarity to uncharacterized protein from higher to lower level of organisms structural elements as a result of convergent evolution (Brzin et al., 2000). As *P. mendocina* protease inhibitor shows 31% similarity to glycine cleavage system H protein it is concluded that the inhibitor is a novel inhibitor of trypsin showing similarity to glycine cleavage system H protein because of the convergent evolution or it may be associated with the particular glycine cleavage H protein to stabilize or regulate the enzyme complex system (Wolfgang et al., 2006). Further detailed studies are needed to establish the precise physiological function of this new inhibitor in bacteria. Similarly the low molecular weight peptide inhibitor marinostatin isolated from marine bacterium *Alteromonas* sp. has no similarity to the known amino acid sequences of terrestrial inhibitors (Imada et al., 1986a; Imada et al., 1985a) and the protease inhibitor (ecotin) isolated from Gram-negative bacteria *E. coli* does not contain any consensus reactive site sequences of known serine protease inhibitor families, suggesting that ecotin is a novel inhibitor (Maurizi, 1992).

The sequence similarity search of inhibitors from *Bacillus brevis* and *Bacillus subtilis* revealed that these are unrelated to any other peptidase inhibitors (Shiga et al., 1992; Shiga et al., 1995). It is possible that the inhibitory edge of serine protease inhibitors could be reproduced by other structural elements as a result of convergent evolution (Brzin et al., 2000). The other possibility is that the inhibitor may be associated to stabilize the enzyme system from the attack of intracellular proteases. Two serine protease inhibitor genes identified in the cellulosome of *Clostridium thermocellum* indicate that protease inhibitor could play unrecognized roles in protein stability and regulation in bacteria (Schwarz et

al., 2006). The mass spectra obtained after tryptic digestion (peptidemass fingerprint) of inhibitor isolated from *Solanum tuberosum* cv. Desirée and analysed with the "MASCOT search tool" also did not match to any of the inhibitors of other plants (Obregón et al., 2012).

Data obtained for the specificity studies conducted for the isolated inhibitor from *P. mendocina* showed remarkable specificity towards trypsin. Specificity studies showed no detectable inhibition against elastase, proteinase K, subtilisin and chymotrypsin. A broad spectrum inhibitor isolated from the bacterium *Photorhabdus luminescens* exhibited inhibitory activity towards proteases like trypsin, elastase, proteinase, cathepsin G, in addition to proteases from *Ph. luminescens and X. nematophila* (Wee et al., 2000). Ecotin is a serine protease inhibitor from the periplasmic space of *E. coli* (Chung et al., 1983). Ecotin is unique among proteinaceous protease inhibitors in that it binds in a substrate-like manner but has a very broad specificity for almost all serine proteases in the chymotrypsin-trypsin-elastase super family (Gillmor et al., 2000). 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). Human WFIKKN-KU2 protein domain which is a Kunitz-type protease inhibitor protein displayed remarkable 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. mendocina* inhibitor was found to be 3.46 x 10^{-10} M from secondary plot. The extracellular protease inhibitor, EPI1, from *P. infestans* having inhibitory constant (Ki) for subtilisin A inhibition was determined at 2.77 +/- 1.07 nM. 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). 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). The serine protease inhibitor protein obtained from a gene *Spi1C* from a metagenomic library of uncultured marine microorganisms exhibited inhibitory activity against α-chymotrypsin and trypsin with *K*i values of around 1.79×10^{-8} and 1.52×10^{-8} M, respectively (Cheng-Jian Jiang et al., 2011). Where as the protease inhibitor (PISC-2002) from culture supernatants of *Streptomyces chromofuscus* inhibited subtilisin, proteinase K, trypsin and proteinase of *S.albovinaceus* strong, with *K*i of micromolar range and weakly inhibited pepsin (Angelova et al., 2006).

The kinetic studies of *P. mendocina* inhibitor with trypsin were carried out. The mode of inhibition (competitive, uncompetitive or non-competitive) was studied by plotting a Lineweaver–Burk curve, 1/*v* versus 1/[s]. For that identical concentration of trypsin (1 nM) was preincubated with enzyme buffer alone and with different concentrations of inhibitor (0.13, 0.325 and 0.65 nM). This 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 3.46 x 10^{-10} M under the assay conditions. The low *K*i values indicated a relatively high affinity of the inhibitor for the enzyme. The kinetic studies suggest the competitive nature of the inhibitor since *V*max is not changing but *K*m changes with the inhibitor concentration. In fact protease inhibitors from plants and microorganisms were characterized by either a reversible or irreversible mechanism (Polgar, 1989).

The trypsin-protease inhibitor interaction studies showed that 1 nM trypsin was completely inhibited by 1 nM of the inhibitor indicating that extrapolation to zero protease activity (100% inhibition) corresponds to 1 nM of

inhibitor. The amount of inhibitor required for 50% inhibition (IC_{50}) of trypsin calculated from the graph, was 0.48 nM. The purified protease inhibitor from the bacterium *Photorhabdus luminescens* inactivated the homologous protease with an almost 1:1 stoichiometry (Wee et al., 2000). The stoichiometry of trypsin-inhibitor interaction of 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), were in the stoichiometric ratio of 1:1 (Avanzo et al., 2009; Bhattacharyya et al., 2006 ; Odani et al., 1999).

5.3 APPLICATION STUDIES

Food spoilage is mainly due to changes in sensory characteristics as a result of protein hydrolysis and is generally an undesirable process. Changes in myofibrillar proteins which affect the quality of muscle have been related to proteolytic activity (Jasra et al., 2001). The protease-producing organisms are responsible for the fish and shrimp muscle degradation during preservation (Chandrasekaran, 1985). They thrive where food and water are present and the temperature is suitable (Kumar et al., 2011) In order to prevent the development of characteristic spoilage off-flavours and off-odours, so many methods are employed. Although the use of chemical preservatives are the most widely used method, an increasingly health conscious public may seek to avoid foods that have undergone extensive processing or which contain chemical preservatives. Therefore, the use of protease inhibitor as preservative will be advantageous and could be exploited by the food industry as a tool to control undesirable bacteria in a food-grade and natural manner, which is likely to be more acceptable to consumers.

Protease inhibitors isolated from Legumes are found to have inhibitory effects on the extracts of fish enzymes (Soottawat et al., 1999). In the present investigation, *P. mendocina* protease inhibitor was analyzed for its efficacy as

shrimp preservative. The experiments were carried out on the basis of the ability of the inhibitor to inhibit microbial proteases responsible for spoilage and resulted in a reduction in microbial population. Total viable microbial flora naturally present along with shrimp was found as producers of protease enzyme. Among the 110 proteases isolated, the inhibitor was found to be active against 55% of spoilage bacterial proteases. A reduction in the number of microbial flora by the action of inhibitor studied at different storage duration and temperatures indicated that *P. mendocina* protease inhibitor has the potential to be used as natural sea food preservative.

Storage experiments on protein degradation were carried out on fresh shrimp tissue in the absence as well as in the presence of inhibitor at various storage conditions. The results demonstrated that the total protein present in the protease inhibitor treated *Peneaus monodon* was higher compared to control. At room temperature the *P. mendocina* protease inhibitor could prevent the proteolysis to a greater extent since a 29% increase in protein content in the inhibitor treated sample was noted compared to the untreated sample after 8 h of incubation. A 16% increase was noted in the protein content of the inhibitor treated shrimp tissue at 4°C after 24 h of incubation compared to the untreated sample and a 14.3% increase at -20^{o}C for 168 h. When the samples were incubated at lower temperature for longer time it was found that the protein degradation is negligible in the case of inhibitor treated samples compared with untreated samples.

Antimicrobial protease inhibitors have captured the attention on account of their therapeutic implications associated with the protection of our body against microbial attack. Microbes are the richest source of anti microbial proteins. The potent anti fungal protein isolated from broad bean is a trypsin-chymotrypsin inhibitor (Banks et al., 2002; Marcela et al., 2000). A bifunctional inhibitor isolated from an extremophilic *Bacillus* sp. was able to inhibit fungal growth (Dash and Rao, 2001). In the present study *P. mendocina* protease inhibitor was

analysed for antibacterial property. Results indicated that the inhibitor is capable of inhibiting *Bacillus cereus* with a minimum inhibitory concentration of 80 µg/well. It has been reported that that the bacterial isolate 6A3 (identified as *Chromohalobacter* sp.) isolated from sponge *X. testudinaria* produced protease inhibitor activity (93.5% activity) against the protease produced by *P.aeruginosa* (Wahyudi et al., 2010). Protease inhibitor monastatin isolated from the marine bacterium *Alteromonas* sp. was found to have inhibitory activity against crude proteases from pathogenic fish bacteria such as *Aeromonas hydrophila* and *Vibrio anguillarum* (Imada, 2004; Imada et al., 1985c).

P. mendocina protease inhibitor together with its broad pH and temperature stability make it an ideal candidate for its exploration in various biotechnological applications especially in food industry and as biocontrol agent in pharmaceutical industry. Being of microbial origin it can be conveniently subjected to various recombinant techniques with minimum genetic manipulations.

Chapter 6

SUMMARY AND CONCLUSION

Bacteria, fungi and actinomycetes of Cochin backwaters, Kerala were screened for their protease inhibitory activity. Among the microorganisms studied, the bacterial strain BTMW 301 was selected as the potential source of protease inhibitor based on its highest activity. The selected bacterium was identified as *Pseudomonas mendocina* by 16S ribotyping. The sequence showed 96% similarity with already available sequences of *P. mendocina* and was deposited in the NCBI GenBank (Accession No: GU139342). The strain was deposited at Microbial Culture Collection unit of National Centre for Cell Science (NCCS), Pune, with the accession number MCC2069.

Time course study revealed that the protease inhibitor production by the bacterium *P. mendocina* BTMW 301 was maximal at 48 h of incubation. Different process parameters affecting protease inhibitor production were optimized individually towards maximal production. Incubation temperature of 30^{o}C, pH 7.0, 3% additional NaCl, 25 mM glucose, 1% malt extract, 4% inoculum and 48 h of incubation were observed to be the optimal conditions that supported maximum protease inhibitor production by *P. mendocina* BTMW 301.

The protease inhibitor isolated from *P. mendocina* BTMW 301 was purified up to homogeneity employing ammonium sulphate precipitation, followed by ion exchange chromatography and affinity chromatography. It was observed that 0-30% saturation of ammonium sulphate was required for the complete precipitation of the protease inhibitor. This fraction was further purified by DEAE Sepharose ion exchange chromatography and trypsin affinity chromatography to get a homogenous protein. The fold of purification of protease inhibitor obtained for ammonium sulphate precipitation, DEAE Sepharose chromatography and trypsin affinity chromatography were 3.257, 4.769 and 106.71 respectively. The purity of the protease inhibitor was further confirmed by

a single band in native PAGE as well as by reverse phase high pressure liquid chromatography, which showed a single peak.

SDS-PAGE under non reducing and reducing conditions yielded a single band which indicated the single polypeptide nature of the inhibitor. Electrophoretic mobility analysis showed that the *P. mendocina* protease inhibitor has an apparent molecular mass of 22 kDa. Whereas, the intact molecular mass of the inhibitor protein was confirmed as 11.567 kDa by MALDI-TOF analysis. MALDI-TOF analysis is a more precise method for molecular weight determination with a mass accuracy of 0.05-0.1% and hence the molecular mass of *P. mendocina* protease inhibitor was considered as 11.567 kDa.

Isoelectric focusing confirmed that pI of the inhibitory protein is 3.8. A single band obtained in 2-D electrophoresis further testified the purity of the protease inhibitor protein. Dot-blot analysis performed on X-ray film testified that the protease inhibitor blocked the gelatin hydrolysis by trypsin similar to that of the soya bean trypsin inhibitor (SBTI) which was used as control.

Maximal stability of the protease inhibitor was observed at 4^{o}C but a progressive decline in activity was evidenced after pre-incubation at temperatures above 40°C. Considerable decrease in activity was observed at 60°C - 90°C after incubation for 1 h and a complete loss of activity at 100°C after 60 min. The protease inhibitor was found to be stable over a broad range of pH showing considerable protease inhibitor activity over a range of 4-11. The inhibitor showed maximum stability at pH 7. However, the activity was observed to get diminished when pre incubated at pH levels above pH 7. Moreover, the protease inhibitor showed sharp decline in stability at pH conditions above pH 10 as the activity reduced steeply at this range.

Additional supplementation of 1 mM Mg^{2+} and 10 mM Ca^{2+} enhanced the protease inhibitory activity. Inductively coupled atomic emission spectroscopy (ICP-AES) analysis of the metal ion concentration of both native and demetallized

protein demonstrated the presence of Mg^{2+} and Ca^{2+} as the integral part of the protein.

The protease inhibitor was completely inactivated in the presence of anionic detergent SDS and cationic detergent CTAB at a concentration of 1%. While, the nonionic detergents Triton X and Tween 80 showed a slight decrease in the protease inhibitory activity and Tween 20 lowered the inhibitory activity to half, compared to the control.

Studies on the effect of oxidizing agents, H_2O_2 and DMSO on protease inhibitory activity indicated that they have a negative effect on protease inhibitory activity. The complete inactivation was noticed at 1.4% H_2O_2 while 6% DMSO caused total loss of inhibitory activity. It was inferred that the oxidation of protease inhibitor by H_2O_2 was stronger than that caused by DMSO.

Studies on the influence of reducing agents on the activity of protease inhibitor revealed that the inhibitory activity was slightly increased by dithiothreitol up to a concentration of 30 mM, and at higher concentrations a considerable inactivation of the inhibitor was noticed. β-mercaptoethanol caused complete inactivation at 350 mM of β-mercaptoethanol.

Chemical modification of amino acid serine in the inhibitor protein molecule led to a reduction in the activity at a lesser concentration and enhancement of activity was noticed at higher concentrations. The modification of cysteine with *N*-Ethylmaleimide led to a drastic reduction in the activity even at smaller concentrations while modification of tryptophan with *N*-Bromosuccinamide did not show any effect on the inhibitory activity. Similarly modification of histidine with DEPC had no effect on the activity of the inhibitor. These results indicated the presence of serine and cysteine residues in the reactive site of the inhibitor.

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. Whereas the complete loss of activity was recorded in 0.08 M HCl.

Fluorescence binding studies of the inhibitor with trypsin endorsed strong binding of protease inhibitor with trypsin as the emission spectra of trypsin was quenched on binding with the inhibitor.

But none of the characteristic features of serine protease inhibitors was found in the peptide similarity search, even though it is a potent inhibitor of trypsin. But relatively high similarity (31%) restricted to two regions of the *P. mendocina* inhibitor for glycine cleavage system H Protein of *P. mendocina.* Hence was concluded that the inhibitor is a novel inhibitor of trypsin and may show similarity to glycine cleavage system H protein because of the convergent evolution or it may be associated with the particular glycine cleavage system H protein to stabilize or regulate the enzyme complex system.

Protease inhibitor was found to show remarkable specificity towards trypsin. Specificity studies carried out with elastase, proteinase K, subtilisin and chymotrypsin showed no detectable inhibition against these proteases.

The inhibitory constant *K*i of the inhibitor was found to be 3.46 x 10^{-10}M and the predicted stoichiometry of trypsin–protease inhibitor interaction were observed as 1:1. Hence 11.567 g of protease inhibitor was 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 was 0.48 nM.

The prospect of *P. mendocina* protease inhibitor as a preservative against proteolysis towards preventing spoilage of shrimp was assessed. Storage experiments on protein degradation were carried out on fresh shrimp tissue in the absence as well as in the presence of inhibitor at various storage conditions. It was observed that the inhibitor could block the protease activity of the spoilage bacteria efficiently evidenced by negligible protein degradation in the case of inhibitor treated samples compared with untreated samples. Further there was a considerable reduction in the microbial load in inhibitor treated samples.

Moreover antibacterial property of the inhibitor analyzed against various standard bacterial cultures indicated that the inhibitor is capable of inhibiting *Bacillus cereus* with a minimum inhibitory concentration of 80 µg/well.

Conclusion

A potential protease inhibitor producing bacterial strain, identified as *Pseudomonas mendocina,* was isolated from the marine environment of Cochin, Kerala. The purified protease inhibitor was recognized to be a new inhibitory protein with novel characteristics. The protease inhibitor was stable over a wide range of pH and temperatures and it is active against spoilage microbes in shrimp. The studies on the antagonistic properties suggested the possible use of this protease inhibitory protein as a biocontrol agent in pharmaceutical industry. Further there is scope for its application in the development as biopesticide, which can withstand alkaline conditions of insect's gut flora. There is ample scope for further research on structure elucidation and protein engineering employing bioinformatics tools to facilitate their use in wide range of applications.

Chapter 7

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LIST OF PUBLICATIONS

a) Peer Reviewed

1. **Sapna. K**, Manzur Ali P. P, Abraham Mathew, Rekha mol K. R, Sarita G. Bhat, Chandrasekaran. M and Elyas K. K. "Marine *Pseudomonas mendocina* BTMW 301 as a potential source for Extracellular Proteinaceous Protease Inhibitor"(2012) *Advanced Biotechnology* 06/2012; 11(12):16-19

2. **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).

3. 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.

4. 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* 02/2010; 27(4):347-51.

5. Sajimol Augustine, P P Manzur Ali, **K Sapna**, K K Elyas, S Jayalekshmi (2013). Size-dependent optical properties of bio-compatible ZnS:Mn nanocrystals and their application in the immobilization of trypsin. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*. 108, 223–228.

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

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

1. **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.**

2. 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 Symposium/Conferences/Seminars

1. **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

2. 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.

3. 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

1. 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.**

2. *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**

3. *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**

4. 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.**

APPENDIX

Zobell Marine Broth 2216 (HiMedia)

Ingredients	**g**
Peptic digest of animal tissue	5
Yeast extract	1
Ferric citrate	0.1
Sodium chloride	19.45
Magnesium chloride	8.8
Sodium sulphate	3.24
Calcium chloride	1.8
Potassium chloride	0.55
Sodium bicarbonate	0.16
Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
pH	7.6
Distilled Water	1000mL