Studies on Procerain B, a Novel Cysteine Protease from *Calotropis procera*

A Thesis
Submitted in Partial
Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

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January 2013

DEDICATED

TO

MY PARENTS AND MY MENTOR

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOTECHNOLOGY

CERTIFICATE

I hereby declare that the matter embodied in this thesis entitled "**Studies on Procerain B, a Novel Cysteine Protease from *Calotropis procera***" is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, India under the supervision of **Dr. Vikash Kumar Dubey**.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred.

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CERTIFICATE

It is certified that the work described in this thesis entitled "**Studies on Procerain B, a Novel Cysteine Protease from *Calotropis procera***" by **Mr. Abhay Narayan Singh** (Roll No: 09610607), submitted to Indian Institute of Technology Guwahati, India for the award of degree of Doctor of Philosophy, is an authentic record of results obtained from the research work carried out under my supervision at the Department of Biotechnology, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree.

Dr. Vikash Kumar Dubey
(Supervisor)

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Abbreviations

Ala	:	Alanine
ANS	:	8-anilino-1-naphthalene Sulfonate
Asp	:	Asparagine
AT	:	Anti Thrombin
ATP	:	Adenosine-5'-triphosphate
BLAST	:	Basic Local Alignment Search Tool
BSA	:	Bovine Serum Albumin
cDNA	:	Complementary Deoxyribonucleic Acid
CM-Sepharose	:	Carboxymethyl Sepharose
DIFP	:	Diisopropylfluorophosphate
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethyl Sulfoxide
DNA	:	Deoxyribonucleic Acid
DTNB	:	5,5-dithiobis-(2- nitrobenzoic acid)
DTT	:	Dithiothreitol
EDTA	:	Ethylene Diamine tetra Acetic Acid
EDX	:	Energy-Dispersive X-ray Spectroscopy
EGTA	:	Ethyleneglycoltetraacetic Acid
FBS	:	Fetal Bovine Serum
FESEM	:	Field Emission Scanning Electron Microscopy
FTIR	:	Fourier Transform Infrared Spectroscopy
Gly	:	Glycine
GSH	:	Glutathione
GuHCl	:	Guanidine hydrochloride
His	:	Histidine
IAA	:	3-Indoleacetic acid
IPTG	:	Isopropyl β-D-1-thiogalactopyranoside
KBr	:	Potassium Bromide
MALDI	:	Matrix-Assisted Laser Desorption Ionization
MG	:	Molten Globule
MMPs	:	Matrix Metalloproteases

mRNA	:	Messenger Ribonucleic Acid
MSA	:	Multiple Sequence Alignment
MTT	:	3-(4,5-dimethyl thiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide
NEM	:	N-Ethylmaleimide
PBS	:	Phosphate Buffer Saline
PCMB	:	p-Chloromercuribenzoic Acid
PCR	:	Polymerase Chain Reaction
PMSF	:	Phenylmethanesulfonyl Fluoride
Pro_B	:	Procerain B
RMSD	:	Root Mean Square Deviation
RNA	:	Ribonucleic Acid
SBTI	:	Soybean Trypsin Inhibitor
SDS-PAGE	:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SPCs	:	Subtilicin-like Proprotein Convertases
STT	:	Sodiumtetrathionate
TCA	:	Trichloro Acetic Acid
TEM	:	Transmission Electron Microscopy
TF	:	Tissue Factor
TFPI	:	Tissue Factor Pathway Inhibitor
Thr	:	Threonine
β-ME	:	2-Mercaptoethanol

Contents

Chapter I

Literature review on proteases: Introduction, current scenario and future scopes*.

1.1 Abstract

Proteases are the hydrolytic enzymes responsible for hydrolysis of long polypeptide chains into shorter peptides and free amino acids. Initially, proteases were thought to be mainly responsible for degradation of nutritional proteins and for the maintenance of protein turnover. However, recent advancement in protease research coupled with the knowledge of crystal structures and detailed reaction mechanism of different proteases, suggest these enzymes to be an indispensable cellular tool for numerous biological functions. Due to bulk commercial unavailability of these crucial proteins, huge scientific talent turned towards the purification and characterization of different proteases from variable sources using several analytical techniques as chromatography, electrophoresis, ultracentrifugation, protein sequencing etc. During evolution, proteases have adapted very diverse range of conditions in different organisms and personalized different catalytic mechanisms for proteolysis. Other than their physiological importance, proteases also share high industrial significance. Thus, the search for new proteases with unique features is still continued.

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* Part of the review is submitted for publication

1.2 Introduction

The molecules like DNA encoding genetic instructions are known to govern the developmental and functional control of all living organisms. The implementation of these genetic instructions is achieved via the synthesis of various RNAs and proteins through the well defined and deep rooted processes known as transcription and translation, respectively. The nature of a cell is defined, not only by the genes it inherits but also by which of those genes are expressed at a time. The gene expression results in the formation of functional gene products which could be anything ranging from functional RNAs to the most amazing group of molecules known as proteins. Proteins are incredibly complex chains of amino acids folded in myriad ways imparting individual protein a unique identity. Proteins are categorized based on their structure and functions with enzymes as one of the grand and valuable class. An enzyme is a biocatalyst which increases the rate of a reaction by lowering the activation energy without disturbing the equilibrium of the reaction. Enzymes are indispensable biomolecules with diverse functions including their essentiality in the basic metabolism of living organisms, usability in different industries, applications in genetic manipulations etc. There are six major classes of enzymes based on the nature of the reactions catalyzed by them: Oxidoreductases, Transferases, Hydrolases, Lyases, Isomerases, and Ligases. Out of these classes, proteases belonging to Hydrolases, catalyze the lysis of different complex molecules into simpler one with help of water molecules. Proteases are one of the extensively explored groups of enzymes with its key pertinence in different industries.

1.3 Literature Review

1.3.1 Proteases: An Overview

Proteases, also called as proteinases, peptidases or proteolytic enzymes are the group of enzymes belonging to 3rd main class of enzyme classification i.e. hydrolases and catalyze the hydrolysis of peptide bonds which link different amino acids to form a polypeptide. This hydrolytic cleavage is achieved by the nucleophilic attack at the carbonyl carbon assisted by the transfer of a proton to the NH group of the scissile peptide bond. The conserved amino acid residues at the catalytic site determine the substrate to be cleaved and ultimately

affecting the rate, time and location of the hydrolysis. Proteases constitute a major portion of cellular toolbox accounting for approximately 2% of human genome (*Puente et al., 2003*). Their ability to cut long strings of amino acid sequences into smaller fragments and thereby controlling synthesis, shape, composition, activation and finally destruction of proteins, made them popular as "Biomolecular version of Swiss army knives" (*Shen & Chou, 2009*). Due to their proteolytic nature, the synthesis, targeting and activation of these molecular scissors are tightly regulated. Uncontrolled action of these enzymes may lead to a disaster for cell, that's why mostly these enzymes are synthesized in inactive form (Zymogens) and activated at the desired time and site of action. The activation may be an autocatalytic process or may depend on other enzymes. Activation generally involves removal of inhibitory sequence (N-terminal or C-terminal) and converts inactive form to active one. Several digestive enzymes (pepsin, chymotrypsin, trypsin etc.) and the proteases of blood clotting cascade are very good examples of this. Blood clotting is a highly regulated multi-steps process including several proteases at different steps which activate one another in a well defined fashion (*Tanaka et al., 2009*) (Figure 1.1). In addition to their role in chopping up polypeptides for digestion and maintaining the protein turnover, they also play a critical role in subtle modifications converting inactive protein precursors into matured and functional enzymes which are necessary for protein assembly and sub cellular targeting, thereby controlling the activity of a variety of regulatory proteins and enzymes. Owing to their ability to act as molecular knives, they are considered indispensable for various developmental, regulatory and physiological processes including blood clotting, digestion, homeostasis, and in etiology of an array of diseases. In recognition to their association with diverse vital processes, the engrossment in proteases was stimulated considerably which then lead to its extensive exploitation for utilization in various industries and drug development. The first protease was christened as pepsin long before the concept of enzyme came into existence. Its recognition is attributed to Theodor Schwann (7 December 1810 – 11 January 1882), the founder of cell theory in animals, as a substance responsible for breakdown of nutritional proteins in stomach. Action of this enzyme leads to conversion of bulky and complex food proteins into smaller oligopeptides and free amino acids required for proper absorption and assimilation.

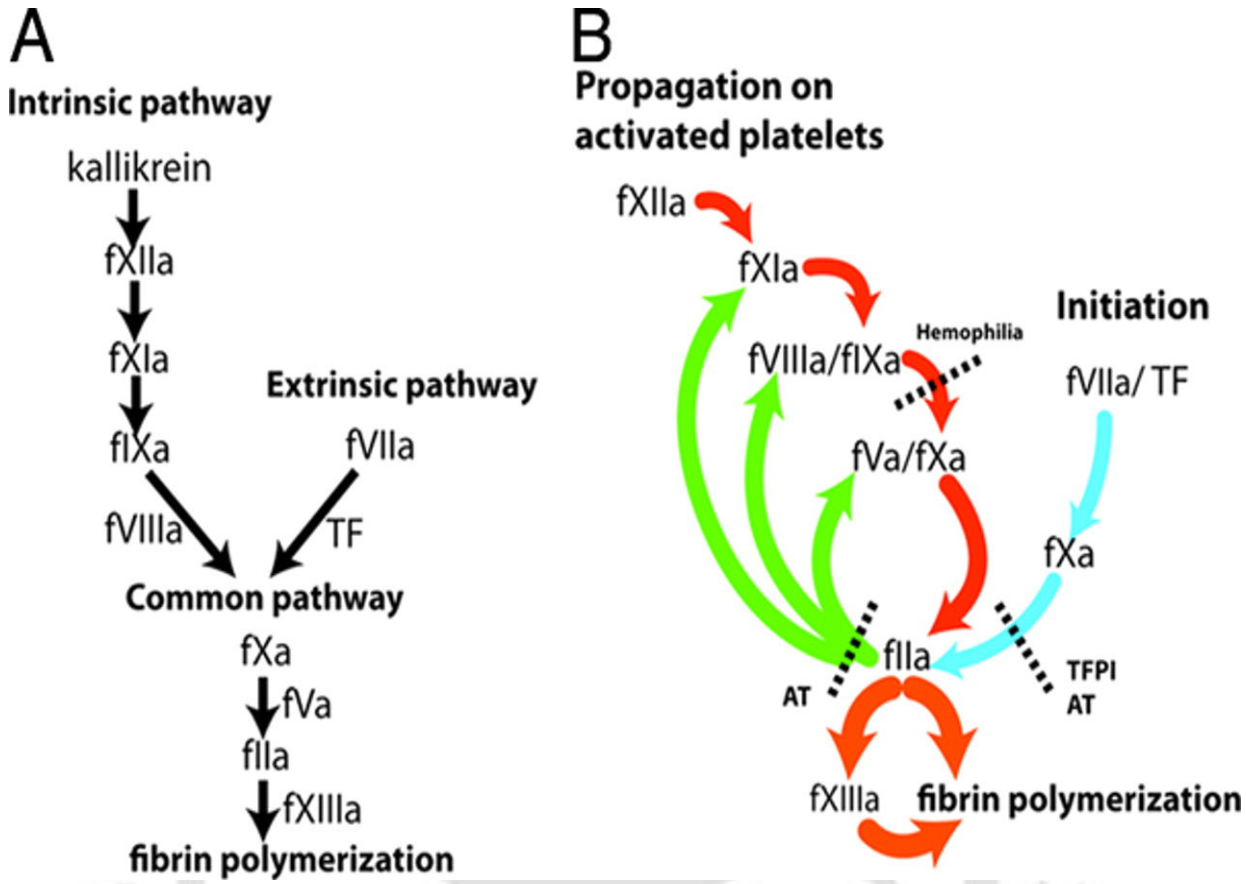

Figure 1.1: To avoid self degradation or undesirable cleavage, proteases are usually synthesized in an inactive form with additional sequences (zymogens). Inactive zymogen form gets activated by other protease whenever the function is required. Blood clotting cascade is a very good example where several proteases are activated in cascade one by other in well regulated manner. **(A)** Conventional cascade model of coagulation. **(B)** Regulation of thrombin generation. Coagulation is triggered (initiation) by circulating trace amounts of fVIIa and locally exposed tissue factor (TF). Subsequent formations of fXa and thrombin are regulated by tissue factor pathway inhibitor (TFPI) and antithrombin (AT). When the threshold level of thrombin is generated, thrombin activates platelets, fV, fVIII, and fXI to augment its own generation (propagation).Some time protease are not synthesized in form of zymogen but activated by allosteric mechanism by activation signal. (Figure and part of figure caption adopted with permission from International Anesthesia Research Society/Wolters Kluwer Health Ref: Anesthesia & Analgesia. 2009, 108, 1433-1446.)

1.3.2 Types of proteases

On the basis of site of action, proteases can be broadly divided into two categories of exopeptidases and endopeptidases. Exopeptidases act by cleaving single amino acids from either end of the polypeptide chain which under appropriate condition may lead to the ultimate reduction of protein to a single amino acid.

They can either be aminopeptidases (target N-terminal peptide bond) or carboxypeptidases (target C-terminal peptide bond). On the other hand endopeptidases act on the peptide bonds in the interior of the polypeptide chain resulting in the formation of smaller oligo and polypeptides (*Rao et al., 1998*; *Parde et al., 2012*) (Figure 1.2b). Based on their catalytic mechanism, proteases are broadly classified into seven major classes: Cysteine proteases, Serine proteases, Threonine proteases, Metallo proteases, Asparagine proteases, Aspartic acid proteases, and Glutamic acid proteases. Out of these proteases cysteine, serine and threonine proteases share a common mechanism (covalent catalysis) whereas aspartic acid, glutamic acid and metallo proteases follow slightly different (acid base catalysis) mechanism (Figure 1.2a).

1.3.2.1 *Cysteine proteases*

Cysteine proteases (thiol proteases) are the proteolytic enzymes whose catalytic activity depends on the nucleophilic attack of cysteine residue present at its catalytic domain. Papain, actinidain, bromelain are some common examples of plant cysteine proteases. Usually in terms of reaction mechanism, the cysteine proteases share significant similarities with serine proteases (*Grudkowska & Zagdańska, 2004*). Although these two classes of enzymes differ in their overall three dimensional structure, certain characteristics of catalytic triad of serine proteases can be seen in cysteine proteases thereby showing remarkable similarities in their mechanism of catalysis (*Chapman et al., 1997)*. The cysteine proteases have a catalytic triad consisting of histidine, cysteine and asparagine which is known to direct their activity. The arrangement of histidine and cysteine residue (His-Cys or Cys-His) in the catalytic triad decides the family of the cysteine proteases. Approximately 20 families of cysteine proteases have been identified so far (*Higaki et al., 1989*). An important feature of cysteine protease is the high degree of nucleophilicity of sulfur atom of cysteine residue present at its catalytic site. This high nucleophilicity is attributed to the fact that the sulfur atom is present as thiolate anion at pH values where the enzyme is active (*Vernet et al., 1995*). Most of the cysteine proteases have maximal activity around neutral pH with exceptions of lysosomal proteases with acidic pH optima (*Parde et al., 2012).*

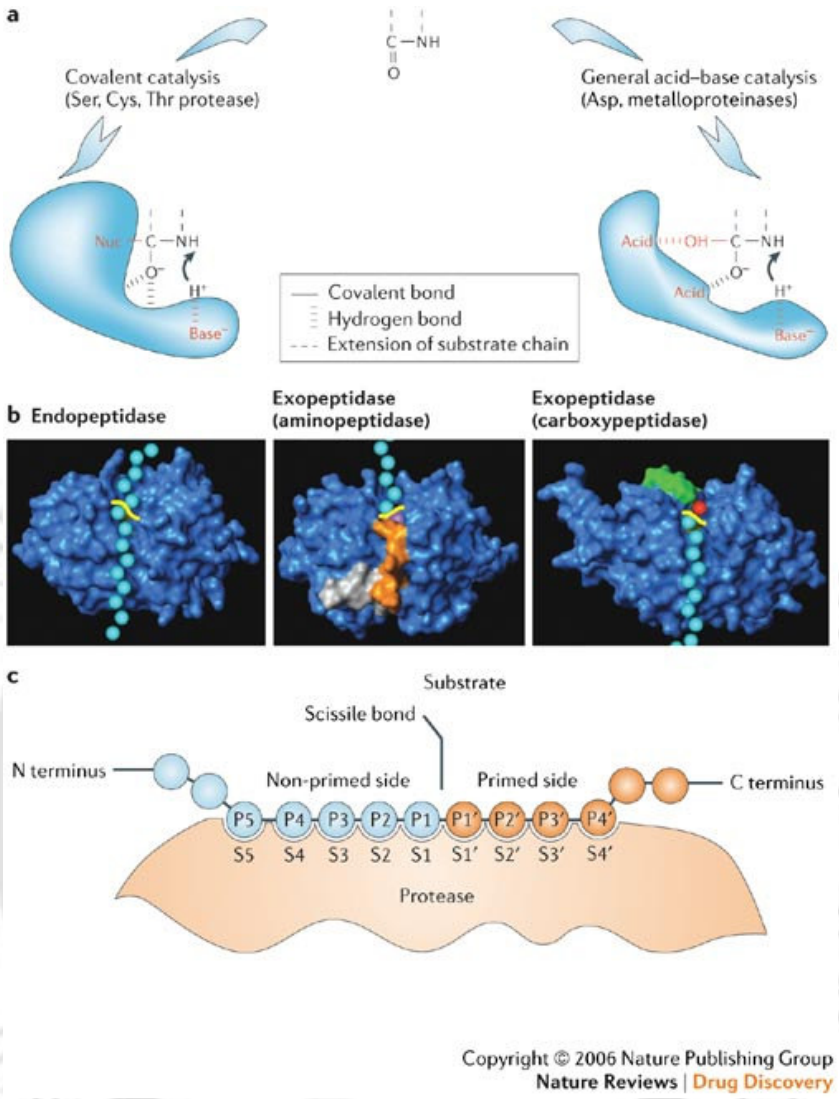

Figure 1.2: (A) Generalized view of proteolysis. Covalent catalysis includes Serine, Cysteine and Threonine proteases where the nucleophiles are either hydroxyl or thiol group of catalytic residue. In case of acid-base catalysis (Aspartic, Glutamic and Metallo proteases), activated water molecule act as nucleophile. **(B)** Based on the mode of cleavage, proteases are of two types, endopeptidase, and exopeptidases. Further, exopetidases are of two types, aminopeptidase and carboxypeptidase. Peptide substrate is represented by cyan balls, runs through the entire length of the active site of an endopeptidase framework shown in blue. The substrate is cleaved in the middle of the molecule in the case of endopeptidae (scissile bond marked yellow) or towards amino or carboxy terminal in case of exopetidases. **(C)** Schematic representation of a protein substrate binding to a protease. The position of amino acid towards N-terminal side of scissile bond is called non-primed position (P1–Pn) while the interaction sites on protease are called non-primed sites (S1–Sn). Similarly, C-terminal side amino acids of scissile bond are called primed position (P1′–Pn′) and respective sites on enzyme are called primed sites (S1′–Sn′). (Adopted with permission from Macmillan Publisher Ltd. Ref: *Nature Review Drug Discovery*. 2006, 5, 785–98.)

1.3.2.1.1 *Mechanism of action of cysteine proteases:*

As mentioned earlier, the mechanism of action of cysteine proteases shares a significant similarity with that of serine proteases. The basic characteristics of the catalytic mechanism includes the formation of a covalent intermediate and the acyl enzyme which results due to the nucleophilic attack of the thiol group (present at the active site) on the carbonyl carbon of scissile bond in substrate. The initial step of catalysis involves the non covalent binding of the substrate to the free enzyme to form a complex intermediate. This is followed by two basic steps known as acylation and deacylation. The acylation of the enzyme along with the generation of the first product of the reaction, R-NH_2, follows the formation of Michaelis enzyme-substrate complex. This is succeeded by the process involving the reaction of acyl enzyme with a water molecule, a process called deacylation, which leads to the formation of next product with the regeneration of free enzyme (Figure 1.3) (*Rao et al., 1998; Dubey et al., 2007; Ma et al., 2007*).

1.3.2.2 *Serine proteases*

Serine proteases are the oldest, most versatile and the largest group of proteases. They are named after the reactive serine residue present at the catalytic triad of the enzyme along with histidine and aspartic acid. This group of proteases comprises two distinct families: the chymotrypsin family and the substilisin family. The chymotrypsin family consists of enzymes of mammalian origin such as trypsin, chymotrypsin etc. while the substilisin family include bacterial enzymes like substilisin. Plant serine proteases have been divided into 14 families. Serine proteases have known to play a crucial role in wound healing, blood clotting, pathogen-host interaction etc. (*Walker & Lynas, 2001; Hedstrom, 2002; Schaller, 2004*).

1.3.2.3 *Aspartic acid proteases*

Aspartic acid proteases are one of the four classical families of proteases. It was discovered accidently by an Arabic traveler during a journey across the desert. He observed coagulation of milk stored in a leather bag. Later the coagulant was identified as rennin, present in the epithelial lining of ruminant stomach. Pepsin, second enzyme crystallized (Northrop, 1930),

is an aspartic acid protease present in the vertebrate stomach and responsible for major protein digestion. The catalytic residues involve are two Asp residues which cause the activation of a water molecule that attacks as nucleophile for the hydrolysis of peptide bond (James, 2004).

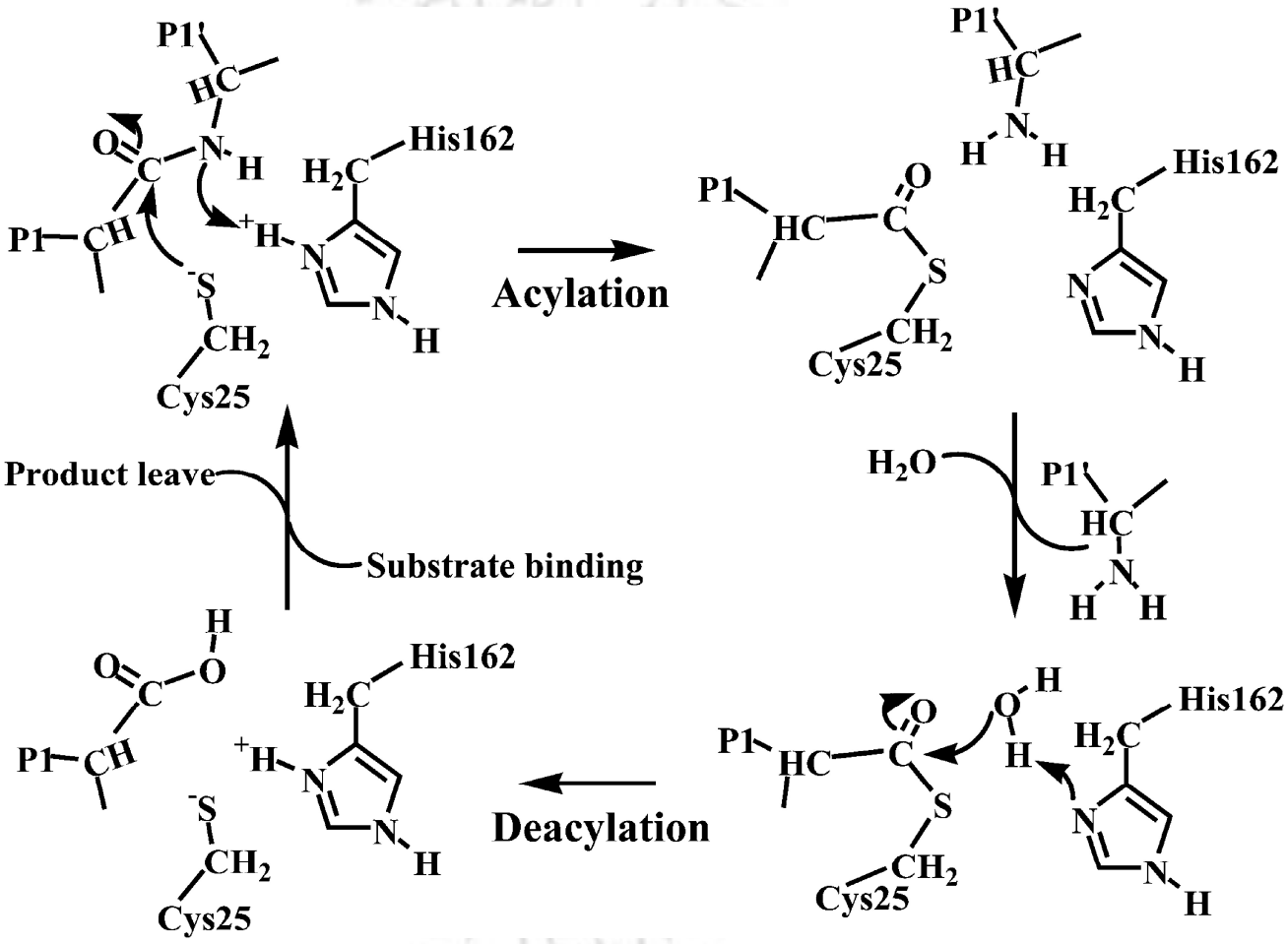

Figure 1.3: Mechanism of action of cysteine proteases. The catalysis by cysteine proteases initiate with the interaction of substrate with free enzyme through noncovalent binding. This is followed by two basic steps known as acylation and deacylation. The acylation of the enzyme along with the generation of the first product of the reaction, R-NH_2 follows the formation of Michaelis enzyme-substrate complex. This is followed by the process involving the reaction of acyl enzyme with a water molecule, which results the deacylation of enzyme and ultimately leads to the formation of another product with the regeneration of free enzyme (Adopted with permission from *American Chemical Society*. 2007, 129, 13633-13645).

1.3.2.4 *Metalloproteases*

Metalloproteases are the most diverse group of enzymes in terms of both structure and function. As the name suggests metalloproteases need a metal ion for their activity. Most of the metalloproteases use Zn^{2+} as cofactor for the activity while some utilize Co^{2+}, Mn^{2+} or Ca^{2+}. Different amino acids as histidine, lysine, arginine, glutamate and aspartate usually hold the metal ion and keep it in proper position for catalysis. These proteases are very crucial for different biological processes as cell proliferation and differentiation, extracellular matrix remodeling mainly for vascularisation and cell migration. All these events are regulated through the proteolytic action for the refining of different growth modulators, receptors and other key molecular players (*Chieh & Zena, 2001*). There are mainly two related families of metalloproteases as: (1). Matrix metalloproteases (MMPs) and (2). Metalloprotease-disintegrins (ADAMs).

1.3.2.5 *Threonine proteases*

Moving to the threonine group of proteases which are the enzymes harboring threonine at their active site were discovered in 1995. Fifth catalytic type of protease was identified as threonine proteases, when the 20S proteasome crystal structure was solved and it was found that out of fourteen different subunits, three subunits were peptidases, with an N-terminal threonine which cause the nucleophilic attack (*Seemuller et al., 1995*). They are responsible for proteasome functions which are self compartmentalizing large protein degrading apparatus.

1.3.2.6 *Glutamic acid proteases*

Glutamic acid proteases are the sixth family of proteases contains mainly fungal and viral peptidases. Initially, it was included in aspartic acid proteases but the current studies of crystal structures of these proteases and detailed molecular study of proteolytic mechanism revealed these as a separate family (*Sims et al., 2004*). The first two alphabets of the name "Eqolisin" were derived on the basis of catalytic residues [E (glutamic acid), Q (glutamine)] at the active site. The hydrolysis involves nucleophilic attack of Glutamic acid activated

water (OH^-) on the carbonyl carbon atom of scissile peptide bond which leads to the formation of tetrahedral intermediate. The side chain of glutamine provides electrophilic assistance and oxyanion stabilization which ultimately results in the cleavage of peptide bond (*Fujinaga et al., 2004*).

Table 1.1 Different classes of proteases and their nucleophile and reaction intermediate involved in reaction mechanism. Cysteine, Serine and Threonine proteases share a common reaction mechanism and involve hydroxyl (-OH) group as nucleophile. In case of Aspartic acid, Glutamic acid and Metalloproteases an activated water molecule act as nucleophile while in Asparagine proteases Asp itself act as nucleophile and initiate the self catalytic process.

Catalytic Class	Nucleophile	Covalent intermediate	Number of clans
Cysteine	Thiole (-SH)	Yes	09
Serine	Hydroxyl (-OH)	Yes	12
Aspartic	Water (H_2O)	No	05
Metallo	Water (H_2O)	No	16
Glutamic	Water (H_2O)	No	02
Asparagine	Asparagine (Asp)	Yes	05
Threonine	Hydroxyl (-OH)	Yes	-

1.3.2.7 *Asparagine proteases*

Asparagine proteases are the latest class discovered when the X-ray crystal structure of the self-cleaving precursor of the Tsh (Temperature sensitive hemagglutinin) auto transporter in *Escherichia coli* was solved. Here the catalytic asparagine causes self-cleavage by nucleophilic attack on own carbonyl carbon atom. It demonstrated that a seventh catalytic type protease exists in which the nucleophile is an asparagine (*Rawlings et al., 2011*). Here the peptide cleavage does not involve water molecule, rather than the lysis is caused by asparagine which can form a stable, five membered succinimide ring with its own carbonyl carbon. Under proper condition it leads to breakage of its own peptide bond. This alternative of normal peptide cleavage was disclosed by Dehart and Anderson (*Dehart & Anderson, 2007*).

1.3.3 Physiological significance of proteases

Presence of proteases in almost all live forms defines their ubiquitous nature which seems to support their intricate role in different physiological and developmental processes. Their relevance in different physiological and pathological processes is reflected by their involvement in processes like cell growth, tissue arrangement, blood coagulation, protein catabolism, secretory pathway, nutrition, protein turnover, germination, regulation of gene expression, in defense mechanism etc.

1.3.3.1 *Proteases in secretory pathways*

Proteases are known to regulate different cellular processes like cell cycle, gene expression, embryogenesis, neural and endocrine functions etc. by proteolysis of precursor proteins. They are the key players in the activation of a variety of secreted proteins. The group of proteases extensively involved in processing of protein precursors traversing the secretory pathway is that of serine proteases. These secretory pathway processing enzymes are popularly called subtilisin-like proprotein convertases (SPCs) as they are calcium-dependent serine endopeptidases related to subtilisin and yeast processing proteases, kexin. These SPCs act to generate active forms of different serum proteins, receptors, prohormones, bacterial toxins and many more. Furin, PC1/3, PC2, PACE4 are some of the examples of these subtilisin related serine proteases. These SPCs share the similar domain structure comprising of the following things:

i. The signal peptide, targeting nascent proteins to the secretory pathways.
ii. The pro-peptide, which drive proper folding of polypeptide.
iii. The catalytic domain related to bacterial subtilisins and contains aspartate, histidine and serine at their active site.
iv. The middle or Homo B-domain, which has a key role in folding and activity.
v. The variable C-terminal region, which play a role in sub cellular routing.

The roles of these SPCs in different physiological processes including receptor and growth factor maturation suggest that loss of their expression and regulation may lead to pathological states like lymphoma and other malignant diseases. So development of SPCs

inhibitors with high specificity and targeted delivery may have valuable therapeutic potential (*Zhou et al., 1999*).

1.3.3.2 *Proteases involved in nutrition*

The nutrient intake and changes in the activities of proteolytic enzymes regulate the metabolism of many proteins and affect the size of protein pool. The extracellular proteases play an important role in nutrition due to their depolymerising activity by which large polypeptides are hydrolyzed in smaller oligopeptides and amino acids which facilitates their absorption by the cells. The extracellular enzymes such as proteases, secreted from pancreas, are involved in providing amino acids pool as nutrition to the cells and help them keeping alive (*Rao et al., 1998*).

1.3.3.3 *Proteases for protein turnover*

In living cells, certain proteases help in facilitating a continuous rate of protein turnover which in turn supports basic cellular functions like growth and differentiation. Degradation of proteins replenishes the pool of amino acids to be further used as precursors for the synthesis of proteins. In addition to this, the catabolism of proteins helps in the removal of inactive polypeptides resulting from translational errors, chemical modifications like oxidation and misfolding of proteins which is important during periods of stress (*Neurath & Walsh, 1976*). Certain selective protein degradation are important for regulating all aspects of plant development which can be appreciated by linking the ubiquitin/proteasome pathway (Figure 1.4), the main proteolytic pathway in eukaryotes, to various developmental processes like embryogenesis, hormone signaling, photomorphogenesis etc. (*Schaller, 2004*). The ubiquitin/proteasome pathway is considered an important proteolytic pathway in plants and other eukaryotes. This pathway is linked to various developmental pathways in plants, including photomorphogenesis, circadian rhythm, embryogenesis, hormone signaling, flower development etc., ultimately directing regulation of plant growth and development.

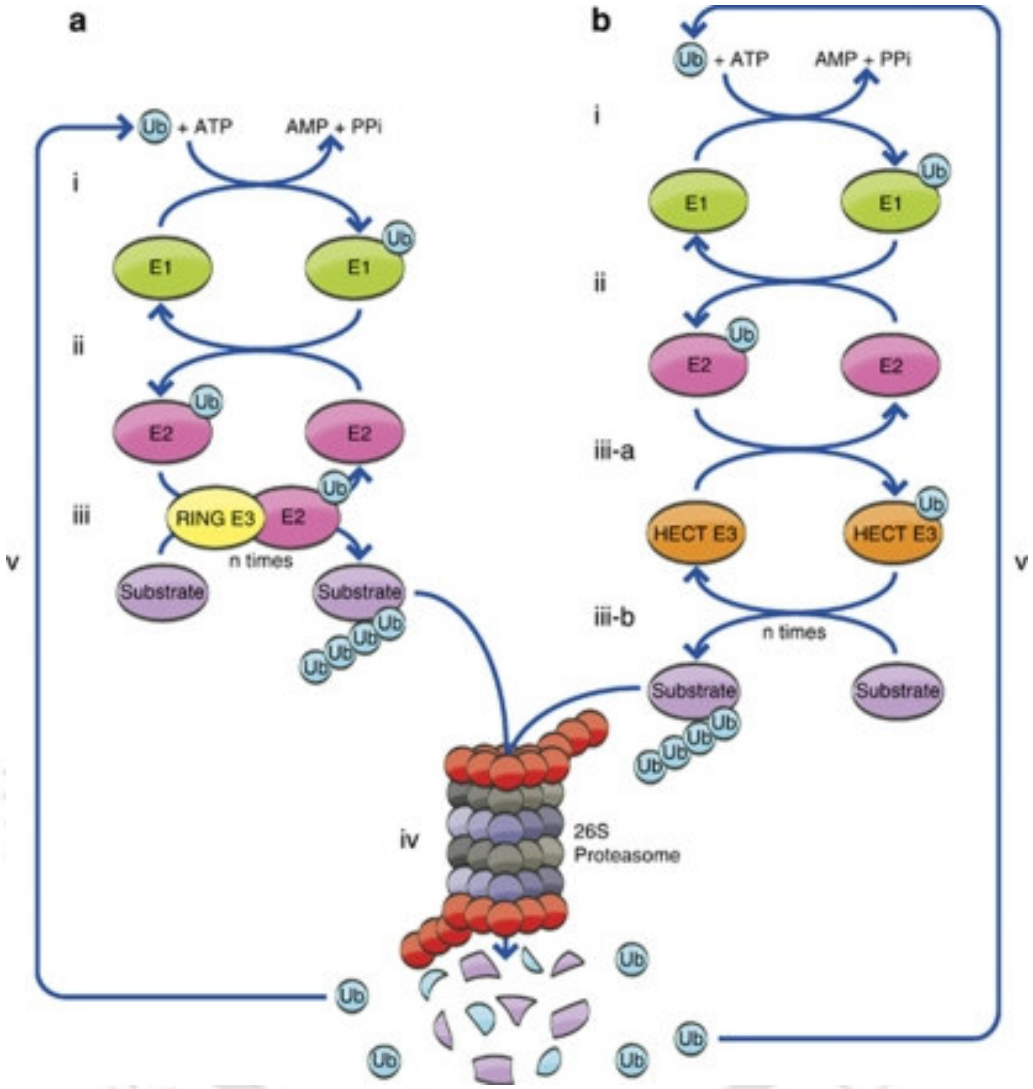

Figure 1.4: Ubiquitination of misfolded proteins and their degradation in proteasome complex. Proteases have role in degradation of proteins that are ubiquitinylated. The ubiquitin-proteasome system is explained in figure. **(A)** Conjugation of ubiquitin catalyzed by RING or **(B)** HECT. **(Ai, Bi)** Domain-containing ligases. **(Aii, Bii)** ATP-dependent activation of ubiquitin catalyzed by the ubiquitin-activating enzyme, E1. Transfer of the activated ubiquitin to a ubiquitin-carrier protein (ubiquitin-conjugating enzyme), E2. **(Aiii)** In the case of a RING ligase, the ubiquitin-charged E2 binds to the E3 and transfers the activated ubiquitin moiety directly to the substrate that is also bound to the E3. **(Biii-A)** In the case of a HECT domain ligase, ubiquitin is transferred from the E2 to a Cys residue in the E3 and then **(Biii-B)** To the substrate. **(IV)** The conjugated substrate is degraded to short peptides by the 26S proteasome. **(V)** With release of free and reusable ubiquitin mediated by DUB(s). **(IV)** Some of the ubiquitin is degraded in this process along with the substrate (Figure and figure caption is adopted with permission from Macmillan Publishers Limited Ref: Cell Death Differ. 2011, 18, 1393–1402.)

This pathway involves targeted degradation of proteins by proteasomes, which is achieved by conjugating ubiquitin to the lysine residues present within the substrate proteins. Of around 1,300 genes are known to be devoted to this ubiquitin/proteasome pathway in arabidopsis. Additionally, more than 600 protease genes are known to be involved in protein turnover, protein trafficking, proprotein processing and regulation of protein activity by proteolysis (*Moon et al., 2004*). The chloroplasts of higher plants consist of various proteases of bacterial ancestry of which ATP-dependent Clp protease localized in stroma was the first to be identified. This Clp protease is assumed to be functional as stromal housekeeping protease. In tobacco, the plastomic ClpP1 protein is reported to be vital for shoot development and cell viability (*Gren et al., 2006*).

1.3.3.4 *Proteases for regulation of gene expression*

Proteases are also known to modulate the expression of genes in living organisms. In viruses, the regulation of gene expression serves to compress the genetic information within the limited area provided by the genome of the virus. In this manner, where only one 3' and 5' untranslated region is required for viral replication, translation, transcription and morphogenesis such as the case of picornaviruses or flaviviruses, the proteases can reduce the genetic space occupied by 3' and 5' untranslated regions and can minimize the signals required for the initiation of mRNA transcription and translation (*Castello et al., 2011*). In enterohemorrhagic *Escherichia coli*, the regulation of flagellar gene expression is controlled by ClpXP which is a member of ATP-dependent protease family (*Kitagawa et al., 2011*). Derepression of a gene can be achieved by proteolysis of repressor by an ATP-dependent protease. It has been reported that the proteolytic modification of β subunit of RNA polymerase of *Bacillus thuringiensis* changes its transcriptional specificity. The proteolytic modification of ribosomal protein has been suggested to be responsible for translational regulation (*Rao et al., 1998*).

1.3.3.5 *Proteases in plant defence*

Proteases also play crucial role in plant defence mechanism. Plants are outfitted with a cosmic range of proteases involved in several processes from germination of seed to senescence of plant. Proteases are found to be involved in defence response against different pathogens and pests, as some subtilisin like proteases get up-regulated on viroid infection (*Tornero et al., 1996*). Similarly the accumulation of some acidic leucine aminopeptidase, metallo, aspartic and cysteine proteases have also been observed (*Pautot et al., 1993; Avrova et al., 1999; Liu et al., 2001; Hückelhoven et al., 2001*). Other than these correlations plant proteases are also involved in hypersensitivity response similar to programmed cell death (*Dangl & Jones, 2001*). Although the presence of caspase like genes in plants have not yet proved, their existence is reflected by some inhibition studies where inhibitors can block the hypersensitivity and other defence responses. There are several papain like cysteine proteases in the latex of plants and were found to be involved in the defence against herbivorous insects. The exact mechanism of defence involving proteases is still not very clear but there are several possibilities through which proteases can contribute in defence against pathogens. Proteases can degrade the elicitors from pathogens which will activate the defence system. The products obtained after degradation of elicitors are also known to up-regulate the components of defence system. In some cases, this degradation of elicitors by proteases is known to suppresss the plant immunity. The defence system can also be induced by attachment of elicitor with the protease. There are few examples where inhibition of protease by elicitors ultimately leads to the activation of plant immune system. Also, the proteases can contribute to defence against pathogens either by directly degrading the pathogenic proteins or by converting them to toxic one for pathogens. The defence against pathogens can also be elicited by degrading the defensive enzymes of pathogens by proteases (Figure 1.5).

1.3.4 Industrial significance of proteases

The potential of proteases has been explored to an extent to cover major shares of the business world. Their promising pertinence in industries proved them to be a resourceful class of enzymes. Their applications cover a broad area varying from basic research to high-throughput processes used in different industries. The industries absorbing various proteases

as key players are food industry, detergent industry, leather industry, pharmaceutical industry and the list goes on and on (Figure 1.6).

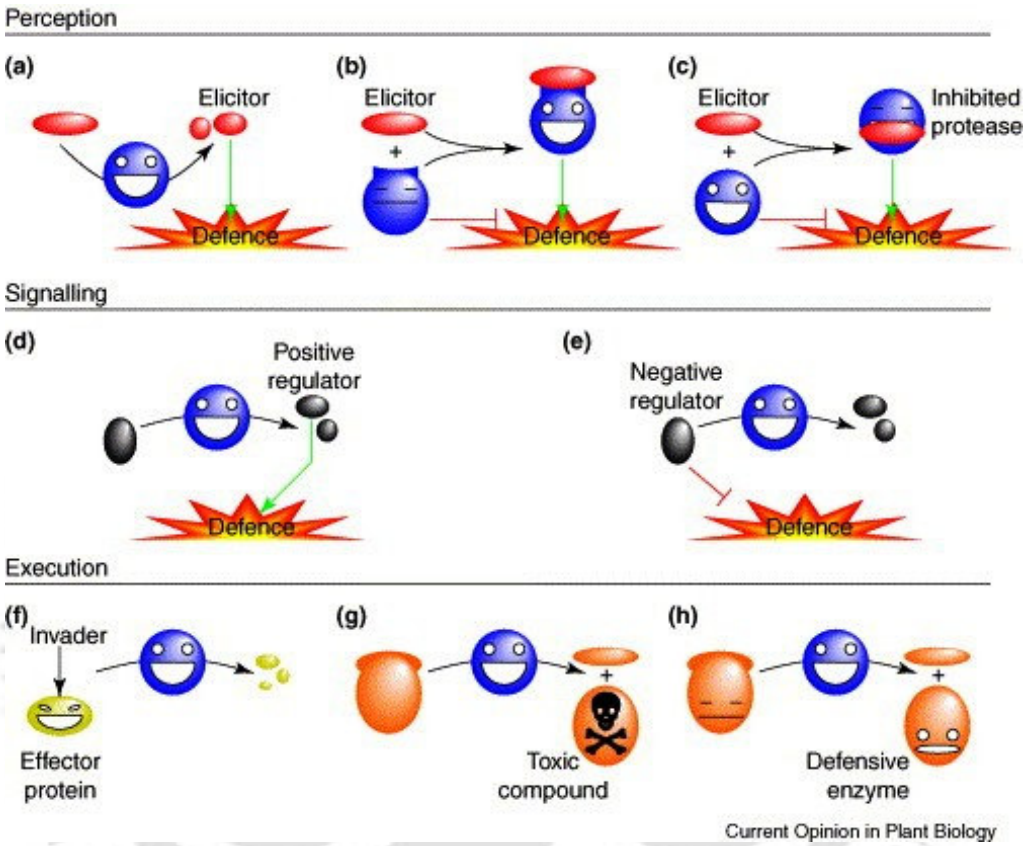

Figure 1.5: Different possible ways through which proteases can participate in defence mechanism. **(A)** Proteases can degrade the elicitors from pathogens which will activate the defence system (Figure 1.5a). **(B)** The protease can induce the defence system after attachment of elicitor (Figure 1.5b). **(C)** The elicitor can inhibit the protease and ultimately activation of defence system (Figure 1.5c). **(D)** The protease can degrade the elicitor and the degraded product can up-regulate the components of defence system (Figure 1.5d). **(E)** The protease can degrade the elicitor, the inhibitor of defence system (Figure 1.5f). **(F)** Proteases can directly degrade the pathogenic proteins. **(G)** Proteases can convert the pathogenic proteins to toxic one for pathogens (Figure 1.5g). **(H)** Proteases can degrade the defensive enzymes of pathogens (Figure 1.5h). (Figure is adopted with permission from Nature Publishing Group: Nature Reviews Drug Discovery. 2006, 5, 785–799).

1.3.4.1 *Proteases in detergent industry*

Proteases are standard ingredients in various kinds of detergents involving those used in household laundering to those indulged in cleaning dentures and contact lenses. Laundry industry is the biggest market for detergents amounting to an annual worldwide production of 13 billion tons where the use of proteases accounts for the 25% of global enzyme sale. Long back in 1913, the first enzymatic detergent known as Brunus was prepared which mainly composed of sodium carbonate and crude pancreatic extract (*Rao et al., 1998; Gupta et al., 2002*). The enhanced usage of these proteases as additives in detergents is due to its cleaning capabilities in environmental friendly and nonphosphate detergents. The use of proteases not only offers improved washing efficiency but also allows shorter period of agitation and higher performance at lower washing temperature (*Kumar & Takagi, 1999; Kirk et al., 2002*). Ideally, a potential protease for detergents should offer broad substrate specificity to facilitate removal of a wide variety of stains including food, blood, grass, body secretions etc. (*Rao et al., 1998*). Activity, stability and compatibility with alkaline environment and varied temperature encountered under harsh washing conditions as well as high concentrations of surfactants, oxidizing and chelating agents are the major prerequisites for the protease to be used in detergents (*Kumar & Takagi, 1999; Gupta et al., 2002*). The pI value is the key parameter for the selection and performance of a protease in a detergent. The detergent protease is found to perform best in a detergent solution with pH value coinciding with the pI of the enzyme. Now a day, the use of detergents at higher washing temperature has been suppressed due to increased use of synthetic fibers which are intolerant to high temperature. This has shifted the washing habits towards the lower washing environment which in turn push the enzyme manufacturer to search for novel enzyme that can act efficiently at low temperatures (*Rao et al., 1998; Gupta et al., 2002*).

1.3.4.2 *Proteases in leather industry*

Leather processing involves a series of operations including soaking, dehairing, batting and tanning (*Choudhary et al., 2004*). Proteins are the major building blocks of animal skin and hairs. In the process of transforming raw hide into leather, collagen which is a major leather making protein present in skin and hide, is freed from non-collagenous constituents

(*Madhavi et al., 2011*). Conventionally, the leather processing involves the use of sodium sulphide and other chemicals which contribute to the environmental pollution and safety hazards. In order to overcome these hazards, leather processing using enzymatic treatment is proved to be a viable alternative technology which can significantly regulate environmental pollution resulting from tannery effluents (*Arunachalam & Saritha, 2009*). Proteases find their application in different phases of skins and hides preparations such as soaking, dehairing and batting.

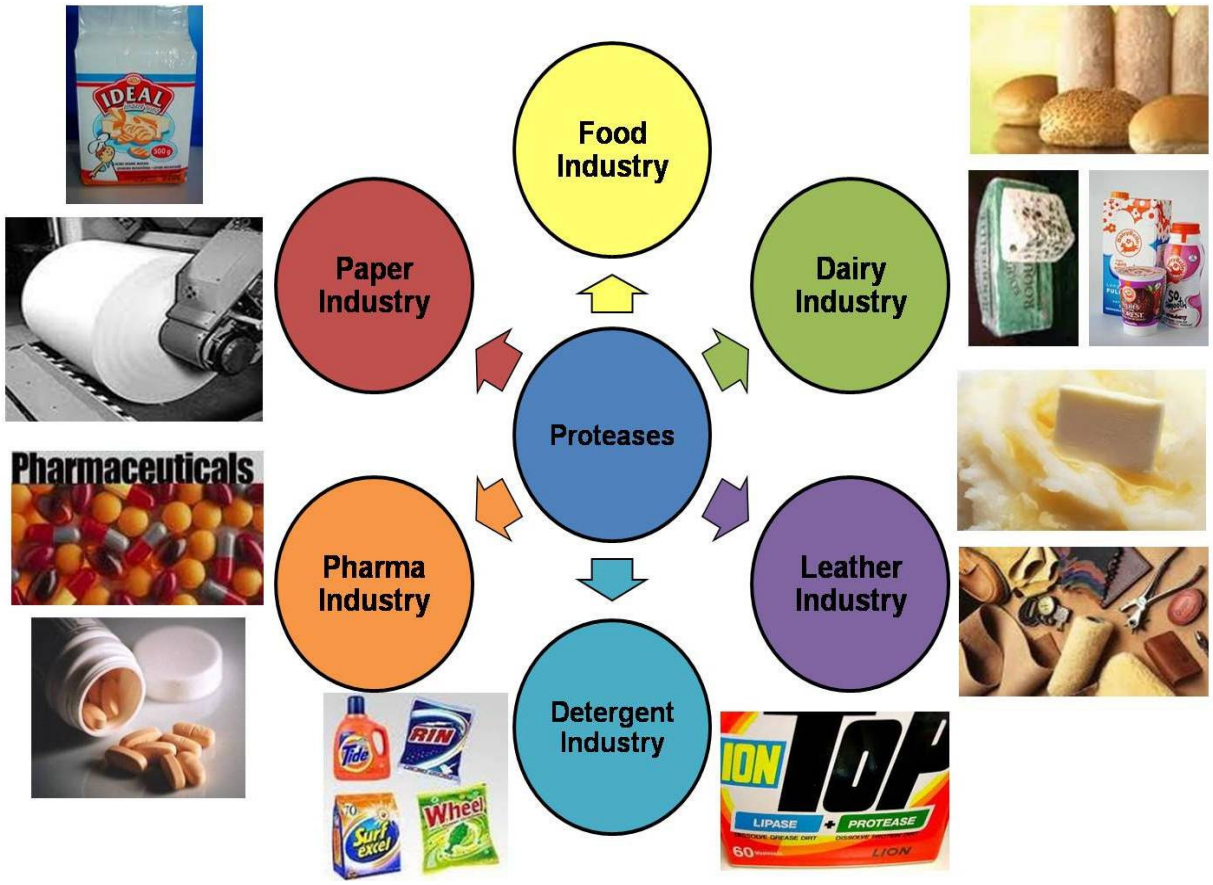

Figure 1.6: Possible industrial applications of proteases. Proteases have various applications in food industry for preparation of partially digested food materials for infants and patients of impaired digestive system. Proteases have applications in dairy industry for cheese and other dairy products. Proteases are also applicable in leather industry for tannin and dehairing of leather. In detergent industry, proteases can be used in combination of detergents for removal of stains with proteinaceous ingredients. In pharmaceutical industry proteases are applicable in different ointments and creams. It can be used in different other formulations for the treatment for certain diseases. Proteases are also applicable in paper industries for recycling of raw materials.

Different combinations of proteolytic enzymes active in neutral and alkaline pH range are used for soaking purpose. Specific proteases such as keratinases found to have potential uses in dehairing. Earlier pancreatic trypsin was used in leather processing which was then replaced by proteases obtained from different sources such as bacteria and fungi. The amount of enzyme used depends on the type of leather, hard or soft, and the selection of enzyme is decided by its specificity for matrix proteins such as keratin, elastin etc. (*Rao et al., 1998*).

1.3.4.3 *Proteases in food industry*

The involvement of proteolytic biocatalysts in food industry dates back to antiquity. The ability of proteases to hydrolyze proteins has been exploited for preparation of protein hydrolysates of high nutritional value. These protein hydrolysates have a key role in preparation of infant food formulations, therapeutic dietary products and the fortification of fruit juices and soft drinks (*Ward, 1985; Neklyudov et al., 2000*). Commercially, the protein hydrolysates are derived from various natural protein substrates such as casein, whey, soy protein etc. with the action of various alkaline proteases (*Gupta et al., 2002*). A commercial protease, Alcalase, has been used to produce debittered enzymatic whey protein hydrolysate (*Kumar & Takagi, 1999; Sumantha et al., 2006*). Sardine muscle when treated with an alkaline protease obtained from *B. licheniformis* has reported to produce protein hydrolysate having angiotensin-I-converting enzyme inhibitory activity (*Matsui et al., 1993*).

Proteases are also known to have prominent role in tenderization of meat which is attributed to their ability to hydrolyze connective tissue and muscle fiber proteins (*Kumar & Takagi, 1999*). Plant protease, papain, is known to tenderize tough meat cuts (*Lyons, 1988; Sengupta & Dasgupta, 2006*). The upgradation of lean meat to edible product by hydrolysis of meat waste using commercial proteases has also been reported (*Meara & Munro, 1984*). Another plant protease, bromelain, present in juice and stem of pineapple, is also used in tenderization of meat. In addition to their role in tenderizing meat, papain and bromelain are known to have role in brewing industry where they are widely utilized in clarification of beer (*Sengupta & Dasgupta, 2006*).

1.3.4.4 *Proteases in dairy industry*

Another important application of proteases is in dairy industry where they are utilized in manufacturing cheese. Acidic proteases have significant pertinence in dairy industry which is supported by their ability to coagulate milk protein, casein, to form curd which can be further processed to produce cheese after the removal of whey (*Sumantha et al., 2006*). Main function of proteases in cheese making is to hydrolyze a specific peptide bond (the Phe105-Met106 bond) to produce para-k-casein and macropeptides. Preference of chymosin in cheese making is anchored by its excellent performance due to its high specificity for casein. Chymosin produced by genetically engineered microorganisms has replaced calf enzyme rennin whose production was limited by animal right issues, thereby facilitating the expansion and development of cheese manufacturing industry (*Rao et al., 1998; Sengupta & Dasgupta, 2006*). Controlling the activity of plasmin, an endogenous protease in bovine milk, has a potential to improve quality of dairy products by effecting their texture and flavor. Increased plasmin activity has been proved advantageous in cheese making as it improves the overall quality and flavor of certain cheeses (*Kelly et al., 2006*).

1.3.4.5 *Proteases in baking industry*

Proteases are widely utilized in baking industry. The properties of the bakery dough is determined by an insoluble protein, gluten, present in the wheat flour which is a major component of bakery industry. Heat labile fungal protease obtained from *Aspergillus oryzae* is used to strengthen gluten and creates more elastic and stronger gluten networks (*Rao et al., 1998; Sengupta & Dasgupta, 2006; Mahajan & Badgujar, 2010*). This enzymatic treatment of dough facilitates its handling and allows the production of an array of products.

1.3.4.6 *Proteases in pharmaceutical industry*

A wide range of proteases have been reported to have therapeutic importance. These enzymes are required at degree of purity and many of them are used topically and orally with a few administered into the blood circulation for the treatment of a variety of diseases. Asparaginases obtained from *E. coli* or *Erwinia chrysanthemi* are important

chemotherapeutic agents used in the treatment of acute lymphoblastic leukaemia and other lymphoid malignancies (*Rao et al., 1998; Sengupta & Dasgupta, 2006*). Proteases obtained from *Aspergillus oryzae* when administered orally work as digestive aid in treating certain syndromes of lytic enzyme deficiency. Proteases are also found to have therapeutic effects in sports medicine where they act to regulate and enhance blood circulation and immune system which in turn helps in muscle maintenance and optimizing workouts. Sport injuries treatment with proteases has shown speedy recovery from inflammation and quick healing of bruises. Proteases from plants are found to be more effective than those from animal sources in wound healing of patients undergone plastic surgery (*Sengupta & Dasgupta, 2006*). Latex of plants like papaya containing proteases like papain, chymopapain, papaya endopeptidases etc. (*Gurung & Skalko, 2009*) and *Calotropis gigantea* containing proteases like calotropin are reported to have wound healing properties (*Nalwaya et al., 2009*). Another protease, plasmin, present in the plasma, is involved in dissolving fibrin clots (*Sengupta & Dasgupta, 2006*).

1.3.4.7 *Proteases in photography industry*

Silver is one of the precious and expensive noble metal used in large quantities for different purposes especially in photography films. This expensive silver can be recovered from the waste X-ray/photographic films containing silver spread in gelatin. It has been reported that 25% of world silver demand is fulfilled by recycling of silver out of which 75% comes from photographic films. The recovery of silver can be achieved by breaking down the gelatin layers on the photographic films using proteases and hence releasing silver from them (*Shankar et al., 2010*). It was found that gelatin gets rapidly degraded by addition of proteolytic enzymes at 50°C at pH-8.0 and the silver particles get separated out (*Mahajan & Badgujar, 2010*). The alkaline proteases from *Conidiobolus coronatus* has been reported to be used for silver removal from X-ray films and its recovery for further use (*Shankar et al., 2010*).

1.3.5 Sources of proteases

The extensive participation of proteases in a variety of physiological and regulatory processes made their ubiquitous presence in diverse living organisms including microbes, animals and plants etc. Plants occupies topmost position with respect to the occurrence of proteases (43.85%) which is followed by bacteria (18.09%), fungi (15.08%), animals (11.15%), algae (7.42%) and viruses (4.41%) (Figure 1.7) (*Mahajan & Badgujar, 2010*).

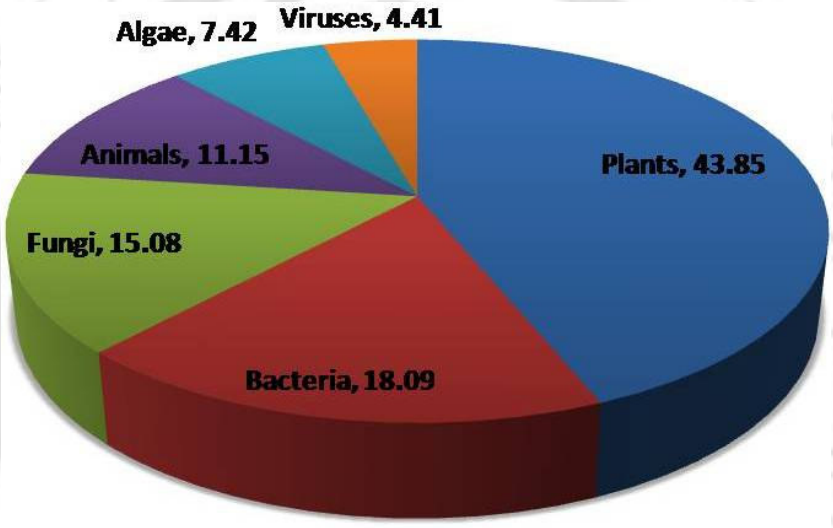

Figure 1.7: Distribution of proteases among various living forms. Proteases are most versatile enzyme and they are found in all life forms. The wide distribution of proteases indicates valuable physiological roles of the enzyme. Plants occupies topmost position with respect to the occurrence of proteases (43.85%) which is followed by bacteria (18.09%), fungi (15.08%), animals (11.15%), algae (7.42%) and viruses (4.41%).

1.3.5.1 *Plant proteases*

In plants, hundreds of genes encoding proteases are elaborately involved in different modes of plant development and physiology. Their role extends from seed germination, embryogenesis, photomorphogenesis, flower development, gametophyte survival, local and systemic defence, and hormone regulation including reactions against auxins, cytokinins, gibberellins, abscisic acid etc. to the ultimate senescence of plants. Proteases degrade

misfolded, harmful and redundant proteins and renew the repertoire of free amino acids necessary for the synthesis of young proteins thereby assisting plant development and growth (*Schaller, 2004*). Some of the well known proteases of plant origin are papain, bromelain, ficin, keratinases etc.

1.3.5.1.1 *Papain:*

Papain is a cysteine protease found in *Carica papaya* (papaya). The latex of the unripe fruit obtained after cutting the surface, is a rich source of papain. It is a thiol dependent single chain protein containing cysteine, histidine and glutamine as important catalytic residues. It preferably cleaves derivatives of basic amino acids such as L-arginine, L-lysine and residues preceded by phenylalanine (*Amri & Mamboya, 2012*). The mechanism of peptide bond breakage by this enzyme involves deprotonation of Cysteine-25 by Histidine-159. This deprotonation is assisted by the Asparginine-175 which helps in the proper orientation of imidazole ring of Histidine-159. After this, Cysteine-25 brings about a nucleophilic attack on the carbonyl carbon present on the backbone of target peptides (*Menard et al., 1990; Tsuge et al., 1999; Amri & Mamboya, 2012*). It is known to show wide range of pH and thermal stability. Papain has several medicinal and industrial applications. Papain is known to treat sport injuries and overcome allergic conditions like leaky gut syndrome, gluten intolerance etc. It is advantageous in a way to be used for chemomechanical removal of dental caries. It offers antibacterial, antifungal and anti-inflammatory properties which make them useful as gel based pharmaceutical products. It acts as clarifying and tenderizing agent in food industries. In leather industry, it is used in tanning of leather thereby making it shinier and smoother (*Amri & Mamboya, 2012*). Their usefulness in textile industry can be attributed to their ability to refine silks and process wools.

1.3.5.1.2 *Bromelain:*

Bromelain is a group of enzymes obtained as an aqueous extract from the stem or fruit of pineapple (*Ananas comosus*). It is classified as stem bromelain or fruit bromelain depending on the source and differs in their specificity and immunological properties. This crude extract

of enzyme consists of complex mixture of thiol proteases and non-proteases like glucosidases, phosphatases, peroxidases, cellulases, carbohydrates and glycoproteins (*Bhattacharya, 2008; Chobotova et al., 2010*). Bromelain is known to have broad substrate spectrum ranging from low molecular weight synthetic amides and dipeptides to high molecular mass substrates such as albumin, casein, fibrin etc. Although not very specific in their catalytic action, they preferentially cleave anayl, leucyl and glycol linkages (*Bhattacharya, 2008*). Bromelain can be absorbed through gastrointestinal tract without showing any sign of degradation and hence retaining its biological activity. Now days, it is attracting attention of researchers worldwide due to its extensive pertinence in medical field. It is known to exhibit anti-cancer properties which are predominantly attributed to its protease components. In association with other enzymes, bromelain is found to exert its anti-inflammatory effects on tissue injuries (*Bhattacharya, 2008; Mahajan & Badgujar, 2010; Chobotova et al., 2010;*). Ability of bromelain to customize the permeability of organs and tissues towards different drugs supported its use for potentiation of antibiotics for several years. It exerts fibrinolytic activity by breaking down cholesterol plaques thereby minimizing severity of angina pectoris and transient ischemic attack (*Bhattacharya, 2008; Chobotova et al., 2010*). Esterase, a non-proteolytic component of bromelain is responsible for its debriding activity and has beneficial effects on acceleration of healing of wounds and elimination of burn debris (*Bhattacharya, 2008*)

1.3.5.1.3 *Ficin:*

Ficin also known as ficain is a cysteine endopeptidase derived from the latex of fig (*Ficus carica*). It has wide range of pH stability and resembles papain with respect to specificity and pH- stability profile, but is found to be less thermostable (*Zhalehjoo & Mostafaie, 2012*). The active site of ficin contains sulfydryl group which is acylated during the reaction. The reaction mechanism of ficin is similar to that of papain (*Hammond & Gutfreund, 1958*). Ficin has more than 10 isozymes with similar molecular weight of 25 KDa and different isoelectric points (*Zhalehjoo & Mostafaie, 2012*). Casein, most abundant protein of milk, is found to be the specific substrate for ficin and can be completely digested in all bufferic conditions especially in phosphate buffers. This makes ficin to be used for making digestive

drugs. It is known to manifest antihelmintic properties by proteolytically digesting the cuticle of helminthes like *Ascaris suum* (*Robbins, 1930*). It can also be used as anti-inflammatory drug and can serve as effective replacement for glucocorticoids and non-steroidal antirheumatics.

1.3.5.2 *Animal proteases*

Most common proteases of animal origin are pepsin, trypsin, chymotrypsin, rennin etc. These proteases are produced in bulk by slaughtering livestock which limits their availability in market.

1.3.5.2.1 *Pepsin:*

Pepsin is an acidic protease found in the mucosal lining of stomach of almost all vertebrates. It is an aspartyl protease and is secreted in the form of zymogen, i.e., pepsinogen. The active form of the enzyme is released from the inactive zymogen form by autocatalysis in presence hydrochloric acid. Its optimal activity is between pH 1-2 and gets inactivated at pH above 6.0 (*Parde et al., 2012*). Pepsin degrades proteins into polypeptides, oligopeptides, peptones, but not to the amino acids. It has broad substrate specificity and is believed to catalyze the peptide bonds involving hydrophobic residues (*Veen & Hallinger, 1947*). Pepsin shows a distinct predilection for some linkages involving phenylalanine and tyrosine residues. Pepsin is known to resemble human immunodeficiency virus type 1 protease which is required for the maturation of HIV-1 (*Parde et al., 2012*).

1.3.5.2.2 *Trypsin:*

Trypsin is a membrane anchored serine protease as for proteolysis it utilizes a specific serine residue present in its catalytic site. It is secreted in the acinar cells of pancreas of the vertebrates in the form of inactive precursor, trypsinogen, to regulate the enzyme activity and to prevent unnecessary destruction of cellular proteins. This inactive trypsinogen gets activated by enterokinases in the gastrointestinal tract. Its catalytic triad consists of serine, aspartic acid and histidine residue. Trypsin specifically cleaves carboxyl-terminal side of

lysine and arginine residues. Peptide bonds involving either lysine or arginine followed by a proline residue are not cleaved by trypsin. The rate of hydrolysis decreases in case of presence of acidic amino acid residues on either side of the cleavage site (*Parde et al., 2012*). It is well known for its critical role in digestion. Its clinical significance can be attributed to its tumor-suppressive role in cancer progression (*Nyberga et al., 2006; Baird et al., 2006*). Excessive activation of trypsinogen to trypsin can lead to the auto digestion of pancreatic cells developing a clinical condition known as pancreatitis. In certain medical applications, trypsin is used for preparing bacterial media. It has a limited application in the food industry as it generates protein hydrolysates having a highly bitter taste (*Rao et al., 1998*). The ability of protease inhibitors to inhibit the enzyme from the gut of insects has focused the potential of trypsin to act as a target for bio control of insect pests.

1.4 Significance of the work

The significance of proteases is intimately interwoven with their intricate role in variety of physiological, developmental and regulatory pathways. Aside from their role in protein turnover and digestion, they are involved in regulation of great many physiological processes such as cell cycle, gene expression, embryogenesis, endocrine functions etc., which considerably stimulated the interest in proteases. The use of proteases dates back to antiquity. Now days, the pertinence of proteases in industries has extended to an extent to account for 60% of the total global enzymes sale. They are intricately involved in different industrial sectors like detergent, food, leather, pharmaceuticals, photography etc. The proteases involved in detergent industries have shown dominance in the world enzyme market. The use of proteases as additives in detergents has proved to considerably reduce risk of the environmental hazards caused by release of chemical constituents of the detergents in surroundings. They are also known to enhance the cleaning capabilities of detergents and increase their performance. Proteases have become an indispensable part of the modern food and feed industries to produce large and diversified ranges of products for human and animal consumption. Proteases have a large variety of applications, mainly in food and detergent industry. In view of the recent trends of developing environment friendly technologies, proteases are found to have extensive pertinence in leather treatement and in several

bioremediation processes. Proteases are also used in pharmaceutical industries for preparation of medicines such as ointments for debridement of wounds, dissolving blood clots in heart diseases etc. The current work reports purification, detailed biochemical characterization, cloning and cleavage specificity studies of a novel protease, procerain B. Further, various possible applications of procerain B was also explored. The work presented in the thesis is divided into four chapters as follow:

I. *Purification and physiochemical characterization of procerain B:* A novel cysteine protease has been purified from the latex of a well known medicinal plant, *Calotropis procera,* and further characterized for its different physiochemical properties. This newly purified enzyme was named as procerain B. Procerain, another cysteine protease, is already reported from the same source. Procerain B shows distinct features compared to procerain, with respect to cleavage recognition site, immunological properties and other physical attributes such as, isoelectric point, molecular weight etc. The newly purified enzyme shows broad pH optima (6.5-8.5) as well as temperature optima (40-60°C). Additionally, this enzyme retains its activity at conditions where most of the other proteases become inactive. Simple and economic purification of procerain B coupled with easy accessibility of latex makes the large-scale production of procerain B feasible. This enables exploration of various industrial as well as biotechnological applications of the enzyme.

II. *Screening of possible applications and determination of cutting site for procerain B:* Procerain B was further characterized by N-terminal amino acid sequencing and peptide mass fingerprinting (PMF). Certain physiochemical analysis such as N-terminal sequencing and peptide mass fingerprinting revealed distinct features of the enzyme. We have also demonstrated that procerain B may be a potential candidate for milk clotting in cheese industry and act as highly efficient digestive agent in food and therapeutic industries. In order to further extend the applications in pharmaceutical and biotech industries, we have determined the cleavage preference of procerain B. The

procerain B was also found to have mitogenic activity which opens a new horizon for its possible applications in angiogenic therapy for ischemic diseases.

III. *Immobilization of procerain B on different matrices and characterization of immobilized product:* Proteases are involved in several crucial biological processes reflecting its physiological importance. In addition to its physiological significances, it also has multifarious applications in food and pharmaceutical industries. Immobilized form of the enzyme further strengthens its applicability by enhancing its reusability and stability. The immobilization of procerain B on glutaraldehyde activated chitosan and amberlite MB-150 beads through covalent attachment were optimized. Besides serving as cross-linking agent, glutaraldehyde also links the procerain B on the surface of chitosan beads by forming schiff-base linkage through primary amine group of either lysine side chain or N-terminal region. The immobilized procerain B was then characterized for optimum functional range and stability with respect to pH and temperature. Better affinity of immobilized (amberlite MB-150) form (Km= ~180.27 µM) was observed as compared to soluble one for azocasein as substrate. The immobilized procerain B retains 50% (chitosan) and 38.6% (amberlite MB-150) activity till 10^{th} use which strongly affirmed its industrial candidature.

IV. *Cloning of cDNA, complete amino acid sequencing and molecular modeling of procerain B:* We report cDNA cloning, complete amino acid sequencing and molecular modeling of procerain B. The derived amino acid sequence shows high homology with other papain like plant cysteine proteases of peptidase C1A superfamily. The three dimensional structure of active procerain B was modeled by homology modeling using X-ray crystal structure of actinidin (PDB ID: 3P5U), a cysteine protease from the fruits of *Actinidia arguta*. The structural aspect of the enzyme was discussed. The cDNA was further cloned in pET-28(a) and pET-22(b) for expression of recombinant protein in *E.coli.*

Chapter II

Purification and physiochemical characterization of procerain B*.

2.1 Abstract

Calotropis procera is a tropical plant belonging to Asclepiadaceae family and has been widely used in Indian traditional medicinal system for the treatment of various diseases as leprosy, ulcers, tumors, piles and diseases of the spleen, liver and abdomen (*Kumar et al., 2006*). Preliminary screening of the latex of the plant showed very high proteolytic activity. A novel endopeptidase has been purified from the latex of *Calotropis procera* and characterized. As one cysteine endopeptidase, "procerain" is already reported from the same source, the newly purified enzyme was named as procerain B. The enzyme shows distinct properties compared to procerain, in terms of cleavage recognition site, immunological properties and other physical properties like molecular weight, isoelectric point etc. The newly purified enzyme has a broad optimum pH (6.5-8.5) as well as broad optimum temperature (40-60°C). Additionally, the enzyme retains its activity where most of other proteases are not active.

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* *Part of the work has been published in* ***Process Biochemistry****, 2010; 45; 399-406.*

2.2 Introduction

Proteases are one of the most versatile enzymes and present in all life forms, from viruses to highly evolved vertebrates. Bioinformatics analysis of the human and mouse genomes indicates that at least 2% genes of the genome code for proteases (*Puente et al., 2003*) which reflects its physiological importance. Endopeptidases are involved in several physiological processes and regulate different biological networks. They have therapeutic and industrial applications as well. The annual sales of proteases accounts for 60% of the total world enzyme market and estimated to reach 220 billion US$ by the year 2009 *(Turk, 2006)*. Based on the catalytic mechanism, there are five main classes (serine, cysteine, threonine, metallo and aspartate) of proteases. The first three classes (serine, cysteine and threonine) share a common catalytic mechanism where a particular amino acid acts as proton-withdrawing group to promote nucleophilic attack on the peptide bond and this is the basis of their nomenclature. The catalytic mechanism of last two classes (metallo and aspartate protease) is different where an activated water molecule acts as proton-withdrawing group.

Occurrence of cysteine proteases is very common in both prokaryotes as well as eukaryotes and in order to properly classify these proteases, total twenty families of cysteine proteases have been identified. Plant cysteine endopeptidases are used in industries owing to their high temperature stability and broad substrate specificity (*Dubey et al., 2007*). Plant genomes encode hundreds of endopeptidases, but little is known about what roles they play in the life of a plant. Endopeptidases are thought to be involved in a range of biological processes, including senescence, implicated in perception, signaling and execution leading to plant defence (*Van & Jones, 2004*). Few other cysteine endopeptidases from the plant sources have been purified and characterized. As usefulness of the endopeptidases depends on its unique cleavage site, stability as well as optimum functional conditions, search for a novel endopeptidase with unique properties is always on.

Calotropis procera is a tropical plant of Asclepiadaceae family. It is cosmopolitan in nature and grows as weed. The plant is of high medicinal importance and has been widely used in Indian traditional medicinal system for the treatment of various diseases namely leprosy, ulcers, tumors, piles and diseases of the spleen, liver and abdomen (*Kumar & Arya, 2006*). Various parts of the plant show anti-microbial, anti-inflammatory, antipyretic and

anti-malarial attributes (*Sharma & Sharma, 1999 & 2001*). The latex of the plant shows antidiabetic, hepatoprotective, antiarthritic, cytotoxic and anticancerous properties (*Singhal & Kumar, 2009*)**.** Preliminary screening of the latex of the plant showed very high proteolytic activity. The literature survey suggests the presence of several endopeptidases in the members of Asclepidaceae family. A cysteine endopeptidase (Procerain) is already reported from the latex of *Calotropis procera* (*Dubey & Jagannadham, 2003*). Here we report purification of another endopeptidase from the plant, which we named as procerain B, with distinct properties and cleavage site.

2.3 Materials & Methods

2.3.1 Materials

Superficial incisions on the young stem of *Calotropis procera* yielded milk like latex. Fresh latex of the plant was collected in chilled buffer with sodium tetrathionate as reversible inhibitor to avoid any autodigestion. CM-Sepharose FF was purchased from GE Healthcare. Azocasein, DTNB (5,5'-Dithiobis-[2-nitro benzoic acid]), DTT (Dithiothreitiol), GuHCl (Guanidine hydrochloride), urea, *o*-phenanthroline, EDTA (Ethylene diamine tetra acetic acid), EGTA (Ethylene glycol-bis [β-amino ethyl ether] N,N,N'N'-tetra acetic acid), leupeptin, SBTI (Soyabean trypsin inhibitor), NEM (N-ethylmaleimide), β-mercaptoethanol, PMSF (Phenylmethanesulfonylfluoride), acrylamide, N, N-methylene bisacrylamide, Coomassie brilliant blue R-250, E-64 (l-trans-epoxysuccinylleucylamide (4-guanidino) butane-N- [N- (L-3-trans-carboxyirane-2-carbonyl)- L-leucyl] agimatine), are obtained from Sigma Chemical Co., (USA). Sodium tetrathionate ($Na_2S_4O_6.2H_2O$) was synthesized by the method of Gilman et al., (*Gilman et al., 1946*). All other chemicals were of highest purity commercially available. Procerain was purified by method of Dubey and Jagannadham (*Dubey & Jagannadham, 2003*).

2.3.2 *Methods*

2.3.2.1 *Purification*

All purification steps were carried out in cold to minimize any complication due to possible autodigestion or unfolding of the enzyme at higher temperature. Most of the purification steps were carried out at 4°C unless stated otherwise. Latex was collected from the young stems of the plant in 0.01 M acetate buffer pH 4.5 containing 0.01 M sodium tetrathionate, a reversible inhibitor of cysteine proteases, and was stored at -20°C. Frozen latex was thawed to room temperature and centrifuged at 24,000 g for 10 min to remove any insoluble materials. The supernatant was then subjected to 50% ammonium sulfate fractionation. The supernatant after 50% ammonium sulfate fractionation found to have more enzyme activity, which was dialyzed extensively and used for further purification. The ion exchange chromatography of the dialyzed supernatant of 50% ammonium sulfate fractionation was performed at 4°C. The protein was loaded on CM Sepharose cation exchange column pre-equilibrated with acetate buffer pH 4.5. The column was washed with the same buffer until no protein was detected in elute and the bound proteins were eluted with a linear gradient of 0-0.8 M NaCl at a flow rate of 3 ml/min. Fractions of 6 ml were collected, the absorbance at 280 nm as well as caseinolytic activities of the protein in all fractions were checked using casein as a substrate. The partially purified fractions of the ion exchange chromatography were further purified by size exclusion chromatography using sephadex S-300 column (1.6 x 40 cm). The loaded protein was eluted by 0.01 M acetate buffer pH 4.5 containing 250 mM NaCl.

2.3.2.2 *Protein concentration*

Protein concentration at different stages of purification was determined by absorbance at 280 nm as well as by the method of Bradford (*Bradford, 1976*) using BSA as a standard.

2.3.2.3 *Protease activity*

The hydrolyzing activity of the endopeptidase was determined using denatured natural substrates casein or azocasein using the method of Dubey and Jagannadham (*Dubey & Jagannadham, 2003*). Enzyme solution (5 µg) was incubated in final volume of 500 µl of 50 mM Tris-HCl buffer pH 7.5 at 37°C for 10 min. Casein solution (1%) (w/v) was prepared in same buffer and added to the enzyme solution making the final reaction volume to 1 ml and the reaction mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 0.5 ml of 10% TCA (w/v), incubated further for 10 min at room temperature and centrifuged (10,000 rpm for 10 min). The absorbance of the soluble peptides in the supernatant was measured at 280 nm. In the case of azocasein as substrate, 0.5 ml of supernatant after TCA precipitation was mixed with equal volume of 0.5 M NaOH and incubated for 15 min. The colour developed was measured spectrophotometrically by taking absorbance at 440 nm. A control assay, without the enzyme was done and used as blank in all spectrophotometric measurements. One unit of enzyme activity was defined as the amount of enzyme, under given assay conditions that give rise to an increase of 0.001 unit of absorbance at 280 nm or an increase of 0.001unit of absorbance at 440 nm per minute of digestion. Number of units of activity per milligram of protein was taken as the specific activity of the enzyme.

2.3.2.4 *Electrophoresis*

Homogeneity and intactness of the enzyme, during purification as well as molecular mass determination of the purified enzyme, was assayed by using SDS-PAGE (*Laemmli UK, 1970*) with little modification. The purified enzyme was inactivated to avoid autolysis by treatment with Diisopropyl fluorophosphate (DFP). The gel was stained with 0.1% Coomassie brilliant blue R-250. Molecular weight markers were used as reference which includes phosphorylase b (97.4 kDa), BSA (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (29.0 kDa), soybean tyrosine inhibitor (20.1 kDa) and lysozyme (14.3 kDa). A plot of log molecular weight *Vs* mobility was generated to extrapolate the molecular weight of the purified enzyme.

2.3.2.5 *Isoelectric point*

The isoelectric point of the purified enzyme was determined by isoelectric focusing in tube gels as described for procerain (*Dubey & Jagannadham, 2003*). Ampholines, in the range of pH 3.5-10.0 were used to generate the pH gradient. A 5% (v/v) polyacrylamide gel containing 2% (v/v) desired ampholine was casted in tube gels. Iminodiacetic acids (0.01 M) and ethylenediamine (0.01 M) were used as anodic and cathodic chamber buffers respectively. The buffers were flushed with nitrogen gas before electrophoresis. The gel was subjected to a pre-run at a constant current of 1 mA per rod for 2 h to develop the pH gradient. A protein sample (100 µg) containing 10% (v/v) ampholine and 25% (v/v) glycerol was loaded on the gel and electrophoresed at constant current of 2 mA per rod for 4 h. Protein bands were stained with 0.04% (w/v) coomassie brilliant G-250 dissolved in 6% (w/v) perchloric acid (*Merril, 1990*).

2.3.2.6 *Carbohydrate content estimation*

The carbohydrate content of procerain B was determined by phenol sulphuric acid method (*Hounsell et al., 1997; Van et al., 2005*).

2.3.2.7 *Tryptophan and tyrosine content*

Total number of tryptophan and tyrosine residues in the purified protein was determined by the method of Goodwin and Morton (*Goodwin & Morton, 1946*). An absorbance spectrum of the purified enzyme in 0.1 M NaOH was recorded between 220-300 nm using a Beckman DU 640B spectrophotometer. Absorbance values at 280 nm and 294.4 nm were obtained from the spectra. For calculations, the formula, $w = (A_{280} – x.\ \varepsilon_y) / (\varepsilon_w - \varepsilon_y)$ was used, where w is the estimated tryptophan content in moles per liter; A_{280} is the absorbance at 280 nm for one molar protein; ε_w and ε_y are molar extinction coefficients of tryptophan and tyrosine in 0.1 M NaOH at 280 nm (ε_y=1576 and ε_w=5225) respectively. The total tyrosine and tryptophan content in the protein, x was calculated using $\varepsilon_{294.4}$= 2375. The number of a particular amino acid residue per molecule of the protein was calculated from the ratio of the molar concentrations of the amino acid residues to that of the total protein.

2.3.2.8 *Measurement of free and total sulfhydryl content*

Free and total cysteine residues of procerain B were determined by using DTNB method of Ellman (*Ellman, 1959*). For free cysteine content determination, the enzyme was activated with 0.05 M β-ME in 50 mM phosphate buffer pH 7.5 at 37°C for 30 min and dialyzed extensively for 24 h against 500 ml of 0.1 M acetic acid with four changes of the dialyzate. After dialysis, 50 µl of the dialyzed enzyme sample was taken in 700 µl of 0.1 M Tris-HCl, pH 7.3 and the sample was allowed to stand for 10 min to attain the pH. Subsequently, 50 µl of 5 mM DTNB solution was added and the reaction mixture was mixed thoroughly. The liberated TNB anion after reaction of sulfhydryl group with DTNB was monitored spectrophotometrically. The numbers of free cysteine residues are assessed using extinction coefficient of TNB of 14150 $M^{-1}cm^{-1}$ at 412 nm (*Creighton, 1989*). For estimation of the total number of cysteine residues, the enzyme was denatured in 6 M GuHCl and reduced with 0.05 M DTT in 0.05 M Tris-HCl, pH-8.0. The excess DTT in the reaction mixture was removed by dialysis against 500 ml of 0.1 M acetic acid with four changes of the dialyzate (*Riddles et al., 1983*). The liberated thiol groups were estimated as described in the case of free cysteine estimation. The numbers of disulfide bonds in the protein were deduced by comparison of the number of free and total cysteine residues. To validate the measurements, similar contents of papain, ribonuclease A, and lysozyme were also determined.

2.3.2.9 *Extinction coefficient*

The extinction coefficient of procerain B was determined using spectrophotometric method (*Aitken & Learmoth, 1997*). The extinction coefficient was determined using the formula, $\varepsilon^{1\%}_{280nm} = 10(5690n_w+1280n_y+120n_c)/M$, where n_w, n_y, n_c are the number of tryptophan, tyrosine, and cysteine residues in the protein; M is the molecular mass of the protein and 5690, 1280, 120 are the respective extinction coefficients of tryptophan, tyrosine and cysteine residues. The total numbers of tryptophan, tyrosine and cysteine residues in the protein are determined as described above.

2.3.2.10 ***pH and temperature optima***

The activity of the purified enzyme is measured as function of varying pH to determine the pH optima of the enzyme. 10 µg of enzyme was used for activity measurement. The buffers used were: 0.05 M KCl-HCl (pH 1.0-1.5); 0.05 M Glycine-HCl (pH 2.0-3.5); 0.05 M Na-acetate (pH 4.0-5.5); 0.05 M Na-phosphate (pH 6.0-7.5); 0.05M Tris-HCl (pH 8.0-10.0) and 0.05 M Sodium carbonate (pH 10.5-12.5). Substrate solution of azoalbumin or hemoglobin was prepared in the respective buffers. Procerain B was equilibrated in 0.5 ml of the buffer at a given pH for 15 min and added to the substrate solution of the same pH. The assay procedure is same as described above. Due to insolubility of azocasein below pH 4.0, hemoglobin was used as substrate for activity measurements at lower pH (*Sarath et al., 1989*).

The effect of temperature on the activity of procerain B was also studied using azocasein as substrate. 10 µg of enzyme was incubated at desired temperature in the range of 10-95°C for 15 min in 50 mM phosphate buffer pH 7.5 and an aliquot was used for the activity measurement at the same temperature. Prior to the assays, substrate solution was also equilibrated at the corresponding temperature in the same buffer. At each temperature, a control assay was carried out without the enzyme and used as a blank.

2.3.2.11 ***Stability***

The ability of purified enzyme to retain its activity under various conditions such as extreme pH, temperatures, strong denaturants and organic solvents were studied. The 15 µg of enzyme was incubated at different pH in the range of pH 0.5-12.0 for 24 h at room temperature and residual activity was measured as described earlier using azocasein as substrate. Similarly, enzyme samples were incubated at temperatures from 10°C to 95°C for 15 min and assayed for residual activity. The enzyme was also incubated for 24 h in presence of chemical denaturants like GuHCl, urea and different solvents such as methanol, acetonitrile, dioxan. The activity was measured after 24 h incubation as previously described.

The thermal stability profile of procerain B against temperature and increase in concentration of GuHCl was also studied with help of fluorescence spectroscopy. The 5 µM

solution of procerain B in Tris-HCl buffer was incubated at different temperatures (10-90°C) for 15 min and then the fluorescence spectra of heated samples were taken immediately. Similarly the 5 μM solution of procerain B in 6 M GuHCl in Tris-HCl buffer was incubated for overnight and the fluorescence spectra was collected next day.

2.3.2.12 *Effect of various compounds on the activity of procerain B*

Effect of protease inhibitors on the proteolytic activity of enzyme was studied. The inhibitors used were sodium tetrathionate (STT), iodoacetic acid (IAA), PMSF, EDTA, EGTA, E-64, p-chloromercuric benzoate, *o*-phenanthroline, NEM, Mercuric chloride, DIFP, Leupeptin, and SBTI. A control assay of the enzyme activity was done without inhibitors and the resulting activity was taken as 100%. The enzyme was incubated with an inhibitor for 30 min at room temperature and an aliquot was used for the activity measurement. The assay was done as described earlier.

2.3.2.13 *Effect of substrate concentration on the reaction velocity*

The effect of increasing substrate concentration on the reaction velocity of the enzyme hydrolysis was studied using azocasein as substrate at pH 7.5 and 37°C. 10 μg of the enzyme was used, and the concentration of azocasein was in the range of 1-400 μM. Assays were performed as already described under proteolytic activity measurements. A blank was used at each specific substrate concentrations without the enzyme. A Lineweaver-Burk plot was plotted and the value of Michaelis-Menten constant (Km) was calculated.

2.3.2.14 *Autocatalysis*

Proteases, in general, are prone to autodigestion and the extent of autolysis depends on enzyme concentration, pH, and temperature. Different concentrations of procerain B ranging from 0.05-0.60 mg/ml were incubated with 0.05 M Tris-HCl buffer pH 7.5 at room temperature. An aliquot containing 2 μg of enzyme was used for the determination of protease activity at different time intervals with casein as substrate. Activity of enzyme after first 10 min of activation was taken as 100% for the calculation of the residual activities.

2.3.2.15 *Substrate specificity*

Equal amount (5 µg) of procerain, procerain B and papain were incubated with 20 µg of BSA at 37°C for 15 min. The resulting fragments were separated on 15% SDS-PAGE. The protein band was stained and gel was destained using normal procedures of protein electrophoresis.

2.3.2.16 *Polyclonal antibodies and immunoassays*

Antibodies against procerain were raised in a male albino rabbit (weight about 1.5 kg) as described by Dubey and Jagannadham (*Dubey & Jagannadham, 2003*). An indirect ELISA was performed (*Friguet et al., 1989*) to check cross reactivity of procerain B and other antigens with anti-procerain serum. Wells of microtiter plate were coated with 1 µg/µl of procerain, procerain B, papain (50 µl/well) in sodium carbonate pH 9.6 and incubated overnight at 4°C. The wells were washed thoroughly with phosphate buffer saline, pH 8.0, containing 0.05% Tween-20 (PBS-T) and coated with 5% BSA to avoid non-specific adsorption. After 1-2 h of incubation at 37°C, the wells were washed thoroughly and primary antibody to procerain appropriately diluted in PBS-T was added to 100 µl/well. The plate was incubated at 37°C for 1-2 h and washed as before. The goat anti-rabbit I_gG, linked to horseradish peroxidase was diluted 5000 times and added to the wells. After 1-2 h of incubation at 37°C, the wells were washed as described earlier and 100 µl of substrate solution prepared by dissolving 100 µl of H_2O_2 and 9 mg of o-phenylenediamine in 25 ml of 0.1 M citrate-phosphate buffer pH 5.0 was added to each well and left at 37°C for 10 min. The colour developed was measured in a microplate reader at 490 nm. Pre-immune serum was used as negative control.

2.3.2.17 *N-Terminal sequencing*

The N-terminal of the procerain B was sequenced to understand homology and evolutionary relationship with other cysteine proteases of its class. Purified protein was transferred to PVDF membrane using the procedure of Choli and Kapp (*Choli & Kapp, 1889*). N-terminal

sequencing was performed using the PROCISE Protein Sequencing System from Applied Biosystems by the method of Matsudaria (*Matsudaria, 1887*).

2.3.2.18 *Peptide mass fingerprinting*

In-gel digestion by trypsin and peptide mass fingerprinting was done by the method of Katayama et al. (*Katayama et al., 2001*) using Ultraflex matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) (Bruker Daltonics). During in-gel digestion, the proteins are reduced in 10 mM dithiothrietol for 45 min at 60°C. The samples are then treated with iodoacetamide (55 mM) in the dark for 45 min to alkylate the newly reduced disulfide bonds. MASCOT search tool (URL, http://www.matrixscience.com) was used for identification of tryptic maps.

2.4 Results

2.4.1 Purification

The purification of procerain B consists of three steps (Figure 2.1). After removal of the gum, crude latex was subjected to 50% ammonium sulphate fractionation. The precipitate and supernatant both revealed protease activity but the supernatant was found to have higher activity and more amount of protein. Thus, the supernatant from previous step was chosen for the further purification using CM-Sepharose at pH 4.5. The bound proteins were eluted with a linear salt gradient of 0-0.8 M NaCl. The elution profile got resolved into two peaks I and II (Figure 2.1A). Another endopeptidase, procerain, is reported from peak I (*Dubey & Jagannadham, 2003*). Thus, proteins in Peak II of the CM-sepharose chromatography were further purified by size exclusion chromatography using sephacryl S300. The loaded proteins got eluted in single asymmetrical peak after size exclusion chromatography. The fractions of descending arm of the peak shown between the arrows were found to be homogeneous (Figure 2.1B). Homogenous fractions after size exclusion chromatography were pooled, concentrated and dialyzed against 50 mM phosphate buffer, pH 7.0 and stored at 4°C for further use. The purification table is summarized in Table 2.1. The relatively low recovery of

protease activity was supposed to be due to presence of several proteases in the crude latex as mentioned in case of other members of the family. It is also important to note that the specific activity of the purified endopeptidase did not increase several folds due to multiple proteases present in the latex. However, characterization of the enzyme in terms of stability and specificity has significant commercial importance.

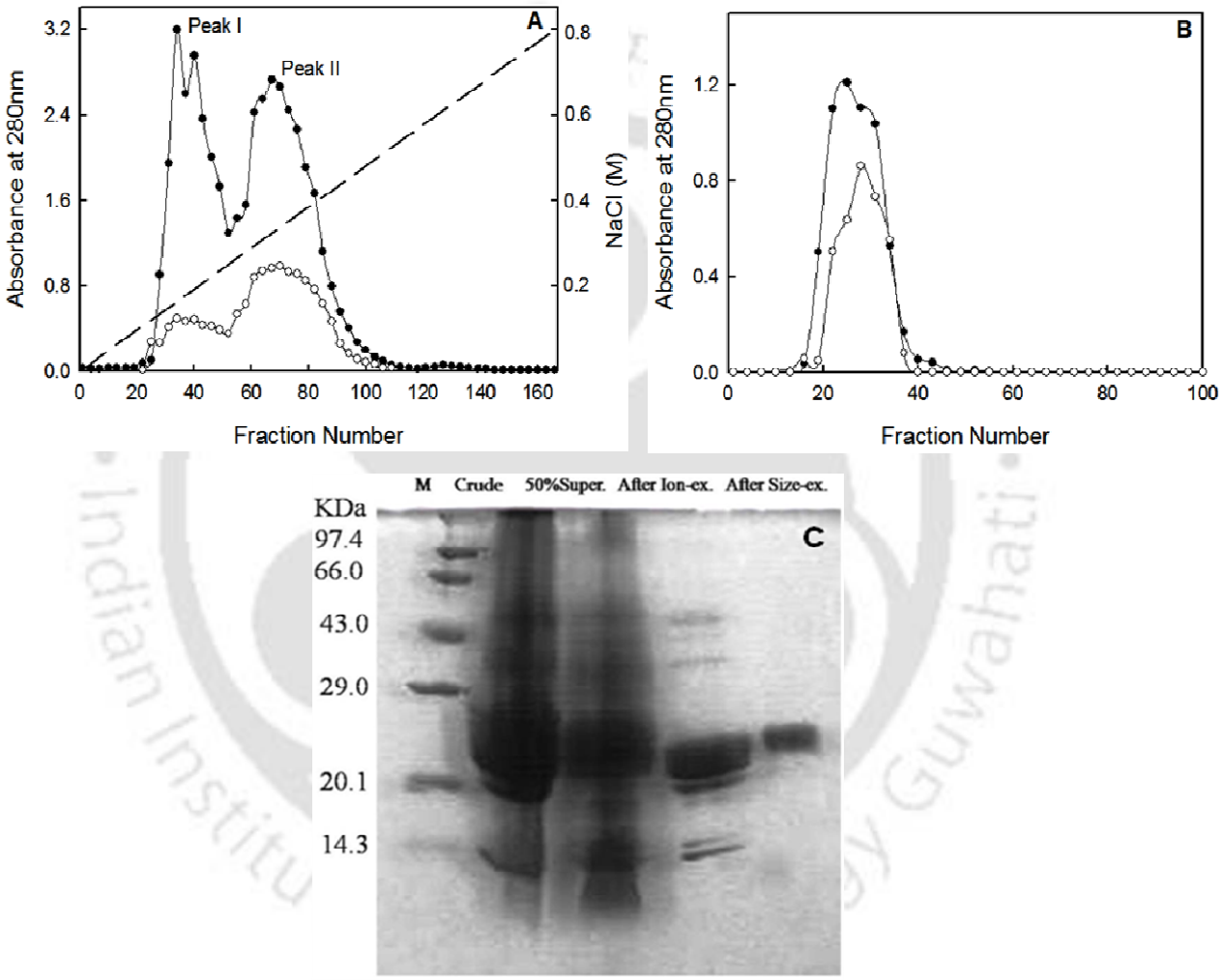

Figure.2.1: Purification of procerain B. **(A)** Elution profile of cation exchange chromatography. Solid circles representing the protein content of different fractions at 280 nm and hollow circles showing the proteolytic activity of corresponding fractions. The proteolytic activity of different fractions was measured by incubating activated procerain B with 1% substrate solution at 37°C for 30 min (Detail is provided in section 2.3.2.3). The bound protein was eluted with linear salt gradient of 0.0 to 0.8 M NaCl. **(B)** Elution profile of gel-filtration chromatography at 1 ml/min flow rate and the fractions of 3 ml were collected. The proteins were eluted in form of single asymmetrical peak and the fractions of descending arm were found to be pure. **(C)** SDS-PAGE profile of different steps of purification. After size exclusion chromatography, the single band represents purified procerain B.

Table 2.1 Purification table of procerain B from the latex of *Calotropis procera.* As the plant has multiple proteases, increase in specific activity was not very high.

Steps	Total protein (mg)	Total activity (units[a])	Specific activity (units/mg)
Crude Latex	780	80,880	103
50% Ammonium sulfate supernatant	384	43,776	114
Ion exchange (peak II descending arm)	120	17,400	145
Purified procerain B	70	15,750	212

[a] Definition of one unit : 1 unit of enzyme activity is defined as the amount of enzyme under the assay conditions described, gives rise to an increase of 0.001 unit absorbance at 280 nm/min of digestion. Casein was used as substrate.

2.4.2 Physical Properties

Procerain B showed a single band on SDS-PAGE confirming its purity (Figure 2.1C). The molecular mass of procerain B as estimated by SDS-PAGE was found to be 25.7 kDa compared to 28.8 kDa for procerain (*Dubey et al., 2003*).

2.4.2.1 *Molecular weight determination*

The molecular weight of procerain B falls well in the range of plant cysteine endopeptidases (Table 2). The isoelectric point of procerain B was found to be 9.52. Most of the cysteine endopeptidases are reported to be basic in nature like procerain (pI 9.32), heynein (pI 10.8), papain (pI 8.75), ficin (pI 9.0), ervatamin C (pI 9.54) and stem bromelain (pI 9.55) (*Dubey et al., 2003 and Patel et al., 2003*). However, some acidic plant endopeptidases like actinidin (pI 3.0) and asclepain (pI 3.11) are also reported (Table 2). The extinction coefficient ($\varepsilon^{1\%}_{280nm}$) of purified enzyme procerain B as determined by spectrometric methods was 10.1 while procerain showed extinction coefficient ($\varepsilon^{1\%}_{280nm}$) of 24.9. The number of tryptophan was found to be five and number of tyrosine was fifteen as determined by spectroscopic analysis. The total cysteine content of procerain B is found to be seven with one free cysteine residue (measured value 0.89), thus forming four disulphide bridges.

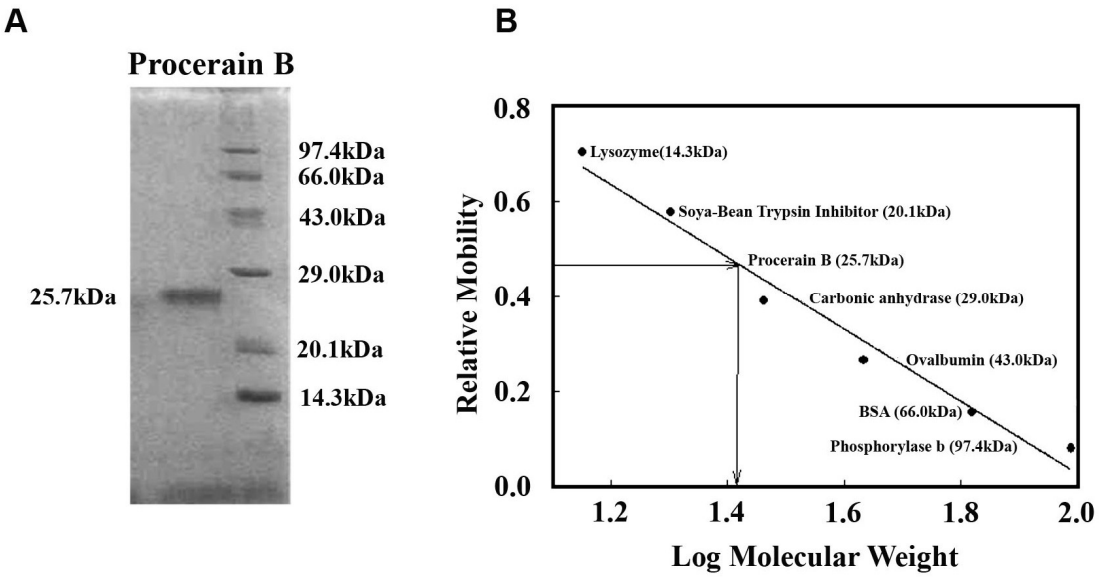

Figure 2.2: (A) SDS -PAGE of protein samples: Lane 1 and 2 represent purified protein and protein molecular weight markers respectively **(B)** Estimation of molecular weight of procerain B on SDS-PAGE by comparing its relative mobility with standard molecular weight markers used in gel. The log molecular weights (X axis) of different markers were plotted against their relative mobility (Y axis) and the molecular weight of procerain B was calculated. The molecular weight of procerain B was found to be 25.7 kDa.

Table 2.2 Comparison of biochemical properties of procerain with other plant cysteine proteases.

Protein	Plant	Mol. weight (kDa)	pH optimum	Temp. Optimum (°C)	Isolectric point (pI)	No of disulfide bond	Carbohydrate	References
Procerain B[1]	*Calotropis Procera*	25.7	6.5-8.5	40-60	9.52	4	No	Current Work
Procerain[2]	*Calotropis Procera*	28.8	7.0-9.0	55-60	9.32	3	No	*Dubey et al., 2003*
Ervatamin A[3]	*Ervatamia coronaria*	27.6	8-8.5	50–55	8.37	3	No	*Pande et al., 2006*
Ervatamin B[4,5]	*Ervatamia coronaria*	26	6.0-6.5	50–55	9.35	3	No	*Bhowmick et al., 2008*
Ervatamin C[6]	*Ervatamia coronaria*	25.5	7.5-8.0	50	9.54	3	No	*Biswas et al., 2003*
Heynein[7]	*Ervatamia heyneana*	23	8.0–8.5	50–55	10.8	5	No	*Sundd et al., 1998*
GP-I[8,9]	*Zingiber officinale*	22.5	NR	NR	4.6	3	Yes	*Patel et al., 2003*
GP-II[8,9]	*Zingiber officinale*	22.5	NR	NR	4.7	3	Yes	*Choi et al., 2002*
Papain[10]	*Carica papaya*	23	6.0 - 7.0	NR	9.6	3	No	*Ohtsuki et al., 1995*
Asclepain c-II[11]	*Asclepias curassavica*	23.6	9.4-10.2	NR	9.3	NR	NR	*Liggieri et al., 2009*

NR: Not reported.

Plant cysteine protease, heynein, is reported to have eleven cysteine residues (five disulfides and one free) (*Patel et al., 2003*). Procerain B, like procerain and many other cysteine endopeptidases, does not contain a detectable carbohydrate moiety (*Dowall, 1970; Dubey et al., 2003; Patel et al., 2003*). Most of the cysteine endopeptidases, including procerain, reported to have seven cysteine residues, out of which six residues are involved in forming three disulfide bonds and one is present at catalytic site (*Brockbank et al., 1979; Pal et al., 1980; Sumner et al., 1993; Sundd et al., 1998; Biswas et al., 2003*). However, few cysteine endopeptidases like ginger endopeptidases GP-I and GP-II reported to have significant carbohydrate moiety (*Choi et al., 2002*).

2.4.2.2 *pH and temperature optimum*

Procerain also shows broad temperature optima in the range of 40-60°C (Figure 2.3A). The enzyme shows 100% activity till 65°C. This unusually high temperature stability, broad thermal and pH optima of procerain B make it a valuable endopeptidase for industrial application. Procerain B shows broad pH optima for hydrolyzing activity and shows optimum activity in the range of pH 6.5-8.5 (Figure 2.3B). Such a broad pH optima range of procerain B may make it a better endopeptidase molecule in various food and biotechnology industries. Further, the optimum pH of procerain B differs significantly with procerain and other cysteine endopeptidase. A sharp decrease in activity in the acidic region below pH 5.0 may be due to the formation of improper ionic forms of the enzyme active site or substrate or combination of these two.

2.4.2.3 *Stability*

The stability of an enzyme under extreme conditions is a decisive factor for its usefulness as a potent industrial enzyme. Procerain B is remarkably stable and retains its activity under the conditions where most of the enzymes lose their activity. The enzyme is fully active till 65°C for 15 min incubation time (Figure 2.3A). The enzyme retains complete activity in 30% methanol while in 80% methanol, enzyme shows 15% activity. Additionally, enzyme is fully active in 35% acetonitrile and 2.0 M GuHCl at pH 7.5. The enzyme retains most of the

activity in 8 M urea. Such a high stability of procerain B under various harsh conditions makes it an excellent enzyme for industrial and biotechnological applications.

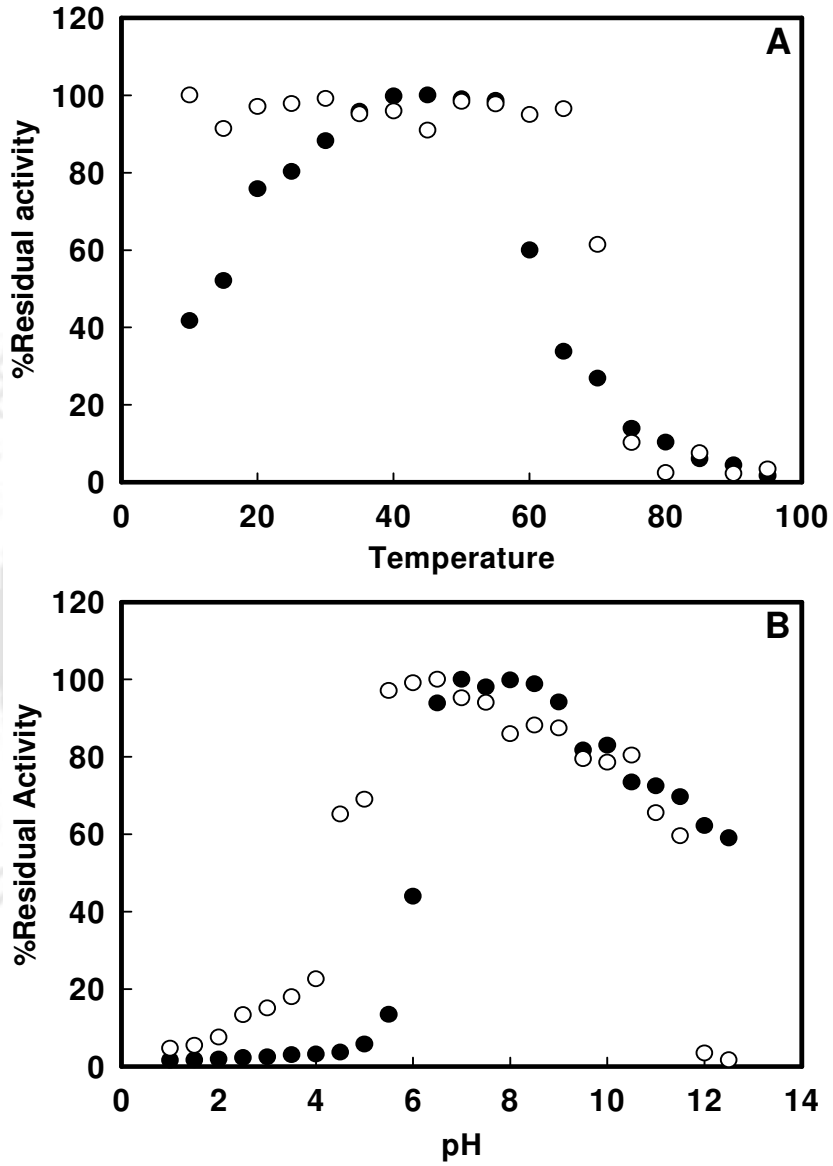

Figure 2.3: Effects of **(A)** temperature and **(B)**, pH on activity (●) and stability (○) of procerain B. The effect of pH on activity of procerain B was determined by equilibrating the enzyme at different pH and then the reaction was also carried out at respective pH. For temperature optima, the enzyme was incubated at different temperatures for 15 min and substrates pre-equilibrated at respective temperatures were added to carry out reaction at corresponding temperatures. Stability was determined by overnight incubating the enzyme at room temperature at different pH conditions and next day activity was taken as described in method section. For temperature stability measurements, enzyme was incubated at required temperature for 15 min and activity was measured at 37°C and pH 7.5.

The thermal stability of procerain B was further studied with help of fluorescence spectroscopy (Figure 2.4). Sharp decrease in intrinsic fluorescence after 60°C (Inset) shows the disruption of native structure of procerain B (Figure 2.4A). Same pattern was observed in unfolding study of procerain B in terms of activity with increasing temperature as shown in Figure 2.3A. After 65°C, the enzyme seems to lose its activity along with native structure disruption. The stability of procerain B against denaturant (GuHCl) was studied with fluorescence spectroscopy (Figure 2.4B) that further indicates high stability of the enzyme.

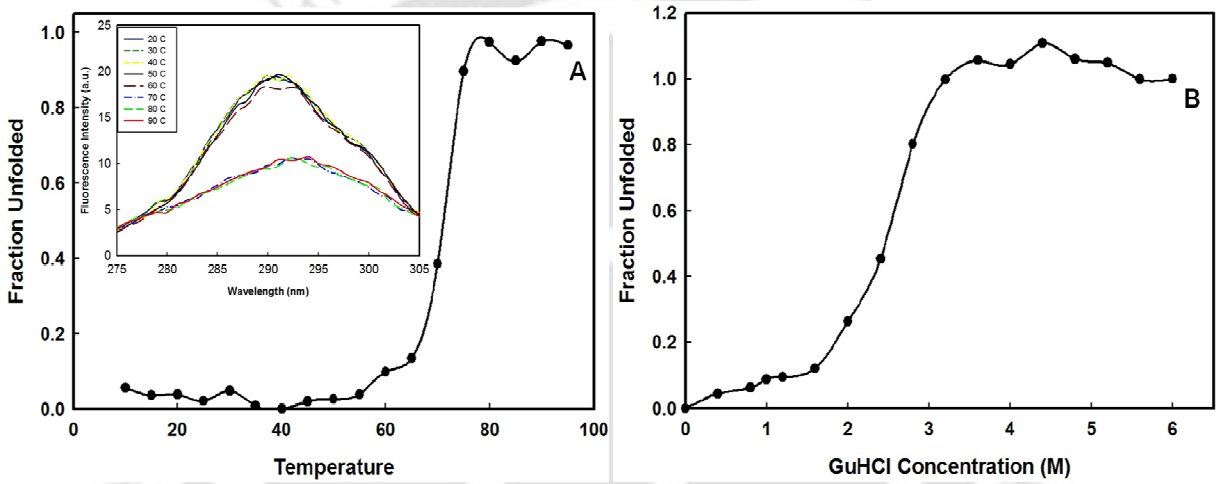

Figure 2.4: Stability profile of procerain B monitored using Fluorescence Spectroscopy, **(A)** Temperature **(B)** GuHCl as denaturant.

2.4.3 Effect of inhibitors on activity of procerain B

Seven catalytic types of proteases are recognized in which serine, threonine, cysteine, aspartic, glutamic, asparagine or metallo groups play primary roles in enzyme catalysis. We have studied effects of inhibitors of various classes of protease to determine the class of the purified endopeptidase, procerain B (Table 2.3). Maximum inhibition of activity of the enzyme observed in the presence of iodoacetic acid, E-64, HgCl2, N-ethylmaleimide (NEM), p-chloromercuribenzoate (PCMB), leupeptine and sodium-tetrathionate (STT). The enzyme did not show significant inhibitions in presence of PMSF, SBTI and DFP. Lack of inhibition of the activity by proteinaceous inhibitors such as SBTI, which are abundant in protein rich

foods like soybean, makes the enzyme a potential endopeptidase for food industries (*Kaneda et al., 1977*). Metalloprotease inhibitors like *o*-phenanthroline, EDTA and EGTA showed no significant effect on the activity of procerain B ruling out the possibility of the endopeptidase being a metalloprotein. The results confirm that the enzyme belongs to cysteine protease family.

Table 2.3 Effect of various compounds on proteolytic activity of procerain B and comparison with procerain.

		Procerain*		Procerain B	
Inhibitor class	Inhibitor	[I]	Residual activity (%)	[I]	Residual activity (%) **
Cysteine protease	Iodoacetic acid	40 µM	12	40 µM	5
	E-64	3 µM	0	3 µM	0
	$HgCl_2$	5µM	8	2 µM	9
	NEM	30 µM	21	50 µM	18
	Sodium-tetrathionate	30 µM	15	30µM	22
	PCMB	5 µM	10	5 µM	12
Ser/Cys	Leupeptin	10 µM	10	10 µM	15.2
Serine protease	DFP	1mM	95	1 mM	98.5
	PMSF	50 µM	85	50 µM	92
	SBTI	1 mM	100	1 mM	100
Metallo protease	EDTA	1 mM	100	5 mM	100
	EGTA	1 mM	100	5 mM	100
	o-phenanthroline	1 mM	100	5 mM	100

* Data taken from Dubey and Jagannadham (2003). ** Average of three measurements

2.4.4 Effect of substrate concentration on the activity of procerain B

The effect of increasing substrate concentration on the activity of procerain B follows the Michaelis-Menten kinetics. The study was carried out using azocasein and casein as substrates. The value of Km was estimated from Lineweaver-Burk plot. The enzyme shows Km value of 210 µM with both the substrates (Figure 2.5).

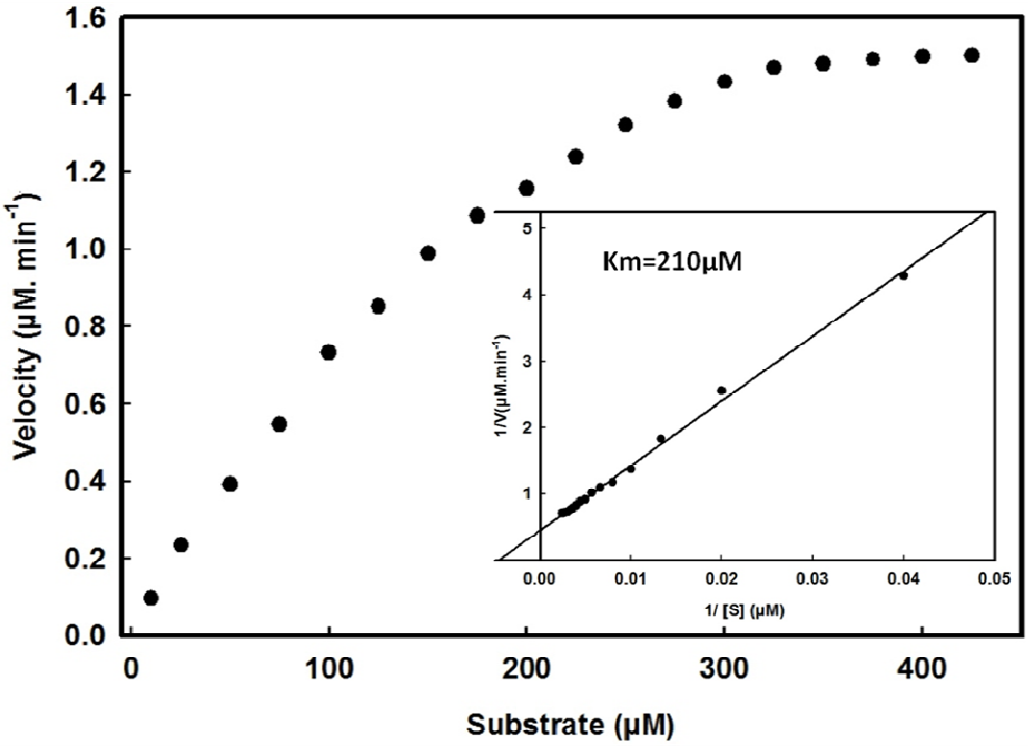

Figure 2.5: Effect of increasing substrate concentration on the activity of procerain B was studied with azocasein as substrate and the km was calculated from the Lineweaver-Burk plot (Inset). The procerain B was found to follow Michaelis Menten kinetics. Initially with increase in substrate concentration the rate of the reaction also increases and after a certain point there was no increase and the velocity remains constant on further increase in substrate concentration. The Km calculated for azocasein was found to be 210 µM.

2.4.5 Substrate specificity

The specificity of the enzyme was further investigated by BSA digestion. Procerain B showed very different cleavage pattern compared to procerain and papain. Thus, procerain B appears to have distinct substrate specificity. Two major digested fragments of 27 kDa and 30 kDa as well as minor bands around 25 kDa are common. However, 21 kDa, 20 kDa and 14 kDa fragments are unique in procerain B (Figure 2.6). This suggests that procerain B can cleave at additional sites which are not recognized by papain and procerain. However, the common fragments indicate that procerain B can also cleave at sites recognized by papain and procerain.

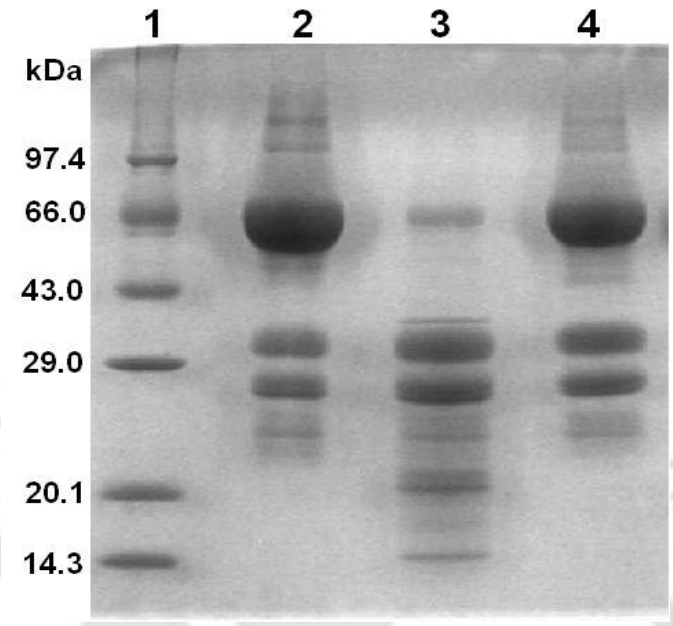

Figure 2.6: Comparative substrate specificity studies using digestion of BSA. Equal amount of BSA was digested with procerain, procerain B and papain. The resulting fragments were electrophoresed on 15% SDS-PAGE as discussed in method section. Lane 1 shows molecular weight marker proteins, Lanes 2 shows BSA digested with procerain, Lane 3 shows BSA digested with procerain B, and Lane 4 represents BSA digested with papain.

2.4.6 Autocatalysis

Further, the autodigestion property of endopeptidases limits its usefulness. Autolysis resistant endopeptidases are considered to be more useful for industrial and therapeutic applications. Autodigestion property of procerain B is in the protein concentration range of 0.05-0.6 mg/ml at pH 7.5 (Figure 2.7). The enzyme shows relatively slow autodigestion and retains above 50% of activity after 24 h upto 0.2 mg/ml concentration. However, relatively rapid autolysis at higher concentrations is observed. The autodigestion property of procerain B is different from procerain and many other endopeptidases where fast autodigestion at lower protein concentrations is reported (*Dowall, 1970; Dubey et al., 2003; Pandey et al., 2006; Bhowmic et al., 2008*). Thus, procerain B appears to resist autodigestion which may make it a valuable enzyme in various food and biotechnology industries.

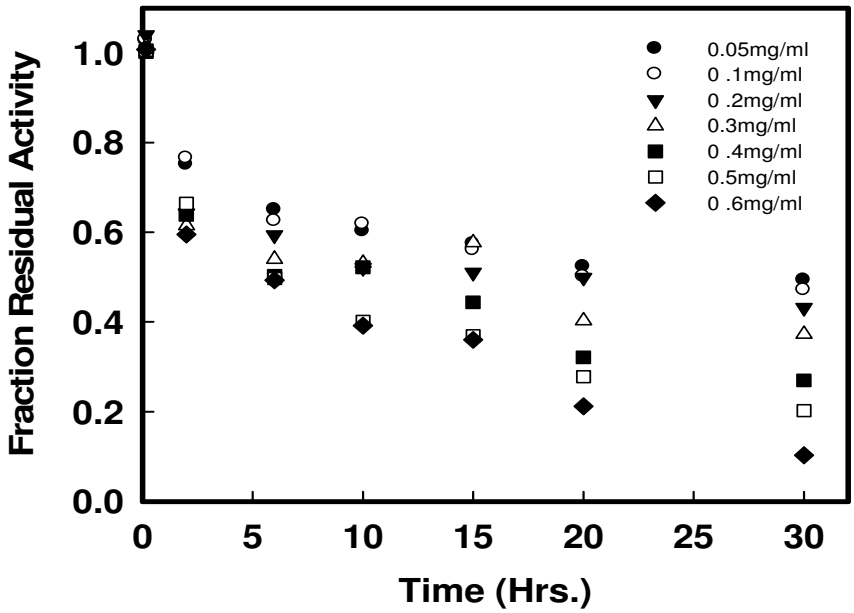

Figure 2.7: Autodigestion of procerain B, as a function of increasing protein concentration was depicted: Enzyme at different protein concentrations (0.05-0.6 mg/mL) was incubated at 37°C in presence of activator (50 mM β-marcaptoethanol). Protein aliquots were taken at various time intervals and proteolytic activity was measured in optimal conditions using 5 µg of enzyme as described in method. The activity of enzyme after 10 min of incubation (time required for enzyme activation) was taken as 100% activity.

2.4.7 Polyclonal antibodies and immunoassays

We have earlier reported procerain from *Calotropis procera* (*Dubey et al., 2003*). As procerain B is the second cysteine endopeptidase reported from the same plant, it was important to show that the procerain B is distinct from procerain. We have shown that procerain and procerain B have distinct physical properties like molecular weight, pH optima, extinction coefficient etc. Additionally, we have used immunological method for the same. Polyclonal antibodies specific to procerain have been successfully raised in male albino rabbit and cross reactivity with procerain B by indirect ELISA was checked. The typical color development in indirect ELISA resulting from procerain anti-procerain complex formation further confirmed the presence of antibodies to procerain (Figure 2.8). The newly purified procerain B and few other common proteases were also checked for cross reactivity against anti-procerain. Like other protease and unlike procerain, procerain B did not show any cross reactivity with anti-procerain. This confirms a distinct nature of procerain B and reveals that purified protein is antigenically different from them.

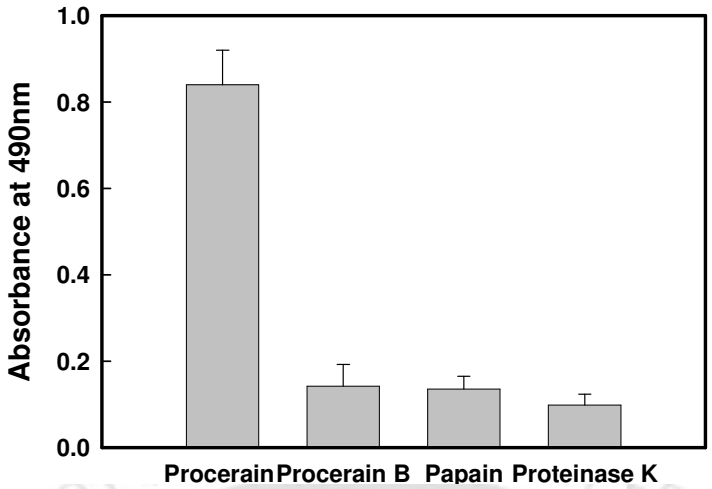

Figure 2.8: Bar diagram for an ELISA to check cross-reactivity of antisera to procerain with procerain, procerain B and other related proteases: ELISA was performed as described in Methods. Antigens were used at a concentration of 1µg/ml and antiserum to procerain diluted 10,000 times was added 100µl/well. The goat anti-rabbit IgG, linked to horse radish peroxidase, were diluted 5000 times in PBS-T. H_2O_2 was used as a substrate and o-phenylenediamine as a colour indicator. The color developed was measured at 490 nm. Background contribution has been deducted.

2.4.8 N-terminal sequencing

First 15 N-terminal sequence of procerain B was compared with other common plant proteases (Table 2.4). The sequence alignment shows distinct nature of procerain B. It is important to mention that another endopeptidase, procerain, purified from the same source with distinct biochemical-biophysical properties has N-terminal blocked (*Dubey et al., 2003*). It is worth mentioning that many proteins cannot be directly sequenced by Edman degradation because their N-terminal amino group is modified by N-acetylserine (which occurs frequently in eukaryotic proteins) or N-acetylthreonine. Procerain B, from the same source does not show modified N-terminal. Among plant cysteine proteases, proline is highly conserved at position two which is reported to be necessary to prevent unwanted proteolysis (*Hounsell et al., 1997*). The characteristic VDWR motif is also present in procerain B. Procerain B shows maximum sequence similarity (80%) with asclepain. In most of other cases, the sequence similarity was above 50% except ervatamin C, heynein and bromelain. However, in case of ervatamin C, heynein and bromelain N-terminal sequences, there are few unidentified residues (X) which are not considered as match with procerain B at respective position in our sequence similarity calculation.

Table 2.4 Amino terminal sequence of procerain B and other cysteine proteases. It is worth mentioning that the N-terminal of procerain is blocked (*Dubey & Jagannadham, 2003*). The procerain B has maximum homology with asclepain and minimum with bromelain, suggesting that procerain B is an asclepain type cysteine protease. Among all plant cysteine proteases, proline is highly conserved at position fifteen while in case of procerain B, it is histidine.

Enzyme	Amino terminal sequence (first 15 residues)															%Homology
Procerain B (*Singh et al., 2010*)	**L**	**P**	**N**	**S**	**V**	**D**	**W**	**R**	**Q**	**K**	**G**	**V**	**V**	**F**	H	100.0
Asclepain (*Lynn et al., 1980*)	**L**	**P**	**N**	**S**	I	**D**	**W**	**R**	**Q**	**K**	N	**V**	**V**	**F**	P	80.0
Papaya proteinase IV (*Taylor et al., 1995*)	**L**	**P**	E	**S**	**V**	**D**	**W**	**R**	A	**K**	**G**	A	**V**	T	P	66.6
Morrenain bII (*Cavalli et al., 2001*)	**L**	**P**	D	**S**	**V**	**D**	**W**	**R**	K	**K**	N	L	**V**	**F**	P	66.6
Morrenain oII (*Cavalli et al., 2001*)	**L**	**P**	D	**S**	**V**	**D**	**W**	**R**	K	**K**	N	L	**V**	**F**	P	66.6
Papain (*Mitchel et al., 1970*)	I	**P**	E	Y	**V**	**D**	**W**	**R**	**Q**	**K**	**G**	A	**V**	T	P	60.0
Papaya proteinase omega (*Revell et al., 1993*)	**L**	**P**	E	N	**V**	**D**	**W**	**R**	K	**K**	**G**	A	**V**	T	P	60.0
Philibertain g I (*Sequeiros et al., 2005*)	**L**	**P**	A	S	**V**	**D**	**W**	**R**	K	E	**G**	A	**V**	L	P	53.3
Ervatamin B (*Kundu et al., 2000*)	**L**	**P**	S	F	**V**	**D**	**W**	X	S	**K**	**G**	A	**V**	N	S	53.3
Ervatamin C (*Sundd et al., 1998*)	**L**	**P**	E	Q	I	**D**	**W**	**R**	K	X	**G**	A	**V**	T	P	46.6
Heynein (*Patel & Jagannadham, 2003*)	**L**	**P**	E	Q	I	**D**	X	**R**	X	X	**G**	A	**V**	N	P	40.0
Bromelain (*Goto et al., 1980*)	V	**P**	Q	**S**	I	**D**	**W**	**R**	N	Y	G	A	**V**	T	S	40.0

Bold letter shows conserved residue.

2.4.9 Peptide mass fingerprinting

Conventional methods to differentiate proteins like protein sequencing and enzymatic characterization is time consuming. Peptide mass fingerprinting is reported to be an excellent tool to differentiate proteins in a fast and unequivocal way. Peptidases even with high similarity have been differentiated with peptide mass fingerprinting. The method has been used for differentiation of the isoenzymes of the latex of *Asclepias curassavica* (*Goodwin & Morton, 1946*), as well as the identification of rAfCP, the recombinant enzyme of *A. fruticosa* (*Ellman, 1959*). MALDI-TOF mass spectra of peptide fragments after in-gel digestion was examined and the peptide mass fingerprinting data was analyzed using MASCOT search tool (http://www.matrixscience.com) (Figure 2.9) and even with high peptide mass tolerance of 2 Da, did not yield any hit with acceptable statistics. The result shows unique sequence of procerain B.

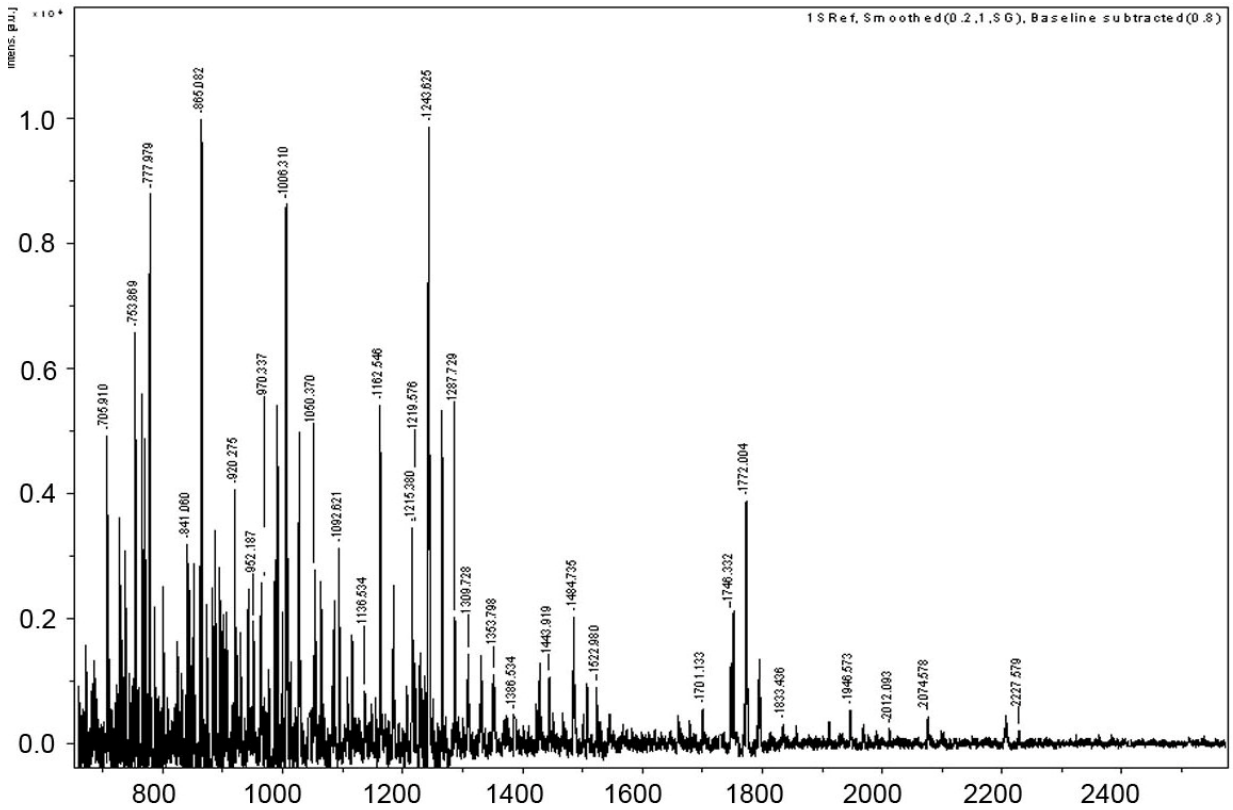

Figure 2.9: Peptide fragment spectra of procerain B. In gel digestion of the protein was performed by trypsin and spectra were collected by using Ultraflex MALDI TOF as explained in method section. The peptide mass fingerprinting data was analyzed using MASCOT search tool (http://www.matrixscience.com) and even with high peptide mass tolerance of 2 Da, the protein did not yield any hit with acceptable statistics. This shows unique sequence of procerain B.

2.5 Conclusions

A novel cysteine endopeptidase was purified from the latex of *Calotropis procera* using cation exchange and gel filtration chromatography techniques and named as procerain B. The physiochemical characterization revealed that procerain B has high temperature stability (upto 65°C) and broad temperature (40-60°C) and pH (pH 6.5-8.5) optima. It differs from the earlier cysteine endopeptidase, procerain, isolated from the same source in several aspects. The N-terminal sequence and peptide mass fingerprinting data indicates unique nature of the enzyme. Further, the enzyme also has unique cleavage specificity compared to other well studied plant cysteine proteases. As the source of the enzyme is easily available, bulk production of procerain B is possible for various applications.

Chapter III

Possible applications and determination of cleavage specificity of procerain B*.

3.1 Abstract

Many plant endopeptidases have been purified and characterized in terms of function and folding. However, not much study has been done to explore industrial applications of these enzymes. We have screened the possible applications for newly purified endopeptidase, procerain B. The enzyme was found to be very efficient in digestion of food proteins as well as coagulation of milk required for cheese preparation. Different metal ions can alter the milk clotting efficiency of procerain B. Metal ions like Ni^{2+}, Co^{2+} and Mn^{2+} can activate the milk clotting efficiency of enzyme while Cu^{2+} and Zn^{2+} have adverse effect. Procerain B was also found compatible with most of the detergents available in market and applicable in detergent industries. As the cleavage specificity of a protease is its characteristic and decides its usefulness in Biotechnology and Pharmaceutical industries, the cleavage preference for the enzyme was determined using integrated biochemical and computational approaches.

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* *Part of the work has been published* ***Applied Biochemistry and Biotechnology****, 2011; 164; 573–580.*

3.2 Introduction

Proteases are one of the most important industrial enzymes and cover major portion of global enzyme sale. We are exploring the hydrolytic property of endopeptidases in different industries. Due to their high thermal stability and broad substrate specificity, plant endopeptidases are the preferred choice for different industries. In previous chapter, we have discussed the purification of a novel cysteine endopeptidase (procerain B) from the latex of a medicinal plant *Calotropis procera* (*Singh et al., 2010*). The plant is used in Ayurveda (Indian traditional medicine) for the treatment of skin diseases, rheumatism, and aches (CSIR Publication and Information Wealth of India: Raw materials III. New Delhi, India: CSIR Publication and Information Directorate; 1992. pp. 78-84). Various extracts of the plant have been reported to have anti-inflammatory, analgesic, and antipyretic activities (*Kumar & Basu, 1994; Dewan et al., 2000; Sangraula et al., 2002; Arya & Kumar, 2005*). Recently, antioxidant and antibacterial activities of the methanolic extract of the plant have also been published (*Yesmin et al., 2008*). Several medicinal properties of the plant may be mediated through endopeptidases present in the plant latex. Moreover, endopeptidases have several applications in food, detergent, leather, and pharmaceutical industries (*Dubey et al., 2007*).

Proteases are used as an additive in various formulations such as detergents, dehairing of leather, in cheese industry, and dietary supplements to aid digestion. Several useful endopeptidases have been obtained from animal sources and used as digestive aids and for predigestion of baby foods. However, large-scale production of animal endopeptidases is difficult. Chymosin is a milk-clotting enzyme from the fourth stomach (abomasum) of the calf and used in the cheese industry. The enzyme is difficult to obtain from a natural source in bulk. Therefore, the search for a new enzyme with chymosin-like milk-clotting action and simple purification method is always on. Similarly, proteases have immense applications in improving digestive function of humans. There is a gradual decrease in the production of digestive enzymes after 50 years of age. In addition, poor eating habits like less chewing and alcohol consumption also cause inadequate production of digestive enzymes. Additionally, the digestive function of an individual gets impaired in several diseased conditions and a digestive supplement is needed. Use of human innate digestive enzymes as digestive agent at large scale is not possible. Alternative sources of proteases could be plants or microbes. We

here report procerain B as a potential candidate for milk clotting in the cheese industry and digestive agent in the food industry. The easy availability of plant material and simple purification method makes industrial production of the enzyme feasible and economical (*Singh et al., 2010*).

3.3 Material and Methods

3.3.1 Materials

Superficial incisions on the young stems of *Calotropis procera* yielded milky latex. Fresh latex of the plant was collected and procerain B was purified using the method of Singh et al. (*Singh et al., 2010*). All other chemicals were of the highest purity, commercially available from Sigma or Merck.

3.3.2 Methods

3.3.2.1 *Digestion of food proteins*

Food protein digestion experiment was performed with egg white albumen protein. Two cubical pieces of boiled egg albumen, 500 mg each, were incubated in 15 ml test tubes with 0.75 ml of Tris-HCl buffer pH 7.5 containing 50 mM of β-mercaptoethanol at 37°C for overnight after addition of 500 µg of enzyme. A control was used for comparison in which enzyme was replaced with distilled water.

3.3.2.2 *Milk coagulation assay*

The clotting activities of procerain B were determined according to the method of Berridge (*Berridge, 1952*). A 10% (w/v) solution of skim milk powder (Sabarkantha District cooperative milk products, India) was prepared in Tris-HCl buffer at pH 7.5 containing 0.01 M $CaCl_2$. Then, 500 µg of enzyme was added to 25 ml of this milk solution and the mixture was incubated at 37°C±0.5. The time required for the appearance of the clot was observed. A control was used by replacing the enzyme with buffer. One unit (1 U) was defined as the

quantity (milligrams) of enzyme needed to coagulate 1 ml of reconstituted skim milk powder in 1 min at 37°C.

3.3.2.3 *Effect of different metal ions on milk-clotting activity of procerain B*

The effect of different metal ions in a range of concentrations on the milk-clotting activity of enzyme was studied. The metals used were Cu^{2+} ($CuSO_4.5H_2O$), Co^{2+} ($CoCl_2.6H_2O$), Ni^{2+} ($NiSO_4.6H_2O$), Zn^{2+} ($ZnSO_4.H_2O$), Mn^{2+} ($MnCl_2.4H_2O$). A control assay was done for the milk-clotting activity without any metal ion and the activity is considered 100%. A 10% (w/v) solution of skim milk was prepared in 50 mM Tris-HCl buffer, pH 7.5 containing 0.01 M $CaCl_2$ and 5 ml of this solution in 15 ml test tubes were mixed with a range of concentrations (10 to 10,000 µM) for each metal. Then 50 µl (2 mg/ml) of enzyme was added in each tube and incubated at 37°C. Control experiments without endopeptidase did not show any effect.

3.3.2.4 *Compatibility with detergents and blood stain removal studies*

Different commercially available detergents like Surf Excel, Tide and Ariel (Procter and Gamble, India) were used to study the compatibility of the purified protease. The enzyme was incubated in 1% of above detergent (w/v) solutions (in tap water) at pH 7.5 and at room temperature for overnight (6-8 h) before measuring the enzyme activity. Enzyme activity without any detergent was taken as 100%. To evaluate the stain removal, clean cotton cloth pieces (5 cm × 5 cm) were soiled with blood, dried and incubated with 1% detergent with 0.1 mg/ml of purified protease for 5 h. Same size cotton cloth with blood stain was soaked only in 1% detergent (without proteases) and used as standard for comparison.

3.3.2.5 *Estimation of cleavage preference for procerain B*

The cleavage preference of procerain B was determined in two steps with degenerate peptide library and computational approach. The specificity towards the C-terminal of scissile bond was determined first with the help of degenerate peptide library of twelve amino acids. The N-terminals of all the peptides were blocked by acetylation and each position of the peptide

library was completely degenerate, which include equimolar mixture of all amino acids except cysteine. The library was dissolved in 20 mM phosphate buffer, pH 7.5 and centrifuged to remove any insoluble fraction. The supernatant was further filtered with 0.22 µm filter and used as substrate. The activated enzyme (<10 pM) was incubated with peptide solution at room temperature for overnight and the digestion of peptides was confirmed with fluorescamine. A control experiment was done without enzyme and used as blank for fluorescence spectroscopy with fluorescamine. The confirmed (digested) peptide sample was sent for N-terminal sequencing. The sequencing data was corrected for biasness of certain amino acids in peptide library and analyzed as described by Turk and Cantley (*Turk & Cantley, 2004*).

On the basis of the results of degenerate peptide library and information available on MEROPS (an online database for proteases and their inhibitors), secondary peptides of five amino acids in length were designed on PyMOL (http://pymol.org/) and docked against the modeled 3D structure of procerain B. The selectivity of procerain B towards the N-terminal of scissile bond was determined by analyzing the docking results as described in results and discussion section (3.4.5).

3.3.2.6 *Determination of mitogenic activity*

The mitogenic activity of procerain B was tested on mouse fibroblast cell line (L929) and different concentrations of procerain B were used for the experiment. Approximately $1x10^4$ cells were seeded in each well of 24-well cell culture plate for 36 h in serum free medium to nullify the proliferation effect of serum. On the day of experiment, cells were washed twice with PBS and then incubated in serum free medium with different concentrations of procerain B (0.1-10 nm) for 24 h at 37°C in 5% CO_2 atmosphere. The proliferation of cells was confirmed by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, tetrazole) reduction assay. After addition of MTT for 4 h, purple colored formazan complex was formed which was dissolved in DMSO and absorbance was measured at 570 nm to calculate percent viability of the cells. Viability of control cells (without procerain B) was considered as 100% and a positive control (with 10% FBS) was used in parallel.

3.4 Results and Discussion

3.4.1 Digestion of food proteins

Proteins are one of the major components of our daily diet and proteases are the enzymes responsible for digestion of proteins. We have selected the egg white albumen protein as a model, in order to check the efficiency of procerain B to digest the food proteins. After overnight incubation of egg white albumen protein with procerain B at 37ºC and pH 7.5, nearly 60% of protein got digested (Figure 3.1), resulting decrease in weight of cubical piece from 500 mg to 195.5 mg in test sample while there was no decrease in weight of control. This property of procerain B makes it a very important enzyme for food and therapeutic industries.

3.4.2 Milk coagulation assay

Endopeptidases are also used in dairy industry for the preparation of cheese and other milk products. We have also estimated the milk clotting efficiency of procerain B. After 14 min of incubation at 37°C, the clotting of milk had started in test which resulted in the precheese formation after 40 min, while there was no clotting in control experiment. The milk clotting activity of procerain B was found to be several folds higher than many commonly used endopeptidases in cheese industry that makes it a potential candidate for dairy industry.

3.4.3 Effect of different metal ions on milk-clotting activity of procerain B

Different metal ions have different affect on milk clotting capacity of enzymes. Some metals increase the milk clotting capacity while other decreases this capacity (*Arima et al., 1968*). Here, Mn^{2+} and Co^{2+} activated the milk clotting property of procerain B. Activation was observed in all concentration range of Mn^{2+} used in the study (10 µM-100 mM), while Cu^{2+} activated above a concentration of 1000 µM (Figure 3.3A). Few ions like Cu^{+2} and Zn^{2+} showed inhibition of milk clotting activity. Although, the mechanism of inhibition by Cu^{2+} and Zn^{2+} ion are not clear at this stage but it might get coordinated with active site residues and inhibit the enzyme (Figure 3.3B).

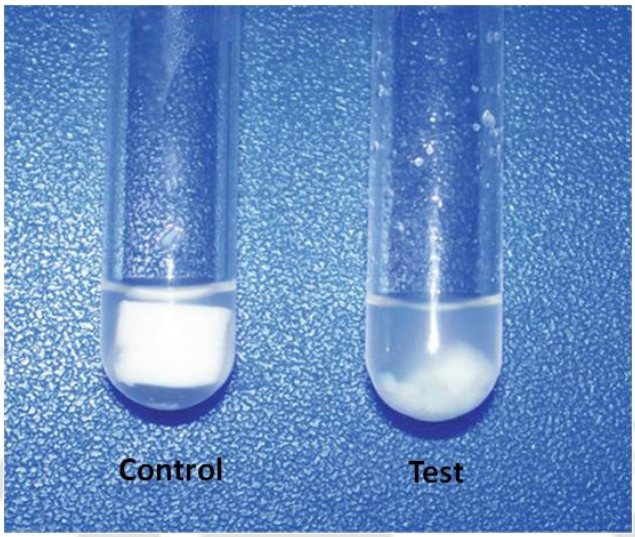

Figure 3.1: Digestion of egg white albumen protein with procerain B. The identical pieces of white portion of boiled egg were incubated with (Test) and without (Control) procerain B for overnight and next day the digestion was observed. Nearly 60% of the protein in test got digested while there was no digestion in control.

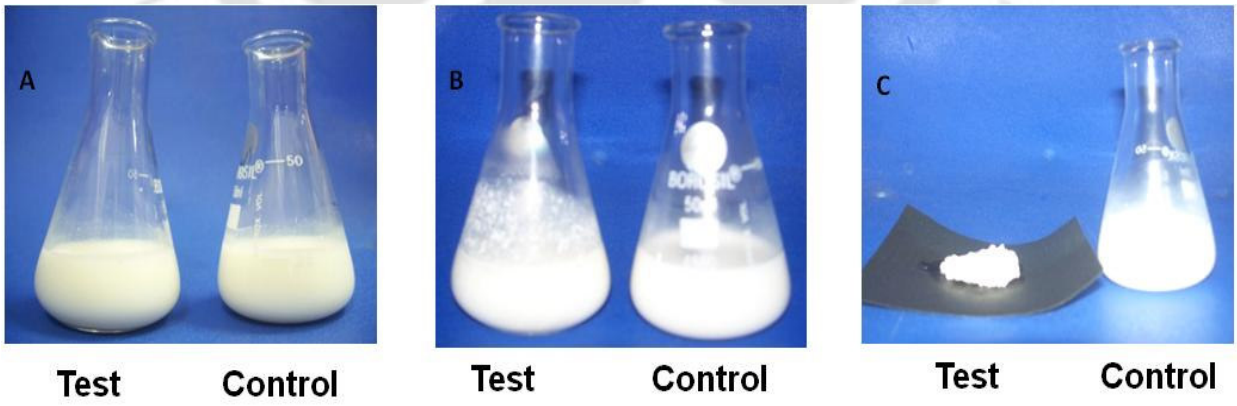

Figure 3.2: Clotting of milk with procerain B. In order to find the application of procerain B in dairy industry, the milk coagulation efficiency of procerain B was tested. 25 ml of 10% skim milk was incubated with (Test) and without (Control) procerain B at room temperature. **(A)** At the time of incubation. **(B)** After 14 min of incubation. **(C)** After 40 min of incubation. After 14 min of incubation the coagulation of milk started in test and after 40 min the milk was coagulated completely and converted into precheese, while there was no coagulation in control.

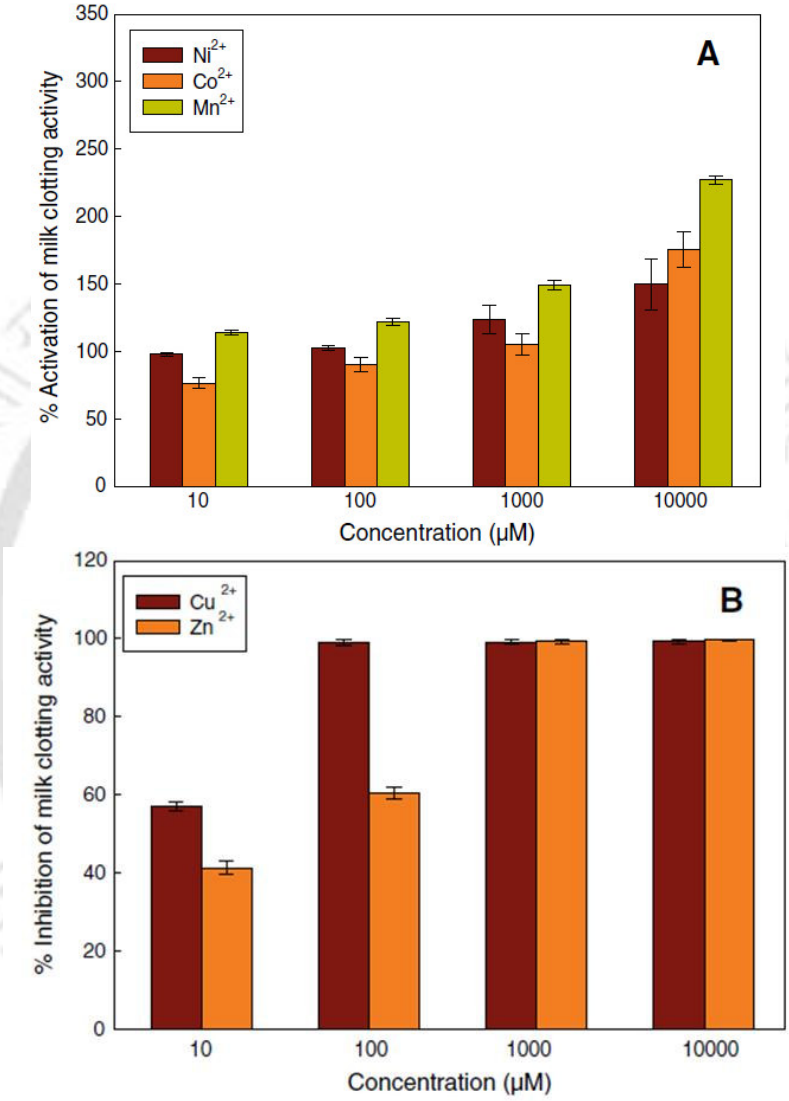

Figure 3.3: Effect of different concentrations of metal ions on milk coagulating activity of procerain B. **(A)** Activation by Mn^{2+}, Co^{2+} and Ni^{2+} at different concentration ranges. **(B)** Inhibition effect of Zn^{2+} and Cu^{2+} at different concentration range. Mn^{2+} activates the coagulation process in all concentration range used in the study (10 µM-100 mM), while Cu^{2+} activates above a concentration of 1000 µM. Cu^{2+} inhibits the coagulation of milk by procerain B completely above the 100 µM concentration while in case of Zn^{2+} this point is achieved at 1000 µM.

3.4.4 Compatibility with detergents and blood stain removal studies

The purified enzyme, procerain B, shows good stability with all detergents used for the study. It shows 100% activity in 1% w/v in all detergents used in the current study. Moreover, it appears to improve blood stain cleaning property of the detergent (Figure 3.4). Thus, procerain B has potential application in detergent industries for improving the cleaning efficiency of detergents.

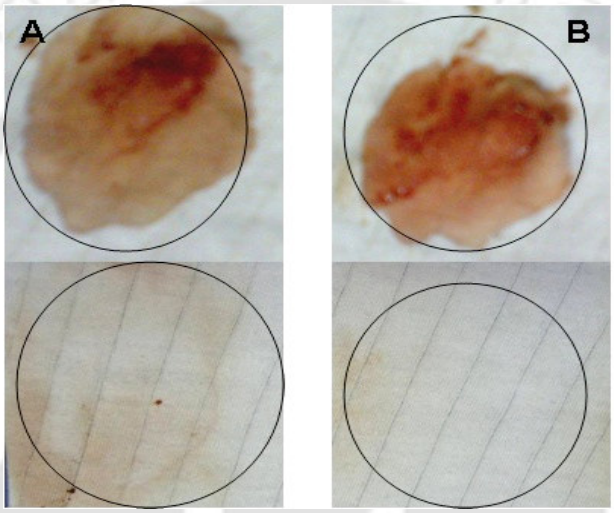

Figure 3.4: To evaluate the compatibility of procerain B and stain removal in combination of detergents available in market, clean cotton cloth pieces were soiled with blood and dried. **(A)** Soaked only in 1% detergent (without procerain B) acts as standard for comparison. **(B)** Incubated with 1% detergent with 0.1 mg/ml of purified procerain B for 5 h. In B part, where procerain B was present, the removal of blood stain was comparatively better.

3.4.5 Estimation of cleavage preference

Proteases (endopeptidases) has its own cleavage specificity which makes it suitable for unique applications. There are several methods for cutting site determination but combinatorial peptide library based method is most common. We have determined cleavage specificity of procerain B in two steps using integrated biochemical and computational approaches. In first step, C-terminal (prime positions) amino acids of scissile bond were determined using experimental methods while in the second step, N-terminal (unprime position) amino acids were determined using computational approach. A completely

degenerate N-terminaly acetylated peptide library of twelve amino acids was digested with procerain B and the cleavage products were subjected to N-terminal sequencing to derive C-terminal amino acids of scissile bond. The brief scheme of the experiment is shown in Figure 3.5 (*Turk & Cantley, 2004*). The undigested population and the N-terminal part generated after digestion of scissile bond will not participate in N-terminal sequencing due to blockage (acetylation) at the N-terminus. Only the C-terminal part generated after digestion of scissile bond will undergo N-terminal sequencing and reflect the primed side (C-terminal) residues for procerain B. The raw data obtained from N-terminal sequencing was processed as described in Turk and Cantley, 2004 (*Turk & Cantley, 2004*) and the selectivity values were plotted against amino acids for each position (Figure 3.6).

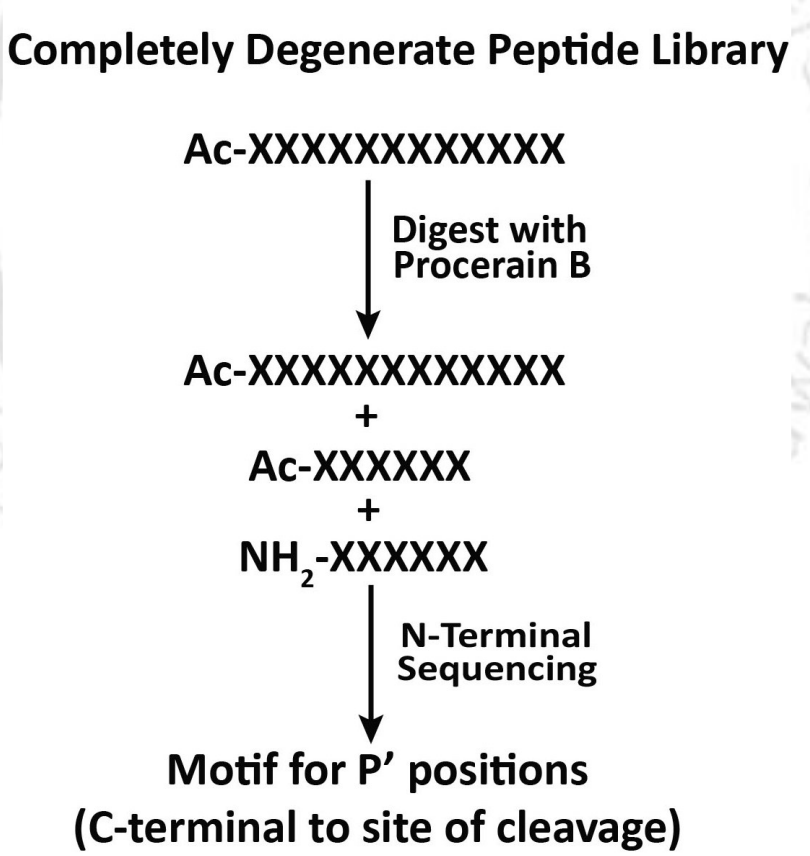

Figure 3.5: Schematic representation of peptide library method for determination of prime side residues of cleavage site of procerain B. A twelve amino acid long, completely degenerate peptide library with blocked (acetylation) N-terminal was incubated with porcerain B and the digestion product was subjected to N-terminal sequencing.

Serine was the most preferred amino acid at P1' position (Figure 3.6A) and threonine was most preferred at P2' position (Figure 3.6B). Procerain B has no clear cut preference for P3' and P4' positions. Histidine, asparagine and phenylalanine has approximately equal preference for P3' position (Figure 3.6C) and at P4' position glutamic acid, phenylalanine and asparagine has comparable selectivity (Figure 3.6D). Subsequently, a computational approach was applied using the informations of MEROPS, an online database for proteases and their inhibitors. Combinations of pentapeptides were generated with two C-terminal residues (Ser & Thr) fixed based on results obtained from earlier step and three N-terminal residues were varied on the basis of knowledge of known cleavage specificity for cysteine endopeptidases (Table 3.1). All possible (630) combinations were generated and docking was done with modeled 3D structure of procerain B. Top hundered peptides were selected and further narrowed down on the basis of docking score (-4.0) and number of conformations. Finally, top sixteen peptides with good statistics (Table 3.2) were analysed to determine the cleavage preference of procerain B. The analysis revealed that glycine is prefered at P1 position as out of top sixteen glycine is present in six pentapeptides. Likewise, alanine (5) and glycine (4) are preferred at P2 position and histidine (12) is most preferred at P3 position (Table 3.3). Alanine and glycine were further compared for P2 position by visual inspection of interaction of pentapeptides with modeled 3D structure of procerain B in electrostatic solid surface view (Figure 3.6) and the alanine was found to be interacting in better way in comparison to glycine.

Table 3.1 Analysis of cleavage preferences for cysteine endopeptidases (MEROPS) and possibilities of pentapeptides as substrate. There are total possibilities of 630 (6 X 15 X 7 X 1 X 1) pentapeptides.

Position	**P3**	**P2**	**P1**	**P1'**	**P2'**
Type of Amino Acid	Basic	Basic	Basic	S	T
Type of Amino Acid	Aliphatic	Small	Small		
Type of Amino Acid		Aliphatic			
Type of Amino Acid		Others			
Total No of Amino Acids	6	15	7	1	1

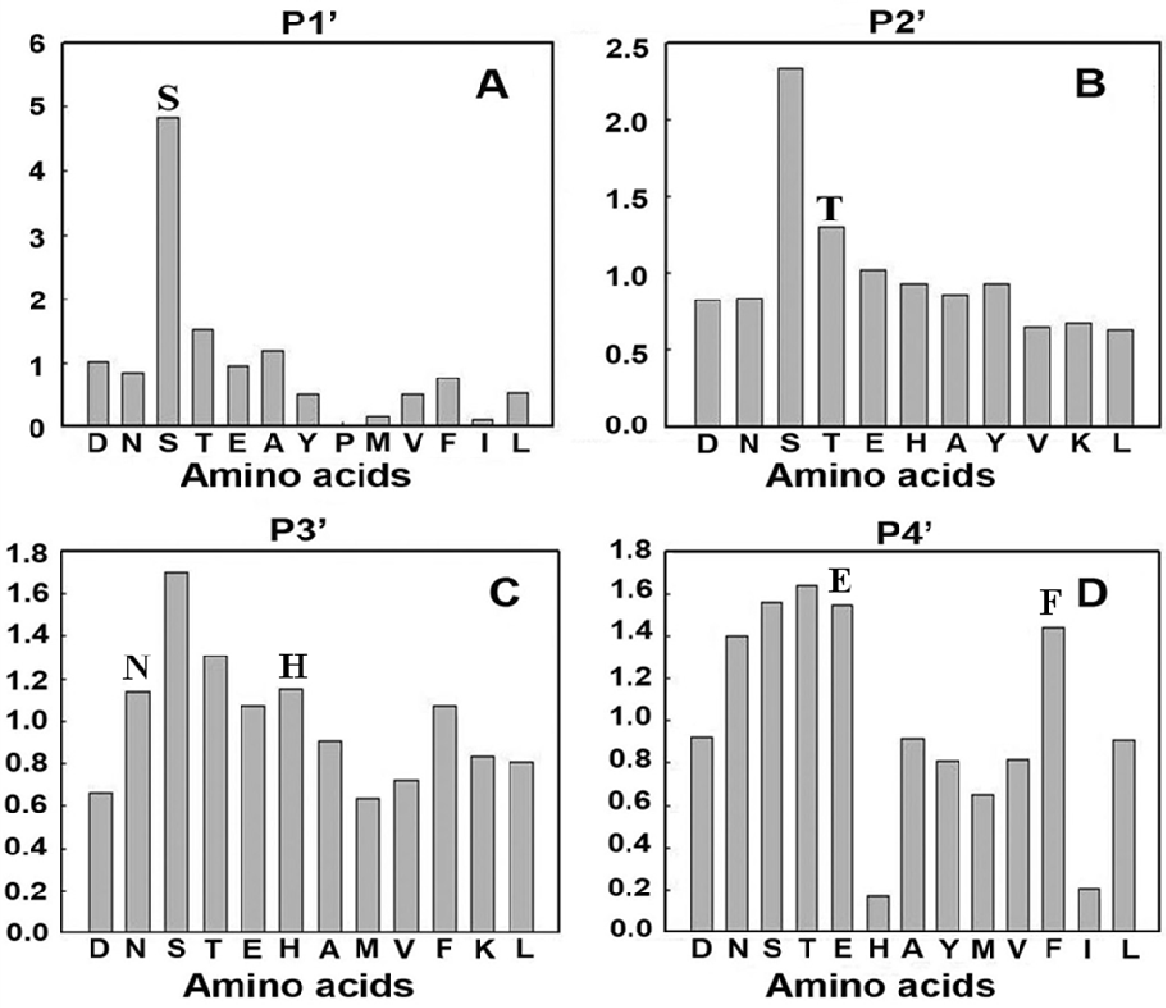

Figure 3.6: Cleavage preference of procerain B for different amino acids at (A) P1', (B) P2', (C) P3' and (D) P4' positions in form of selectivities value. Serine is the most preferred amino acid at P1'(A), Threonine has highest selectivity at P2' (B), although the bar for serine is longer than the threonine, we are considering it as the carry over of first cycle. At P3' and P4' positions there were no clear cut selectivity, however Asparagine (N) and Histidine (H) showed higher preference than other amino acids at P3'. At P4' position Glutamic acid (E) and Phenylalanine (F) were more preferred.

Table 3.2 Top hits (16) of pentapeptides as substrate for procerain B resulted from the virtual screening of all 630 pentapeptides against the 3D modeled structure of procerain B. Top 100 peptides from the screening result were selected and further narrowed down to 16 on the basis of doc score (≥ -4.0) and number of conformations.

Peptides	Score	Average	Conformations
HAHST	-4.96	-4.84	90
HGHST	-4.79	-4.54	88
IPAST	-4.52	-4.46	91
HAGST	-4.37	-4.2	74
HGGST	-4.35	-4.28	96
HACST	-4.23	-4.19	87
HTGST	-4.17	-4.14	79
HGCST	-4.16	-4.08	96
VLAST	-4.14	-4.03	84
HHGST	-4.14	-4.06	78
HAAST	-4.12	-4.06	75
VLSST	-4.09	-3.96	81
HASST	-4.07	-3.99	80
LPGST	-4.01	-3.99	99
HGSST	-4.01	-3.96	91
HVGST	-3.99	-3.97	97

Table 3.3 Analysis of top hits (16) for the presence of same amino acid residues at different specificity positions and final cleavage preference for procerain B.

Position	P3	P2	P1	P1’	P2’
Preferred amino acids	H (12)	A (5)	G (6)	S	T
Preferred amino acids	V (2)	G (4)	A (3)	S	T
Preferred amino acids	I (1)	L (2)	S (3)	S	T
Preferred amino acids	L (1)	P (2)	H (2)	S	T
Preferred amino acids		T (1)	C (2)	S	T
Preferred amino acids		H (1)		S	T
Preferred amino acids		V (1)			

Table 3.4 Final amino acid preference for procerain B derived using integrated computational and biochemical approaches. Procerain B will cleave the peptide bond in between P1 and P1’.

Position	P3	P2	P1	P1’	P2’
Final amino acid preference	**H**	**A**	**G**	**S**	**T**

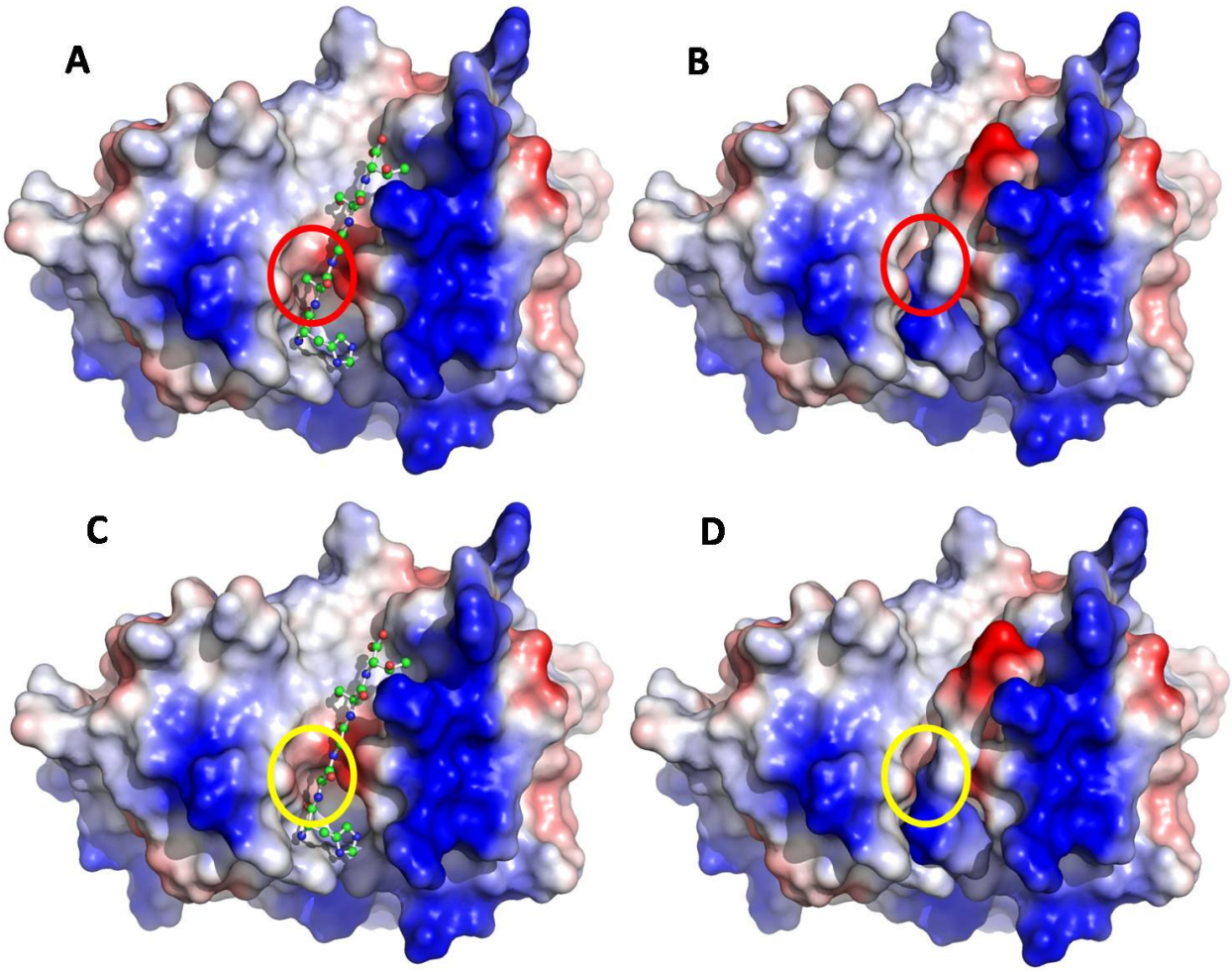

Figure 3.7: Interaction of pentapeptides (HAGST and HGGST) with procerain B. The interaction of pentapeptide with Alanine (in red circle) at P2 position **(A & B)** is better in comparison to pentapeptide with Glycine (in yellow circle) at P2 position **(C & D)** Side chain of Alanine is filling the free space in the groove.

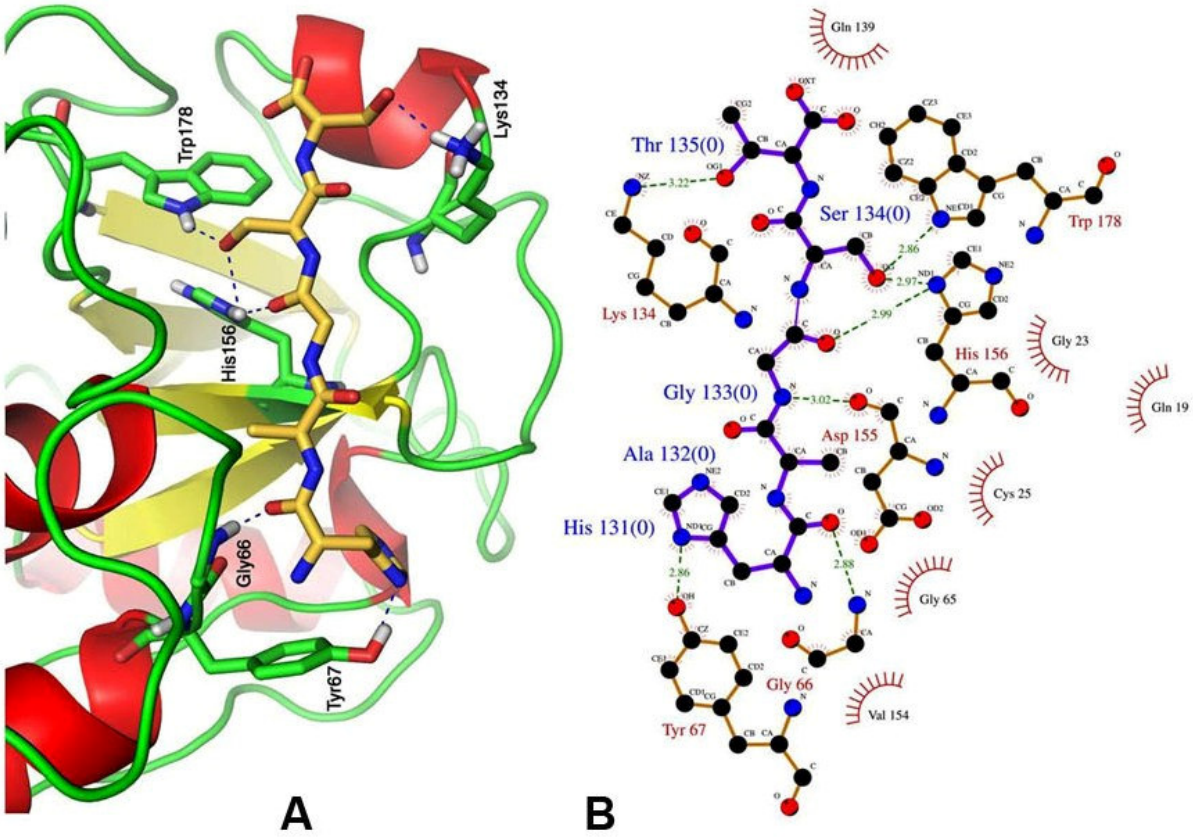

Figure 3.8: **(A)** Detail interaction of pentapeptide with Aln at P2 position with procerain B. **(B)** The catalytic residues His156 is interacting with both Gly (P1) and Ser (P1'). The scissile peptide bond is present in between Gly (P1) and Ser (P1').

3.4.6 Determination of mitogenic activity

Further, the mitogenic activity of procerain B was tested on mouse fibroblast cells (L929) and was found to activate the proliferation of fibroblast cells. The mild angiogenic potential of procerain B suggests its potential application in treatment of various ischemic diseases. The optimal mitogenic activity was found to be at 1 nM concentration and beyond this concentration there is decrease in mitogenic potential (Figure 3.8). The mitogenic activity of native and heat denatured procerain B suggest that the proteolytic activity is required for the mitogenic activity (Inset of Figure 3.8).

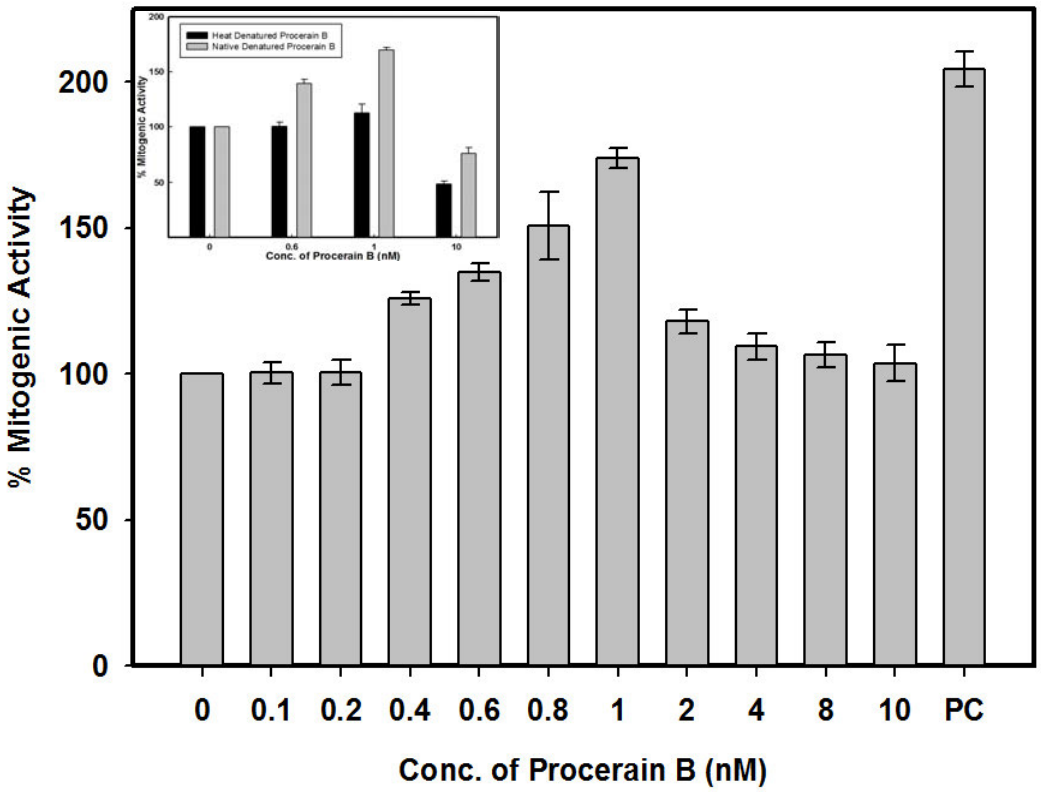

Figure 3.9: Evaluation of mitogenic activity of procerain B. Different concentrations (in nM range) of procerain B were tried to check the mitogenic activity of procerain B on mouse fibroblast cell lines (L929). At 1.0 nM concentration, the procerain B cause maximum proliferation of fibroblast cells. Further at higher concentrations of procerain B the proliferation get inhibited. This might be due to excess proteolysis by procerain B at higher concentraions. PC is the positive control where 10% FBS was used. Inset of the figure shows mitogentic activity of native and heat denatured procerain B. The data indicates that the protease activity seems to have some role in mitogenic activity.

The precise mechanism of mitogenic activity of procerain B remains unclear and further studies required to understand the molecular basis of the same. Procerain B may activate some or other protein in the pathways that leads to mitogenic activity. There is a previous report about plant cysteine protease, CMS2MS2, showing mitogenic activity (*Gomes et al., 2009*). However, the previous report suggests that the mitogenic activity is independent of protease activity and mitogenic activity is mediated through ERK phosphorylation and phospholipase C activation. Recently Correa et al., 2011 has also reported mitogenic potential of a cysteine protease from *Carica candamarcensis* (*Correa et al., 2011*). Recombinant protease from *Carica candamarcensis* showed mitogenic potential despite lack of proteolytic activity. It triggers a mitogenic response in L929 cells by stimulating ERK1/2 phosphorylation (*Correa et al., 2011*). These, studies along with our finding suggests that the application of proteases as potent mitogens are not well explored.

3.5 Conclusions

We have evaluated various possible applications of procerain B. The enzyme has potential for food, dairy and detergent industries as it can effectively digest the egg white albumin protein, causes coagulation of milk and compatible with most of the detergents available in market. We have also found this enzyme as a mitogenic agent. The mitogenic activity of the enzyme is related to its protease activity as the denatured form of the enzyme did not show mitogenic potential. Further, the cleavage specificity of the enzyme was derived using biochemical and computational approaches. The information about cleavage specificity of the enzyme will further extend its application in Biotechnology and Pharmaceutical industries.

Chapter IV

Immobilization of procerain B on glutaraldehyde activated chitosan and amberlite MB-150 beads and characterization of immobilized product*¥.

4.1 Abstract

Immobilization is the attachment of enzymes with inert insoluble matrix through which substrate can easily pass and product can diffuse out. Immobilized enzymes are usually more advantageous than the soluble form. There are multiple limitations of soluble enzymes, such as structural instability after isolation from natural environment, narrow functional range, sensitivity towards trace amount of inhibitors and contamination by end product, mainly in food and pharmacy industries where purity of products is of utmost importance. Use of immobilized enzymes can not only circumvent these problems but also has some added advantages, such as repeated use of same batch of enzyme and better control over catalytic period. In earlier chapters we have discussed the purification, characterization and screening of possible applications for procerain B.

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* *Part of the work has been published in* ***Journal of Food and Agricultural Chemistry (ACS)****, 2011; 59 (11); 6256–6262.* ¥ *Part of the work has been submitted for publication.*

It was found to be applicable in several industries as food, dairy, detergent, pharmaceutical and biotech industries. In order to widen the horizon of its applications in industries, here we are reporting the immobilization of procerain B on glutaraldehyde activated chitosan and amberlite MB-150 beads through covalent attachment. The immobilization parameters were optimized and the immobilized products were characterized in terms of functional optima and stability.

4.2 Introduction

Chitosan is the de-acetylated product of chitin, the second most abundant polysaccharide after cellulose in nature (*Bailey et al., 1999*). Chitin, the main constituent of outer skeleton of insects and crustaceans yields chitosan after removing acetyl groups by alkaline treatment (*Muzzarelli, 1977; Roberts, 1992*). Waste shells of shrimps, prawns, crabs, lobsters and crayfish are commonly used as raw material for commercial production of chitin and chitosan. Fungi and insect larvae are being explored to serve as starting material for chitin production (*Kuhlmann et al., 1999; Struszczyk et al., 1999*). People have also used recombinant approach to clone the gene responsible for production of chitosan. Chitinases (EC 3.2.1.14) are enzymes capable of hydrolyzing chitin, and are produced by several micro-organisms which include actinomycetes (*Aranaz et al., 2009*), other bacteria (*Jamcela et al., 1995; Krajewska, 2004*) and yeast (*Sawayanagi et al., 1982; Kawashima et al., 1985*). Due to its easy availability, biocompatibility, inert and hydrophilic nature, it has several applications in diverse fields. It can be used as encapsulating agent for slow and prolonged release of drugs (*Jayakrishnan et al. 1990; Li et al., 1992; Stone et al., 2000; Wang et al., 2005*), as a chelating agent for cleaning of industrial waste (*William et al., 1983*), in surgery and grafting (*Xianfang et al., 1998; Safarikova et al., 2000; Hua et al., 2010*) and in purification techniques such as ion-exchange and affinity chromatography (*Katzir & Kraemer, 2000; Krajewska, 2004; Bhandari et al., 2010*). Chitosan is an ideal matrix for immobilization of enzymes. It can be used in the form of gel, membrane, bead or powder. Hydroxyl and amino groups present in chitosan favors the immobilization process by adsorption and covalent linkage.

Endopeptidases have several applications in food, dairy and detergent industries. It also has a wide range of applicability in medicines, energy production and environmental control (*Clarkep & Traceym, 1956; Monreal & Reese, 1969; Watanabet et al., 1990*). Industries usually prefer immobilized biocatalysts rather than the traditional chemical methods as it offers reusability, high specificity, easy product separation and negligible byproducts (*Hanefeld et al., 2009*). Due to increasing industrial demand of biocatalysts, different measures are explored to enhance their utilization and reduce their cost. Immobilization proved out to be the best remedy. Proteases are one of the most common industrial enzymes which have wide range of applications varying from food to pharma industries (*Correaj et al., 1982; Elangon et al., 1982; Sawayanagi et al., 1982; Jamcela & Jayakrishnan, 1995*). Slowly with increasing demand it became an integral part of our daily life. Particularly in food industry the proteases are used in processing of foods. The most common example is the papain from unripe fruits of *Carica papaya* used for meat tenderization. Some neutral and alkaline proteases are used for the recovery of meat from butchering which are used in several canned soups (*Jayakrishnan & Thanno, 1990; Li et al., 1992*). Proteases are also used for predigesting gluten protein of wheat in baking industries (*Wang et al., 2005*). Proteases are also applicable in dairy industry for production of several dairy products. Certain proteases are used as ingredients of chocolates, cakes and some canned drinks to enhance the flavor.

Endopeptidases from different plants, animals and microbial sources are known. Plant cysteine endopeptidases are well known for their high thermal stability which makes them a potential candidate for several industries where high temperature is required at certain stages (*Stone et al., 2000*). Since the applicability of any endopeptidase depends on its functional and stability range and cutting sites, the search for new endopeptidases with unique cutting sites and broad operational range still continues. We have also purified and characterized a novel cysteine endopeptidase from the latex of a medicinal plant *Calotropis procera (Singh et al., 2010).* We have tested its applicability in food, detergent and dairy industries and found it as a potential candidate for several industrial applications (*Singh & Dubey, 2011*). In order to increase its industrial applicability, we have immobilized procerain B on glutaraldehyde activated chitosan matrix (*Singh et al., 2011*) but due to the fragile

nature of chitosan beads and leaching problem we get inspired to have a comparatively stable alternate. Amberlite MB-150 beads were used as stable alternate and the immobilization of procerain B on glutaraldehyde activated amberlite beads was optimized. The immobilization product was characterized and compared with soluble form.

4.3 Materials and Methods

4.3.1 Materials

The enzyme was purified from the latex of plant *Calotropis procera* by the method of Singh et al. (*Singh et al., 2010*). Chitosan (having 75% degree of deacetylation), amberlite MB-150 beads, BSA, azocasein, protease inhibitor and Bradford reagent were purchased from Sigma Aldrich, India. Tris buffer, dialysis tubing and glutaraldehyde were obtained from Fluka Biochemika, Germany. The trichloroacetic acid (TCA) was purchased from Hi-Media Laboratories, India. All other chemicals used were of the highest purity and commercially available. All reagents were prepared in Milli Q water (Millipore, USA).

4.3.2 Methods

4.3.2.1 *Protein concentration*

Protein concentration at different stages of purification was determined by absorbance at 280 nm as well as by method of Bradford (*Bradford, 1976*) using BSA as a standard.

4.3.2.2 *Protease activity*

The hydrolyzing activity of the protease was determined with denatured natural substrate casein and azocasein using the method of Dubey and Jagannadham (*Dubey & Jagannadham, 2003*). Enzyme solution (5 µg) was incubated in final volume of 500 µl of 50 mM Tris-Cl buffer pH 7.5 at 37°C for 10 min. Casein solution (1%, w/v) was prepared in same buffer at the same pH and added to the enzyme solution making the final reaction volume to 1 ml and the reaction mixture was incubated for the 30 min at 37°C. The reaction was stopped by

adding 0.5 ml of 10% (w/v) TCA, incubated further for 10 min at room temperature and centrifuged (10,000 rpm for 10 min). The absorbance of the soluble peptides in the supernatant was measured at 280 nm. In the case of azocasein as substrate, 0.5 ml of supernatant after TCA precipitation was mixed with equal volume of 0.5 M NaOH and incubated for 15 min. The development of colour was measured spectrophotometrically by taking absorbance at 440 nm. A control assay, without the enzyme was done and used as blank in all spectrophotometric measurements. One unit of enzyme activity was defined as the amount of enzyme, under given assay conditions that give rise to an increase of 0.001 unit of absorbance at 280 nm or an increase of 0.001 unit of absorbance at 440 nm per minute of digestion. Number of units of activity per milligram of protein was taken as the specific activity of the enzyme. In case of immobilized enzyme, seven chitosan beads or 50 mg of amberlite beads with immobilized procerain B were used in all experiments.

4.3.2.3 *Preparation of beads*

Due to high solubility of chitosan at lower pH, the chitosan beads of uniform size were prepared by dissolving powdered chitosan in milli Q water containing 1.5% (v/v) glacial acetic acid. The different concentrations (1.0-3.0%, w/v) of chitosan were tried for bead preparation and the most appropriate concentration was used for further experiments. Uniform beads were prepared by casting the stock solution of chitosan with a 12 ml syringe. The syringe was fixed at appropriate height (from larger height the beads became disk shaped) and the stock solution was added drop wise in 1 N KOH solution containing 25% (v/v) ethanol under stirring to form spherical beads and solution was allowed to stand for 1-2 h for ripening of beads. The beads of diameter nearly 2.0 mm and uniform shape obtained were immediately washed with Milli Q water and stored in water at 4°C till activation with glutaraldehyde. In case of amberlite the readymade beads were purchased as discussed in material section.

4.3.2.4 *Immobilization of procerain B*

The immobilization of procerain B on chitosan and amberlite MB-150 beads was optimized. The beads were activated with different concentrations of glutaraldehyde, in the range 0.5-3.0% (v/v) at 37°C for different time interval (0.5-4.0 h). After activation, the beads were washed extensively with 50 mM Tris-HCl buffer, pH 8.5 for complete removal of unreacted glutaraldehyde till the absorbance of washing solution was lower than 0.01 at 280 nm after washing and beads were incubated with varying concentrations (0.2-0.8 mg/ml) of purified procerain B at 4°C for different time interval (8.0-32.0 h). The beads were washed with 50 mM Tris-HCl buffer, pH 8.5 to remove unbound procerain B. Same procedure was used to optimize the immobilization of purified procerain B on glutaraldehyde activated amberlite MB-150 beads. The beads were washed extensively with distilled water and then equilibrated with 50 mM Tris-HCl buffer, pH 8.0. The equilibrated amberlite MB-150 beads were treated with different concentration (1-5 %, v/v) of glutaraldehyde for varying time interval (1-8 h). The activated amberlite MB-150 beads were washed extensively with 50 mM Tris-HCl buffer, pH 8.0 to remove unused glutaraldehyde. The activated beads were incubated at 4°C with different enzyme concentrations (02-1.0 mg/ml) for varying time interval (8-32 h). The beads were washed with 50 mM Tris-HCl buffer, pH 8.0 to remove unbound procerain B. The activity and protein content of chitosan and amberlite MB-150 beads were determined. The percent immobilization (percent enzyme activity retention) was calculated as follows:

$$\text{Immobilization } (\%) = \frac{\text{Total activity of immobilized protease}}{\text{Total activity of soluble protease}} \times 100$$

Note: Total activity of immobilized protease was determined by subtracting total activity of unbound protease from total activity of soluble enzyme.

4.3.2.5 *FTIR spectra of beads*

In order to confirm the activation of beads with glutaraldehyde, the FTIR spectra of normal and glutaraldehyde activated beads were taken with UNICAM Mattson 1000 FTIR spectrophotometer and compared. For FTIR spectra, samples were crushed with potassium

bromide (KBr) to form a very fine powder. The powder was compressed into thin disc for analysis.

4.3.2.6 *Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) analysis of beads for immobilization*

The detailed surface morphology of the normal, glutaraldehyde treated and immobilized beads (Chitosan and amberlite MB-150) were analyzed by scanning electron microscopy. Samples were subjected to gold coating, then imaged and photographed by using scanning electron microscope (LEO 1430 VP) operated at an acceleration voltage of 10.00 kV. An EDX spectrum was also collected to analyze percentage elemental composition of bead surface.

4.3.2.7 *pH and temperature optima*

The activity of the immobilized enzyme was measured as function of varying pH to determine the pH optima of the immobilized procerain B. In all activity assay experiments the amount of chitosan (07 beads) and amberlite MB-150 beads (50 mg) were kept constant. The buffers used were 0.05 M Glycine-HCl (pH 2.0-3.5); 0.05 M Na-acetate (pH 4.0-5.5); 0.05 M Na-phosphate (pH 6.0-7.5); 0.05 M Tris-HCl (pH 8.0-10.0) and 0.05 M sodium carbonate (pH 10.5-12.5). Substrate solution of azocasein or hemoglobin was prepared in the respective buffers. Immobilized procerain B on beads were equilibrated in 0.5 ml of the buffer at a given pH for 15 min and added to the substrate solution of the same pH. The assay procedure was same as described above. Due to insolubility of azocasein below pH 4.0, hemoglobin was used as substrate for activity measurements at lower pH (*Sarath et al., 1989*).

The effect of temperature on the activity of immobilized procerain B was also studied using azocasein as substrate. Beads were incubated at desired temperature in the range of 15 to 95°C for 15 min in 50 mM Tris-HCl buffer pH 7.5 and then used for the activity measurement at the same temperature. Prior to the assays, substrate solution was also equilibrated at the corresponding temperature in the same buffer. A control assay was carried out without the enzyme at each temperature and used as a blank.

4.3.2.8 *Stability*

The ability of immobilized enzyme to retain its activity under various conditions such as extreme pH and temperatures were studied. Chitosan (07 beads) and amberlite MB-150 beads (50 mg) were incubated at different pH in the range of pH 1.0-12.0 for 12 h at room temperature and residual activity was measured as described earlier using azocasein as substrate. Similarly, beads were incubated at temperatures from 10°C to 95°C for 15 min and assayed for residual activity.

4.3.2.9 *Effect of substrate concentration*

The effect of increasing substrate concentration on the reaction velocity of the immobilized procerain B was studied using azocasein as substrate in the range of 1-160 µM at pH 7.5 and 37°C. Same amount of beads, Chitosan (07 beads) and amberlite MB-150 beads (50 mg) were used. Assays were performed as already described under proteolytic activity measurements. A blank was used at each substrate concentration without the enzyme. Lineweaver-Burk plot was generated and the value of Michaelis-Menten constant (Km) was calculated.

4.3.2.10 *Operational stability of immobilized protease*

The operational stability of the immobilized enzyme with repeated use of beads (chitosan and amberlite MB-150) was tested. The assay was performed as described in previous section. After each reaction, the beads with immobilized procerain B were removed and washed with 0.1 M Tris-HCl buffer, pH 7.5 to remove any residual substrate within the beads and stored in the same buffer at 4°C till further use.

4.4 Results and Discussion

Enzymes have been used for industrial purposes from a long time and proteases are one of the most important industrial enzymes. Use of immobilized enzymes rather than free form is more fascinating and offers added advantages. We have already studied the possible

applications of recently purified cysteine endopeptidase, procerain B (*Singh et al., 2010*). Immobilization can further increase its pertinence in various industries. In this chapter, we mainly focused on the optimization of immobilization conditions on chitosan and amberlite MB-150 beads and characterization of immobilized product. The chitosan beads were prepared and then activated with glutaraldehyde. Glutaraldehyde not only activates the beads but also cross-links the chitosan to provide resistance against lower pH. The two terminal aldehyde groups of glutaraldehyde react with amino groups of D-glucosamine units of different chains resulting in cross-linking of those chains through glutaraldehyde (Figure 4.1). The irreversible Schiff's base linking of aldehyde with amino group provides operational stability to beads. Spherical chitosan beads of uniform size were prepared and the immobilization of procerain B on glutaraldehyde activated chitosan beads and commercially available amberlite beads were successfully optimized.

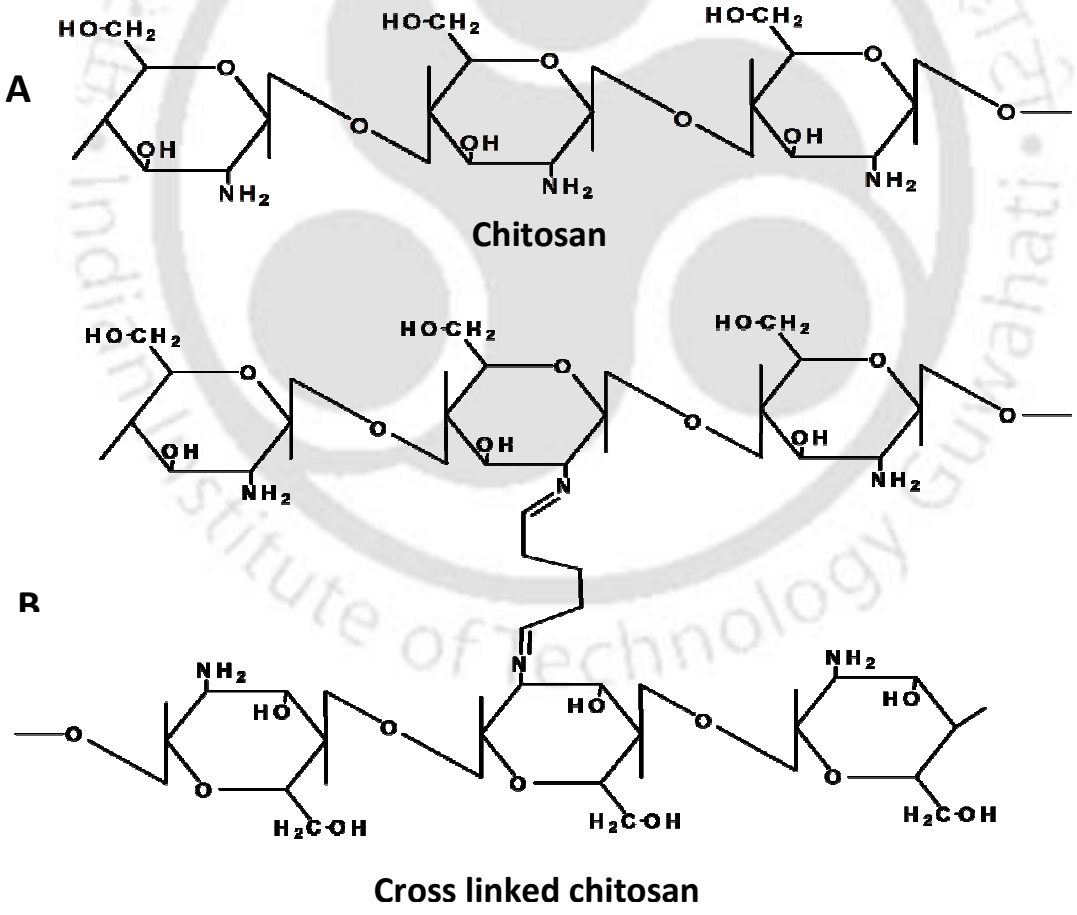

Figure 4.1: (A) Single chain of chitosan showing different subunits. **(B)** Cross linking of chitosan subunits through glutaraldehyde.

4.4.1 Effect of pH on immobilization of procerain B

For immobilization of enzymes through amino group on a matrix, pH plays an important role. The ionization state of different functional groups present on the surface of any enzyme mainly depends on the pH of enzyme solution. The effect of pH on the immobilization of procerain B on chitosan and amberlite beads was studied in the range of pH 5-10. The optimum pH for chitosan was found to be 8.5 with nearly 65% immobilization while in case of amberlite beads maximum immobilization of 52.65% was observed at pH 8.0 (Figure 4.2). At lower pH the immobilization was comparatively less, that may be due to lower stability of chitosan beads and improper ionization state at the surface of amberlite beads at lower pH.

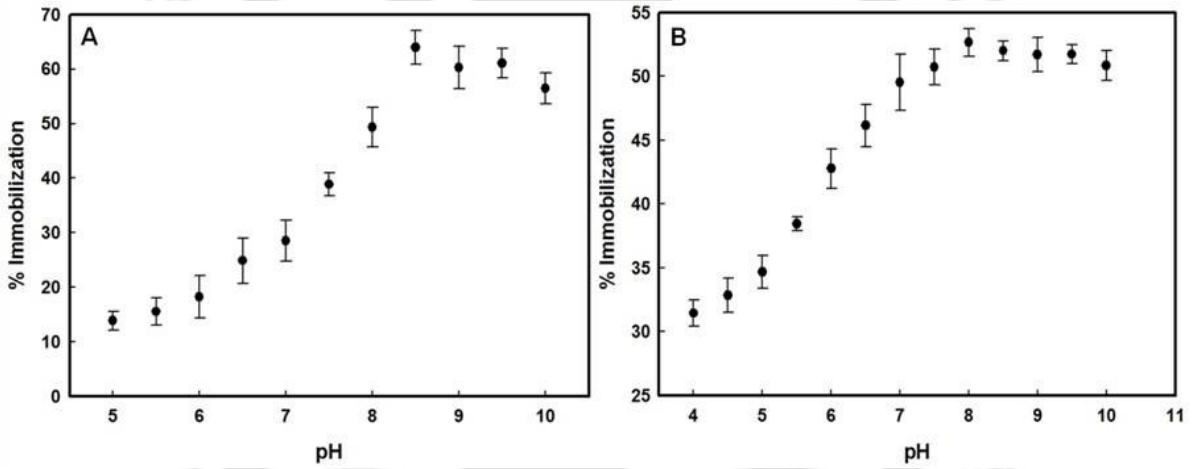

Figure 4.2: Effect of pH on immobilization of procerain B on **(A)** Glutaraldehyde activated chitosan beads, in the range of pH 4-10. **(B)** Glutaraldehyde activated amberlite MB-150 beads, in the range of pH 2-12. The optimum pH for immobilization of procerain B was nearly 8.5 with 65% immobilization and for amberlite MB-150 beads it was 8.0 with 52.65 % immobilization.

4.4.2 Optimization of immobilization conditions

The immobilization of procerain B on chitosan and amberlite beads were optimized in terms of glutaraldehyde concentration (3% & 4%), time of activation (3 & 4 h), coupling time (24 & 24 h) and concentration of enzyme solution (0.2 & 0.8 mg/ml) in immobilization mixture and finally 87.50% (chitosan) and 52.65% (amberlite) immobilization were achieved with all optimized parameters (Table 4.1 & 4.2). Different glutaraldehyde concentrations (1-5%)

were tried to activate the beads for immobilization of enzyme. The optimum concentrations were 3% glutaraldehyde with 84.39% immobilization and 4% glutaraldehyde with 54.51% immobilization on chitosan and amberlite beads respectively (Table 4.1 & 4.2). The time for activation with glutaraldehyde was also optimized in the range of 1-8 h and the maximum immobilization was observed at 3 h for chitosan and 4 h for amberlite beads with 86.69% & 55.86% immobilization respectively (Table 4.1 & 4.2).

Table 4.1: Optimization of immobilization conditions for procerain B on glutaraldehyde activated chitosan beads. The immobilization was optimized for glutaraldehyde concentration, activation time, coupling time and procerain B concentration in final immobilization solution.

	Glutaraldehyde Concentration (%)	Activation Time (h)	Coupling Time (h)	Protein Concentration in Immobilization mixture (mg/ml)	Immobilization (%)	Activity per Bead
Variation of glutaraldehyde concentration	1	2	20	0.2	58.33 ± 0.79	0.069±0.006
	2	2	20	0.2	72.58 ± 1.23	0.085±0.013
	3	2	20	0.2	**84.39 ± 0.32**	**0.097±0.015**
	4	2	20	0.2	84.51 ± 0.62	0.099±0.008
Variation of activation time	3	0.5	20	0.2	66.05 ± 0.44	0.083±0.009
	3	1	20	0.2	70.49 ±0.93	0.090±0.015
	3	2	20	0.2	84.56 ±0.69	0.100±0.023
	3	3	20	0.2	**86.69 ± 0.41**	**0.102±0.027**
	3	4	20	0.2	85.17 ± 1.07	0.101±0.017
Variation of coupling time	3	3	8	0.2	43.55 ± 0.35	0.052±0.007
	3	3	16	0.2	65.60 ± 0.92	0.069±0.011
	3	3	24	0.2	**86.98 ± 0.72**	**0.102±0.031**
	3	3	32	0.2	64.46 ± 0.45	0.068±0.019
Variation of protein concentration	3	3	24	0.2	**87.50 ± 1.15**	0.105±0.015
	3	3	24	0.4	74.02 ± 0.73	0.139±0.087
	3	3	24	0.6	48.60 ± 0.65	0.161±0.073
	3	3	24	0.8	24.05 ± 1.98	**0.163±0.083**

At longer time of activation, there was no further increase in immobilization. The next parameter was coupling time for attachment of enzyme through free amino group with aldehyde of glutaraldehyde. The coupling time was varied from 8-32 h and maximum immobilization of 86.98% & 56.98% was observed at 24 h for both chitosan and amberlite beads (Table 4.1 & 4.2). For economic use of enzyme in immobilization, the enzyme concentration in immobilization mixture was also optimized by varying the concentration of procerain B from 0.2 mg/ml to 1.0 mg/ml. Maximum immobilization of 87.50% &

62.07±1.13% was achieved with 0.2 & 0.8 mg/ml enzyme concentrations for chitosan and amberlite beads respectively (Table 4.1 & 4.2).

Table 4.2: Optimization of immobilization conditions for procerain B on glutaraldehyde activated amberlite beads. Immobilization was optimized for glutaraldehyde concentration, activation time, coupling time and procerain B concentration in final immobilization solution.

	Glutaraldehyde Concentration (%)	Activation Time (h)	Coupling Time (h)	Protein Concentration in Immobilization mixture (mg/ml)	Immobilization (%)
Variation of glutaraldehyde concentration	1	6	12	0.2	49.56 ± 0.43
	2	6	12	0.2	51.39 ± 1.90
	3	6	12	0.2	52.66 ± 2.71
	4	6	12	0.2	**54.51 ± 0.62**
	5	6	12	0.2	51.89 ± 0.83
Variation of activation time	4	1	12	0.2	46.15 ± 2.38
	4	2	12	0.2	50.31 ± 1.25
	4	4	12	0.2	**55.86 ± 0.58**
	4	6	12	0.2	54.19 ± 0.16
	4	8	12	0.2	53.93 ± 0.87
Variation of coupling time	4	4	8	0.2	43.55 ± 0.35
	4	4	16	0.2	56.30 ± 0.79
	4	4	24	0.2	**56.98 ± 0.72**
	4	4	32	0.2	55.62 ± 1.53
Variation of protein concentration	4	4	24	0.2	57.05 ± 0.36
	4	4	24	0.4	58.23 ± 1.35
	4	4	24	0.6	60.18 ± 1.53
	4	4	24	0.8	**62.07 ± 1.13**
	4	4	24	1.0	60.86 ± 1.71

4.4.3 FTIR spectra of beads

The cross-linking and activation with glutaraldehyde was confirmed by comparison of FTIR spectra of normal and activated beads, in case of both chitosan (Figure 4.3A) and amberlite beads (Figure 4.3B). The peak at 1412 cm^{-1} represents the C-N bond formed by cross-linking reaction. Other peaks at 1646 cm^{-1} and 1054 cm^{-1} may be attributed to C=N and polysaccharide structure (Figure 4.3A). In case of normal amberlite beads the peaks at 3427, 2925 and 1121 cm^{-1} in normal amberlite beads was due to amberlite composition and the corresponding peaks were also present in glutaraldehyde activated beads (Figure 4.3B). The increased peak intensity at 1633 cm^{-1} in graph B might be due to activation of beads with glutaraldehyde. Other peaks at 1453 cm^{-1} in graph-A, 1413 and 1475 cm^{-1} in graph-B was due to acid groups (Figure 4.3B).

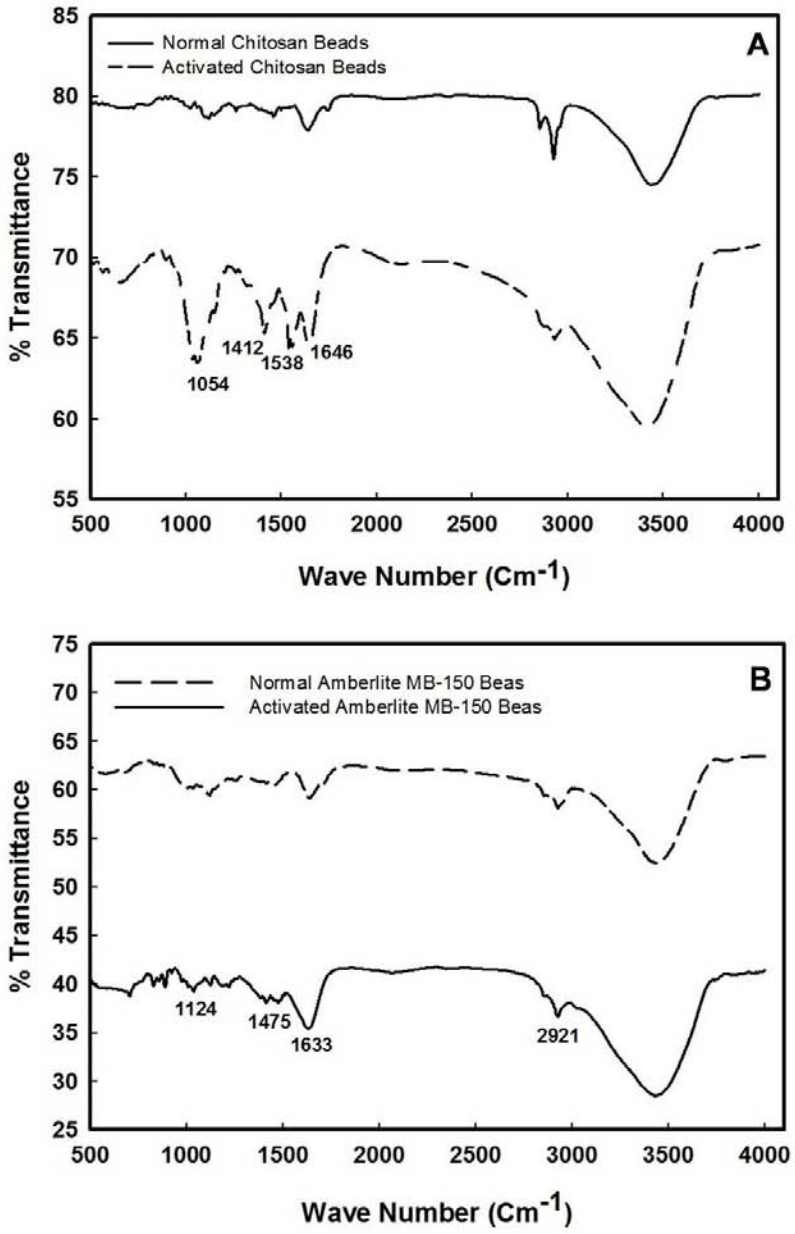

Figure 4.3: (A) Comparison of FTIR spectra of normal and glutaraldehyde activated chitosan beads. The peak at 1412 cm^{-1} represents the C-N bond formed by cross-linking reaction. Other peak at 1646 cm^{-1} is representing the C=N due to activation and the peak at 1054 cm^{-1} may be due to polysaccharide structure of chitosan **(B)** Comparison of FTIR spectra of normal and glutaraldehyde activated amberlite MB-150 beads. The peaks at 2925, 1453 and 1121 are due to amberlite. The increase in 1637 peak intensity is due to activation of bead with glutaraldehyde.

4.4.4 Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) analysis of beads for immobilization

The surface morphology of activated and immobilized beads got changed after activation and immobilization as observed under scanning electron microscope (Figure 4.4 & 4.5). The EDX analysis of the bead surface showed increase of nitrogen percentage in case of immobilized beads. This was due to presence of proteins (procerain B) on the surface of beads.

4.4.5 pH and thermal optima of immobilized procerain B

After immobilization, chitosan immobilized procerain B showed very interesting two pH optima, one lies in acidic (pH 3.0-3.5) and other around neutral pH (pH 6.0-8.0) range which shows the better applicability of immobilized procerain B in comparison to soluble form having only one pH optima in the range of pH 6.5 to 8.5. Immobilized procerain B displays its maximum activity in the temperature range 45-65°C while soluble form showed maximum activity in the range of 40-60°C (*Singh et al., 2010*). In case of amberlite beads, the pH optimum was shifted toward alkaline pH after immobilization. The immobilized enzyme showed pH optima in range of pH 8.5 to 10.0. It can be used in the reactions where alkaline pH is required. In terms of temperature, the functional optima of procerain B immobilized on the surface of amberlite beads was 45-65°C which is higher than the soluble form (*William et al., 1983*). Increased thermal optima make it suitable for applications with higher temperature.

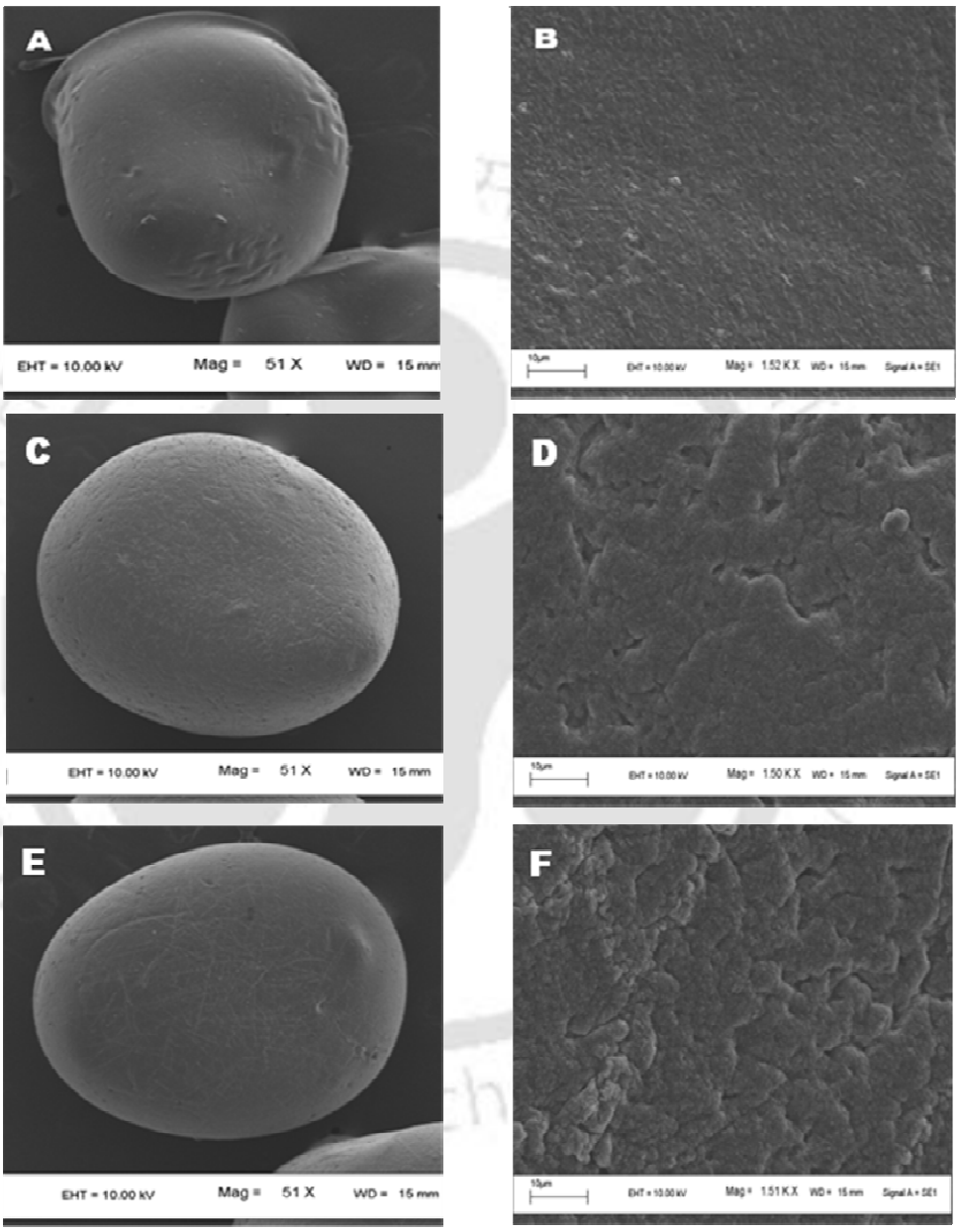

Figure 4.4: SEM images of beads and detailed surface view of **(A and B)** normal chitosan beads, **(C and D)** glutaraldehyde activated chitosan beads, **(E and F)** immobilized chitosan beads. After glutaraldehyde activation and immobilization of procerain B on activated beads the surface morphology of chitosan beads got changed.

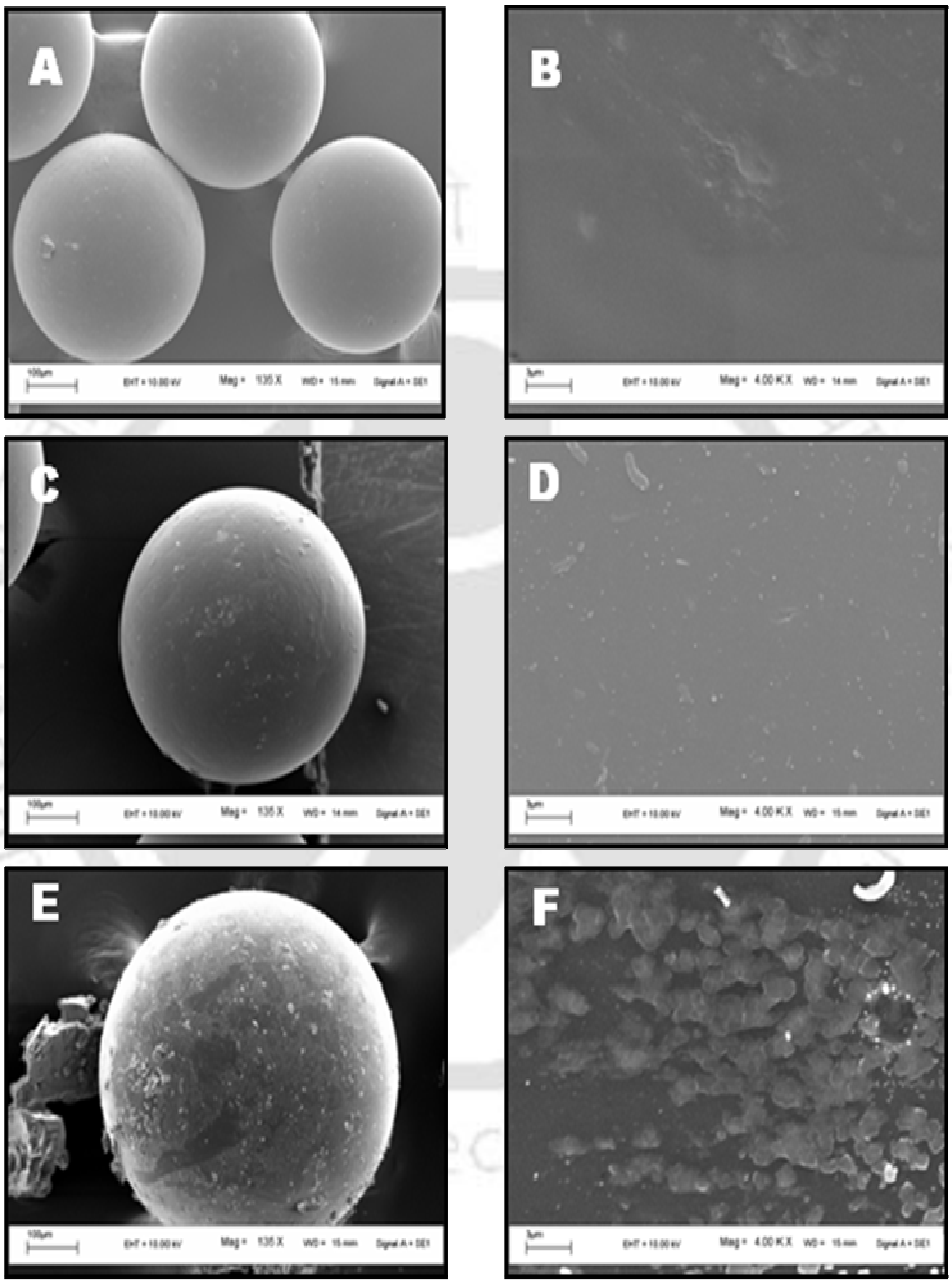

Figure 4.5: SEM images of beads and detailed surface view of **(A and B)** normal amberlite MB-150 beads, **(C and D)** glutaraldehyde activated amberlite MB-150 beads, **(E and F)** immobilized amberlite MB-150 beads. After glutaraldehyde activation and immobilization of procerain B on activated beads the surface morphology of amberlite MB-150 beads got changed.

4.4.6 Stability

The conformational flexibility of procerain B was affected by immobilization. Increase in temperature optima is the clear reflection of conformational rigidity which makes it resistant to denaturation. Plant cysteine endopeptidases are well known for their higher thermal stabilities and widely used in the process where higher temperature is required. Immobilization has further increased its stability and industrial importance. Immobilization on chitosan beads causes restricted deformation in three dimensional structure of procerain B due to temperature and leads to increased thermal stability. The temperature stability of immobilized procerain B was investigated and found that, it shows more than 80% activity upto 70°C while soluble procerain B shows similar activity upto 65°C only (*Singh et al., 2010*). In case of amberlite MB-150 beads, the thermal stability of immobilized enzyme was similar to soluble form (Figure 4.6). Thus chitosan immobilization leads to slight increase in thermal stability which makes it industrially more valuable.

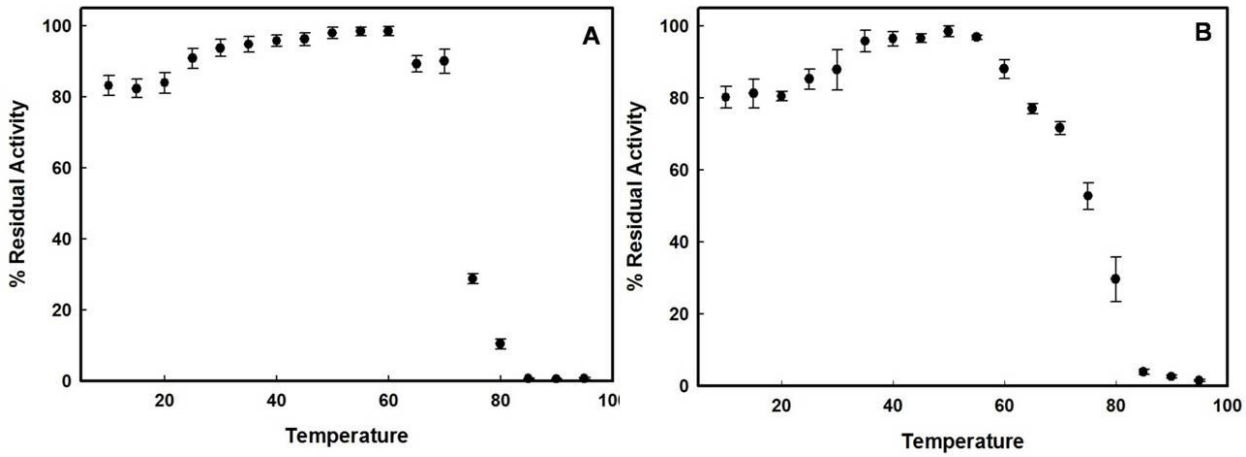

Figure 4.6: Effects of temperature on activity stability of immobilized procerain B on **(A)** Chitosan beads and **(B)** Amberlite MB-150 beads. For temperature stability measurements, enzyme was incubated at required temperature for 15 min and activity was measured at 37°C and pH 7.5.

4.4.7 Effect of substrate concentration on the activity of procerain B

The immobilized procerain B followed Michaelis-Menten kinetics with azocasein as substrate. At higher concentrations of substrate the enzyme activity gets saturated. In case of chitosan the Km of immobilized enzyme was ~148.6 µM, which is lower than the soluble enzyme (~210 µM) (*Singh et al., 2010*). It shows that the chitosan immobilized procerain B has more affinity for azocasein as substrate in comparison to soluble form. In case of amberlite MB-150 immobilized procerain B the Km observed was ~180.27 µM for azocasein as substrate, which is lower than the soluble form but higher than the chitosan immobilized procerain B (Figure 4.7).

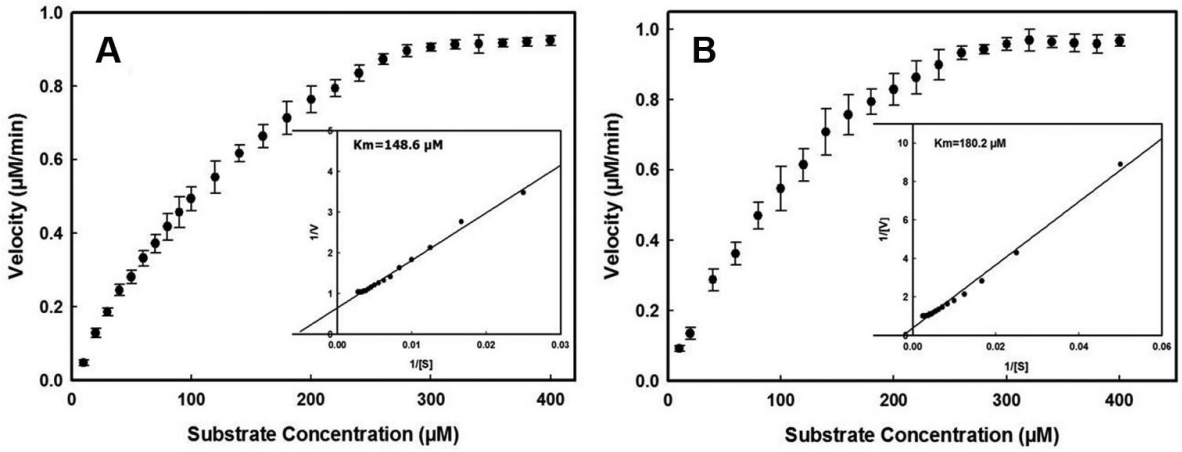

Figure 4.7: Effect of increasing substrate concentration on the activity of immobilized procerain B on chitosan beads **(A)** and amberlite MB-150 beads **(B)** The Km value for azocasein as substrate was calculated from the Lineweaver-Burk plot shown in subset of the graphs.

4.4.8 Reusability of immobilized procerain B

Reusability is one of the most important advantages of immobilization. The chitosan immobilized procerain B showed nearly 50% activity till 10th use (Figure 4.8A) and amberlite immobilized procerain B shows nearly 38.60% activity till 10th use (Figure 4.8B). In case of chitosan beads the activity of immobilized procerain B got increased initially with repeated use of beads and then decreased to 50% of the maximum till 10th use.

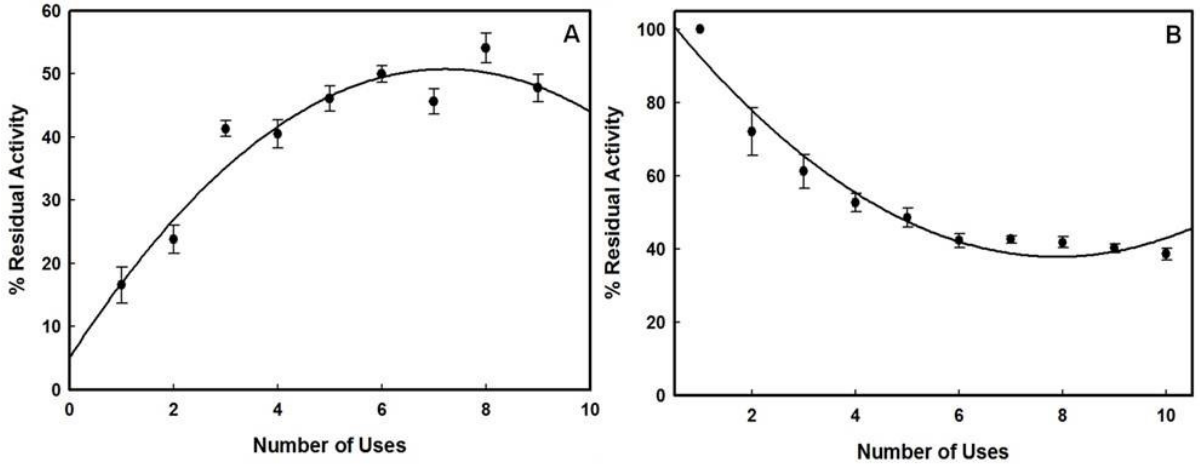

Figure 4.8: Reusability of **(A)** chitosan immobilized procerain B and **(B)** amberlite immobilized procerain B. After each reaction, the beads with immobilized procerain B were removed and washed with 0.1M Tris-Cl buffer, pH 7.5 to remove any residual substrate within the beads and stored in the same buffer at 4°C till further use.

In case of amberlite MB-150 beads the activity of immobilized procerain B was found to be maximum at first use and then decreased to 38.6% till 10^{th} use.

4.5 Conclusions

Immobilization of procerain B may further extend its applicability. Spherical chitosan beads of uniform size were prepared and the immobilization of procerain B on glutaraldehyde activated chitosan beads was successfully optimized. Overall 87.50% immobilization was achieved after optimization of different parameters. The immobilized product was characterized for optimum functional range and stability. The chitosan immobilized procerain B was found to be thermodynamically more stable as compared to soluble form. Due to leaching problem and lower storage stability of chitosan beads, the procerain B was further immobilized on robust Amberlie MB-150 beads. After glutaraldehyde activation nearly 62% of immobilization was achieved with optimized parameters. The amberlite immobilized form of enzyme showed comparatively broad pH and temperature optima with better stability in alkaline pH range which proves its candidature as a potential candidate for different industrial purposes.

Chapter V

Cloning of cDNA, complete amino acid sequencing and molecular modeling of procerain B*.

5.1 Abstract

Procerain B is a novel cysteine endopeptidase isolated from *Calotropis procera,* a medicinal plant of Asclepiadaceae family. Purification, biochemical characterization and potential applications of the enzyme have already been discussed in previous chapters. Here, we report the cDNA cloning, complete amino acid sequencing and molecular modeling of procerain B. The analysis of derived amino acid sequence showed high sequence homology with other papain like plant cysteine proteases of peptidase C1A super family. The three dimensional structure of active procerain B was modeled by homology modeling using X-ray crystal structure of actinidin (PDB ID: 3P5U), a cysteine endopeptidase from the fruits of *Actinidia arguta*. The structural aspect of the enzyme was discussed. The recombinant protein was expressed in form of inclusion body. Extensive attempts have been made to solubilize the inclusion body.

** Part of the work has been submitted for publication in **Plos One (Under revision)***

5.2 Introduction

Proteases are the single class of enzymes which occupy a crucial position with respect to physiological role as well as various industrial and therapeutic applications (*Chapman et al., 1997; Liu et al., 2004; Dubey et al., 2007*). Endopeptidases can modify different proteins in highly specific and selective manner for different physiological processes such as activation of zymogenic forms of enzymes by limited proteolysis, blood clotting, lysis of fibrin clots, processing and transport of secretory proteins across the membranes (*Chapman et al., 1997; Liu et al., 2004*). In plants, senescence of leaves, ripening of fruits, seed germination, defence mechanisms against pathogens and several other processes are protease dependent (*Hagel et al., 2008; Grudkowska et al., 2004Liggieri et al., 2009*). Papain like cysteine endopeptidases is modifying enzymes present in wide range of organisms (*Mladenovic et al., 2008*). Initially, the significance of cysteine endopeptidases was limited and they were believed to be involved in some house-keeping activities. However, owing to its presence in all organisms and involvement at several key steps of physiological and pathological processes to regulate the complex biological networks, the attention on cysteine endopeptidases are magnifying day by day with expanding knowledge and facilities in terms of scientific expertise (*Mottram et al., 1998; Sajid et al., 2002; Grudkowska et al., 2004*). Other than their physiological relevance, proteases are of high commercial value. Proteases are one of the most commercialized enzymes across the globe covering nearly half of the global enzyme sale (*Turk, 2006*). Proteases are being utilized in different industries like food industry for preparation of foods for infants, tenderization of meat, clarification of beer, preservation of spices, in several caned juice and soups for enhancement of flavor (*Bernholdt, 1975; Lyons, 1982; Warren, 1992*). In dairy industry proteases are used for preparation of several dairy products (*Uchikova & Kaneda, 1996*). Likewise in leather and textile industries, proteases are applicable for tannin of leather and smoothening of silk and wool fibers (*Galante & Formantici, 2003*). Proteases with detergents are used for removal of blood and other stains containing proteinaceous ingredients in detergent industry (*Aaslyng et al., 2007*). In pharmaceutical industries, endopeptidases are used with different formulations such as several ointments and lotions, as blood coagulant and as food additives (*Rao et al., 1998; Kirk et al., 2002; Gupta et al., 2002*).

The plants of Asclepiadaceae family are well known for their latex, containing different components such as alkaloids, terpenoids, tannins, proteins, vitamins, gum etc. and play an important role in first line of defence against pathogen (*Hagel et al., 2008; Liggieri et al., 2009*). *Calotropis procera* of family Asclepiadaceae has been explored for proteases by Dubey and Jagannadham (*Dubey & Jagannadham, 2003*) and purified a novel cysteine endopeptidase "Procerain" from the latex of this medicinal plant and also characterized it in terms of physiochemical properties. As discussed in earlier chapters we have also purified another cysteine endopeptidase, "procerain B" with high thermal stability and broad pH optima which pronounce its commercial importance (*Singh et al., 2010*). We have also screened some possible applications and found it very applicable in food, dairy, detergent and several other industries (*Singh & Dubey, 2011*). Since procerain B has such a variety of applications, it is desirable to obtain this protein in adequate amount for both basic research and industrial use. The enzyme preparation from plant source depends on several factors such as climatic conditions for the growth of plant and the techniques involved in purification of enzyme, which make traditional purification methods less reliable and efficient for preparation of pure form of enzyme in large quantity. Recombinant production of wild type enzyme is the only option which further offers the possibilities of protein engineering in desired fashion. The recombinant proteins can be engineered in different ways to make it more stable and resistant for autodigestion (common problem in protease research). The specificity of the enzymes can also be altered with selective modifications in recombinant proteins which will further elaborate their applications.

In this study we aim to find the cDNA sequence of active procerain B for the first time. After confirmation of sequence the *in silico* analysis was performed and the corresponding cDNA was cloned in two T_7 promoters based expression vectors (pET-28a and pET-22b) and the three dimensional structure of protein was modeled using a suitable template by homology modeling.

5.3 Materials and methods

5.3.1 Materials

The young leaves were collected freshly from the plant grown in IIT Guwahati campus (Guwahati). Trizol, DEPC, isopropanol, DNase, His-Tag Ni affinity beads, IPTG, azocasein and nuclease free water were purchased from Sigma Aldrich (USA). PCR master mix, cDNA synthesis kit, NheI, BamHI, XhoI restriction endonucleases and T_4 DNA ligase were purchased from New England Biolabs (NEB, England). Plasmid extraction kit, Gel elution kit and PCR cleanup kits were obtained from QUIAGEN (USA). All other chemicals and reagents were of highest purity available.

5.3.2 Methods

5.3.2.1 *RNA isolation and cDNA synthesis*

Total RNA was isolated from young leaves of *Calotropis procera* by trizol method. The yield and purity of RNA was determined spectrophotometrically and the quality of RNA was tested on 1% formaldehyde agarose gel. The first strand of cDNA was synthesized by the process of reverse transcription using 1 µg of total RNA as template under following conditions; 40 U M-MuLV reverse transcriptase (NEB, USA) with 0.5 µg of oligo $dT_{(18)}$ primer, 1 mM dNTPs and 20 U RNase inhibitor in a final volume of 20 µl. An aliquot of the first cDNA strand was used as template in PCR reaction for the synthesis of second strand of cDNA and then subsequent amplification of double stranded cDNA was performed with degenerate forward (DEG-N) and oligo $dT_{(18)}$ as reverse primers. The forward degenerate primer was designed on the basis of first seven N-terminal amino acid sequence of procerain B. The PCR was carried out as follows; predenaturation at 95°C, 3 min; 25 cycles of denaturation at 95°C, 30 sec; annealing at 50°C, 45 sec; extension at 72°C, 1 min and a final extension step at 72°C for 10 min.

The amplified products were separated on 1% (w/v) agarose gel and the product of expected size was extracted from the gel using QIAquick gel elution kit (QIAGEN, USA).

The gel eluted product was ligated to the pTZ57R/T vector using a T/A cloning kit (Fermentas, USA) and the ligation product was transformed into competent *E. coli* DH5α cells. Transformed colonies carrying the TA plasmid with insert were screened by α-complementation of lacZ gene and the plasmid DNAs were isolated with QIAprep Spin Miniprep kit (QUIAGEN, USA). The presence of insert DNA in the isolated plasmids were confirmed by PCR with M_{13} primers and finally sequenced from both directions with M_{13} forward and reverse primers.

5.3.2.2 *cDNA sequence analysis and construction of expression vector*

The cDNA sequence was analyzed to identify its homologous sequences with Basic Local Alignment Search Tool (BLASTx) against non redundant protein sequences in NCBI. The cDNA was translated in all six possible reading frames using ExPASy translation tool (http://web.expasy.org/translate/) to determine the corresponding amino acid sequence. The correct frame was used for BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis. New specific primers were designed from the sequence and used for subsequent PCR amplification of cDNA. The restriction sites for NheI and BamHI restriction endonucleases (underlined) were introduced at the 5' of forward (pET28aProB-F) and reverse (pET28aProB-R) primers respectively. The cDNA fragment was amplified by PCR with Dream Taq Green PCR Master Mix (Fermentas, USA) using above mentioned specific primers and checked on 1% agarose gel with EtBr staining. The conditions used for PCR were as follows, predenaturation at 95°C, 3 min; 25 cycles of denaturation at 95°C, 30 sec; annealing at 62°C, 45 sec; extension at 72°C, 1 min and a final extension step at 72°C for 10 min. The PCR product and pET-28a(+) expression vector (Novagen, USA) was digested with NheI (NEB, USA) and BamHI (NEB, USA) restriction endonucleases for the generation of complimentary sticky ends and then ligated with T_4 DNA ligase (NEB, USA). The ligation product was then transformed in competent *E. Coli*, DH5α cells by heat shock method. The transformed colonies containing recombinant clones were confirmed by PCR with gene specific primers and double digestion (NheI/BamHI). The recombinant plasmids were isolated from the confirmed colonies and named as pET28(a)-ProB. In order to confirm the orientation and presence of the insert in proper frame for the synthesis of complete

recombinant protein, the new construct (pET28(a)-ProB) was sequenced from both directions with T_7 promoter and terminator primers.

Simultaneously, the cDNA of procerain B was also cloned in pET-22b(+) expression vector (Novagen, USA) having a pelB leader sequence at N-terminal for periplasmic localization of recombinant protein and His-tag at C-terminal. The cDNA was amplified by PCR with gene specific primers. The restriction sites for BamHI and XhoI restriction endonucleases (underlined) were introduced at the 5' of forward (pET22bProB-F) and reverse (pET22bProB-R) primers respectively. The recombinant construct (pET22(b)-ProB) was confirmed as above and sent for sequencing with T_7 promoter and terminator primers.

5.3.2.3 *Expression and purification of fusion protein*

For the expression of fusion protein, *E. Coli* expression strain BL21(DE3) competent cells were transformed with confirmed pET28(a)-ProB and pET22(b)-ProB recombinant plasmids by heat shock method. Further, the transformed cells from a single colony were grown overnight in Luria Bertani (LB) broth (1% Tryptone, 0.5% Yeast Extract, 1% NaCl), supplemented with 50 µg ml^{-1} kanamycin antibiotic. The overnight grown culture was sub cultured in fresh LB medium with 1:100 dilutions and grown at 37°C with continuous shaking at 180 rpm, until the OD was 0.6-0.8 at 660 nm. In order to optimize the expression of recombinant procerain B, the transformed culture was induced with 0.1-1.0 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 20, 25 and 37°C. Samples were withdrawn at 2, 4, 6, 8 and 10 h intervals and the expression profile was checked on SDS-PAGE.

After small scale optimization the expression was scaled up with following conditions. The transformed colonies were grown till mid log phase at 37°C with continuous shaking at 180 rpm, 1.0 mM IPTG was added and then the growth was continued for 6 h at 25°C. The bacterial cells were harvested by centrifugation at 5000 rpm, 4°C for 10 min. The cell pellet was suspended in ice chilled Tris-HCl buffer, pH 7.4 supplemented with sodium tetrathionate (2mM) and lysozyme (0.2%, w/v) and incubated at 4°C for 30 min. The culture was then subjected to sonication with 5 sec on and 9 sec off cycles for 10 min and lysate was

centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant (soluble protein) was collected and the pellet (inclusion bodies) were dissolved in solubilization buffer pH 7.4 (20 mM Tris-HCl, 0.5 M NaCl, 6 M urea) at 4°C by continuous stirring and then centrifuged at 12,000 rpm for 20 min at 4°C to remove the insoluble traces.

The supernatant of last step was applied on Nickel-affinity column (Ni Sepharose FF, GE Health Care Life sciences) pre-equilibrated with 10 column volume of solubilization buffer and then washed with five column volume of same buffer with 5 mM imidazole. The bound protein was eluted in same buffer with 250 mM imidazole. The eluted protein was tried for refolding with dilution (10 times) by adding drop wise in refolding buffer (20 mM Tris-HCl, pH 8.0, 300 mM arginine, 20 mM cysteine, 5 mM EDTA, 15%, v/v glycerol) at 4°C and left for overnight with slow stirring and then concentrated by Amicon.

5.3.2.4 *Determination of protease activity*

The proteolytic activity of recombinant procerain B was determined by the method of Dubey and Jagannadham with slight modifications using azocasein as substrate (*Dubey & Jagannadham, 2003*). The purified recombinant enzyme (20 µg) was incubated at 37°C for 10 min in 500 µl of Tris-Cl buffer pH 7.5 containing 50 mM β-Mercaptoethanol as reducing agent. Azocasein solution (1%, w/v) was prepared in same buffer without β-Mercaptoethanol and added in enzyme solution to make the final volume 1 ml. The solutions were mixed properly and incubated at 37°C for 1 h. TCA (10%, w/v) was added to the reaction mixture to stop the reaction and incubated at room temperature for 5 min. The mixture was centrifuged at 10,000 rpm for 10 min. In case of azocasein as substrate, 500 µl of supernatant was mixed with equal volume of 50 mM NaOH and the color developed was quantified spectrophotometrically at 440 nm. A control assay was performed without enzyme and used as blank in all spectrophotometric experiments.

5.3.2.5 *Sequence analysis*

The molecular weight, isoelectric point (pI), extinction coefficient, number of cysteine, tyrosine and tryptophan residues and the number of disulphide bonds in procerain B were

calculated with pepstats (http://emboss.bioinformatics.nl/) server and the *in silico* data was compared with biochemical data (*Singh et al., 2010*).

5.3.2.6 *Homology modeling*

The predicted amino acid sequence was used as input for finding the homologous protein sequences with 3D structure using BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) program against PDB database in NCBI. The amino acid sequence of procerain B was aligned with the identified homologous proteins using Clustal X (*Thompson et al., 1994*) for understanding the conservation of amino acids throughout the protein family.

The three dimensional structure of procerain B (hereafter referred as Pro_B) from the latex of *C. procera* was predicted using actinidin from *Actinidia arguta* (PDB Id: 3P5U) (*Yogavel et al., 2010*) with Modeller 9v7 software (http://www.salilab.org/modeller) using sequence alignment and structural coordinates of the template as input. The initial hundred models generated for procerain B were prioritized on the basis of MOLPDF, discrete optimized protein energy (DOPE) and GA341 score. The final model with reasonable statistics (MOLPDF and DOPE) was validated in SAVES server (http://nihserver.mbi.ucla.edu/SAVES/) and subsequently energy minimized by Gromacs version package (http://www.gromacs.org/). The final model was analyzed with PROCHECK (*Laskowski et al., 1993*) and ERRAT plot and visually inspected with PyMOL (http://pymol.org/).

5.4 Results and Discussion

5.4.1 RT-PCR and cloning in pTZ57R/T vector

Total RNA from the young leaves of *Calotropis procera* was extracted by trizole method as described in section 5.3.1 and used as template for first strand cDNA synthesis by RT-PCR with oligo $dT_{(18)}$ primer. The subsequent PCR using first strand of cDNA as template with degenerate forward primer and oligo $dT_{(18)}$ as reverse primer yielded approximate 750 bp

fragment (Figure 5.1) which was ligated in pTZ57R/T vector to form a pTZ57R/T-ProB recombinant construct.

Table 5.1 Primers used for PCR amplification and cloning of procerain B in pET-28a(+) and pET-22b(+) vectors. Degenerate primer was designed on the basis of N-terminal sequence of procerain B.

S. No.	Name	Sequence (5'-3')
1	DEG-N	CTRCCNAAYTCYGTNGAYTGG
2	Oligo $dT_{(18)}$	TTTTTTTTTTTTTTTTTT
3	pET28aProB-F	CGGGCTAGCCTGCCTAACTCCGTT
4	pET28aProB-R	CCGGATCCTCGAGTCAATAAACAGGATA
5	pET22bProB-F	ATGGATCCACTGCCTAACTCCGTTG
6	pET22bProB-R	CACCTCGAGATAAACAGGATAAGCAGC

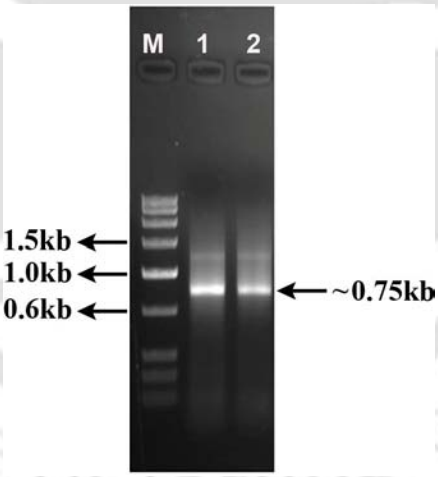

Figure 5.1: Amplified fragment from a *Calotropis procera* cDNA pool using degenerate forward (based on first seven N-terminal amino acids of procerain B) and oligo $dT_{(18)}$ reverse primer. Lane M represents Low range DNA ruler (Bangalore Genei). Lane 1 and 2 represent ~ 0.75 kb cDNA fragment on 1% agarose gel stained with ethidium bromide.

The competent *E. coli* (DH5α) cells were transformed with ligation product and the plasmids isolated from transformed colonies were sequenced. The DNA fragment of 639 nucleotides was identified as a putative cysteine protease by BLASTx analysis as described in section 5.3.2. The resulting cDNA sequence, named as Pro_B was submitted to nucleotide database (accession number: KC128816). The BLASTx analysis revealed a strong homology with asclepain, asclepain cI and asclepain cII isolated from Gomphocarpus fruticosus subsp. fruticosus (E value= 2×10-103) and A. curassavica (E value= 4×10-101 and 5×10-93). According to the BLAST results, the putative protein belongs to peptidase C1A super family and the corresponding translated sequence is given in Figure 5.2.

```
ctgcctaactccgttgattggcgagaaaaagatgttgttttcctattagaaatcaagga
 L  P  N  S  V  D  W  R  E  K  D  V  V  F  P  I  R  N  Q  G
caatgtgggagttgctggacattctcggctgttgcttcaattgaaactctaattggaatt
 Q  C  G  S  C  W  T  F  S  A  V  A  S  I  E  T  L  I  G  I
aaagaagatcgtatgattgcattatccgagcaagagctattggactgcgaaagaacaagt
 K  E  D  R  M  I  A  L  S  E  Q  E  L  L  D  C  E  R  T  S
tacgggtgtaaaggaggttactatactaatgcattcgcatatgtagccaagaaaggtctt
 Y  G  C  K  G  G  Y  Y  T  N  A  F  A  Y  V  A  K  K  G  L
acctctcgggaaaaatatccatatatatttcagcaaggacaatgttatcaaaaggaaaag
 T  S  R  E  K  Y  P  Y  I  F  Q  Q  G  Q  C  Y  Q  K  E  K
gtggtcagaatttctggttataggagaatacctaaaaacgacgagaaaaaacttcaaagt
 V  V  R  I  S  G  Y  R  R  I  P  K  N  D  E  K  K  L  Q  S
gttgtagcacaacaagtagtgagtgttggcgtaaaatctaaaagtagagatttccagcac
 V  V  A  Q  Q  V  V  S  V  G  V  K  S  K  S  R  D  F  Q  H
tatcgttcgggtgtatttagtggagtttgcggtccacgagtggatcatgcagtgaatatt
 Y  R  S  G  V  F  S  G  V  C  G  P  R  V  D  H  A  V  N  I
gttggatatggttctgaaggtggagttaattattggatcgtgagaaactcttggggtaca
 V  G  Y  G  S  E  G  G  V  N  Y  W  I  V  R  N  S  W  G  T
aattggggagagaatggatatatgagaattccaaggaattcaaatcagtccggaggttat
 N  W  G  E  N  G  Y  M  R  I  P  R  N  S  N  Q  S  G  G  Y
tgtggagttgctgttcaggctgcttatcctgtttattga
 C  G  V  A  V  Q  A  A  Y  P  V  Y  -
```

Figure 5.2: Nucleotide and deduced amino acid sequence of mature Procerain B from *Calotropis procera*. The amino acid sequence was predicted using TRANSLATE tool of ExPASy server (http://expasy.org/tools/). The (–) denotes the stop codon.

5.4.2 *In silico* sequence analysis

The physiochemical properties of procerain B was calculated with pepstats server (Table 5.2). Procerain B has seven cysteine and five tryptophan residues in it. Out of seven, six cysteine were involved in the formation of disulphide bonds while the active site cysteine (Cys_{25}) was free. The enzyme has fifteen tyrosine residues. Isoelectric point of procerain B from sequence analysis was found to be 9.25 which reflect the net positive charge on the surface of procerain B at neutral or lower pH. The physiochemical parameters of procerain B slightly differs from biochemical studies reported earlier as the biochemical studies are prone to errors (*Singh et al., 2010*). Moreover, the sequence analysis does not consider various possible post translational modifications. It is worth mentioning that the enzyme does not have any carbohydrate moiety attached (*Singh et al., 2010*) but possibility of other modifications can't be ruled out.

Table 5.2: Physiochemical properties of procerain B derived from amino acid sequencing data.

S.No.	Properties	Sequence Analysis
1	Isoelectric Point	9.25
2	Number of Cysteine Residues	7
3	Number of Disulphide Bonds	3
4	Number of Tryptophan Residues	5
5	Number of Tyrosine Residues	15

We have designed degenerate primer using first seven amino acids sequence from Edman's degradation. The c-DNA was amplified using oligo-dT and the degenerate primer. Only two c-DNA were amplified one 1.25kb and other 0.75kb (corresponding to expected mass of procerain B) as shown in Figure 5.1. Product of 1.25kb was also sequenced and found to be tubulin protein while sequence of 0.75kb matched well with procerain B. The experiment was

repeated multiple times with reproducibility. Thus, we concluded that the mismatch at couple of positions in chemically derived sequence and sequence from c-DNA is likely to be error of protein sequencing.

5.4.3 Sub cloning in expression vectors (pET-28a(+) and pET-22b(+))

In order to express the recombinant endopeptidase, the cDNA fragment was amplified with gene specific forward and reverse primers containing appropriate restriction sites at their 5' ends and sub-cloned in pET-28a(+) and pET-22b(+) expression vector as described in section 5.3.2. The plasmids were isolated from the recombinant colonies. Further, the presence of cDNA fragment in recombinant pET28(a)-ProB and pET22(b)-ProB vectors was confirmed by PCR and double digestion (Figure 5.3). The amplification of 639 bp fragments in PCR (Figure 5.3A) and release of same size of fragment by restriction digestion (Figure 5.3B and Figure 5.3C) confirms the presence of cDNA fragment in recombinant pET28(a)-ProB and pET22(b)-ProB vectors. The presence of same cDNA fragment in correct frame for complete expression of recombinant protein was confirmed by sequencing with T_7 forward and T_7 reverse primers which resulted in frame sequence with proper 5'-3' orientation. The *in silico* translated cDNA sequence predicted an open reading frame of 212 amino acids.

5.4.4. Expression and purification

The recombinant endopeptidase (procerain B) was expressed in BL21 (DE3) strain of *E. coli* at optimized induction with 1.0 mM IPTG at 25°C for 6 h and then the culture was centrifuged and sonicated to release the protein. The SDS-PAGE analysis of lysate reflected the newly synthesized recombinant protein as major fraction of total lysate protein. For soluble expression of recombinant protein the endopeptidase was expressed with (Figure 5.3E) and without (Figure 5.3D) pelB leader sequence. The pelB sequence at N-terminal was added for translocation of recombinant protein to periplasm where the microenvironment will be less reducing in comparison to cytoplasm for disulphide bond formation which will favor the proper folding of protein. Most of the recombinant endopeptidase was expressed in form of inclusion body present in pellet. Different other modified strains (*Rosetta-gami, R2*-(DE3)

were also tried for soluble expression of recombinant endopeptidase but the protein was expressed in form of inclusion body. The inclusion body was dissolved with 6 M urea and purified by Ni-affinity chromatography. The approximate yield of recombinant procerain B was nearly 2.5 mg L^{-1}. The purified recombinant protein was refolded by dilution with refolding buffer (20 mM Tris-HCl, pH 8.0, 300 mM arginine, 20 mM cysteine, 5 mM EDTA, 15%, v/v glycerol) as described in previous section without success. Further, attempts were made to solubilize recombinat procerain B from inclusion body using different concentrations of denaturants like urea, Na-deoxycholate and guanidine hydrochloride. It was found that 6 M urea was most effective in solubilising procerain B from inclusion body. Moreover, attempts to oxidative refolding of enzyme were also made using the combination of reduced and oxidised glutathione (this experiment was performed by Ms Prity Yadav, M. Tech. student of the Department). In order to determine the biological activity of recombinant procerain B, the proteolytic assay was performed with both refolded and solubilized recombinant protein, but enzyme was still found to have insignificant activity or no activity. A control experiment with native form of procerain B was proteolytically active. The lack of proteolytic activity may be attributed to misfolding of protein due to the absence of proper folding conditions in host system. Literature survey suggests the importance of pro-peptide region in proper folding of several cysteine endopeptidases. The absence of pro-peptide region could be another possibility for lacking of proteolytic activity in recombinant enzyme. Another reason which could affect the proper folding of protein is the presence of non cysteine protease region (from vector) in the recombinant protein. Although these regions are very small in size but may prevent the proper folding of protein. The other possibility is the requirement of a unique folding condition different from the other known cysteine endopeptidases. Therefore the reason for absence of biological activity in recombinant Pro_B is not clear and further research is required in this direction.

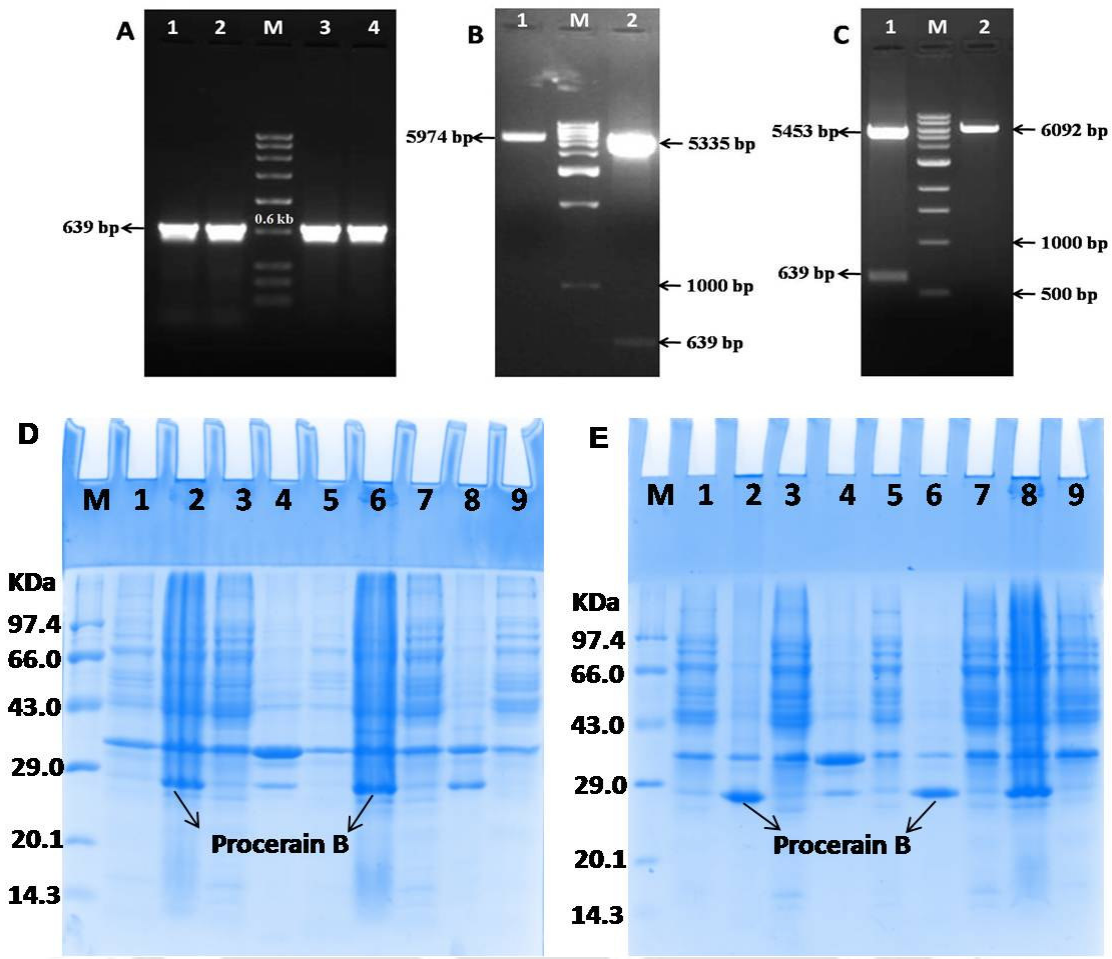

Figure 5.3: Confirmation of pET28(a)ProB and pET22(b)ProB clones with PCR and double digestion. **(A)** Lane 1 & 2 represents PCR amplified 639 bp fragment using pET28(a)ProB construct as template. M, low range DNA Ladder (Bangalore Genei). Lane 3 & 4 represents PCR amplified 639 bp fragment using pET22(b)ProB construct as template. **(B)** Lane 1 represents pET28(a)ProB construct digested with Nhe1. The DNA fragment of 5974 bp represents the combined size of pET-28(a) and procerain B cDNA. Lane M represents 1 kb DNA ladder (NEB). Lane 2 represents release of 639 bp fragment (procerain B cDNA) on double digestion (Nhe1/BamH1) of pET28(a)ProB construct. **(C)** Lane 1 represents release of 639 bp fragment (procerain B cDNA) on double digestion (BamH1/Xho1) of pET22(b)ProB construct. Lane M represents 1 kb DNA ladder (NEB). Lane 2 represents pET22(b)ProB construct digested with BamH1. The DNA fragment of 6092 bp represents the combined size of pET-22b(+) and procerain B cDNA. **(D)** SDS-PAGE showing the over expression of recombinant procerain B in BL21 transformed with pET28(a)ProB. Lane M represents medium range protein molecular weight marker (Bangalore Genei). Lane 1 and 2 represent supernatant and pellet of induced culture (1 mM IPTG) at 37°C. Lane 3 and 4 represent supernatant and pellet of un-induced culture at 37°C. Lane 5 & 6 represents supernatant and pellet of induced (1 mM IPTG) at 25°C. Lane 7 and 8 represent supernatant and pellet of un-induced culture at 25°C. Lane 9 represents pellet of BL21 transformed with pET-28a(+) at 25°C. **(E).** SDS-PAGE showing the over expression of recombinant procerain B in BL21 transformed with pET22(b)ProB. Lane M represents medium range protein molecular weight marker (Bangalore Genei). Lane 1 and 2 represent supernatant and pellet of induced culture (1 mM IPTG) at 25°C. Lane 3 and 4 represent supernatant and pellet of un-induced culture at 25°C. Lane 5 & 6 represents supernatant and pellet of induced (1 mM IPTG) at 20°C. Lane 7 and 8 represent supernatant and pellet of un-induced culture at 20°C. Lane 9 represents pellet of BL21 transformed with pET-22b(+) at 25°C.

5.4.5 Homology modeling

Sequence analysis of Pro_B showed maximum homology with plant cysteine proteases of peptidase C1A super family (Figure 5.4), which are highly similar to papain with endopeptidase activity and have preference towards substrates having bulky hydrophobic or aromatic residues at P2 position. Pro_B showed 68% similarity (52% identity) with actinidin, a cysteine endopeptidase from the fruits of *Actinidia arguta* belonging to Asclapidaceae family which has multiple crystallized structures (PDB ID: 3P5U, 3P5V, 3P5W, 3P5X) (*Yogavel et al., 2010*). Owing to its high sequence similarity with query coverage of 99%, conservation of catalytic domains and resolution of crystal structure (1.5 Å), actinidin from *Actinidia arguta* (PDB ID: 3P5U, A chain) was selected as template to model the three dimensional structure of procerain B. The complete structure of Pro_B was modeled except the C-terminal tryptophan, which was believed not to participate in structure or function. The quality of final model was validated by Ramachandran plot using PROCHECK and ERRAT plot. PROCHECK analysis revealed that 84.9% residues were falling in most favored regions, 14% in additionally allowed regions and only 0.6% (2 residues) was found in generously allowed and disallowed regions. The overall quality factor of the model from ERRAT plot was 90.15 which reflect that the modeled structure has low steric hinderance and is well in the range of good statistics (most of the residues were below 95% cutoff of error-value). The 3D model of Pro_B was energy minimized to eliminate steric hinderence in the modeled structure, shown in Figure 5.5.A. The closer structural analysis revealed the Pro_B belong to α+β class of protein with characteristic cysteine proteinase fold that consists of one α-helix and four strands of anti-parallel β-sheet which holds the catalytic triad (Cys_{25}, His_{156}, Asn_{176}). Structural comparison of Pro_B with actinidin showed that both the proteins were superimposed globally with RMSD (Root mean square deviation) of 0.54 Å (Cα atoms of 180) and 0.81 Å (Cα atoms of 199).

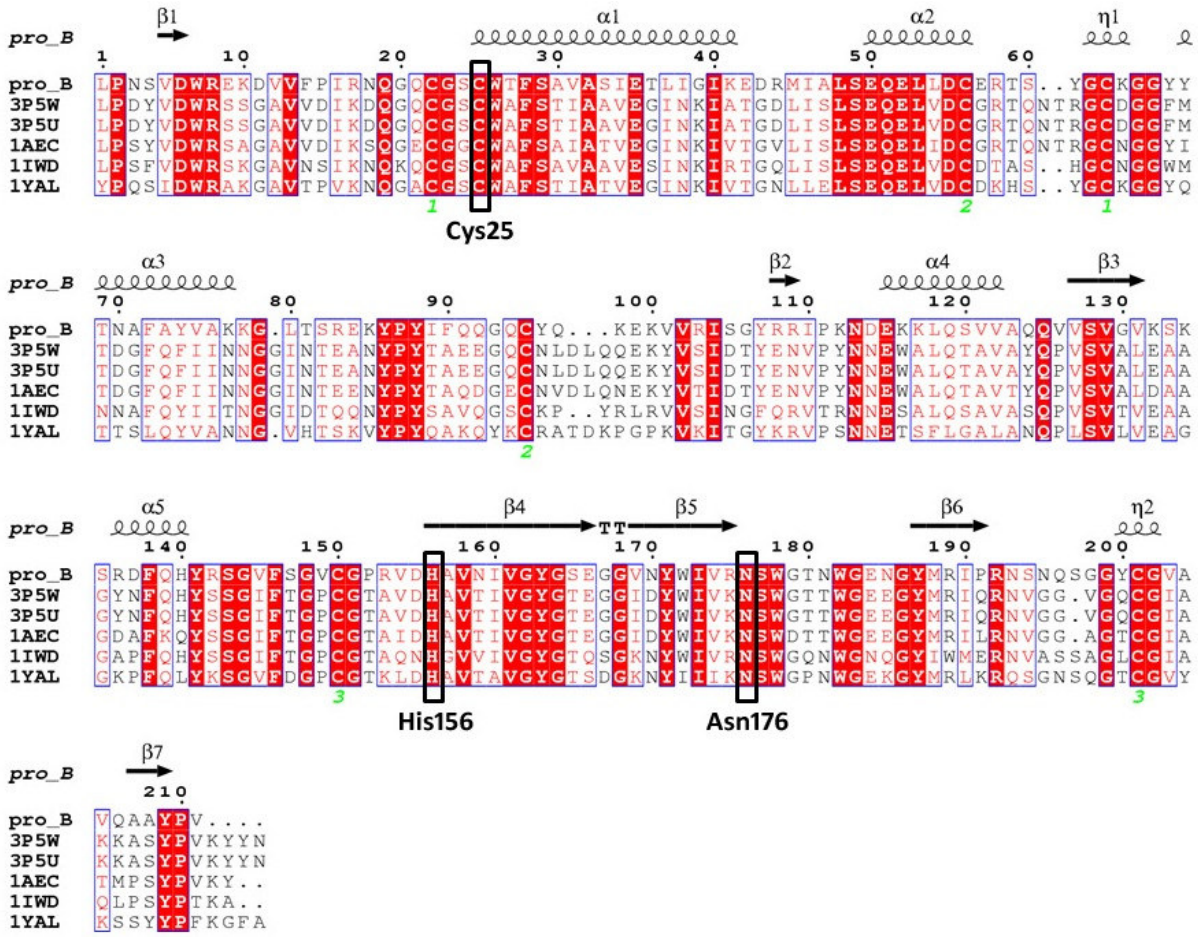

Figure 5.4: Multiple sequence alignment of translated Pro_B sequence with five top hits of BLASTp analysis (PDB ID: 3P5W, 3P5U, 1AEC, 1IDW and 1YAL) by CLUSTAL V. The active site residues (Cys_{25}, His_{156}, Asn_{176}) and other crucial conserved amino acids are highlighted in red. Three disulphide binding sites are marked in green as 1-1, 2-2, 3-3. The regions of α-helix, β-sheets and η-turns are marked on the top.

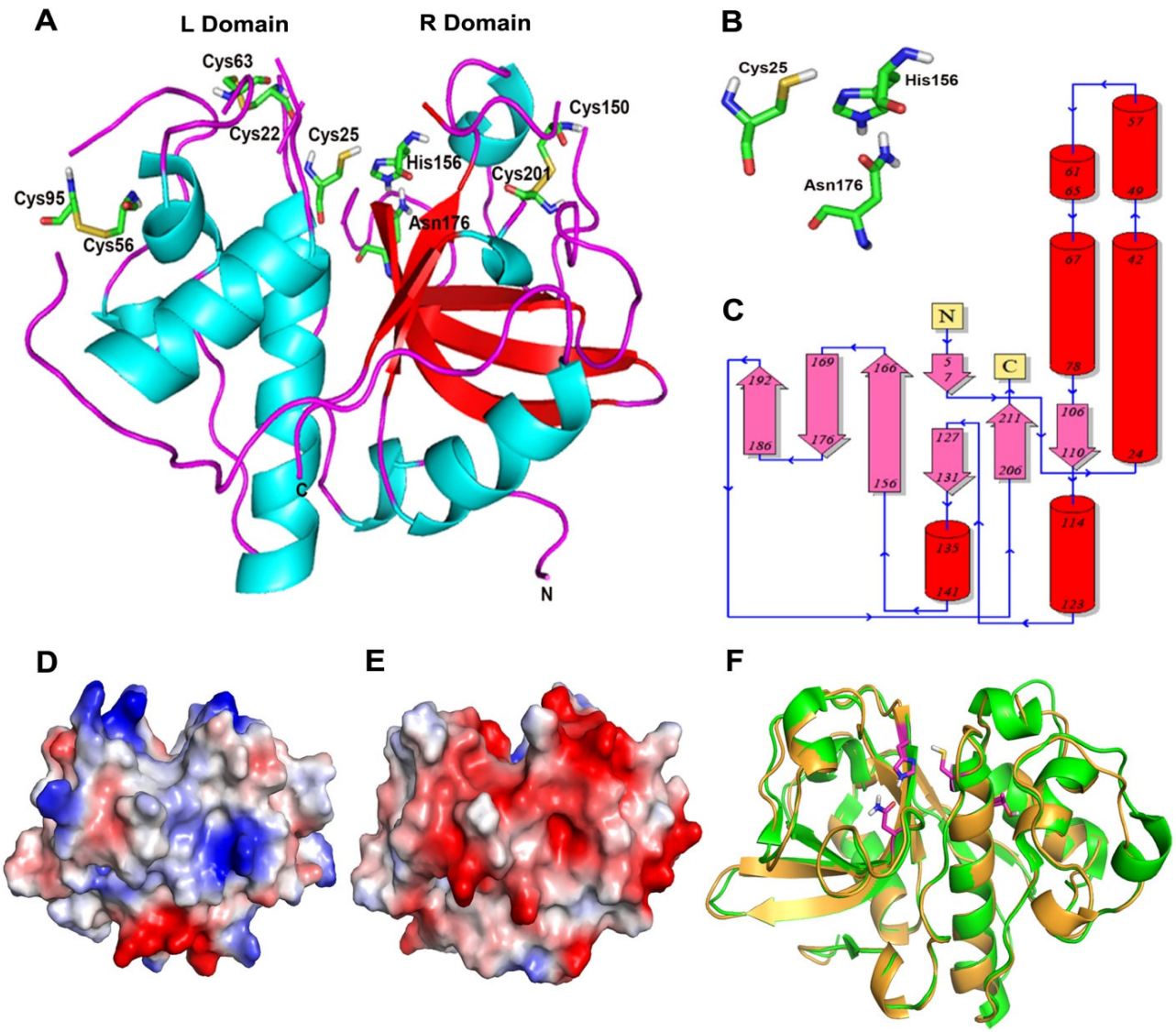

Figure 5.5: The three dimensional structure of procerain B. **(A)** Modeled structure with Modeller software using the X-ray crystal structure of actinidin (PDB ID: 3P5U) from the fruits of *Actinidia arguta* as template for homology modeling. The structural analysis of the model revealed that the Pro_B belong to α+β class of proteins with characteristic cysteine proteinase fold consists of one α-helix and four strands of anti-parallel β-sheet which holds the catalytic triad (Cys_{25}, His_{156}, Asn_{176}). The model contains two typical L (N-terminal) and R (C-terminal) domains of papain like cysteine endopeptidases sharing "V" shaped active site. **(B)** The active site residues (Cys_{25}, His_{156}, Asn_{176}) of procerain B. **(C)** Predicted secondary structure of procerain B using PDBSUB server showing two domains of protein. The N-terminal domain is dominated by α-helixes (red) while the C-terminal domain is dominated by anti-parallel β-sheets (pink). The electroststic sufrace charge distrebution of procerain B **(D)** and actinidin **(E)**. In comparison to actinidin the surface of procerain B was found to be more elecropositive. **(F)** Superimposition of 3-D structures of procerain B (green) with actinidin (golden) in cartoon representation. The active site analysis around catalytic Cys_{25} revealed Thr_{27} at S_2 position which is responsible for substrate specificity. The substrate binding pocket of Pro_B was found to be less hydrophobic (Thr_{27}) as compared to actinidin (Ala_{27}).

The model contains two typical L and R domains of papain like cysteine endopeptidases sharing "V" shaped active site (Figure 5.5.B). The topology of the modeled structure was determined by the secondary structure model from PDBsum (http://www.ebi.ac.uk/pdbsum/) (Figure 5.5.C). The L domain (N-terminal) is populated by three α helices (Ser_{24}-Glu_{42}, Ser_{49}-Arg_{57}, Tyr_{67}-Lys_{78}) while the R domain (C-terminal) mainly composed of a β-motif with anti-parallel β-strands. The active site cysteine (Cys_{25}) of Pro_B was present at the N-terminal of longest α helix (17 amino acids) which has more freedom to form ion pair with His156 located in the β-barrel domain of other side. High thermal stability of procerain B (upto 70°C) may be attributed to more number of γ-turns (Gly_{20}-Cys_{22}, Glu_{42}-Arg_{44}, Gly_{144}-Phe_{146}, Pro_{152}-Val_{154}, Gly_{183}-Asn_{185}) as compared to actinidin (Gly_{20}-Cys_{22}, Cys_{22}-Ser_{24}, Thr_{158}-Val_{160}, Gly_{189}-Glu_{191}). The increased thermostability of proteins with γ-turns may be explained by intra molecular hydrogen bonding in short γ-turns (*Chakravorty et al., 2011*). The L domain was supported by two disulphide bonds (Cys_{22}-Cys_{63}, Cys_{56}-Cys_{95}) where as the third disulphide bond (Cys_{150}-Cys_{201}) was present in R domain is conserved as it was observed in other cysteine endopeptidases (*Ghosh et al., 2008*). The active site analysis around catalytic Cys_{25} with 4 Å radius revealed Thr_{27} as S_2 position, responsible for substrate specificity. The substrate binding pocket of Pro_B was found to be less hydrophobic (Thr_{27}) as compared to actinidin (Ala_{27}) and several other endopeptidases of this family.

5.5 Conclusions

Cloning and expression of a cysteine endopeptidase from *Calotropis procera* is reported for the first time. The derived cDNA sequence of procerain B (named as Pro_B) was submitted to nucleotide database (Accession number: KC128816). The recombinant protein was expressed as a fusion protein with N-terminal His-tag and with N-terminal pelB sequence for periplasmic translocation. The protein was expressed in form of inclusion body. We made extensive attempts to solubilize the inclusion body of the recombinant protein. The three dimensional structure of protein was modeled using actinidin X-ray crystal structure (3P5U) as template and analyzed for conservation of domains with catalytic residues specific for papain like cysteine endopeptidases.

Chapter VI

Summary

6.1 Abstract

Bioinformatics analysis of the human and mouse genomes indicates that at least 2% genes of the genome code for proteases which signifies physiological role of proteases. Proteases have therapeutic and industrial applications as well. The annual sales of proteases accounts for 60% of the total world enzyme market and estimated to reach 220 billion US$ approximately. Plant cysteine proteases have wide range of applications in industries, which are attributed to their high temperature stability and broad substrate specificity. Plant genomes encode hundreds of proteases, but little is known about their roles in the life of a plant. Proteases are thought to be involved in a range of biological processes, including senescence, implicated in perception, signaling and execution leading to plant defence. Few other cysteine proteases from the plant sources have been purified and characterized. As

usefulness of the proteases depends on its unique cleavage site, stability as well as optimum activity conditions, search for a novel protease with unique properties is always in demand. *Calotropis procera* (family Asclepiadaceae) is a tropical plant that has been widely used in Indian traditional medicinal system for the treatment of various diseases namely leprosy, ulcers, tumors, piles and diseases of the spleen, liver and abdomen. Various parts of the plant show anti-microbial, anti-inflammatory, antipyretic and anti-malarial activities. The latex of the plant shows antidiabetic, hepatoprotective, antiarthritic, cytotoxic and anticancerous properties. Preliminary screening of the latex of the plant showed very high proteolytic activity.

We report purification of a novel cysteine endopeptidase from the latex of the plant, which we named as procerain B. The enzyme was characterized for its physiochemical properties and the possible applications in different industries were screened. In order to further increase its industrial applicability, the endopeptidase was immobilized on different matrices and the cDNA of this protein was cloned in TA (pTZ57R/T) and expression vectors (pET-28a(+) & pET-22b(+)) for complete amino acid sequencing and production of recombinant enzyme.

6.2 Purification and physiochemical characterization of procerain B

A novel cysteine endopeptidase was purified from the latex of a well known medicinal plant, *Calotropis procera,* and further characterized for its different physiochemical properties. Since another cysteine endopeptidase "Procerain" is already known from the same source, this newly purified enzyme was named as "Procerain B". For purification of procerain B, the latex was collected from the young stems of plant by making superficial incision. The milky liquid oozing out was collected in chilled 10 mM acetate buffer with sodium-tetrathionate as reversible inhibitor to avoid any complication due to proteolysis. The purification includes ammonium sulphate fractionation, cation-exchange chromatography (CM-Sepharose) and gel filtration chromatography (Sephacryl S300). The fractions of descending arm of single asymmetrical peak of gel filtration chromatography were found to be homogeneous. The purity was checked on 12% SDS-PAGE stained with coomassie brilliant blue R-250.

The purified enzyme was characterized in terms of molecular weight, pH and thermal optima and stability, isoelectric point, inhibition and kinetic properties, stability against different chemicals and solvents etc. Different properties of procerain B were compared with procerain and the identity of two proteases (procerain and procerain B) as separate proteins were confirmed by immunological assay. The newly purified enzyme (procerain B) showed broad pH (6.5-8.5) as well as temperature optima (40-60^{o}C). Additionally, this enzyme retains its activity at conditions where most of the other proteases are not active. Procerain B was further characterized by N-terminal amino acid sequencing and peptide mass fingerprinting (PMF). Certain physiochemical analysis such as N-terminal amino acid sequencing and peptide mass fingerprinting, revealed distinct features of the enzyme. Simple and economic purification of procerain B coupled with easy accessibility of latex makes the large-scale production of procerain B feasible. This enables exploration of various industrial as well as biotechnological applications of the enzyme.

6.3 Possible applications and determination of cutting site for procerain B

Proteases are one of the most important industrial enzymes and according to recent market survey it covers more than half of global enzyme market. We are exploring the hydrolytic properties of proteases in different industries. In food industries proteases are applicable for preparation of several partially digested foods, in bakery products, in beverage preparation, in dairy industry etc. Proteases are also used in leather industry for tanning and dehairing process of leather. In detergent industry, proteases are useful for removal of proteinaceous stains. In pharmaceutical industries, proteases are used in several ointments and lotions specially recommended for pain relief. Proteases are also applicable for recycling of raw materials in paper industry, cleaning of eye contact lenses, as digestive supplements, in reducing the inflammation etc. Due to high industrial importance of proteases, I was very enthusiastic to screen some possible applications for procerain B.

Procerain B was first checked for efficiency of digestion of food proteins and egg white albumin protein from boiled egg was used as model protein. Procerain B showed high efficiency for digestion of food proteins and more than 60% protein got digested in overnight incubation at room temperature. It shows the applicability of procerain B in food industry.

The milk coagulation efficiency of procerain B was also checked and effect of different metal ions on the coagulation of milk in presence of procerain B was investigated. Procerain B coagulates the milk and converts it into precheese within 40 min, which reflects its applicability in dairy industry for preparation of cheese. The compatibility of procerain B with detergents available in market was tested and the removal of blood stain in combination with detergents was observed. Procerain B was found to be compatible with most of the detergents for removal of blood stain. In order to further extend its applications in pharmaceutical and biotech industries, we have determined the cleavage preference of procerain B (Table 3.4). Procerain B was also found to have mitogenic activity for mouse fibroblast cells (L929) which was dependent on proteolytic activity of enzyme. It opens a new horizon for its possible applications in angiogenic therapy for ischemic diseases and healing of wounds.

6.4 Immobilization of procerain B on different matrices and characterization of immobilized product

Proteases are involved in several crucial biological processes reflecting its physiological importance. In addition to its physiological significances, it also has multifarious applications in food and pharmaceutical industries. Immobilized form of the enzyme further strengthens its applicability by enhancing its reusability and stability. Immobilization is the attachment of biocatalyst to an inert matrix through which substrate can freely pass and the product can easily diffuse out. The immobilization of procerain B on glutaraldehyde activated chitosan and amberlite MB-150 beads through covalent attachment were optimized. Besides serving as cross-linking agent, glutaraldehyde also links the procerain B on the surface of chitosan beads by forming schiff-base linkage through primary amine group of either lysine side chain or N-terminal region. The immobilized procerain B was then characterized for optimum functional range and stability with respect to pH and temperature. Better affinity of immobilized (amberlite MB-150) form (Km=~180.27 μM) was observed as compared to soluble one for azocasein as substrate. The immobilized procerain B retains 50% (chitosan) and 38.6% (amberlite MB-150) activity till 10^{th} use which strongly affirmed its industrial candidature.

6.4 Cloning of cDNA, complete amino acid sequencing and molecular modeling of procerain B

A novel cysteine protease was purified from the latex of *Calotropis procera* and characterized extensively in terms of its physiochemical properties. Several applications were screened for this newly purified enzyme (procerain B) and it was found to be applicable in several industries. Further for determining the complete amino acid sequence and production of recombinant enzyme, which will offer the possibilities of protein engineering we cloned the cDNA of procerain B in TA cloning vector, pTZ57R/T (Fermentas, USA). The complete amino acid sequence of procerain B was determined which shows high homology with other papain like plant cysteine proteases of peptidase C1A superfamily. The three dimensional structure of active procerain B was modeled by homology modeling using X-ray crystal structure of actinidin (PDB ID: 3P5U), a cysteine protease from the fruits of *Actinidia arguta*. The structural aspect of the enzyme was discussed for the presence of two domains (L & R) in the protein and in terms of catalytic residues (Cys_{25}, His_{156}, Asp_{178}). The cDNA was further cloned in pET-28(a) and pET-22(b) for expression of recombinant protein in *E.coli.*

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PUBLICATIONS

A. Publications in peer reviewed international journals from PhD work:

1. **Abhay Narayan Singh**, Sushant Singh, Neeraj Suthar and Vikash Kumar Dubey. Glutaraldehyde activated chitosan matrix for immobilization of a novel cysteine protease, procerain B. ***Journal of Food and Agricultural Chemistry*** **(ACS)**, **2011**; 59 (11); 6256–6262.

2. **Abhay Narayan Singh** and Vikash Kumar Dubey. Exploring applications of procerain B, a novel protease from *Calotropis procera*, and characterization by N-terminal sequencing as well as peptide mass fingerprinting. ***Applied Biochemistry and Biotechnology***, **2011**; 164; 573–580**.**

3. **Abhay Narain Singh**, Anil Kumar Shukla, M.V. Jagannadham and Vikash Kumar Dubey. Purification of a novel cysteine protease, procerain B, from *Calotropis procera* with distinct characteristics compared to procerain. ***Process Biochemistry***, **2010**; 45; 399-406.

4. **Abhay Narayan Singh**, Prity Yadav and Vikash Kumar Dubey. cDNA Cloning and Molecular Modeling of Procerain B, a Novel Cysteine Endopeptidase isolated from *Calotropis procera*. **PLOS ONE**, 2013, 8(3): e59806. doi:10.1371/journal.pone.0059806

5. **Abhay Narayan Singh**, Sushant Singh and Vikash Kumar Dubey. Immobilization of Procerain B, a Cysteine Endopeptidase, on Amberlite MB-150 Beads. **PLOS ONE**, 2013, Accepted. doi: 10.1371/journal.pone.0066000

B. *Publications in peer reviewed international journals from* other work:

6. Bankapallai Leela Krishna, **Abhay Narayan Singh**, Sanjukta Patra and Vikash Kumar Dubey. Purification, characterization and immobilization of urease from *Momordica charantia* seeds, **Process Biochemistry**, **2011**, 46, 1486-1491.

7. Nandini Sarkar, **Abhay Narain Singh** and Vikash Kumar Dubey. Effect of curcumin on amyloidogenic property of molten globule like intermediate state of 2, 5-Diketo-D-Gluconate Reductase A. **Biological Chemistry**, **2009**, 390, 1057-1061.

8. Sushant Singh, **Abhay Narayan Singh** and Vikash Kumar Dubey. A novel superoxide dismutase from *Cicer arietinum* L. seedlings: purification and characterization. **Protein and Peptide Letters**, Accepted.

Publications in conference presentations

1. **Abhay Narayan Singh** and Vikash Kumar Dubey. Purification, characterization and cloning of procerain B: an industrially important plant cysteine protease. 18th International conference (Post ISCBC-2012) Perspective and Challenges in Chemical and Biological Sciences Innovation Cross Roads. Organised by IASST, Guwahati & Indian Society of Chemist and Biologist, Lucknow, India, 28-30th Jan., 2012. ***[Poster presentation]***
2. **Abhay Narayan Singh** and Vikash Kumar Dubey. Physiochemical characterization, immobilization and cloning of procerain B; an industrially important plant cysteine protease. 80th Annual Meeting of Society of Biological Chemists, India held at Central Institute of Medicinal and Aromatic Plants, Lucknow, India, 12-15 Nov, 2011. ***[Poster presentation]***
3. Prity Yadav, **Abhay Narayan Singh** and Vikash Kumar Dubey. Heterologous expression of a novel plant cysteine protease: procerain B in E. coli and site directed mutagenesis to study the amino acids at catalytic triad. 80th Annual Meeting of Society of Biological Chemists, held at Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India, November 21-24, 2011. ***[Oral presentation]***
4. **Abhay Narayan Singh** and Vikash Kumar Dubey. Procerain B a potential candidate for protease industry. World Congress on Biotechnology, held at HICC, Hyderabad, India, 21-23 March 2011. ***[Poster presentation]***
5. **Abhay Narain Singh** and Vikash Kumar Dubey. Procerain B a novel cysteine protease from the latex of medicinal plant *Calotropis procera*. 78th Annual Meeting of Society of Biological Chemists, India held at National Centre for Cell Science and University of Pune, Oct 30-Nov 1, 2009. ***[Poster presentation]***

C. Workshop attended:

- "Scientific Paper Writing and Research Methodology Workshop" organized by IAMM (Guwahati Chapter) in association with Omeo Kumar Das Institute of Social Change & Development and ICMR, held on 24th April 2009 at Gauhati Medical College, Guwahati, Assam.