Studies on Proteases of Pathogenic Bacteria with Special Emphasis on Elastase and Natural Anti Elastase Factors

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By

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CERTIFICATE OF DECLARATION BY THE CANDIDATE

I, DEENA CHRISTABEL MENDEZ do hereby declare that the research work presented in the thesis titled **"Studies on Proteases of Pathogenic Bacteria with Special Emphasis on Elastase and Natural Anti Elastase Factors"** is an original work under the guidance of Dr. A.V.M. Kutty Professor Department of Biochemistry and co – guidance of Dr. S.R. Prasad Professor Department of Microbiology Sri Devaraj Urs Medical College in partial fulfillment of the requirements for the degree of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associate ship, fellowship or any other similar title or recognition.

Deena C. Mendez

CERTIFICATE

This is to certify that the research work presented in the thesis entitled **"Studies on Proteases of Pathogenic Bacteria with Special Emphasis on Elastase and Natural Anti Elastase Factors"** is based on the original work done by Mrs. DEENA CHRISTABEL MENDEZ (Reg No 11Ph. D 301) under the guidance of Dr. A.V.M. Kutty Professor Department of Biochemistry and co – guidance of Dr. S.R. Prasad Professor Department of Microbiology Sri Devaraj Urs Medical College in partial fulfillment of the requirements for the degree of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associate ship. fellowship or any other similar title or recognition.

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Deena Christabel Mendez

ABBREVIATIONS

1) PAR – Protease Activated Receptors
2) PASP – Pseudomonas aeruginosa small protease
3) HNE – Human Neutrophil Elastase
4) NE - Neutrophil Elastase
5) NET – Neutrophil Extracellular Traps
6) AAT – α1 Antitrypsin
7) AMG – α2 Macroglobulin
8) API - α1 Proteinase Inhibitor
9) WAP – Whey Acidic Proteins
10) SLPI – Secretory Leukocyte Protease Inhibitor
11) Serpins – Serine Protease Inhibitors
12) UPAR – Urokinase Type Plasminogen Activation Receptor
13) P. aeruginosa – Pseudomonas aeruginosa
14) B. coagulans - Bacillus coagulans
15) ATBI – Alkalo Thermophilic Bacillus Inhibitor
16) AFLEI – Aspergillus Flavus Leukocyte Elastase Inhibitor
17) PI – Proteinase Inhibitor
18) SET – Streptomyces Erythrase Trypsin
19) ATEE – Acetyl Tyrosine Ethyl Ester
20) STANA – Succinyl Tri alanyl Para nitroanilide
21) BAPNA – Benzoyl Arginine Para nitroanilide
22) S. aureus – Staphylococcus aureus
23) B. subtilis – Bacillus subtilis
24) A. aceti – Acetobacter aceti
25) GRAS – Generally regarded as safe
26) ^{0}C – Celsius
27) TCA – Trichloro aceti acid
28) ECM – Extracellular Matrix
29) P. guajava – Psidium guajava
30) PGG – Pentagalloyl glucose
31) MAP kinase –Mitogen Activated Protein kinase

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Introduction

Proteases are the enzymes that can hydrolyze peptide bonds within peptides and proteins (1). For a long time the function of proteases were solely attributed to turn over or digestion of proteins as nutritional source. However, it is now clear that hydrolysis of peptide bonds of protein is very vital and brings about wide range of biological functions critical for life processes. (2)

Proteases constitute a very large and complex group of enzymes present in both prokaryotes and eukaryotes. They are classified in to six different catalytic forms viz; serine proteases, aspartic proteases, cysteine proteases, threonine proteases, glutamate proteases and metalloproteases. This classification is based on the critical amino acid residues present at their active sites and which involve in catalytic processes. (2,3). Proteases are also classified as acidic, alkaline and neutral proteases based on the pH optima or range of pH at which they are active (4).

Common examples of animal proteases are pancreatic trypsin, chymotrypsin, pepsin and rennin. Papain, Bromelin, Keratinase and

Ficin are some of the well characterized proteases of plant origin. Though, plants and animals are known to have proteases and many of them have been studied extensively, microorganisms are preferred over these for the large scale production of proteases. This is on account of their fast growth and easiness with which microorganisms could be cultivated. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. A variety of proteases are produced by microorganisms depending on, the genera and species or strains belonging to the same species. Among the proteases of microbial origin, neutral and alkaline proteases hold prominence.

Sequencing of the human genome revealed that more than 2% of the genes encode proteases, signifying that these enzymes have functions which are complex and vital (5). Proteases perform a large variety of well regulated and diverse physiological functions such as protein metabolism, coagulation, cell growth and migration, tissue arrangement, morphogenesis, gene expression, , activation of zymogens, release of hormones, formation of pharmacologically

active peptides from precursor proteins and transport of secretor proteins across membranes. Disturbances in the regulatory processes could lead to onset of disease conditions like inflammation, tumor growth metastasis etc.

There are two types of secreted proteases intracellular and extra cellular. Intracellular proteases are vital to sustain various cellular and metabolic processes, such as protein turn over, maturation of enzyme and hormones and also in activation of inactive precursor molecules. Extracellular proteases carry out protein hydrolysis in natural environment enabling the cells to absorb and utilize hydrolytic products (1,6). In contrast to the multitude of the roles considered for proteases, knowledge about their mechanisms of actions is still an area of exploration. Extensive research is being carried out to unravel the pathways in which proteases play integral roles.

The actions of proteases are highly regulated at various levels starting with transcription, translation, and in many cases extensive

post-translational modifications. After the biosynthesis as proteins, proteases are generally restricted to specific organs, tissues and sub-cellular locations, or circulate in the vascular or lymphatic systems. To control the spatial and sequential location of protease activity, almost all proteases are synthesized initially as zymogens, which have no catalytic activity. These zymogens are activated by specific processing involving other highly specific proteases. The activation of zymogens are many a times very crucial in bringing about the desired physiological effects and hence are often found in the pathways involving in cascade or sequential protease activation. Protease activity is regulated also by protease inhibitors that modulate the effective concentration of active enzyme .

Proteases could be potentially damaging when over expressed or when present in high concentrations as uncontrolled secretion can trigger off destructive processes associated with various chronic diseases such as rheumatoid arthritis, emphysema and psoriasis (7). Hence, they are well regulated and controlled by a number of naturally occurring protease inhibitors such as α_1 Antitrypsin(α_1 AT) which is the major circulating serine protease inhibitor(Serpin)

and a potent inhibitor of multiple serine proteases with high activity against neutrophil serine proteases, neutrophil elastase and proteinase 3 (8). This regulation of protease activity by endogenous anti proteases represent a major mechanism limiting host tissue destruction. Many of the protease inhibitors interact with the target protease by contact with the active site of the protease, resulting in formation of a stable protease inhibitor complex that is incapable of showing enzymatic activity (9,10).

As in the case of eukaryotes, there are different types of proteases in bacteria, which are broadly grouped into two major classes: cell-associated protease complexes and extracellular enzymes. While cell associated or intracellular proteases involve in various cellular processes like protein turn over, enzyme maturation, sporulation etc. the extracellular enzymes perform protein hydrolysis to enable bacteria to absorb and utilize the products. Several of these extracellular proteases have been isolated and well characterized from *Bacillus*, *Staphylococcus, Streptococcus*, *Pseudomonas* etc. It is noteworthy that the spectra of proteases include all the catalytic types of the proteases with serine proteases leading the list. These

enzymes also exhibit bond specificities and are capable of acting on variety of proteins. These properties of microbial proteases make them molecules of interest not only of commercial importance but also in health and disease

The proteases of microorganisms are also involved in the pathogenesis of disease, by acting as virulence factors (3). Most of the secreted proteases are unique to specific pathogens and are single-subunit enzymes that target a specific protein or set of substrates in an unregulated fashion. Pathogenic bacteria need to overpower the host resistance to establish and successfully maintain an infection. A successful pathogen has an arsenal of virulence factors in which proteases play an important role by destroying host defense lines by degradation of virulence regulators and indirectly by providing tolerance to adverse conditions such as those experienced in the host (3,11). Once the host cells are broken, the bacteria enter into an environment needed for growth. The proteolysis adopted by bacterial pathogens takes place at multiple levels to ensure the success of the pathogen in contact with the host. Some of these

proteases are elastase A and B, protease IV, alkaline protease and PAS P (*Pseudomonas Aeruginosa* Small Protease) which break down cellular matrix protein allowing spread of infection (12,13,14).

Of these various proteases involved in bacterial pathogenesis, elastase an important virulence factor aids in tissue invasion by degrading elastin and can also degrade proteins of the human immune defense system and surfactant protein(13,14). Elastase also has been reported in a few nonpathogenic bacteria (15). Bacterial pathogenicity however, depends on several factors which vary widely. The bacteria have to counter the defense mechanism of the host. HNE (Human Neutrophil Elastase), stored principally in neutrophils is released when neutrophils encounter foreign substances. Neutrophil elastase (NE) contributes to killing of Gram negative bacteria and plays a role in bacterial killing as it comprises a key component of neutrophil extracellular traps (NET) which are involved in host defense (16). They bind Gram positive and Gram negative bacteria and allow neutrophils to deliver high concentrations of proteases which kill bacteria. This elastase released

during the invasion of microorganisms can be inhibited by anti-proteolytic substances produced by the microorganism, which can facilitate the invasive process. Recently, bacterial virulence that counteracts NE Traps has been identified and they are capable of degrading the NET backbone (17,18).

In almost every pathogenic bacterial species that has been examined, proteases have been found to act as master regulators of crucial aspects of virulence, including initial colonization and the prevention of killing by host responses. Establishment of infection often involves the upward regulation of cellular processes that facilitate adherence, invasion and survival inside the host environment. To survive the unfavorable conditions during infection and in order to avoid elimination by the host's innate immune system, pathogenic bacteria effectively utilize proteolytic defense mechanisms in order to rapidly adapt to new environments.

Anti-proteases exhibit diverse roles in nature, from regulating protease activity and as a defense against proteases of microorganisms. The imbalance between proteases and their inhibitors has substantial significance in the pathogenesis of diseases. Human α_1 AT inhibitor which maintains a tight control of elastase activity is known to be cleaved by the elastase of *P. aeruginosa* (19). Hence, both these factors the defense, by HNE against the invasive microorganism and the virulence factors that counter acts HNE have to be taken into consideration in the establishment of an infectious disease or its prevention.

It is the capabilities of bacterial proteases and pathogenicity that make these proteases particularly interesting molecules for diagnostic and therapeutic purposes because many of these enzymes are secreted in to the surrounding micro-environment of the bacterium, making them easy to get detected. Indeed several recent publications have shown the proteolytic activity can be detected using specific peptide substrates coupled to fluorogentic or chromogentic labels, and that such substrates could potentially

provide a rapid and simple technique for the detection of proteases secreting bacteria (20). Further, the development of bacterial protease-related and simple detection and virulence monitoring devices could potentially allow rapid and cheap monitoring of patient-specific changes to bacterial virulence over time, possibly leading to personalized medical treatment regimens. Finally, research in to bacterial proteases and their substrates will allow the development of novel protease inhibiting compounds that could potentially be used to limit the action of destructive proteases secreted by bacteria during clinical infections (21, 22).

A comprehensive understanding of the multiple biological roles of proteases at both physiological and pharmacological levels is therefore an important component for advancing their therapeutic use. Protein engineering to improve and focus protease activity to only one or a few targets or sites is a promising avenue for future research.

Proteases also represent promising therapeutic targets as they are susceptible to inhibition with pharmacologically active compounds.

In fact, several proteases have been successfully targeted in the treatment of a wide range of diseases. These targets include accessible extracellular proteases that function in blood pressure regulation blood coagulation (thrombin and factor X a) as well as intracellular viral proteases that process viral poly-proteins into the mature proteins that mediate viral packaging and replication.(23,24)

As outlined above, proteases are clearly important for many cellular processes. In some cases, disruption of proteases could block cell growth; in others, modulation could limit virulence. Targeting virulence pathways using small molecules, to prevent colonization in the host and removal of bacteria by the host immune system and other clearance mechanisms, represent a promising strategy for the development of novel antimicrobial agents in contrast to traditional antibiotics.

Regulation of proteolytic activity is an important factor in the application of proteases as therapeutics. Human lysosome proteinase is currently in development for re-modelling scar tissue. A mixture

of trypsin and chymotrypsin obtained from the gastrointestinal tract of north Atlantic cod digests the outer layers of the skin for the treatment of psoriasis, among other dermatological conditions. Serine, aspartic, and cysteine peptidases are found in various viruses and these Viral proteases have gained importance due to their functional involvement in the processing of proteins of viruses that cause certain fatal diseases such as AIDS and cancer (25,26,27,28,29) .

It is evident from the foregoing description/overview that proteases play a significant role in normal and Pathophysiology in all living forms. It is noteworthy that the proteases are highly heterogeneous molecules with broad and highly specific target substrates. The regulations of their activity are a critical component to maintain normal cell Physiology. Proteases therefore could be a target for modulation either by activation or by inhibition to achieve desired effects. The role of Proteases have also been noted as tissue, organ or organisms specific. Accordingly, they find a special place in diagnostics and as a therapeutic substance in treating cardio vascular wound healing, debridement, psoriasis, digestive disorders

inflammation and other diseases. It is therefore necessary that an understanding of the biology and molecular mechanism of proteases would enhance their utility in diagnosis and treatment. Thus this opportunity keeps the isolation, characterization, mechanism of action and inhibitory studies on proteases still an interesting area of research.

Proteases perform profound and diverse functional Physiology and Pathophysiology roles. Therefore it was felt appropriate to study the proteases profile of pathogenic and non-pathogenic microorganism to get an insight into the spectra and specificity of the proteases. This study was performed to know the (enzymes) proteases characteristic to pathogenic and non-pathogenic organisms. The study was then extrapolated to the purification of the enzymes from these organisms, characterizing them to know their substrate specification and inhibition characterization and to extrapolate the outcome to anything unique to the proteases of various substrates. The proteases are gaining attention not only for their versatile nature but for its

potential as a diagnostic, as well as therapeutic targets and possibility of manipulation to achieve desired quality through protein engineering.

Review of Literature

Proteases are Hydrolases (EC 3.4.21-24 and 99) and are degradative enzymes which cleave proteins into peptides and amino acids(30). They represent a class of enzymes which occupy a central position with regard to their biological functions and commercial applications. As they are physiologically necessary for living organisms, they are present in all forms of life such as plants, animals and microorganisms which are the major sources.

Animal proteases- The most familiar proteases of animal origin are trypsin, chymotrypsin and pepsin. Trypsin and chymotrypsin are the major intestinal digestive enzymes which are responsible for the hydrolysis of proteins in food. Pepsin is an acidic protease found in the stomach of most vertebrates(31).

Plant proteases- Some of the well known proteases of plant origin are papain and keratinase. However production of plant proteases depends on selection of suitable climatic conditions for cultivation. Moreover, the concentration of enzymes in plant tissues is low(31).

Microbial proteases- Microorganisms represent an excellent source of proteases. Microbes are an attractive source of proteases and protease genes from bacteria have been cloned and sequenced with the aim of overproduction of the enzyme by means of gene amplification, delineation of an enzyme in cases of pathogenicity and altering enzyme properties to suit a commercial purpose (1). In addition, they have the potential to induce enzyme production depending upon the nutritional status/environment. It is estimated that approximately 3% of the genome of bacteria have genes capable of producing proteases of various functional diversities (32). Microorganisms live in an environment where the nutrients are macromolecular nature and these nutrients have to be cleaved into smaller molecules by extracellular proteolytic enzymes which enables it to use the hydrolytic products. Microbes also produce intracellular proteases which are important for various metabolic processes such as protein turnover and maintenance of cellular protein pool(33). Thus, proteases occupy a pivotal role in microorganisms and Protein engineering techniques have been exploited to obtain proteases which can show unique specificity and

enhanced stability to extreme pH and temperature. In fact, of all the industrial enzymes almost 60% are proteases (1).

Classification of proteases- According to the International Union of Biochemistry and Molecular biology , classification of enzymes depends on the chemical name of the substrate and the type of reaction that it undergoes Proteases are included in sub group 4 of group 3 (Hydrolases). They are classified into groups according to the type of reactions catalysed , chemical nature of the catalytic site and evolutionary relationship. As mentioned in the introduction, they are classified into at least six different catalytic forms: serine proteases, aspartic proteases, cysteine proteases, threonine proteases, glutamate proteases and metalloproteases depending on the amino acid residue present at their catalytic/ active sites and involved in catalysis (1,3).

A brief classification of Proteases with their general mechanism of action is represented in Table -1

Table 1- mechanism of action Classification of proteases with their mechanism of action

Protease	Mechanism
Exopeptidases	Cleaves peptide bond proximal to amino or carboxy terminals
Aminopeptidases	Acts at free N terminal and liberates a single amino acid
Carboxypeptidases	Acts at C terminal and releases single residue
Endopeptidases	Cleaves bonds which are inside the polypeptide chain
Serine Protease	Has serine in the active center
Cysteine Protease	Has cysteine in the active center
Aspartic Protease	Possesses Aspartic acid residue required for its catalytic activity
Metallo Protease	Uses a metal ion for its catalytic mechanism

They are also classified as acidic, alkaline and neutral proteases depending on the pH at which they are active (4).

Acid proteases – They are most active in a pH range of 2.0 to 6.0. Some of the metallo proteases and cysteine proteases are also categorized as acidic proteases. Common examples are aspartic proteases of the Pepsin family (1).

Neutral proteases – They are active at neutral, weakly alkaline or weakly acidic pH. Majority of cysteine proteases, metallo proteases and some other proteases come under this class (34).

Alkaline proteases – They are optimally active in an alkaline pH range of 8.0 to 13.0, though they do maintain some activity in neutral pH as well. In most cases, the active site has a serine residue(1).

Specificity of Proteases-Proteases exhibit a high degree of specifity such as substrate specificity (distinguish between different substrates), region specificity (discriminate between similar parts of molecules) and stereo specificity (distinguish between optical isomers).Of these, substrate specificity characterization provides invaluable information on understanding biological pathways and is also the basis for studying enzyme activity by provision of selective substrates. Many of the Serine proteases can be further categorized

based on their substrate specificity as trypsin-like, chymotrypsin-like or elastase-like. Trypsin-like proteases cleave peptide bonds following a positively charged amino acid (lysine or arginine). The S1 pocket of chymotrypsin-like enzymes is more hydrophobic than in trypsin-like proteases which results in a specificity for medium to large sized hydrophobic residues, such as tyrosine, phenylalanine and tryptophan Elastase-like proteases have a much smaller S1 cleft than either trypsin- or chymotrypsin-like proteases. Consequently, residues such as alanine, glycine and valine are preferably cleaved.

Proteases are also divided into exo and endopeptidases based on their action on the peptide bonds at or away from the termini of the protein molecules. Exopeptidases act only near the ends or extremities of polypeptide chains and they cleave the peptide bonds proximal to the amino or carboxy terminals of the substrate while Endopeptidases act on peptide bonds inside the polypeptides ie. they act on the inner region of peptide chains away from the carboxy and amino terminals(1,35).

Substrates used for estimation of serine proteases -

For studying protease activity, a suitable assay for the protease and a reasonable source of activity is essential. The chosen assay should be quantifiable, experimentally convenient and sensitive.

Casein as substrate :

Casein is used generally as a substrate for proteases in assay systems. When the protease digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin and Ciocalteu's (FC) reagent primarily reacts with free tyrosine to produce a blue colored chromophore, which is quantifiable and measured as an absorbance value on the spectrophotometer. The more tyrosine that is released from casein, the more the chromophores are generated and the stronger the activity of the protease. Absorbance values generated by the activity of the protease are compared to a standard curve, which is generated by reacting known quantities of tyrosine with the FC reagent. From the standard curve the activity of protease samples can be determined

in terms of Units, which is the amount in micromoles of tyrosine equivalents released from casein per minute (36).

Use of synthetic substrates:

While casein is used as a general substrate for proteases, assays of different serine proteases are also performed using synthetic substrates. The use of synthetic substrates to measure protease activity has some advantages over the use of whole proteins as substrates, because each peptide bond in a protein is a potential substrate for different proteases. So comparison of different proteases using whole proteins as substrates is not simple task. Synthetic substrates are designed so that only a specific bond is cleaved and the progress of the hydrolytic reaction can be monitored spectrophotometrically(37).

Trypsin can be assayed with Benzoyl arginine 4-nitrophenylaanine and the substrate is hydrolyzed with a preference for basic amino acids as the acyl donor (38). Elastase cleaves Succinyl Tri Alanyl p-Nitroanilide (STANA) between second and third alanine residue and release of p-nitroaniline is estimated by use of this substrate.

Chymotrypsin catalyzes hydrolyses of peptide bonds at carboxy terminal of aromatic amino acids and synthetic substrates used are Acetyl Tyrosine ethyl ester (ATEE)and benzoyl 1 tyrosyl glycinamide (39).

Mechanism of action of proteases :

Initially proteases were classified into 4 major classes ie. Serine, Aspartic, Metallo and Cysteine proteases and their mechanism of action is well documented. Glutamic acid proteases and Threonine proteases were discovered later as part of a proteosome complex.

Serine proteases (EC.3.4.21) are characterized by the presence of a serine residue in the active site of the enzyme. The catalytic triad consists of serine, histidine and aspartic acid residues. They are widely distributed in prokaryotes and eukaryotes and include exopeptidases, endopeptidases and oligopeptidases. They are generally active at neutral to alkaline pH with the pH optimum between pH 7.0 and 11.0 (1,2). Bacterial serine proteases are divided into two families the chymotrypsin family which shares

sequence homology with mammalian enzymes and the subtilisin family which do not share homology to mammalian enzymes(2). The catalytic mechanism of serine proteases follows a two-step reaction. The nucleophilic attack by the serine hydroxyl group on the carbonyl carbon atom of the substrate is catalyzed by a histidine imidazole group as general base. This leads to the formation of a tetrahedral intermediate and an imidazolium ion. The intermediate breaks down to an acyl enzyme, an imidazole base and an amino product. During acylation the imidazole group transfers proton of serine hydroxyl to the amine leaving group and the acyl enzyme is deacylated(1). The mechanism is as represented in Fig -1

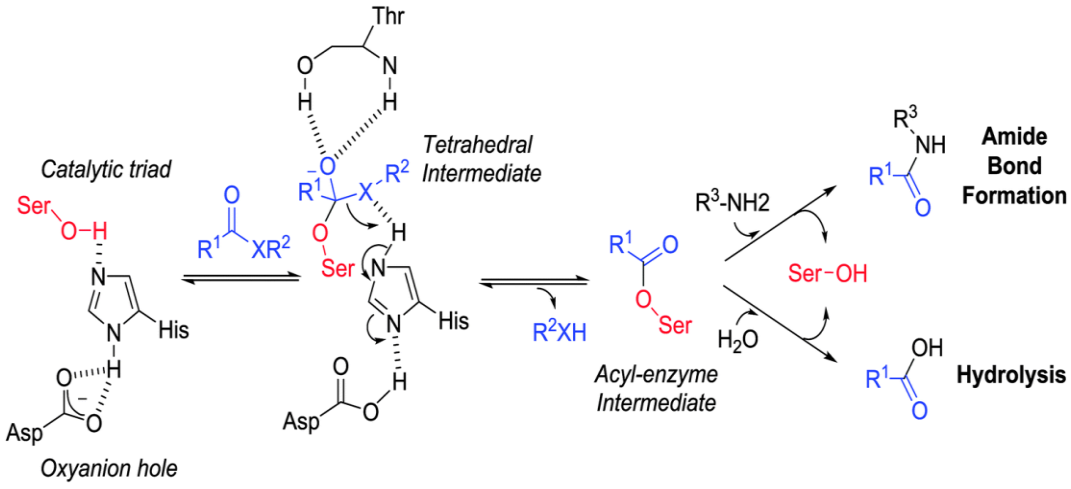


Fig -1 Mechanism of action of serine proteases (From Protein Mutations Database)

Aspartic proteases (EC 3. 4. 23) depend on aspartic acid residues for the catalytic activity and are characterized by their acidic pH optima and are specifically inhibited by pepstatin. Most of them show optimum activity at a low pH of 3.0 to 4.0. The active site aspartic residue is situated within the motif Asp – Xaa – Gly in which Xaa can be serine or threonine (40). Mechanism is as represented in Fig-2

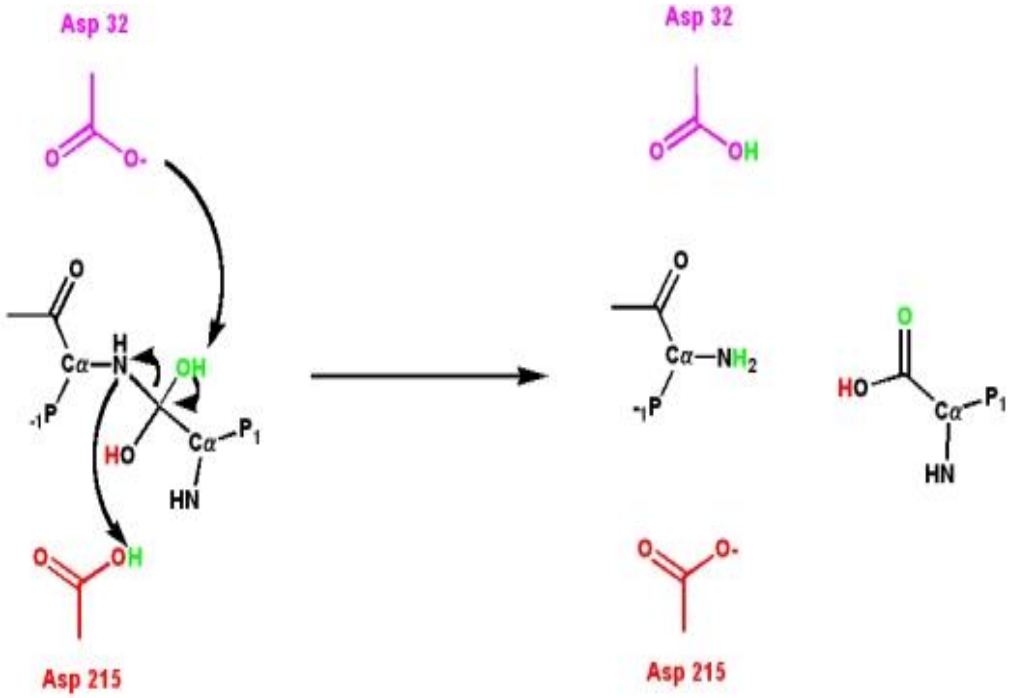


Fig -2 Mechanism of action of aspartic proteases (From Types of Enzyme Mechanism Dr. Blaber)

Cysteine proteases (EC 3. 4. 22) depend upon a catalytic diad consisting of cysteine and histidine and catalyse the hydrolysis of peptide, amide, ester and thiol ester bonds (35,40). Papain is the best known cysteine proteases. These proteases have neutral pH optima although some of them like lysosomal proteases are maximally active at acidic pH (35). Mechanism is as represented in Fig-3

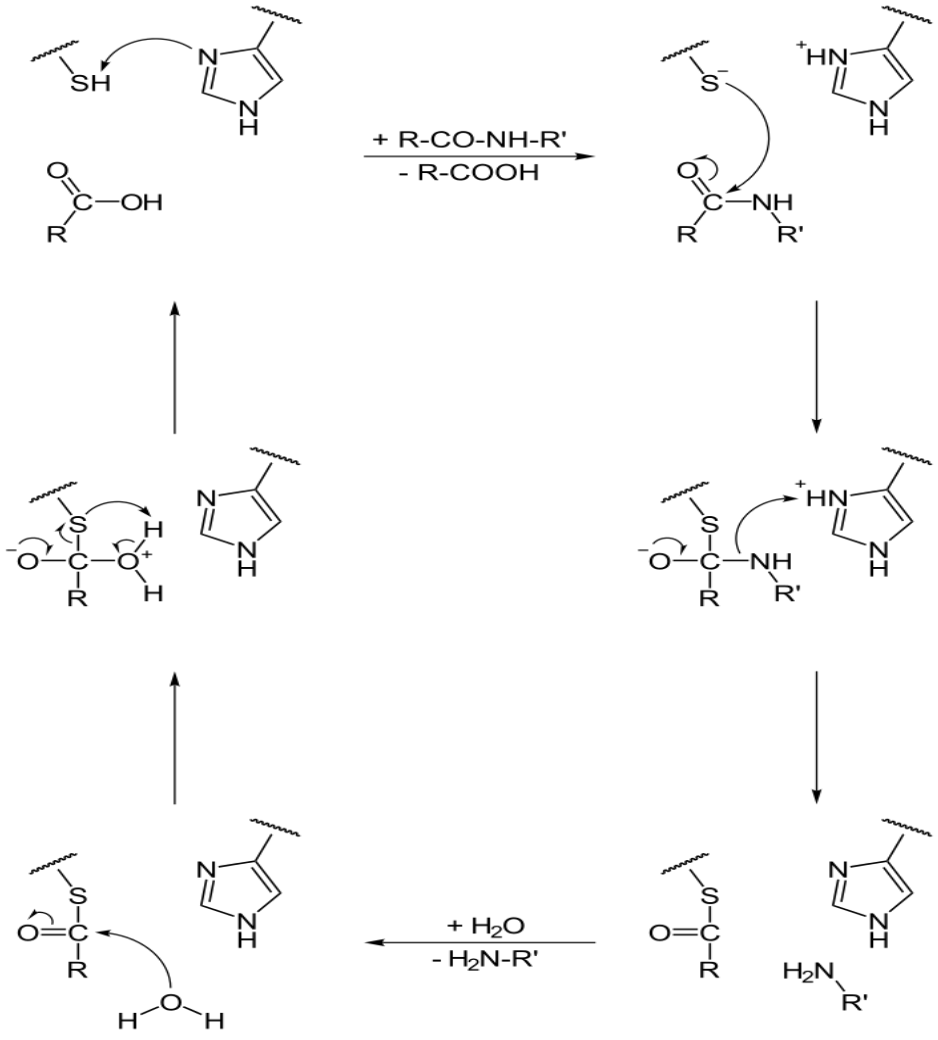


Fig -3 Mechanism of action of cysteine proteases(From Structural Biochemistry of Cysteine Proteases)

Metallo protease (EC 3. 4. 24) depends on the presence of bound divalent cations for catalytic activity. Metallo proteases are divided into neutral proteases which shows specificity towards hydrophobic

amino acids and alkaline proteases which have a broad specificity. Metallo proteases are enzymes containing His – Glu – Xaa – Xaa – His (HEXXH) motif to form a part of the site for binding of the metal. Mechanism is as represented in Fig-4

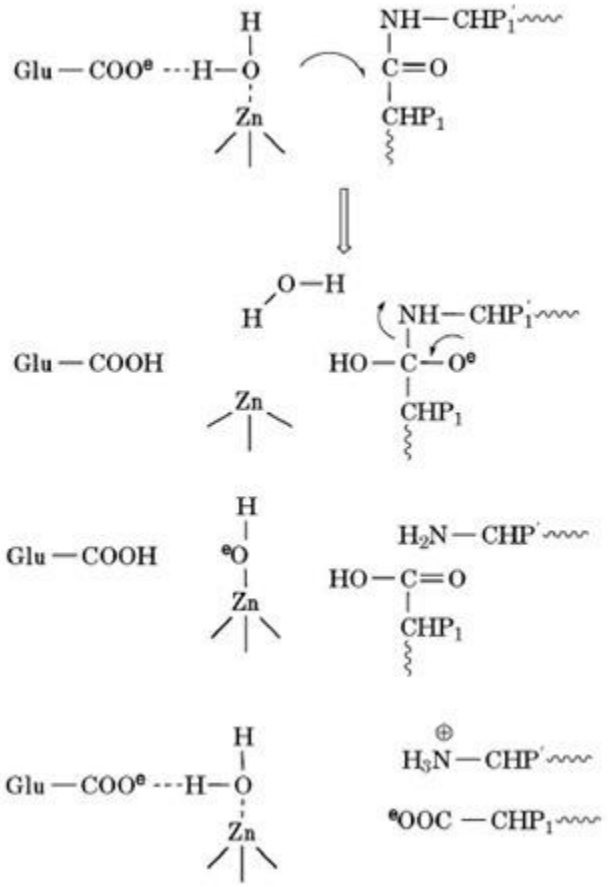


Fig -4 Mechanism of action of metallo proteases (From Molecular Biology-Metallo proteinases)

Glutamic acid proteases (EC 3.4.23.19) and Threonine proteases were discovered in 1995 as part of a proteosome complex. Glutamic acid proteases were discovered in *Aspergillus niger* and *Stylidium lignicola.* The active site diad Glutamic acid and Glutamine play a critical role in substrate binding and catalysis (41).

Threonine proteases (EC 3.4.25) are part of a multi component proteosome complex in microbial cells. The active site nucleophile is the hydroxyl group on the threonine at the N – terminus of the β subunit. Replacement of the terminal threonine by serine allows complete proteolytic activity (41).

Physiological Role of Proteases –

While, microbial proteases play a role in carbon nitrogen cycle and catalyse metabolic reactions within the bacterial cell such as cellular respiration proteases are also involved in many aspects of human biology. In the small intestine, proteases digest dietary proteins to allow absorption of amino acids. Other important processes mediated by proteases in the human system include blood coagulation, immune function, maturation of proteolytic hormones, bone

formation, programmed cell death and the recycling of cellular proteins that are no longer needed. Besides, they also digest the cell walls of unwanted harmful organisms in the body and break down unwanted wastes such as toxins, cellular debris, and undigested proteins. Even in the nervous system, proteases such as thrombin, trypsin and tryptase can bring about depolarization in myenteric neurons so that there is increased excitability (42,43). Proteases are produced during tissue damage and are involved in tissue responses to injury, such as haemostasis, repair, cell survival, inflammation and pain(44).

Pathological Role of Proteases -

The success of a microbe during pathogenesis depends on its ability to respond to the environment in the host cell. This requires the use of a repertoire of genetic function's on the part of microorganisms which are independently regulated in response to environmental signals encountered inside the infected host. This regulation is very important during the natural course of infection

(45). Many pathogens cause disease in a single or limited number of host species as a consequence of a long coevolutionary history. Although, many bacterial virulence components are thought to be host-specific, several studies have demonstrated the existence of a virulence mechanisms used by diverse bacterial species (46). The ability of these bacteria to survive different environments and establish an infection is due to its ability to synthesize exoproducts such as lipase, phospholipase, alkaline phosphatase, exotoxin and proteases (47). There have been extensive studies on virulence factors in microorganisms which cause a variety of disease and the role and presence of these proteases which help in establishing and maintaining an infection have been well documented (26). So, besides their physiological role, a number of disease states are also attributed to microbial proteases as, they are involved in the cycle of disease causing organisms.

Many of these bacterial proteases are not only resistant to human plasma protease inhibitors but also inactivate them. These extracellular proteases, can also activate the host cascade system and

accelerate disease process (48). Microbial proteases activate kinin generating cascade system such as that of Bradykinin leading to pain seen in periodontal diseases (49). Bradykinin generated as a result of this cascade system is responsible for the pain, edema and inflammation seen during an infection. Microbial proteases activate the blood coagulation cascade system and the triggering of this cascade results in Disseminated Intravascular Coagulation (DIC) one of the serious consequences of an infectious disease(50). There are several bacterial proteases which interact with their hosts during a pathogenic infection. The highly lethal Anthrax Toxin of *Bacillus anthracis* consists of 3 different proteins of which one is called the lethal factor, which is a metalloprotease and cleaves and inactivates Mitogen Activated Protein (MAP) kinase (51). Botulinum neurotoxin is one of nature's most lethal toxins obtained from *Clostridium botulinium*. It is also a metallo protease which is able to block acetylcholine release at peripheral nerve endings (52). Other toxins such as diphtheria toxin and cholera toxin require proteolytic activation by either bacterial or host proteases for exertion of toxin activity (53). Proteases of Streptococci and Nessiria also cleave Ig A

so the complement system can be inactivated by microbial proteases. (54,55). There are several proteases that play a role in pathogenic interaction between bacterium and host as seen in *Pseudomonas aeruginosa* species which include Elastase B, Elastase A, Protease 1V and Alkaline protease. (3).

Elastase is the proto type virulence factors of *P. aeruginosa* and has a dominant role. It is an endo peptidase which was characterized by Morihara in 1963(56). This elastase release is regulated by quorum sensing cascade which is a kind of bacterial cell to cell communication via small molecular chemical signals (57). Elastase B can degrade human elastin as well as collagen Type III and IV found in extracellular matrix and basement membranes (58). Pathological studies showed a loss of endothelial integrity and these pathological effects are said to be due to fibrinogenolytic and fibrinolytic activity of elastase B which causes haemorrhage and muscle damage. (59). Elastase B can also interact with proteins of human immune defence system and degrade Ig A and Ig G (13, 60). Elastase is also able to inactivate key components of the complement system (43) and inactive Proteinase activated receptor -

2 (PAR 2) which is a transmembranic receptor which can be activated after cleavage by a variety of proteases (61). Activation of PAR 2 leads to the triggering of prostanoids, cytokines and metalloproteases (43) which is disarmed by Elastase B by cleaving the exodomain and this leads to lower secretion of interleukin- 8 and prostaglandin $E_{2.}$ Elastase B also cleaves Urokinase - type Plasminogen Activation Receptor (UPAR). UPAR has high affinity for proteins involved in cell adherence and migration. Elastase B inactivates the binding of UPAR to substrate by cleavage (62). This cleavage of different substrates show that elastase has a broad substrate specificity.

Elastase A (Las A) also known as staphyolysin is a zinc metalloprotease. It has low elastolytic and high staphylolytic activity (63). Besides its intrinsic elastolytic activity, Las A increases the virulence of Las B by increasing the elastolytic activity of Las B and other proteases (64). The staphylolytic activity caused lysis of *S. aureus* cells and probably is a defense strategy to out compete *S.aureus* during colonization (63,65).

Protease IV of *P. aeruginosa* a can degrade a whole range of proteins such as fibrinogen, plasminogen and immunoglobulin G (54) though it mainly contributes to the virulence of corneal infection. Alkaline protease also known as aeruginolysin like Elastase B, inhibits the function of neutrophils by interfering with chemotaxis, thus helping the bacterium escape from phagocytes of the host defense systems (66). Furthermore, elastase and alkaline protease can interfere with human lymphocyte function due to degradation of 1L-2 (67). It has also been demonstrated that the strain having high proteolytic activity is significantly more invasive than strains that produce little or no protease activity (68) .

Microbial infections proceed rapidly and devastatingly due to the involvement of protease activity. As mentioned above, the inactivation of immunoglobulins, protease inhibitors and other defense proteins of the host by bacterial proteases are critically important for pathogenicity of bacteria. The vast range of actions of bacterial proteases in pathogenesis is highly complex and should receive more attention in treatment of infectious diseases.

Industrial and Commercial role of microbial proteases - The industrial and commercial applications of microbial proteases are huge. Proteases are widely used in food industry and their use dates back in time. They are used for cheese making to hydrolyze the peptide bonds to generate para casein, to modify wheat gluten in the baking industry thereby improving the extensibility and strength of the dough, and is also used to recover proteins from parts of animals which could otherwise go waste after butchering (69,70,71). They are also used for dehairing in the leather industry (72). Most of the studies on proteases were directed on those which could become attractive industrial tools especially in the detergent industry which accounts for nearly two thirds of the enzyme market (6). Recent advances have enabled surgical instruments to be cleaned by enzyme based formulations to remove microbes that are trapped on or within surgical instruments and also as denture and contact lens enzyme cleaners.(71, 73). Besides, these commercial uses, microbial proteases are also used for diagnostic and therapeutic purposes. The involvement of proteases in the life cycle of disease causing organisms has led to become a potential target for developing

therapeutic agents against fatal diseases such as cancer and AIDS (1). Virus such as HIV depends on proteases for their replication. Protease inhibitors are being developed to be used as antivirals as they prevent viral replication by selectively binding to viral proteases and blocking proteolytic cleavage of protein precursors that are necessary for the production of infectious viral particles. Proteolytic enzymes are used in the treatment of cancer by boosting cytokines particularly interferon and tumor necrosis factor and decreasing inflammation. Proteases are also known to dissolve fibrin which Cancer cells use to stick together which increase the chances of metastasis(1,74). Microbial proteases are increasingly used in the treatment of various disorders like inflammation, cardiovascular disorders and necrotic wounds (75,76,77). They are also used as immune stimulatory agents (78). It has also been found that there is increased antibiotic concentration at a target site when a protease is used concomitantly with an antibiotic (79). Proteases are used widely in ointments for debridement of wounds (1). They are effective therapeutic agents. Oral administration of proteases from *Aspergillus oryzae* is used as a digestive aid, while Clostridial

collagenase or subtilisin is used for treatment of burns and wounds (71,80,81). Subtilisin is used in combination with broad spectrum antibiotics in treatment while asparaginase isolated from *E. coli* is used in the treatment of lymphocytic leukemia (82). Besides,a variety of natural proteases are used in treatment such as in thrombolysis (urokinase), haemophilia (factor VII a), sepsis (activated protein C), muscle spasms (botulininium toxin A and B) (83, 84).

Besides these, industrial and medical applications they are also used in molecular biology practices. They are used for tissue dissociation, cell isolation, cell culturing, the removal of affinity tags from recombinant proteins and specific protein digestion in the field of proteomics mainly for protein sequencing (85). Recent trends have involved isolating proteases from extremophilic organisms, so as to mimic these properties in commercial applications (1,86).

Relevance of protease purification and characterization -

There are number of important reasons of purifying proteases as by the process of purification, its biological activity its affinity for particular substrates and its specificity can be known. This latter property can be used, to determine protease activity in a clinical specimen with the use of a particular substrate and this offers both diagnostic and therapeutic options. Determination of increased proteolytic enzyme activity in pancreatic diseases have been determined with the use of BAPNA as a substrate (87). So, determination of protease activity in clinical specimens can offer diagnostic and therapeutic options. Proteomic screens for protease/substrate are experiencing an impressive progress (74). With the ability to isolate and purify the enzymes, it is also possible to identify mutant forms. The advent of site directed mutagenesis, makes it possible to purify a protease with essentially any mutation. By the use of genetic engineering, novel proteases can be produced to serve a specific function. It is even possible to modify proteases to have desirable characteristics like heat stability. Once isolated and

purified, suitable antibodies/reagents can be designed to test for their function in vivo.

Media for Proteases – There is no defined medium established for best production of proteases from different microbial sources. Each organism or strain has its own special conditions and the extracellular enzyme production varies with different strains (31). Production of an enzyme exhibits a characteristic relationship with regard to the growth phase of an organism. To achieve suitable environmental conditions for maximum release of proteases, there are several major factors such as selection of a suitable microorganism, suitable substrate, temperature, pH, proper agitation and humidity control (88). Nutrient media must provide all the elements that most bacteria need for growth. The most common growth media for microorganisms are *nutrient broths* (liquid nutrient medium). A broth is a medium that contains a carbon source such as glucose for bacterial growth, water, various salts needed for bacterial growth and a source of amino acids and nitrogen (e.g., beef, yeast extract). Liquid media are often mixed with agar and poured

via sterile media dispenser into Petri dishes to solidify. These agar plates provide a solid medium on which microbes may be cultured. They remain solid, as very few bacteria are able to decompose agar. Broth is convenient, as most bacterial samples can be easily introduced into and grown in this type of medium, even those with widely different aero tolerance (oxygen requirements) (89).

Methodology of purification of Proteases –

When a protease is purified the starting material is fractionated using one of a large number of physical and biochemical approaches. In a well designed purification procedure, each step should result in removal of contamination material and a progressive increase in specific activity.

After separation of cells from the culture broth, the supernatant is usually concentrated and the enzyme precipitated by salting out with the use of solid ammonium sulfate crystals or solvent precipitation by use of acetone. They are many reports of using different concentrations of ammonium sulfate precipitation ranging from 20% to 90% for precipitation of microbial proteases (90, 91).

Further purification are carried out by chromatography techniques such as ion exchange chromatography, affinity chromatography and gel filtration chromatography. Alkaline proteases are usually positively charged and therefore cation exchanges are used. The different resins used for ion exchange chromatography contains functional groups such as Di Ethyl Amino Ethyl. DEAE cellulose chromatography has been performed for purification of alkaline proteases belonging to the *Bacillus* family and Pseudomonas species (92, 93). Gel filtration chromatography technique has been used for purification of alkaline proteases of the *Bacillus* family (94). Sephadex G – 100 and Sephadex G – 75 have been used for purification of the proteases of Pseudomonas species (95,96). Elastase from microorganisms has been purified from culture broth by use of ammonium sulfate precipitation, ion exchange chromatography, fast performance liquid chromatography and gel filtration methods (97). Quantification methods include quantification of clearance zones on elastin plates, quantification of dye labeled elastin hydrolysis and ELISA methods for direct

evaluation of elastase from culture supernatents and the use of synthetic substrates(98,99).

Properties of microbial proteases –

pH and temperature kinetics – The optimum pH of most proteases ranges from pH 8.0 to 11.0 (30,100). A few exceptional cases of pH stability have also been reported (101,102). Optimal temperature ranges from 40°C to 60°C (30, 92). Extremely thermostable proteases are stable upto 80°C (103).

Molecular mass of proteases – Serine proteases to which a large number of bacterial proteases belong to, have a wide range of molecular mass though they are usually in the range of 18 to 35 kDa (104). Some of the subclasses of serine proteases such as chymotrypsin like proteases which shows structural homology to trypsin and elastase have a molecular mass of around 20 kDa (41). Subtilisin like proteases which are more specific for aromatic, hydrophobic residues have a molecular weight range of 15 to 30 kDa (1). Molecular mass of *Bacillus* species varies from 25 to 40 kDa (31). *Stapphylococcal* proteases have a molecular mass of

around 12 kDa and are specific for peptide bonds with acidic amino acids (56). Molecular mass of *P. aeruginosa* ranges from 20 to 50 kDa. With a exception of Las A, all proteases are found as preproenzymes with a length of more than 400 amino acids and with a molecular mass of 20 to 50 kDa when they are in the form of mature extracellular protease (105).

Elastase – Structure and Characteristics :

Very few enzymes are known to degrade elastin and proteases that can use elastin as a substrate are of either bacterial or mammalian origin. Elastase of *P. aeruginosa* is of bacterial origin while pancreatic elastase and elastase from granules of polymorphonuclear leucocytes are of mammalian origin (56,106). The ability of elastase to degrade elastin which gives elasticity to tissues, collagen and several complement factors emphasizes its potential importance though the mechanism as to how elastase

degrades and inactivates such a wide variety of substrates is not known.(107).

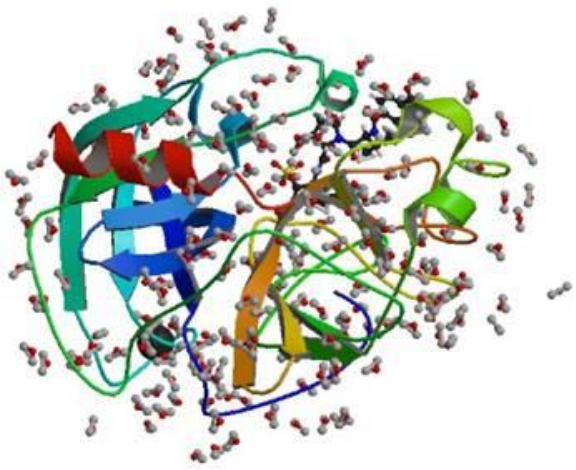


Fig 5-High resolution neutron and x-ray analysis of Elastase

Elastase has an active site that pulls in the substrate to create an enzyme-substrate complex, which then leads to a product. As it is a serine protease it has a structure that is designed to cleave proteins (specifically elastin). The structure of elastase is quite similar to some other serine proteases, such as chymotrypsin, with which it has about 40% identical sequence, and a very similar overall structure.

These two proteins are obviously homologs, and are divergent in evolution. As a serine protease, elastase has an active site with three main amino acids: aspartate, histidine, and serine, which make up the catalytic triad that work together to create a nucleophilic catalysis. Elastase has bulky valine residues that aid in closing off the pocket of the cleaving site (108). The catalytic triad of nucleophilic elastase is shown in Figure -6 which illustrates the molecular model showing a substrate (yellow) bound to the active site of human neutrophil elastase (magenta). A substrate histidine (red) at the P2 but not P1 position can be virtually superimposed upon the catalytic histidine, H57 (green), and mimic the interactions of H57 with the other members of the catalytic triad, S195 and D102 (green) (109).

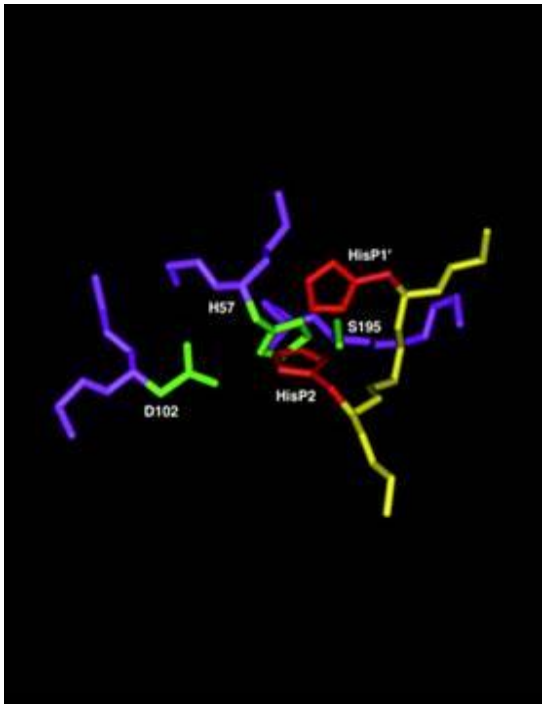


Figure 6: Catalytic triad of Elastase

The catalytic triad is formed by three hydrogen-bonded amino acid residues (H71, D119, and S214). The polypeptide chain is composed of two antiparallel beta-barrel domains, which form a crevice containing the catalytic triad, and a small proportion of alpha-helices(110). During the reaction in which elastase breaks down the substrate (elastin), two phases occur: a burst phase during which the amino side of the peptide bond is released, and a steady-state phase, in which the acyl side of the substrate is released. The elastin is

positioned on the enzyme elastase so that the catalytic triad has access to the peptide bond. After this occurs, serine nucleophilically attacks the carbonyl of the peptide bond. A tetrahedral intermediate is formed and decomposed. Water enters the active site and attacks the reaction, causing the nitrogen terminus to leave. Water then attacks the acyl-enzyme intermediate and causes the release of the carboxylic acid component (*1*). Elastase is thus successful in breaking apart elastin, aiding in the digestion of this protein. Due to its activity on elastin, elastase is used commercially for tissue dissociation to dissociate tissues that contain extensive intercellular fiber networks. For this purpose, it is usually used with other enzymes such as collagenase, trypsin, and chymotrypsin. It is also used for Membrane protein solubilization and Protein sequence studies.

Regulation of proteases

Proteases are involved in many aspects of human biology and their roles are multi fold. However, Proteases may be potentially damaging when over expressed or when present in high concentrations so they are tightly regulated. Many enzymes especially those of the digestive tract and those involved in blood clotting are present as zymogens. For instance, pepsinogen is the inactive precursor (zymogen) and pepsin is the activated form of the enzyme. The purpose of this regulation is to prevent the Pepsin from digesting proteins in the body before it is introduced into the digestive tract. Another example is seen in blood clotting which is carried out by a cascade of proteolytic activations to achieve a rapid response. For blood clotting, the response time must be fast in order to achieve clotting at the right spot and time to prevent excessive bleeding. A cascade of zymogen activations activates a clotting factor, which is then responsible for activating another clotting factor and so forth until the final clot is achieved. When trauma exposes tissue factor, thrombin, also a serine protease and a key enzyme in

clotting, is synthesized. More thrombin is produced by positive feedback which then activates enzymes and factors such as fibrinogen and forms fibrin, the key part in blood clotting (111).

There are certain protein hormones which are synthesized as inactive precursors such as proinsulin which is then converted to the activated form insulin by proteolytic cleavage of a specific peptide. Collagen, a fibrous protein and the major component of skin and bone and which makes up the majority of the components of connective tissues in animals is made from the zymogen procollagen. Programmed cell death is also mediated by proteolytic enzymes(111).

Once activated proteolytic enzymes are irreversible so there are specific inhibitors present such as pancreatic trypsin inhibitor. This inhibits trypsin by binding firmly to the active site of the enzyme. There is formation of hydrogen bonds between the inhibitor and the active site resulting in firm binding on the active site. Trypsin inhibitors are essential as they prevent severe damage such as inflammation in the pancreas ie. pancreatitis. Because trypsin

engages in activation of several zymogens, inhibiting trypsin can prevent unwanted or premature cascades such as inflammation. Elastase, can destroys alveolar walls in the lungs by breaking down elastic fibers and other connective tissue proteins in the lungs leading to emphysema. Neutrophils also produce large quantities of elastase at sites of injury. This Neutrophil elastase is a 29 kDa serine protease expressed by neutrophils from the gene ELANE located on chromosome 19 (112). It also contributes to tissue damage and can digest elastin, type III, type IV Collagen and proteoglycan (113,114). So, excess proteases must be inhibited by anti proteases /protease inhibitors which maintain a homeostatic balance with proteases thus preventing inflammatory damage from excess protease activity. This balance is maintained by serine protease inhibitors (serpins) of which the principal ones are α1 Antitrypsin, α_2 Macroglobulin and the Whey acid protein family.

α1 Antitrypsin is a serine protease inhibitor active against trypsin, plasmin, elastase and cathepsin G. It is a 52 kDa mature peptide (10). Oxidation of its methionine 358 residue at its active site

makes it inactive. Upon binding to a target protease it acylates the protease and there is cleavage of P –P_1 bond. In the serpin – protease complex the active site of the protease gets distorted. The inhibition process is due to the suppression of the deacylation process rather than protease translocation. α_1 AT has a reactive center loop that function as a bait for a target protease. Following the formation of a complex and cleavage of the Met 358 – Ser 359 bond, α_1 AT undergoes extensive conformational rearrangement that traps the protease before completion of its catalytic cycle and the inhibition of the trapped protease is virtually irreversible (115).

α_2 Macroglobulin is able to inactivate an enormous variety of proteases. It inactivates serine, cysteine, aspartic and metallo proteases. Proteases bind and cleave the bait region that is a segment particularly susceptible to proteolytic cleavage which initiates a conformational change so that the α_2 MG collapses about the protease and forms an α_2 MG – protease complex. This complex is recognized by macrophage receptors and cleared from the system. α_2 MG also functions as an inhibitor of fibrinolysis by inhibiting

plasmin and as an inhibitor of coagulation by inhibiting thrombin. It also acts as carrier protein as it binds to numerous growth factors and cytokines (116).

The Whey acid protein family are multi functional host defense proteins. The majority of which are transcribed from genes on chromosome 20. SLPI and elafin are the best characterized members. SLPI is a serine protease inhibitor and is produced by cells such as neutrophils, macrophages and serous cells of bronchial submucosal glands (117). They are expressed in response to stimuli such as bacterial lipopolysaccharides, HNE and cytokines (118). Elafin has a mass of 6 kDa and possesses antiprotease activity against HNE, trypsin and chymotrypsin. (119). Serpins are thought to be suicide inhibitors, though there have been examples of serpins becoming substrates for serine proteases. For example C1 inhibitor is a good inhibition of α kallikrien at 38^0 C but becomes a substrate for it at 4^0C (120).

Protease inhibitors were unknown in the bacterial kingdom until recently. Just like protease inhibitors in humans, microorganisms are also now known to have protease inhibitors in them and these have been shown to be highly active against neutrophil elastase (121). These microbial elastase inhibitors might be a factor affecting the degree of tissue injury associated with pyogenic infections. One of the obvious reasons, for the presence of these proteases inhibitors is that they help the bacteria to protect themselves against neutrophil elastase and the elastase protease secreted by bacteria. The coexistence of protease inhibitors in microorganisms suggests that it is a defense against host resistance mechanisms and enables the organism to counter the defense mechanism of the host. (122,123).

Relevance of protease inhibition studies and its potential application -

Protease inhibitors of microorganisms are the potential target for development of therapeutic agents such as the protease from *Aspergillus oryzae* which is used as a digestive aid while Clostridial

collagenase or subtilisin is used for treatment of burns and wounds as mentioned earlier. (1, 80, 81) Protease inhibitors of *E. Coli* is said to have inhibitory activity on pancreatic proteases (27,28). Protease receptors are also a potential target for HIV treatment. HIV - 1 Protease was found to be inhibited by a peptidic inhibitor invitro (21). Pepstatin was isolated from various species of Streptomyces and is an inhibitor of pepsin (124). Pepstatin is reported to be effective against muscle dystrophy and inhibits ascites accumulation in carcinoma (24). There is evidence suggestive that an inhibitor isolated from. *Aspergillus flavus* inhibits leukocyte elastase which is helpful in controlling inflammatory responses caused by leukocyte elastase (125). This novel elastase inhibitor from *Aspergillus flavus* (AFLEI) was isolated and was found to inhibit elastase of *A. flavus, A. fumigatus*, pancreatic elastase, trypsin and chymotrypsin(126). This AFLEI was isolated purified and it was found to be useful in treatment of aspergillosis (126) . This showed that an elastase inhibitor isolated from the microorganism itself is a potent inhibitor against aspergillosis. Alkalo Thermophilic Bacillus Inhibitor (ATBI) is a aspartic protease inhibitor isolated from an alkalothermophilic

Bacillus sp and is involved in crop protection against plant pathogens(22, 127). Proteases are known to be involved in tumor progression, and tumor specific proteases are released into the blood during metastasis. So, development of protease inhibitors to stop this protease are in progress. Synthetic protease inhibitors are being developed to treat anemia, inflammatory artherosclerosis and other chronic diseases (128,129).

Anti proteases (serpins) besides their role in inhibition of human sera proteases, also inhibit bacterial extra cellular proteases. Human Neutrophil Elastase (HNE) is stored principally in neutrophils and are released when neutrophils encounter foreign substances. Neutrophil elastase (NE) contributes to killing of Gram negative bacteria and can play a role in bacterial killing by comprising a key component of neutrophil extracellular traps (NET) which are involved in host defense (16). They bind Gram positive and Gram negative bacteria and allow neutrophils to deliver high concentrations of proteases which then degrade virulence factors and kill bacteria. Neutrophil elastase cleaves microbial peptides liberated

during phagocytosis. HNE is found associated with DNA structures secreted from activated neutrophils called Neutrophil Extracellular Traps (NET). They are produced as a result of reactive oxygen species. Proteinase 3 (PR 3) is a 29 kDa protease expressed from PRTN3 gene by activated neutrophils (130). Its biological role is similar to HNE.

Elastase which is released during the invasion of microorganisms can be inhibited by anti-proteolytic substances produced by the microorganism, which can facilitate the invasive process. Serpins counter effect of serum proteases as well as proteases of microorganisms. As mentioned earlier, the neutrophil elastase is inhibited by α_1 protease inhibitor, elafin inhibitor, and secretory leukocyte inhibitor (131). Interestingly, *Pseudomonas aeruginosa* elastase does not inactivate alpha I proteinase inhibitor in the presence of leukocyte elastase (132). This rationalizes the fact that when α_1 proteinase inhibitor (α_1 PI, also known as α_1 anti trypsin) is faced with its target enzyme ie leukocyte elastase, it will perform its physiologic antielastase function even if bacterial

elastase is present in excess (132). *P. aeruginosa* produces a substance that by inactivating α_1 PI, can enhance tissue damage by leukocyte elastase and other indigenous serine proteases released during Pseudomonas induced diseases (133). Pseudomonal elastase is a potent inactivator of α1 PI if the inhibitor is exposed to elastase before the inhibitor complexes with trypsin. This shows that bacterial elastase can disrupt the balance between serine protease and α1 PI (134). Another bacterial protease, *Streptomyces erythraeus* trypsin (SET) a serine protease secreted extracellularly is not inhibited by α1 PI. SET not only is resistant to α1 PI but also inactivates it (135). The mechanism of how SET escapes inhibition however is still not discovered. Alkaline protease and Elastase B of *P. aeruginosa* inhibits the function of neutrophils by interfering with chemotaxis, thus helping the bacterium escape from phagocytes of the host defense systems (66). More recently bacterial virulence that counteract NET'S have been identified; they are capable of degrading the NET backbone, reduce bacterial trapping by capsule expression and modulating the charge on the cell surface (17,18) .

However, α2 Macroglobulin can inactivate Pseudomonal proteases (136).

In addition to the antiprotease and anti-inflammatory functions both SLPI and elafin have antimicrobial activity against Gram negative and Gram positive organisms such as *P. aeruginosa* and *Staphylococcus aureus*.(137). Elafin also reduces cytokine expression (138). Just like SLPI, elafin is cleaved by excess HNE in patients with *P. aeruginosa* infections (138). Besides protease inhibition SLPI inhibits inflammatory responses by binding to bacterial lipopolysaccharides. SLPI is cleaved by excessive levels of HNE seen in *P. aeruginosa* infections. (120) So, successful establishment of an infection depends on the ability of the bacteria to overcome the host defence or the efficiency of the host to destroy the invasive microorganism.

Plants have developed mechanisms to fight pathogenic organisms. One important line of defense is through various inhibitors which can act against proteolytic enzymes of pathogens. These inhibitors are thus active in endogenous as well as exogenous

defense systems. These protease inhibitors (PIs) are particularly effective against insects and microorganisms. Plants extracts are also reported to have analgesic, antimicrobial, antinflammatory, antitumor, antimotility and antioxidant activities as well as antidiabetic activities. There have been various attempts to isolate compounds which are present in plants. In a detailed study by Ayyanar et al (139) of the Entomology Research Institute, results of wound healing treatments among tribal people of southern India, documented the therapeutic use of plant products against wounds, cuts, burns and bruises. Of these plant products, leaves were the most frequently utilized plant part. They were ground to a paste and applied externally. Infection of wounds takes place due to poor hygienic conditions (140) . The process of wound healing is promoted by a number of active principles present in plant products such as triterpenes, alkaloids, flavonoids and biomolecules (141). Essential oils of plants are also very effective in treating small or medium wounds, skin abrasions and skin infection (142). Many of these traditional remedies are based on systematic observations which has been time tested but however scientific evidence is

lacking and only a few randomized controlled trials have proved the clinical efficacy of these traditional wound healing plants (143). The major problem with pharmacological validation of these wound healing plants is that the exact mechanism is not clearly understood. Most researchers restrict the screening of plants to simple healing of wounds and show the antimicrobial effect by Agar well diffusion methods, but do not go into details. These naturally occurring inhibitors in plants probably can inhibit HNE activity and invading microorganisms. However, the inhibitory mechanisms, and the inhibitory kinetics is not clear in literatures review. The PI's are less toxic when compared to synthetic HNE inhibitors, but whether these substances used for wound healing inhibit both the bacterial elastase and the defensive HNE released is not clear. Several non toxic protease inhibitors from barley seeds, cabbages etc. have been purified and now are available for preventing peri anal dermatitis (144). A trypsin protease inhibitor has been isolated from potato tuber and has strong antimicrobial activity (144). So protease inhibitors if isolated from leaves of plants used for wound healing will have the potential for development of a new antimicrobial drug.

Recently there have been several incidences of microbial drug resistance around the microbial drug resistance around the world (145). These drug resistant microorganisms are due to the overuse of antibiotics in medicine, as well as the dairy, poultry and agriculture industries. So, there is a continuous need to develop newer drugs.

So, a comprehensive evaluation on the plants with wound healing activity, an identification of the active compounds and the mechanism of their action would help in using these compounds as therapeutic agents and lead to their more widespread use for treatment of burns and wound.

One of the plants widely reported for antimicrobial activity is the *Psidium guajava* (146). The strong bactericidal activity exhibited by the leaf extract of *Psidium gujava* is possibly due to protein degrading activities of the extracts. Tannins present both in aqueous and ethanolic extracts have protein binding activities and can interfere with many substances (147).

The leaves of the guava plant (*Psidium guajava*) is widely used for wound healing. During wound infection, bacteria at the wound site release proteolytic enzymes to improve their invasive powers. The bacteria present also stimulate protease production via activation of the immune system. Elastase of *P. aeruginosa* has also been shown to degrade proteins in human wound fluids and is commonly found in chronic ulcer infections (148) .In a defensive mechanism, HNE is secreted against the invasive microorganisms and is the first line of defence as they kill bacteria. (149) . The leaves of *Psidium guajava* collected in Korea is said to have pancreatic elastase inhibition property (144). There however has been no attempt to categorize the mechanism of the inhibitory effect of the leaves or their effect on bacterial proteases or HNE.

It is evident from the above review of literature, that there exists a high functional relationship between the extracellular proteolytic enzymes of pathogenic microorganisms and development of diseases. To combat the effect of the proteases on the host cells, the host produces anti protease substances which have been

characterized as peptides and proteins. These have been shown to be active in endogenous and exogenous systems as a defense against proteolytic damage. These antiprotease not only have an inhibitory activity against excessive amount of proteases but also against invasive microorganisms such as bacteria and fungi. From the review of literature it can also be seen that there is a lacunae in the role of elastase in non-pathogenic organisms and the ability of other microorganisms to produce elastase or elastase like enzymes and the role of sera anti proteases to other bacterial proteases . There is also a paucity of details on the protease – inhibitor interactions and their mechanisms. There has not been any study on whether there is a preferential inhibition by these plant extracts on bacterial elastase or HNE. Many of the studies on anti microbial activity of plant extracts have been done by well diffusion methods and disc diffusion methods. Inhibitory action against bacteria by these plant extracts are shown by development of a zone of inhibition. Taking all the above facts into consideration, this study was designed to achieve the following objectives.

Screening of both pathogenic and non pathogenic strains of bacteria for production of proteases and their substrate specificities.

Isolation and purification of the elastase enzyme from one each, of pathogenic and non-pathogenic bacteria, showing highest proteolytic activity.

Study the kinetic and physiochemical properties of these proteases, the inhibitory effect of human anti proteases α_1 anti trypsin and α_2 macro globulin on pathogenic bacterial elastase and compare it with the inhibitory effect on pure human neutrophil elastase.

Study the inhibitory effect of a plant extract on bacterial elastase and human neutrophil elastase.

Even though proteases of microbial origin have been studied extensively, there is no comparative study available in respect of the ability of non-pathogenic and pathogenic groups to produce proteases under given set of conditions and their comparative analysis with regard to the spectra and specificities. This study would outline the potential of an organism in producing proteases

which are detrimental of beneficial to mankind or could be of commercial nature.

Further discovery of novel select protease inhibitors can proceed through combination screening. The rapid emergence of microbial pathogens that are resistant to currently available antibiotics has triggered considerable interest in isolation and investigation of protease inhibitors. The exploitation of the vast microbial diversity will also help in identifying more of these inhibitors which would have a high degree of success in being of clinical use in the fields of medicine as well as in agriculture and biotechnology. The importance of developing potent protease inhibitors with a broad antiprotease spectrum should be realized.

CHAPTER III

SCREENING

Introduction -

The Review of Literature shows that proteases play a pivotal role in normal and abnormal physiology. However, many of the studies on bacterial proteases, have been discrete as most studies have focused on a single strain which was then isolated, purified and characterized. Some studies isolated and compared different strains of the same microorganism. In screening studies of non pathogenic bacteria, focus was on maximum production of proteases particularly by different members of the *Bacillus* family as the sole focus of these studies was on protease production for industrial purposes. Though, there have been reports of elastase production by *Bacillus* species, there does not appear to have been any attempt to characterize the enzyme or study its role, whether it could be a potential pathogen under favorable conditions. Considerable efforts have been made to screen elastase producing pathogenic strains but, there does not appear to be a systematic screening of pathogenic and non pathogenic bacteria for protease and elastase production as was done in the current study. Since ,the objective of this study was screening

for proteases in pathogens and non pathogens especially elastase, all strains were cultured in the same nutrient broth i.e. a fixed media was used as adding various nutrients to stimulate protease production was not the purpose of the study.

With the objective of selecting strains for protease/elastase production, a systematic screening of bacteria belonging to pathogenic and non pathogenic strains were done. The micro-organisms were obtained in a lyophilized form from Microbial Type Culture Collection and Gene Bank, Chandigarh, India in order to ascertain the purity of the strains and to maintain consistency in the experimental system.

Materials and Methods:

Materials:

The organisms selected for screening are as in Table 2.

Table: 2 Microorganisms used for the study.

Sl.No.	**Pathogenic Organisms**
1.	*Pseudomonas aeruginosa* a) Strain MTCC-3541 b) Strain MTCC-424
2.	*Staphylococus aureus* a) Strain MTCC 9886 b) Strain MTCC 9011
	Non- Pathogenic Organisms
1.	*Bacillus subtilis* a) Strain-MTCC 2616
2.	*Bacillus coagulans* a) Strain - MTCC-4823 b) Strain -MTCC-2302
3.	*Acetobacter aceti* a) Strain -MTCC-3246 b) Strain -MTCC-3347

1) ***Pseudomonas aeruginosa***- It is a gram negative opportunistic pathogen and requires an alteration or defect in the hosts' defense system to establish an infection (151). The virulence factors of *Pseudomonas aeruginosa* include Pili, Flagellum, Phospholipase,

Lipopolysaccharide, Exotoxin A and proteases, such as as Las B , elastase, Las A elastase and Alkaline protease. Las B elastase (pseudolysin) is a metalloprotease which has both proteolytic and elastase activities and its proteolytic activity exceeds its elastolytic activity (151). .Las A (staphylolysin) is a serine protease which just nicks elastin making it susceptible to elastase activity of Las B (152). Alkaline protease (aerugolysin) is a zinc metaloprotease which causes tissue damage. This possession of an array of proteases makes it a highly virulent organism. Clinical studies have shown that the infection site and duration of infection can alter the production of these proteases (153) . It has also been reported that despite the fact that Las A and Las B genes were present in 145 *P.aeruginosa* isolates, some of them failed to express elastase A and elastase B activity(154). The protease profiles also depend on the strains used as well as the bacterial culture media (155) . Clinical strains also differ from ATCC strains in their proteinase pattern, probably due to the diverse requirements for expression of proteolytic activities by the strain studied in tissues (156).

2) ***Staphylococcus aureus*:** These are non motile gram positive cocci. *S.aureus* is always considered a potentially pathogenic bacteria responsible for cutaneous infections, toxin mediated infections as wells as in other diseases such as pneumonia, bacteremia, endocarditis, and septic arthritis (157)

The proteolytic enzymes of *Staphylococcus aureus* have not been studied in detail when compared to *P. aeruginosa*. Extracellular proteases however have been described and a few have been purified. *Staphylococcus* species produces some industrially important extracellular enzymes such as lipase and several extracellular proteases of the serine, cysteine and metallo protease type which is important in the pathogenity of bacteria (158) (159). The proteases of S*taphylococcus* are insensitive to the plasma protease inhibitors of humans and also inactivate some of them (159). V_8 protease was found to inactivate α_1 proteinase inhibitor in human plasma (160).. Auerolysin a metalloprotease was also isolated from *S. aureus* but is not as efficient as $V_{8.}$ The proteases of *Staphylococci* are known to have effects in the virulence of the

bacteria and interact with the hosts defensive mechanisms causing deleterious effects (161). Staphylococcal elastase has been isolated from *S. epidermis*. However a relationship between the elastase of this species and the proteases of *S. aureus* have not been determined though there are similarities (162).

3) Bacillus subtilis- is a gram positive, bacillus which has been known to produce several proteases. Many of the species secrete large amounts of proteases required for their physiological activities and have been widely exploited in the detergent industry. The *Bacillus* species are preferred due to their high growth rate, short fermentation time, and their capacity to secrete extracellular proteases into the media and their GRAS (Generally regarded as safe) status by the food and Drug administration (163). One of the noteworthy characteristics of this species is its ability to modulate the environment, so that they can convert a neutral or highly alkaline medium in their favour so as to optimize pH for their growth (164). The presence of alkaline proteases has been widely reported in this

species and there has been a report of elastase extraction from a *Bacillus* species (15).

4) ***Bacillus coagulans*:** It also belongs to the *Bacillus* species known to produce alkaline proteases and has been used profitably for large scale production of alkaline protease. Almost half, of the present production of bulk protease enzymes are from strains of Bacillus (165). The main features of *Bacillus coagulans* are its acid and heat resistance and easy culture.

5) ***Acetobacter aceti*** : *Acetobacters* are unique gram negative organisms that can oxidize ethanol to acetic acid and show a high resistances to acetic acid and ethanol (166). Due to this characteristic it is widely used for the industrial production of vinegar. This bacteria has been historically utilized for vinegar production and is least explored for its ability to produce proteases.

However, it was felt worthwhile investigate the production of proteases by this microorganism also, since it already has an industrial application.

Chemicals/ Reagents:

All chemicals/ reagents used in this study were of analytical grade. Media for microbiology culture such as beef extract, peptone agar, mannitol and yeast extract were obtained from Himedia. Bovine casein and synthetic substrates STANA (succinyl tri alanyl p-nitroanilide), BAPNA(L-N-Benzoyl DZ arginine p-nitronilide) and ATEE(N-Acetyl L-tyrosine ethyl ester) were obtained from Sigma (C7078,S4760,B3133 and A6751).

Methods:

Screening for ability to produce proteases:

All the above mentioned strains used in the study were purchased from the Microbial Type Culture Collection and Gene Bank Chandigarh, India in a lyophilized form.

The microorganisms were rehydrated and cultivated as per the suppliers' instructions and routinely used procedures (The ampule containing lyophilized bacteria was first disinfected with 70% ethanol. It was then broken at the pre scored area. 0.5 ml of sterile

water was added and with a sterile pipette, the contents were aspirated several times to mix the suspension thoroughly. The suspension was kept for 30 minutes and then inoculated into the media).

The strains of *Bacillus coagulans, Pseudomonas aeruginosa, Bacillus subtilis* and *Staphylococcus aureus* were cultivated on Nutrient Agar plates (Nutrient agar prepared with 0.3 gm beef extract, 0.5 gm peptone and 1.5 gm agar in 100 ml distilled water) while *Acetobacter aceti* was plated on Mannitol Agar plates. (Mannitol agar prepared with 0.5 gm yeast extract, 0.3 gm peptone, 2.5 gm of mannitol and 1.5 gm of agar in 100 ml of water).

Fig -7 Mannitol agar plate

After inoculation, all the plates were incubated at 37^{o}C for 1-2 days. Plates of caseinate agar were prepared and proteolytic activities of the strains were detected by the appearance of a clear zone around the bacterial colonies (2).Rao MB

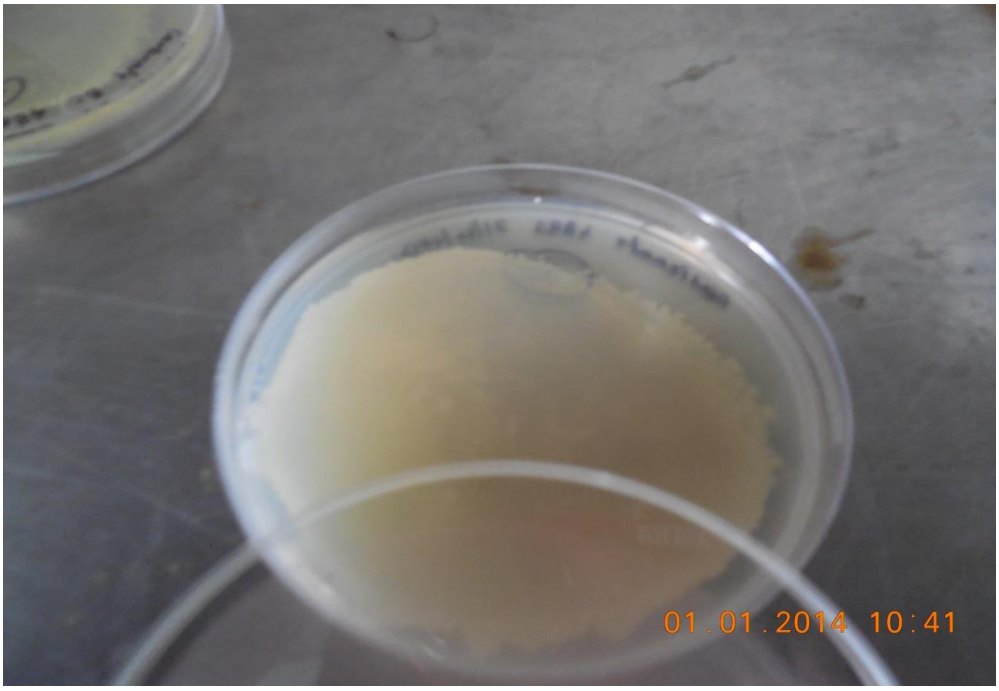


Fig -8 Caseinate agar plates with zone of hydrolysis

Preservation of the Isolates:

Glycerol slants were prepared and samples of each strain enriched in the nutrient broth were added with 50% Glycerol and stored at - 80 ^{o}C for future use.

Protease production: A nutrient broth was prepared containing 1% glucose 0.5% Casein, 0.5% yeast extract, 0.2% K_2HPO_4, 0.2% $KHPO_4$ and 0.1% $MgSo_4H_2O$ (167)Rao K This media was used for all the strains of the microorganisms throughout the study. They

were inoculated into the above nutrient broth and were kept in a rotatory incubator at 120 rpm a 37^{o}C.

Samples were drawn at intervals of 24 hours for a period of 5 days with aseptic precautions. It was centrifuged at 10000rpm for 10 minutes at 4 ^{o}C and the crude clear supernatant was used as the source of the enzyme.

The capabilities of protease production of all the 9 strains were examined and one strain each from a pathogenic and a non pathogenic organism which showed maximum proteolytic activity were selected for further studies.

Assay of proteolytic activity:

Total protease activity was measured using Casein substrate as described by Sumathi et al (168) The assay stem consisted of 1% Casein, 0.2M Phosphate buffer pH 7.6. The reaction was initiated by addition of the crude clear supernatant as the enzyme source. After 10 minutes incubation at 37^{o}C, the enzyme action was arrested by the addition of 3ml of 5% Trichloro acetic acid (TCA) solution. The

reaction mixture was allowed to stand for 30 minutes at room temperature and then centrifuged at 3000rpm for 10 minutes. One ml of the clear supernatant was analyzed for TCA soluble fragments and colour developed with Folin –Ciocalteu reagent (169,170). One unit of enzyme was defined as the amount of enzyme required to liberate TCA soluble fragment which produced a color intensity equivalent to 1µmole of tyrosine per minute .

Protein estimation:

Total protein content was estimated using Bovine Serum Albumin (BSA) as the standard by the method described by Lowry et al (169).

Use of synthetic substrates:

The substrate specificity studies were initially performed by using the crude supernatant. Specific synthetic substrates used were STANA (succinyl trialanyl p-nitroanilide) for elastase activity, BAPNA (L-N-Benzoyl DZ arginine p-nitronilide) for trypsin like activity and ATEE (N-Acetyl L-tyrosine ethyl ester) for

chymotrypsin like activity as per the methods of Bieth et al, Swaminathan et al and Rao et al respectively (171,172,173).

STANA as the substrate: To determine if the protease had elastase like activity the substrate STANA was used and the activity determined by the method of Bieth etal (171). The assay system comprised of crude supernatant, 200 mM of STANA in 200 mM of Tris Hcl buffer pH 8.0. The reaction was initiated by the addition of aliquots of enzyme from the supernatant of all the bacteria used for screening for a consecutive period of 5 days. After a 15 minute incubation at 37^{o}C, the reaction was stopped by the addition of 1.0 ml of 30% Acetic acid. The optical density of p-nitroaniline liberated was measured at 410 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µ mole of p-nitroaniline per unit time under standard assay conditions.

BAPNA:

The crude supernatant of all the screened bacteria was assayed to see if it had trypsin like activities by the use of BAPNA as the substrate, by the method of Swaminathan et al (172). The assay system consisted of 50m M of BAPNA in 50mM phosphate buffer pH 7.6. The reaction was initiated by aliquots of the enzyme obtained from both culture supernatants of these microorganisms. After 15 minute incubation at 37^{o}C, the reaction was stopped by addition of 1 ml of 30% acetic acid and intensity of p-nitroaniline liberated was measured at 410nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1µmol of p-nitroaniline per unit time under standard assay conditions.

ATEE:

The chymotrypsin like activities in the crude fraction of the screened organisms was determined by the method of Rao et al (173). The assay system consists of 10mM of acetyl tyrosine ethyl acetate, 100mM of phosphate buffer, pH 7.6 and the reaction was initiated by the addition of aliquots of the crude enzyme. After 10 minutes

incubation at 37^{o}C, the reaction mixture was extracted with 3ml of ethyl acetate. Acetyl tyrosine in the aqueous phase was estimated by the method of Lowry et al (169). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1µmol of tyrosine equivalents (acetyl tyrosine) from ATEE under standard assay conditions .

Selection of the high yielding strains:

As mentioned above, the bacterial strains were inoculated into a nutrient broth and the protease production was assayed at regular intervals for a period of 5 days. Statistically analyzed by SPSS software version 22 . Results expressed as Mean ± SD

Results and Discussion:

Table 3 – Maximum protease activity and days on which it was expressed

Organisms	Days on which highest proteolytic activity was recorded	Highest recorded Proteolytic Activity on Casein U/ml/min
.*P.aeruginosa* MTCC-3541	1st	80.60±8.8
P.aeruginosa MTCC-424	2nd	30.9±1.85
S.aureus MTCC 9886	3rd	21.4±2.99
S.aureus MTCC 9011	1st	27.5±2.97
B.subtilis MTCC 2616	5th	90.1±3.79
B.coagulans MTCC-4823	1st	69.1±3.29
B.coagulans MTCC-2302	3rd	52.9±4.33
A.aceti MTCC-3246	4th	67.6±4.78
A.aceti MTCC-3347	4th	19.5±2.35

Table 4 – Total proteolytic activity of crude enzymes on casein as substrate from day 1 to day5

Sl. No.	Organisms	Days	Enzyme Activity U/ml/min	Day wise protein content in mg/ml	Specific Activity in U/mg
1	*P.aeruginosa* MTCC-3541	1	80.6±8.84	6.680±0.08	12.06
		2	56.7±3.31	5.96±0.20	9.51
		3	27.9±1.69	5.44±0.26	5.12
		4	19.5±1.64	5.10±0.25	3.82
		5	9.5±1.73	4.86±0.08	1.95
2	*P.aeruginosa* MTCC-424	1	3.1±0.76	5.64±0.16	0.54
		2	30.9±1.85	5.06±0.08	6.10
		3	28.0±1.61	4.90±0.10	5.71
		4	11.9±1.18	4.94±0.16	2.40
		5	8.2±0.98	4.98±0.13	1.64
3	*S.aureus* MTCC 9886	1	4.3±0.82	4.24±0.18	1.01
		2	18.1±1.03	4.24±0.18	4.26
		3	21.4±2.99	4.08±0.13	5.24
		4	18.6±2.53	4.20±0.07	4.42
		5	8.6±0.75	4.00±0.07	2.15
4	*S.aureus* MTCC 9011	1	27.5±2.97	4.66±0.11	5.90
		2	22.9±1.90	4.66±0.11	4.91
		3	18.2±2.31	4.50±0.07	4.04
		4	10.8±0.92	4.18±0.08	2.58
		5	4.0±0.58	4.00±0.07	1.00
5	*B.subtilis* MTCC 2616	1	11.7±0.75	3.02±0.21	3.87
		2	56.5±4.24	3.02±0.13	18.54
		3	63.0±4.91	2.98±0.08	21.14
		4	76.5±5.73	3.16±0.05	24.20
		5	90.1±3.79	3.12±0.17	28.87

6	*B.coagulans* MTCC-4823	1	69.1±3.29	6.98±0.14	9.89
		2	24.0±2.32	6.16±0.23	3.89
		3	19.3±1.65	5.30±0.23	3.64
		4	13.4±1.08	4.84±0.20	2.76
		5	7.0±0.37	4.60±0.25	1.52
7	*B.coagulans* MTCC-2302	1	26.3±4.21	5.20±0.14	5.05
		2	33.8±3.36	5.00±0.07	6.76
		3	52.9±4.33	4.92±0.13	14.81
		4	39.7±3.17	4.82±0.10	8.23
		5	19.7±1.61	4.66±0.08	4.22
8	*A.aceti* MTCC-3246	1	6.8±0.76	3.86±0.08	1.76
		2	34.0±4.25	3.98±0.13	8.54
		3	43.0±5.06	3.96±0.08	10.85
		4	67.6±4.78	3.98±0.08	16.98
		5	50.4±8.83	4.00±0.07	12.6
9	*A.aceti* MTCC-3347	1	2.4±0.44	3.76±0.05	0.63
		2	10.0±0.86	3.64±0.08	2.74
		3	13.6±1.83	3.62±0.08	3.75
		4	19.5±2.35	3.60±0.07	5.41
		5	4.6±0.47	3.28±0.08	1.40

The results showed that, all the bacteria screened had proteolytic activity. The maximum protease activity and the days on which it was expressed are as shown in table 3 .The total proteolytic activity expressed in U/ml/min, on all 5 days using, casein as substrate with day wise protein concentration in mg/ml and the specific Activity in

U/mg of protein of the crude enzyme of all the microorganism screened are as shown in Table 4.

Among the pathogenic bacteria screened, *Pseudomonas aeruginosa* MTCC 3541 was the highest protease yielding strain with a maximal proteolytic activity of 80.6±8.84 U/ml/min on the 1st day. The other strain of *Pseudomonas aeruginosa* MTCC 424 had a maximal proteolytic activity of 30.9±1.85 U/ml/min on the 2nd day while, *S. aureus* MTCC 9886 had maximum protease production of 21.4±2.99 U/ml/min on 3rd day and *S. aureus* MTCC 9011 had maximum protease production of 27.5±2.97 U/ml/min on the 1st day.

Among the non pathogenic bacteria screened, *Bacillus coagulans* MTCC 4823 showed the highest proteolytic activity of 69.1±3.29 U/ml/min on the 1st day. *Bacillus coagulans* MTCC 2302 had maximum protease production of 52.9±4.33 U/ml/min on the 3rd day and *B.subtilis* MTCC 2616 had maximum protease production of 90.1±3.79 U/ml/min on the 5th day. *Acetobacter aceti* MTCC 3246 and *Acetobacter aceti* MTCC 3347 had maximum protease

production of 67.6±4.78 U/ml/min and 19.5±2.35 U/ml/min on the 4th day respectively.

Table 5 – Activity of the crude enzymes on synthetic substrates

Organisms	**Activity on STANA U/dl/min**	**Activity on ATEE U/dl/min**	**Activity on BAPNA U/dl/min**
*P.aeruginosa*MTCC-3541	1.006±0.10	207.0±20.2	NR*
P.aeruginosa MTCC-424	0.214±0.03	89.8±8.46	NR*
S.aureus MTCC 9886	0.174±0.03	NR*	NR*
S.aureus MTCC 9011	0.160±0.02	NR*	NR*
B.subtilis MTCC 2616	NR*	NR*	NR*
B.coagulans MTCC-4823	0.008±0.00	156.2±19.71	NR*
B.coagulans MTCC-2302	NR*	65.2±9.5	NR*
A.aceti MTCC-3246	NR*	413.0±42.5	NR*
A.aceti MTCC-3347	NR*	51.4±11.28	NR*

Table 6 – Activity of the crude enzymes on synthetic substrates from day 1 to day 5

Sl. No.	Organisms	Days	Activity on STANA U/dl/min	Activity on ATEE U/dl/min	Activity on BAPNA U/dl/min
1	*P.aeruginosa* MTCC-3541	1	1.00±0.10	207.0±20.2	NR
		2	0.20±0.44	159.4±19.1	NR
		3	0.006±0.00	127.2±9.47	NR
		4	0.005±0.00	82.3±6.74	NR
		5	0.003±0.00	50.0±9.29	NR
2	*P.aeruginosa* MTCC-424	1	0.11±0.01	37.6±6.88	NR
		2	0.21±0.03	89.8±8.46	NR
		3	0.19±0.01	61.6±8.96	NR
		4	0.07±0.00	32.5±4.38	NR
		5	0.01±0.00	13.7±3.23	NR
3	*S.aureus* MTCC 9886	1	0.04±0.01	NR	NR
		2	0.13±0.01	NR	NR
		3	0.17±0.03	NR	NR
		4	0.15±0.01	NR	NR
		5	0.08±0.02	NR	NR
4	*S.aureus* MTCC 9011	1	0.16±0.02	NR	NR
		2	0.14±0.01	NR	NR
		3	0.12±0.02	NR	NR
		4	0.08±0.01	NR	NR
		5	0.04±0.01	NR	NR
5	*B.subtilis* MTCC 2616	1	NR	NR	NR
		2	NR	NR	NR
		3	NR	NR	NR

		4	NR	NR	NR
		5	NR	NR	NR
6	*B.coagulans*	1	0.00±0.00	156.2±19.7	NR
	MTCC-4823	2	0.00±0.00	123.9±6.51	NR
		3	0.00±0.00	99.7±8.00	NR
		4	0.00±0.00	46.8±5.69	NR
		5	0.00±0.00	15.3±4.35	NR
7	*B.coagulans*	1	NR	19.3±2.03	NR
	MTCC-2302	2	NR	43.5±7.81	NR
		3	NR	65.2±9.54	NR
		4	NR	38.0±3.73	NR
		5	NR	22.7±4.43	NR
8	*A.aceti*	1	NR	174.5±29.4	NR
	MTCC-3246	2	NR	265.7±19.79	NR
		3	NR	307.5±6.30	NR
		4	NR	413.0±42.5	NR
		5	NR	360.3±28.29	NR
9	*A.aceti*	1	NR	7.8±1.72	NR
	MTCC-3347	2	NR	25.8±3.53	NR
		3	NR	37.5±3.81	NR
		4	NR	51.4±11.2	NR
		5	NR	14.1±2.40	NR

Figure 9 - Crude enzyme Activity Of *P. aeruginosa* and *B.coagulans* on STANA Day 1

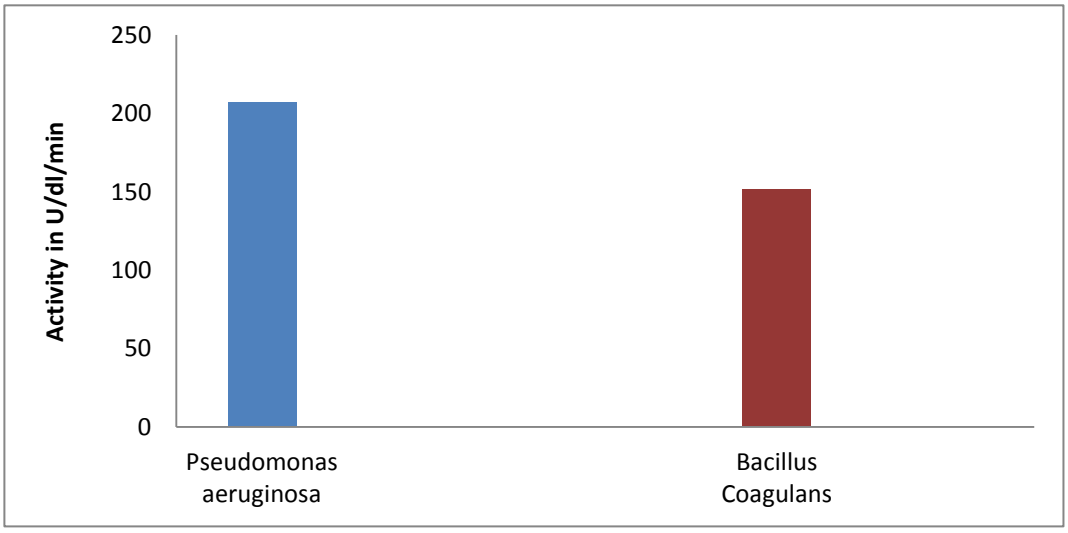


Figure 10 - Crude enzyme Activity Of *P. aeruginosa* and *B.coagulans* on ATEE Day-1

Results of the screening studies on synthetic substrates are as detailed in Tables 5 and 6. Results indicated that the presence of elastase enzyme is restricted to pathogenic bacteria especially *Pseudomonas aeruginosa* as reported earlier. It had elastase activity of 1.006±0.10U/dl/min. Though elastase production by *Staphylococus aureus* has, been reported in the present study, the expression of elastase was minimal in comparison to *Pseudomonas aeruginosa*. This could be attributed to the fact that conditions which were provided in the media were not suitable for release of elastase or the property of the organism itself.

Among the non-pathogenic organisms, none showed elastase activity but all exhibited protease activity. Though, there are few reports on elastase presence in non pathogenic bacteria,(15) none of the screened non pathogens had elastase under the given set of conditions. One of the salient observations of the study was production of proteases by *Acetobacte*r *aceti*. This organism has not been explored for protease production and is known more for its ability to produce acetic acid from ethanol. Another important observation was that, the proteases of both strains of *Bacillus*

coagulans had chymotrypsin like activity which was not seen in the case of *Bacillus subtilis* though both belong to the same Bacillus family.

Both strains of *P. aeruginosa, B. coagulans* and *A. aceti* had proteases which had a chymotrypsin like activity as shown by the hydrolysis of ATEE. The proteases of both strains of *Staphylococus aureus* also did not have any hydrolytic activity on ATEE.

Bacteria are known to produce extracellular proteases and several of them having characterized and studied extensively. However, many of the studies concentrate on individual enzymes with specific characteristics. Studies on the potential of bacteria belonging to both pathogenic and nonpathogenic strains show that they produce extracellular proteases but information on their substrate specificities/preferences are scarce.

In view of this a simple approach to assess the spectra of protease production/ secretion was designed using specific substrates for specific enzymes types, broadly belonging to the serine protease class viz. elastase, trypsin and chymotrypsin. The substrates

used were STANA for elastase like activity, BAPNA for trypsin like activity and ATEE for chymotrypsin like enzyme activity.

This approach has yielded interesting data and the analysis of the results indicate that the majority of the microorganisms studied, had ATEE hydrolyzing / chymotrypsin like activity irrespective of whether they belong to pathogenic or nonpathogenic group.

In the screening process, for the first time we could identify an organism *Acetobacter aceti* which though usually used for acetic acid production could hydrolyze ATEE indicating that a new environment induces it to produce a protease which has a chymotrypsin like activity. (the findings of this study was published (Publications).

The presence of elastase in *P. aeruginosa* has been well documented (174). In case of *Staphylococcus aureus*, though several extracellular proteases of the serine, cysteine and metallo protease type which are important in the pathogenicity of bacteria were reported in earlier studies, (11) the strains used in the current study exhibited relatively low proteolytic activity. Protease profiles have

been shown to depend on the strains used as well as the culture media (11). It is a known fact that bacteria adapt to the environment by gene on and off mechanisms. The reason for some of these organisms showing peak production of proteases on 4^{th} and 5^{th} days are probably due to the organism adapting to the new environment. The fall in activity seen is due to decreased availability of nutrients or auto degradation of proteases (148,155).

In case of non-pathogenic organisms, no elastase enzyme was released under the given set of conditions. Though, these non-pathogenic bacteria secrete large amounts of protease these proteases are for primarily attacking proteins in the media for nutritional gain.

It is obvious from the analysis of the results obtained that the strain of *Pseudomonas aeruginosa* 3541 has elastase activity(Fig-9). The production of elastase is important in all the types of *Pseudomonas aeruginosa* infections (175) However, interestingly *Pseudomonas aeruginosa* isolates from canine-otitis externae are found to be elastase deficient (176) But, most of the *Pseudomonas aeruginosa* strains produce a protease with elastase activity .

The activity of the crude supernatant of all the screened bacteria on BAPNA were low and barely detectable. *Pseudomonas aeruginosa* 3541 also did not hydrolyse BAPNA and the observation in this study is similar to that of Izarel Zivkovic etal (47). ATEE a synthetic substrate for chymotrypsin was hydrolysed by the enzyme of *P aeruginosa* (EA = 207 U/dl/min)(Fig-10). This is contrary to the findings of Izrail Ziovkovic etal (47) who reported low but detectable activity on ATEE. It should be emphasized that various strains show varying substrate specificity; though usually the protease of *P aeruginosa* prefers small to medium sized substrates (177). *P aeruginosa* elastase is known to share significant Amino Acid sequence homology with neutral protease of *Bacillus* species as it shares 28% sequence identity with thermolysin. So, *P aeruginosa* elastase appears to be an evolutionary distant relative of the neutral proteases of the Bacillus species (35).

The protease of *B. coagulans* had barely recordable hydrolytic activity on STANA and BAPNA but hydrolysed ATEE (EA = 156.2 U/dl/min)(Fig -10).

Members of the *Bacillus* species are known to secrete alkaline proteases. As the major use of the proteases of this species is for industrial purposes, all studies have focused on the use of additives, the effect of major medium ingredients such as carbon and nitrogen sources as well as metal ions for enhancing the production of the protease. There does not appear to be any attempt to categorize these proteases by use of synthetic substrates. This study had attempted to categorize the protease by use of synthetic substrates, and the conclusion was that the protease of *B. coagulans* 4823 had ATEE hydrolyzing activity.

Conclusion:

All organisms studied expressed proteolytic activity under the given set of conditions. However, the spectra and specificities were found to be varied among the organisms studied. One of the important findings of this study was the absence of elastase among the nonpathogens screened and this could be attributed to pathogenic bacteria secreting proteases like elastase in order to increase their virulence. The presence of elastase is based on the hydrolysis of the

substrate STANA. However, in case of non-pathogenic organisms, they do not need elastase as they are not invasive. Though, these non-pathogenic bacteria secreted large amounts of protease this appear to be more for their physiological role. *Bacillus coagulans* 4823 which was selected for further purification due to its release of protease did not hydrolyze STANA, even after further levels of purification.

Chapter IV

Purification and Characterization of Proteases of Pathogenic and Non Pathogenic Bacteria

Introduction –

Proteases from microbial sources are preferred over that of plant and animal sources for reasons mentioned earlier such as the ease with which microbes can be cultivated and their properties altered to suit commercial purpose (1). The purpose for purification of proteases is to study their physiochemical properties and develop them for use in the fields of medicine and industry. The major objective of purification and characterization of microbial sources is usually, to isolate proteases which can be used for industrial applications and to select those proteases exhibiting stability on exposure to a wide range of pH, temperature and salt concentration so that they can be used for varied industrial conditions. The enzymes from *Bacillus* species are widely used as; they are stable and active under varied extreme conditions. Isolation and purification of proteases of members of the pathogenic *Pseudomonas* and *Staphylococcus* species have been performed as a knowledge of their enzyme characteristics are of medical importance as some of these enzymes have been reported as virulence factors (3,157). So, the characterization and

purification of these bacterial proteases make it an active area of research for commercial as well as diagnostic and therapeutic purposes.

The screening studies of the pathogenic and non pathogenic organisms for protease production and specificity of substrate indicated that all of the organisms studied exhibited protease activities which included elastase and other enzymes like trypsin and chymotrypsin based on the substrate specificities as seen in the previous chapter. The screening studies indicated presence of two enzymes in *P. aeruginosa* MTCC 3541. One of it was elastase as reported earlier which had been purified and characterized. This enzyme has been attributed to its virulence as it facilitates the invasion of the organism during infection (175). Elastase is a neutral metallo protease having a zinc atom (153) but not much is known on the other proteases of *P. aeruginosa*. Hence, the second enzyme which has not been studied for its substrate specificity and other characteristics was to be studied further. In view of the paucity of the knowledge on the second enzyme it was considered worthwhile to purify these enzymes to ensure that the proteolytic activities were due to two distinct enzymes. Various strains of *P. aeruginosa* are known to show varying substrate specificity

though the proteases of this organism usually prefer small to medium substrates (177).

The proper choice of a starting material is the key for a successful purification process. As seen from the screening studies among pathogenic organisms, *P.aeruginosa* MTCC 3541 secreted the largest amount of protease and elastase. Moreover, the protease also exhibited chymotrypsin like activity which has not been reported in literature reviews and hence this microorganism was selected for further purification studies. *B.coagulans* MTCC 4823 belonging to *Bacillus* species did not release elastase but its protease had a chymotrypsin like activity which has not been characterized in earlier studies so, this microorganism was also selected for further purification. Besides, most of the studies on proteases of this family have been focused on proteolytic production for industrial purposes. Hence, further studies in this chapter were on the proteases of *P. aeruginosa* and the protease of *B. coagulans*.

Materials and Methods:

Materials:

Organisms used were - *P. aeruginosa* and *B. coagulans*

DEAE cellulose(30477), Tris(252859), Sephadex G-100(G100120), Acrylamide(A6050), TEMED(T7024), Bromophenol blue(11391), Coomassie Brilliant Blue R-250(B8522) and Blue Sepharose CL 6 B(R9903) were obtained from Sigma. Standard Protein markers from New England Bio Labs(2-212kDa) and Bio Rad (14-97kDa) .

All chemicals/ reagents used in this study were of analytical grade.

Methods:

Ammonium sulfate precipitation:

The crude clear supernatant obtained from *P. aeruginosa* and *B. coagulans* by the method mentioned previously (under Protease production in the previous chapter) was subjected to Ammonium sulfate precipitation. This procedure was carried out in a cold room at 4^0C. Crystals of Ammonium sulfate were added with constant stirring until 70% saturation was obtained and was left overnight at 4^0C. Then, it was centrifuged at 10,000rpm for 20 minutes. In the case of *P. aeruginosa*, the precipitate obtained was

collected and dissolved in 0.2M Tris-HCl buffer pH 8.0 and centrifuged again at 10,000rpm for 10 minutes. The supernatant was collected and dialyzed against 0.2M Tris-HCl buffer pH 8.0 for 24 hours with a change of buffer at the 12th hour of dialysis.

Following this, the dialyzed sample was collected, measured for protein content and proteolytic activity using casein as the general substrate and the synthetic substrates STANA, BAPNA and ATEE for elastase , trypsin and chymotrypsin like activities.

The methods followed are as mentioned earlier (in the previous chapter) except that the source of the enzyme here was the Ammonium sulfate fraction (dialyzed sample.) A similar procedure was followed for the protease of *B. coagulans* but the precipitate was dissolved in 0.2M phosphate buffer pH 7.6 and it was dialyzed against the same buffer for 24 hours with a change of buffer at the 12th hour of dialysis.

DEAE- cellulose chromatography- The column used was Bio-Rad column length 25 cm. Bed volume was 15 ml. The required DEAE cellulose (Sigma) was kept for swelling overnight. Activation was as per the manufacturer's instructions. It was suspended in 0.1 NaOH containing

0.5 M NaCl for 10 minutes and this was repeated twice. The procedure was repeated by suspension in 0.1 M HCl containing 0.5M NaCl. It was then washed with 5 bed volumes of deionized water and pH was tested. After this, it was suspended in the buffer i.e. 0.2M Tris -HCl buffer pH 8.0 and the column was packed. The entire procedure was carried out in a cold room at 4^0C. The ammonium sulfate fraction(1 ml containing 13 mg of protein) was loaded onto the DEAE cellulose column equilibrated with 0.2M Tris -HCl buffer pH 8. Elution was then carried out in a step wise manner with varying concentrations of NaCl (0.2M – 1.0M) in the same buffer. Fractions of 2.0 ml were collected and monitored for protein by reading absorbance at 280nm. Fractions which had proteolytic activity were pooled, stored and used for further substrate specific studies.

The column was washed with 5 bed volumes of deionized water and equilibrated with 2 bed volumes of 0.2M Sodium Phosphate buffer pH 7.6. Ammonium sulfate fraction of *Bacillus coagulans* obtained after dialysis (1 ml containing 12mg of protein) was applied to the column. Elution was done in a step wise manner using NaCl (0.2M-1.0M) in the same Sodium Phosphate buffer. Fractions of 2.0ml were collected and monitored for

protein by reading absorbance at 280nm. Fractions which had proteolytic activity were pooled, stored and used for further substrate specific studies. After each step in the purification process, specific activities in U/mg of protein were determined.

Gel Filtration:

The homogeneity of the proteolytic enzyme obtained from *P. aeruginosa* was determined by Gel filtration using Sephadex G-100 in a Bio-Rad Echno column (50 x 0.7cms). The gel was mixed with deionized water with constant stirring until a thick slurry was formed and was equilibrated with 5 bed volumes of 0.05M Tris-Hcl buffer pH 8.0 and kept overnight. The bed volume was 33ml and the flow rate adjusted to 8 ml/hour. The entire procedure was carried out in a cold room at 4^0C.

The elastase of *P. aeruginosa* obtained after DEAE cellulose chromatography was applied along the walls of the column. (1.0 ml contained 1.7 mg of protein).Buffer for elution was 0.05M Tris-Hcl buffer pH 8.0 containing 0.1 M NaCl. Fractions of 1 ml were collected and estimated for protein at 280nm. After proteolytic activity was determined using casein, STANA was used as substrate for elastase determination.

The column was washed with 5 bed volumes of deionized water and equilibrated with 0.2M sodium phosphate buffer pH 7.6. The protease of *B. coagulans* obtained after DEAE cellulose chromatography was then applied along the walls of the column. (1.0 ml contained 1.9 mg of protein) . Elution was done using 0.2M sodium phosphate buffer pH 7.6 containing 0.1M NaCl. Fractions of 1 ml were collected and estimated for protein at 280nm. Total proteolytic activity was determined using casein and then ATEE was used as substrate.

SDS-PAGE:

The fractions with peak activities obtained after Gel chromatography was pooled and concentrated and were subjected to SDS-PAGE electrophoresis (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) using 12 % resolving gel(30% acrylamide,1.5M Tris,10.0% SDS, 10.0% Ammonium per sulfate, TEMED) and 5% stacking gel (30% acrylamide,1.0M Tris,10.0% SDS, 10.0%Ammonium per sulfate, TEMED) under reducing conditions as per the method of Sambrook and Russell.(178) Dissociation and reduction of proteins was performed by heating for 15 minutes at 100^0C. It was then centrifuged in a microfuge at 12000 rpm for 2 minutes.

After polymerization was complete, the gel was mounted on the electrophoresis apparatus. Tris Glycine buffer was the electrophoretic buffer (25mM Tris, 250mM glycine ph 8.3 with 0.1% w/v SDS) and samples were loaded onto the gel. Bromophenol blue was used to visualize the run and when the run was completed, the plates were pried apart and the gel stained with Coomassie Brilliant Blue R-250. Destaining was done with a mixture of Methanol/ acetic acid/ water solution for a period of 4 hours. A standard protein marker with the range of 2-212kD (New England Biolab) was used along with the loaded samples in order to calculate the molecular weight of the enzymes.

Purification using Affinity Chromatography-Dye binding method (Use of Blue Sepharose CL-6B):

After using conventional methods of purification such as salt fractionation and Ion exchange chromatography in the case of *P. aeruginosa* there was loss of ATEE hydrolyzing enzyme in the washing after DEAE chromatography. In view of this, the ammonium sulfate fraction was also subjected to a dye ligand pseudo affinity chromatography using Blue Sepharose (CL – 6B Sigma). Blue Sepharose is a cibacron blue dye

covalently attached to Sepharose CL-6B. The structure of the blue dye makes it versatile to separate many proteins and bind to several enzymes.

The required amount of the Blue Sepharose was weighed and suspended in distilled water. The medium swelled and was washed for 15 minutes (20 ml of distilled water per milligram).The binding buffer 0.2M Sodium Phosphate buffer pH 8.0 was used to prepare the slurry. The slurry was poured into the column in one continuous motion. The column used was Bio Rad Echno column (5 cm length with bed volume 3.5 ml).The column was then filled with buffer and mounted. The entire procedure was carried out in a cold room at 4^0C.

The ammonium sulfate fraction of *P. aeruginosa* (1 ml containing 13 mg of protein) was dialyzed against 0.2M Sodium Phosphate buffer pH 8.0 and loaded on to column. The pH of the sample was the same as that of the binding buffer.

After the sample was loaded, the medium was washed with 2 bed volumes of the binding buffer. Flow rate was adjusted to 15 ml per hour. For elution, the binding buffer with 0.5M NaCl was used and 5 bed volumes of fractions of 1.0 ml were collected. The fractions were analyzed for

presence of protein. Fractions showing presence of protein were pooled and assayed for proteolytic activity.

pH stability studies

After purification by the stability of the protease to various pH were determined. The pH dependent enzyme stability studies were performed by incubating the protease at a pH ranging from pH 5.0 to 10.0. The buffers used to test the pH stability were 0.2M citrate buffer (pH 5.0) 0.2M Phosphate buffer (pH 6.0, 7.5, 7.6) and 0.2M Tris Hcl buffer (pH 8.0, 8.5, 9.0, 10.0). Here a fixed concentration of the enzyme was preincubated in the different pH ranges from pH 5.0 to pH 10.0 at 37^0C for 30 minutes. The residual caseinolytic activity of the enzyme was then assayed under the standard conditions as mentioned previously.

Thermal stability studies:

For thermal stability the purified enzyme preparations was incubated at different temperatures ranging from 28^0C to 80^0C for 30 minutes and the residual enzyme activity was measured under standard assay conditions using casein as the substrate by the method mentioned previously.(Sumathi et al 168)

Kinetic parameters-

Km and Vmax: The Km and Vmax values for hydrolysis of casein were determined by plotting varying substrate concentrations vs velocity at 37^0C pH 7.6. The substrate was prepared in various concentrations ranging from 0.5 mg/ml to 16mg/ml. A fixed amount of the purified elastase of *P. aeruginosa* and protease *B. coagulans* was added to varying concentrations of casein solution until the enzymes was fully saturated with substrate.

Result and Discussion:

Table 7 – Total proteolytic activity of the Ammonium Sulfate Fraction and after DEAE cellulose chromatography

Organisms	Total proteolytic activity on Casein (Amm Sulf fraction) U/ml/min	Total proteolytic activity on Casein (DEAE cellulose) U/ml/min
P.aeruginosa MTCC-3541	370.4±17.03	473.6±28.33
B.coagulans MTCC-4823	258.1±14.10	378.7±13.87

The results showed that after Ammonium sulfate precipitation, the enzyme activity of both the organisms on casein as substrate increased due to the further degree of purification brought about being 370.4 and 258.1U/ml/min respectively as seen in Table 7 while the crude enzymes of both organisms had an EA of 80.6 U/ml/min and 69.1 U/ml/min. After DEAE cellulose chromatography, the activity was 473.6 and 378.7 U/ml/min respectively. (Table 7)

Table 8 – Activity of Ammonium Sulfate Fraction and DEAE cellulose fraction on synthetic substrates

Organisms	Activity on STANA (Amm Sulf fraction) U/dl/min	Activity on STANA (DEAE cellulose) U/dl/min	Activity on ATEE (Amm Sulf fraction) U/dl/min	Activity on ATEE (DEAE cellulose) U/dl/min
P.aeruginosa MTCC-3541	6.5±0.30	11.2±1.11	892.3±10.78	-------
B.coagulans MTCC-4823	---------	------	598.0±10.59	312.3±12.66

The extracellular enzyme of *P. aeruginosa* showed increased hydrolysis on STANA showing that it retained its elastase activity with EA-6.5 U/dl/min(while in the crude form it was EA-1.006 U/dl/min). It also retained its ATEE hydrolyzing activity (EA-892.3 U/dl/min) (Table -8 and Fig- 12) with non recordable activity on BAPNA. So, *Pseudomonas aeruginosa* showed presence of 2 proteases one being elastase (STANA hydrolyzing)

and the other having ATEE hydrolyzing activity which was seen both in crude and after ammonium sulfate precipitation.

The extracellular enzyme of *Bacillus coagulans* did not have any hydrolytic activity on STANA and BAPNA. It retained its ability to hydrolyze ATEE, though its hydrolytic activity on ATEE(EA -598.0 U/dl/min) was less when compared to *P. aeruginosa* (Table 8 and Fig- 12).

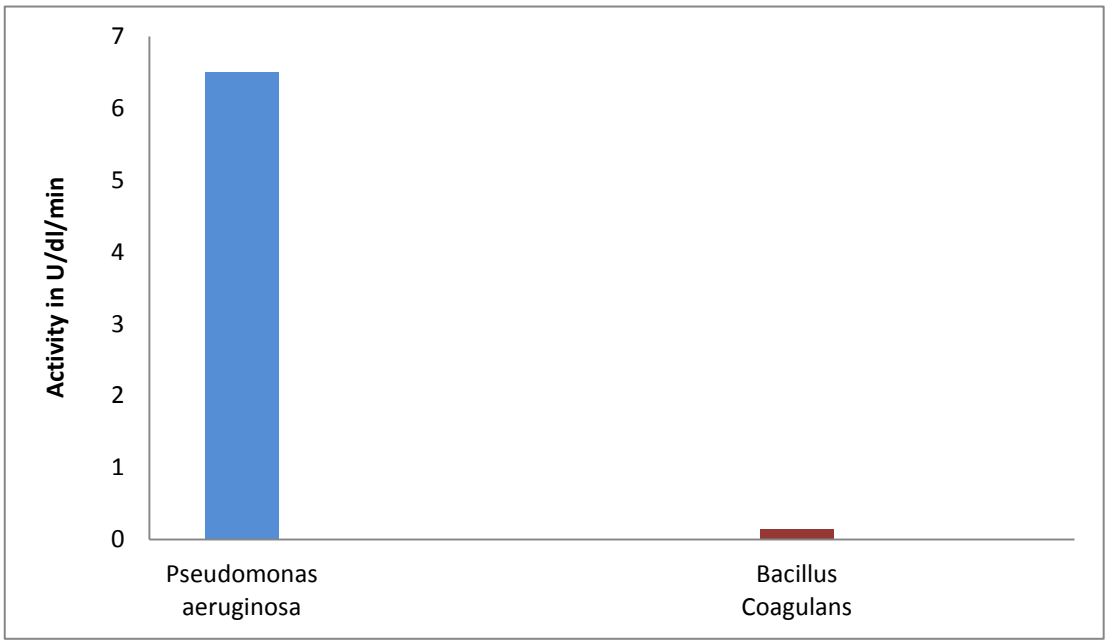


Figure 11 - Activity of Ammonium Sulfate fraction Of *P. aeruginosa* and *B. coagulans* on STANA

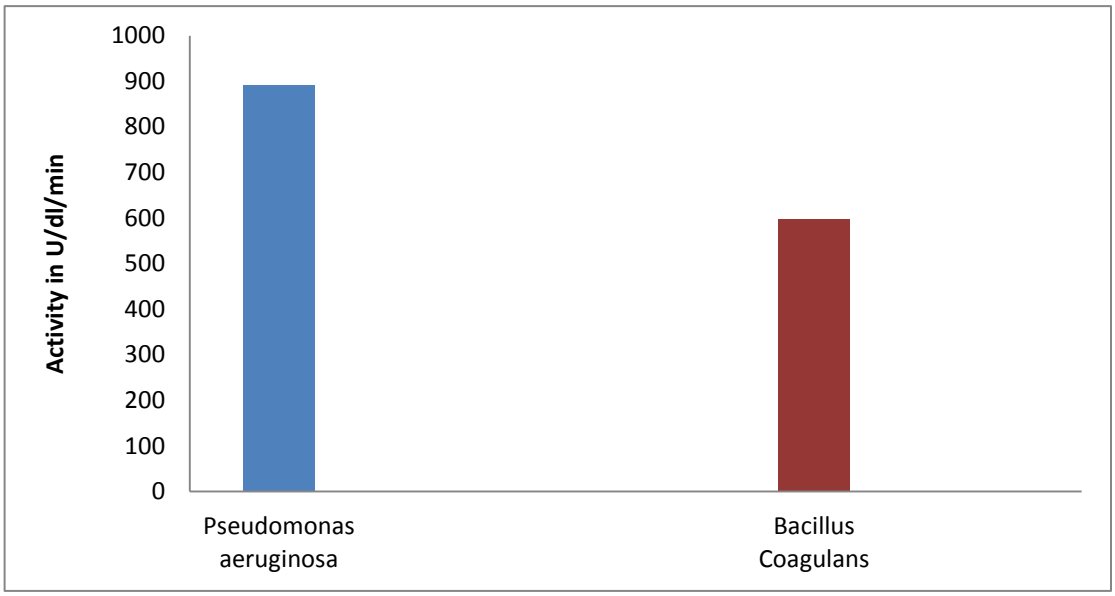


Figure 12 - Activity of Ammonium Sulfate fraction Of *P. aeruginosa* and *B. coagulans* on ATEE

After the ammonium sulfate fraction of *P. aeruginosa* was subjected to DEAE chromatography it was found that elastase was bound to the resin and was eluted with 0.2M NaCl in 0.2M Tris HCl buffer pH 8.0. Fractions collected were examined for presence of protein by measurement of absorbance at 280nm (FIG 13) . Fractions containing protein were pooled and were then assayed for proteolytic activity using casein and synthetic substrates. (TABLE 7 and 8) (FIG -13). The total proteolytic activity was 473 U/ml/min and its activity on STANA was 11.2 U/dl/min. The pooled fractions in the maximal peak area had the highest specific activity of 21.3 U/mg and this fraction was used for gel chromatography.

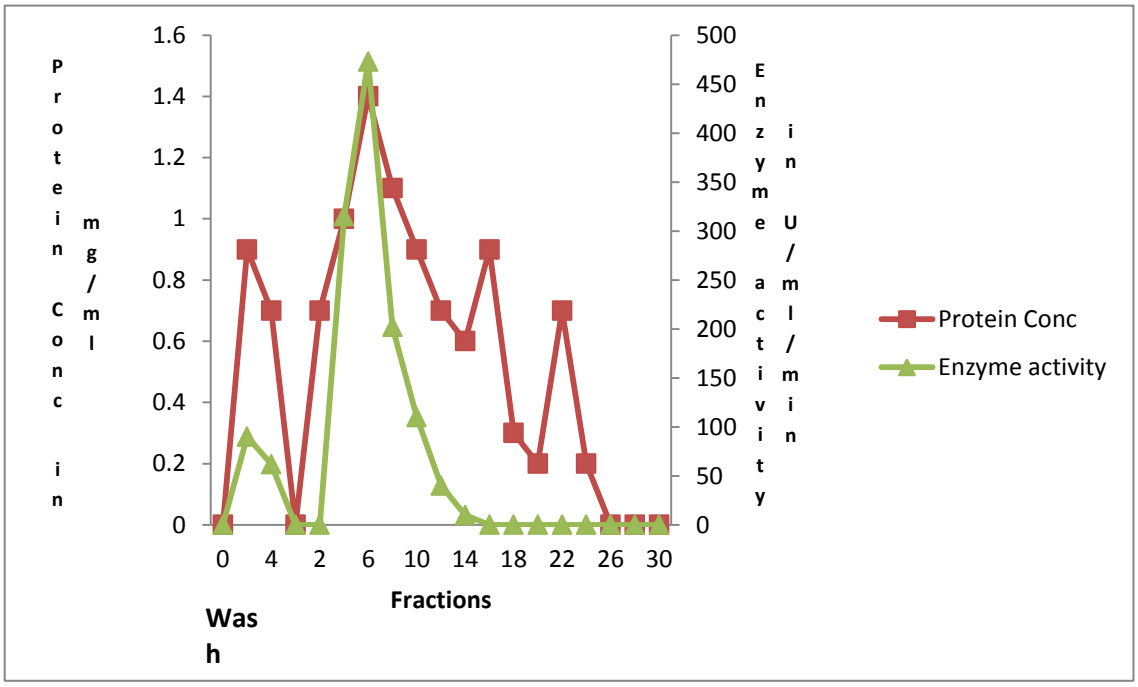


Fig- 13 – Elution pattern of protein and proteolytic activity present after DEAE chromatography of *P. aeruginosa*

The purification protocol of elastase of P.aeruginosa is represented in Table 9.

Table 9 - Purification protocol of the elastase of *P. aeruginosa*

	Volume ml	**Total Activity in U**	**Total Protein in mg**	**Specific Activity in U/mg**	**Yield %**	**Purification Fold (%)**
Crude	1000	1900±43.63	6700±115.3	0.28±0.00	100	1
Ammonium sulfate fraction	15	1120±17.3	197±2.64	5.6±0.05	59	20
DEAE cellulose chromatography	5	190±11.25	8.9±0.15	21.3±0.37	17	76

It can be seen from the results(Table 8) that in the case of *P.aeruginosa* ,after being subjected to DEAE chromatography, the resin could bind only elastase of *P. aeruginosa* which was eluted by 0.2M NaCl in 0.2M Tris Hcl pH 8.0. Only STANA was hydrolyzed by the bound fraction while the other chymotrypsin like enzyme was not recovered. Hence, the resin could not bind the other protease present which had ATEE hydrolysing activity.

Due to the non binding of the chymotrypsin like enzyme of *P. aeruginosa* the ammonium sulfate fraction was subjected to Pseudo Ligand Affinity Chromatography the protocol of which is shown in Table 10 and pattern of elution in Figure 14

Table 10 - Purification by Dye ligand Chromatography of ammonium sulfate fraction of *P. aeruginosa*

Step	Vol of sample in ml	Total Protein (mg)	Total Activity in Units	Specific Activity in U/mg	Fold Purification	% Yield
Amm. Sul. fraction of *P. aeruginosa*	15	197	1120	5.6	20	59
Sample on Blue Sepharose Column* *only1 ml of Am sul fraction taken	1	13	74.6	5.6	-----	-------
Bound fraction*	5	4.2	24.8	5.9	1	33.2

* Volume includes all the pooled fractions having proteolytic activity. Specific activity was the same in all the pooled fractions.

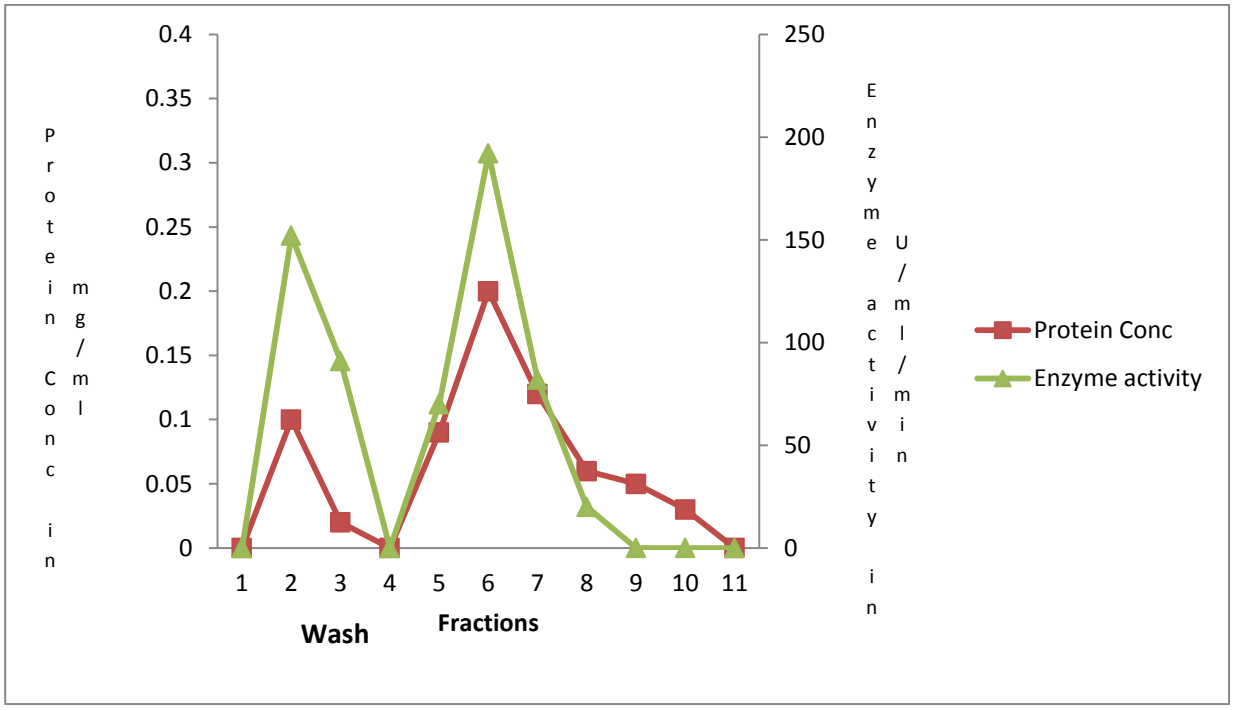


Fig- 14 – Elution pattern of protein and proteolytic activity after Blue Sepharose chromatography of *P. aeruginosa*

Table 11 – Activity of protease of P. aeruginosa on synthetic substrates after dye ligand chromatography

Fraction	Activity on STANA U/dl/min	Activity on ATEE U/dl/min
Bound fraction	-------	760

This purification method used was a one step purification process. When this procedure was applied, two fractions were separated. The dye did not bind Elastase but the ATEE hydrolyzing enzyme was bound by the resin. The bound fractions which showed presence of protein were pooled and assayed for proteolytic activity.(Fig -14) The fractions within the peak had the same specific activity. The bound fraction was eluted by sodium phosphate buffer 0.2M pH 8.0 containing 0.5M NaCl. This fraction hydrolysed ATEE with an activity of 760 U/dl/min and a yield of about 33%. (Tables 11)

The purification protocol of protease of *B. coagulans* used in this study is as shown in Table 12.

Table 12 - Purification protocol of protease of the *B. coagulans*

	Volume ml	Total Activity in U	Total Protein in mg	Specific Activity in U/mg	Yield %	Purification Fold(%)
Crude	1000	1820±25.53	7100±145.4	0.25±0.00	100	1
Ammonium sulfate fraction	15	1050±10.0	193±12.76	5.4±0.05	58	22
DEAE cellulose	5	201±5.03	9.7±0.26	20.7±0.41	19	82

When the partially purified protease of *Bacillus coagulans* obtained after ammonium sulfate fractionation was subjected to DEAE cellulose chromatography, the protease which was bound by the resin could be eluted from the column with the use of 0.4M NaCl in sodium phosphate buffer pH 7.6.(Fig- 15) It had chymotrypsin like activity. (EA=312.3 U/dl/min).(Table- 8) The pooled fractions in the peak had high specific activity of 20.7 U/mg of protein (Fig 16).

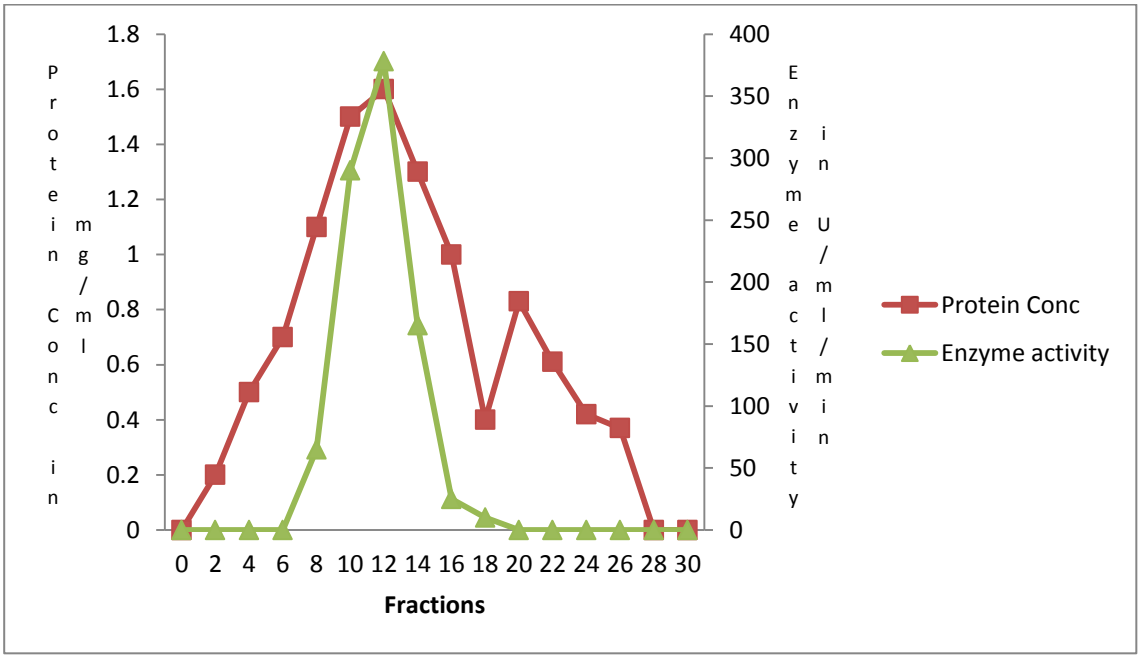


Fig- 15 – Elution pattern of protein and proteolytic activity present after DEAE chromatography of *B. coagulans*

The increase in specific activity of elastase of *P.aeruginosa* and protease of *B. coagulans* used in this study is represented in Tables 9,12 and Figure 16.

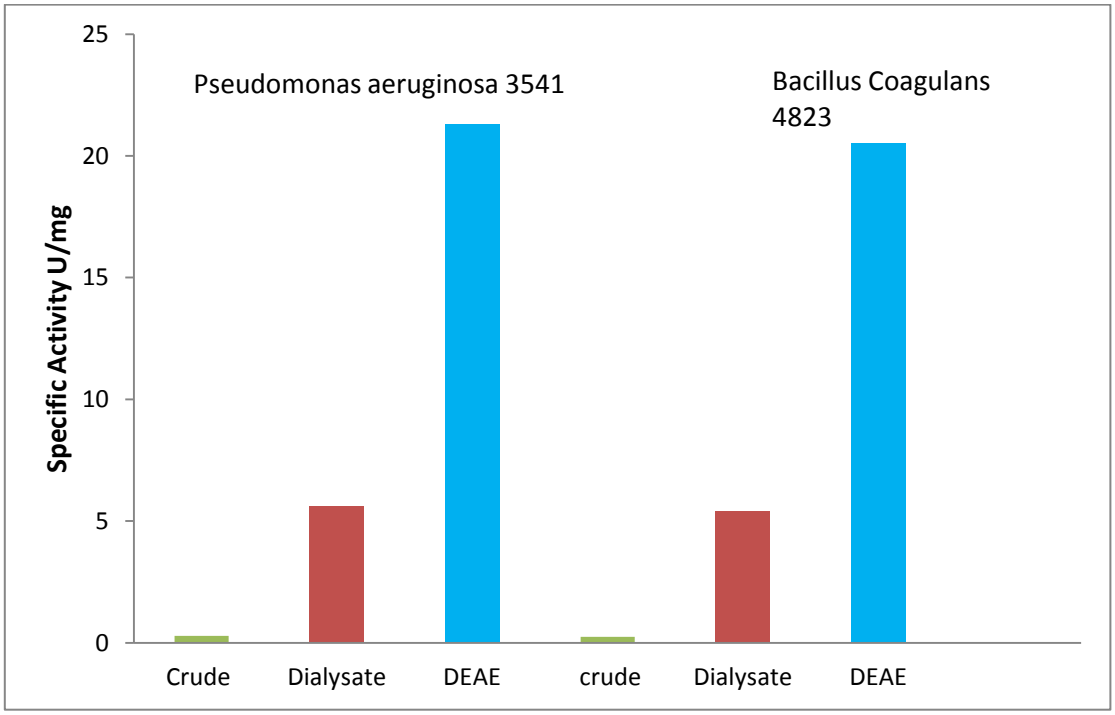


Figure 16 - Increase in Specific Activity after purification steps of the enzymes of P. *aeruginosa* 3541 and B. *coagulans* 4823

As can be seen from the purification protocol in Tables 9 and 12 the elastase of *P. aeruginosa* was purified about 80 fold starting from the culture filtrate , followed by ammonium sulfate precipitation and ion

exchange chromatography .The specific activity increased from 0.28 U/mg in crude form to 21.3 U/mg after DEAE chromatography (Fig -16). The protease from *Bacillus coagulans* was purified to about 80 fold (Table 12,Fig 16)after DEAE cellulose chromatography and its specific activity increased from 0.25 U/mg to 20.7 U/mg.

The elastase of *P. aeruginosa* after DEAE chromatography was subjected to Gel filtration and a single peak was seen . (Fig 17) Fractions containing protein were pooled and estimated for activity on STANA. By this method, elastase of *P. aeruginosa* was recovered with an activity of 9.7 U/dl/min (Table 13).

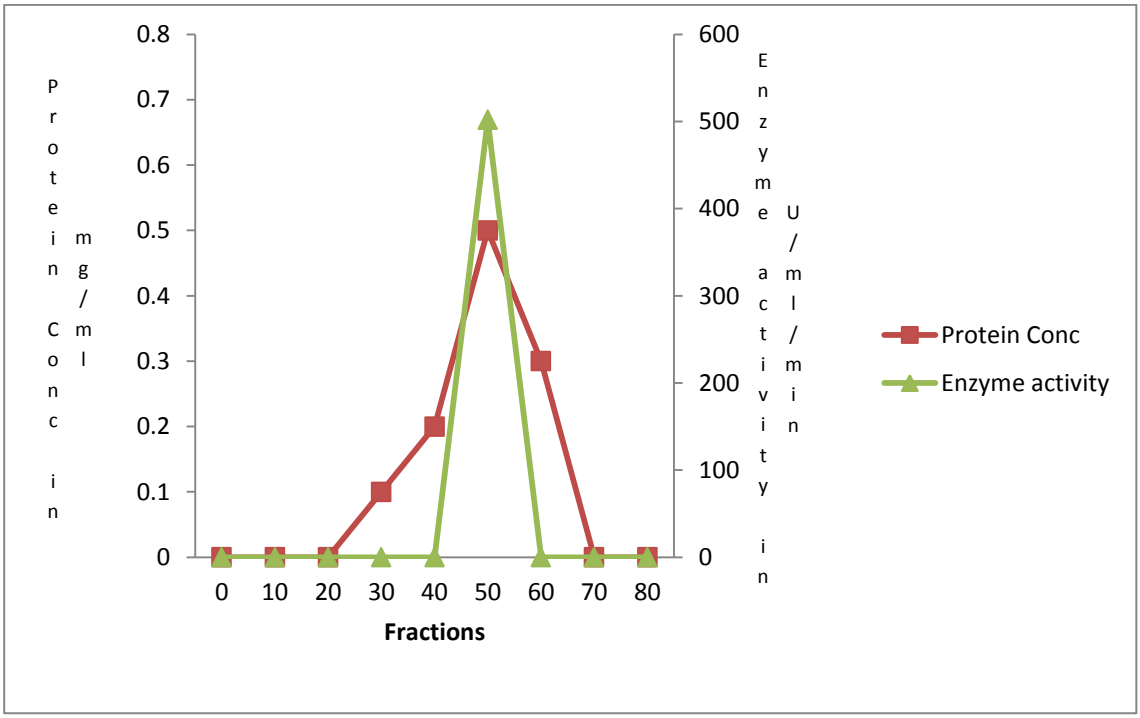


Fig -17 Elution profile of protein after Gel Chromatography of elastase of *P. aeruginosa*

The protease of *B. coagulans* purified by Gel filtration also showed a single peak and elution profile is as shown in Fig 18. It had ATEE hydrolyzing activity with 262.0 U/dl/min on ATEE (Table13).

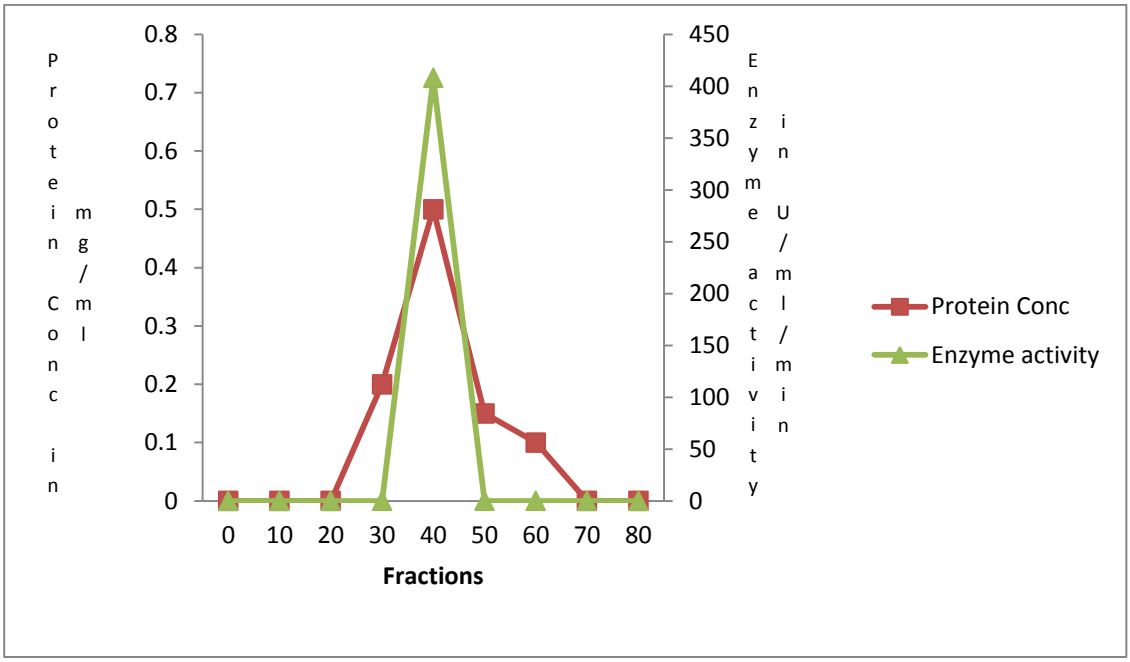


Fig -18 Elution profile of protein after Gel Chromatography of protease *B. coagulans*

Table 13– Activity on synthetic substrates after Gel chromatography

Organism	Activity on STANA U/dl/min	Activity on ATEE U/dl/min
P. aeruginosa	9.7	----------
B.coagulans	----------	262.0

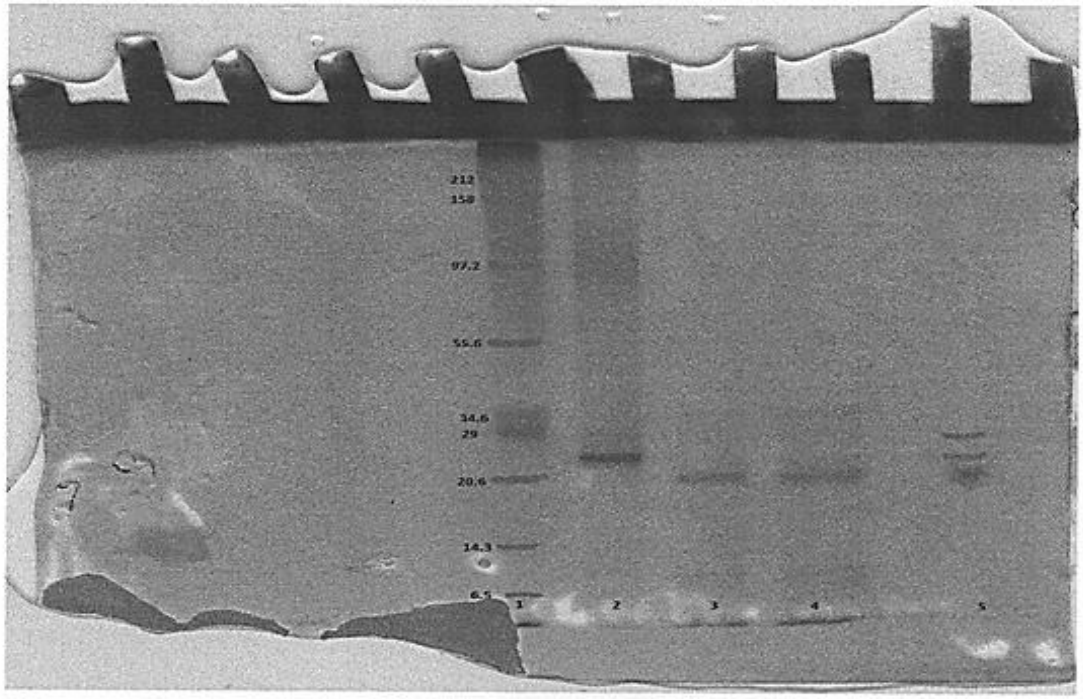
The specific activities of elastase of *P. aeruginosa* and protease of *B. coagulans* were 26 U/mg and 24.7 U/mg of protein after gel filtration and achieved near homogeneity with 95% fold purification.

After DEAE cellulose chromatography, the protease bound by the resin in the case of *P. aeruginosa* was found to be elastase as it hydrolyzed STANA (Table 8). This elastase after gel filtration chromatography was subjected to SDS -PAGE Gel Electrophoresis. When subjected to SDS - PAGE Gel Electrophoresis the elastase of *P. aeruginosa* showed a single band. A single band was also seen with the ammonium sulfate fraction (fig 19). However, it was known from the activity on the synthetic substrates that both STANA and ATEE were hydrolyzed so, probably *P. aeruginosa* had two proteases having a molecular weight very close to each other which was seen as a single band on SDS PAGE gel after ammonium sulfate fractionation.

The ammonium sulfate fraction and the protease of *B. coagulans* after gel filtration were also run on SDS PAGE Gel and showed a single band after Gel chromatography (fig 19). A standard protein marker (New England Biolabs) was run along with the samples. A plot of the logarithm the

molecular weights of the standard proteins against mobility gave a straight line (Fig 20). By interpolation the molecular weight of the elastase of *P. aeruginosa* was around 24.6kDa. For *B. coagulans* the protease was larger with the molecular weight of 28kDa.

So, to determine the molecular weight of the ATEE hydrolyzing enzyme, of *P. aeruginosa* the bound fraction having ATEE hydrolyzing activity obtained after Pseudo Ligand chromatography was concentrated and another SDS PAGE Gel was run. A low grade protein marker (Bio Rad marker range 14-97kDa) was used for this electrophoretic run. A plot of the logarithm the molecular weights of the standard proteins against mobility gave a straight line and by interpolation the molecular weight of the enzyme of *P. aeruginosa* with ATEE hydrolyzing activity was also found to be around 25.2kDa (Figs -21,22).



Lane 1 – Known protein marker (Range 2-212kDa)

Lane 2 – Protease of *B. coagulans* after Gel Chromatography

Lane 3 – Elastase of *P. aeruginosa* after Gel Chromatography

Lane 4 – Protease of *P. aeruginosa* after Ammonium sulfate fractionation

Lane 5 – Protease of *B. coagulans* after Ammonium sulfate fractionation

Fig-19 SDS-PAGE Gel Electrophoresis after Gel chromatography

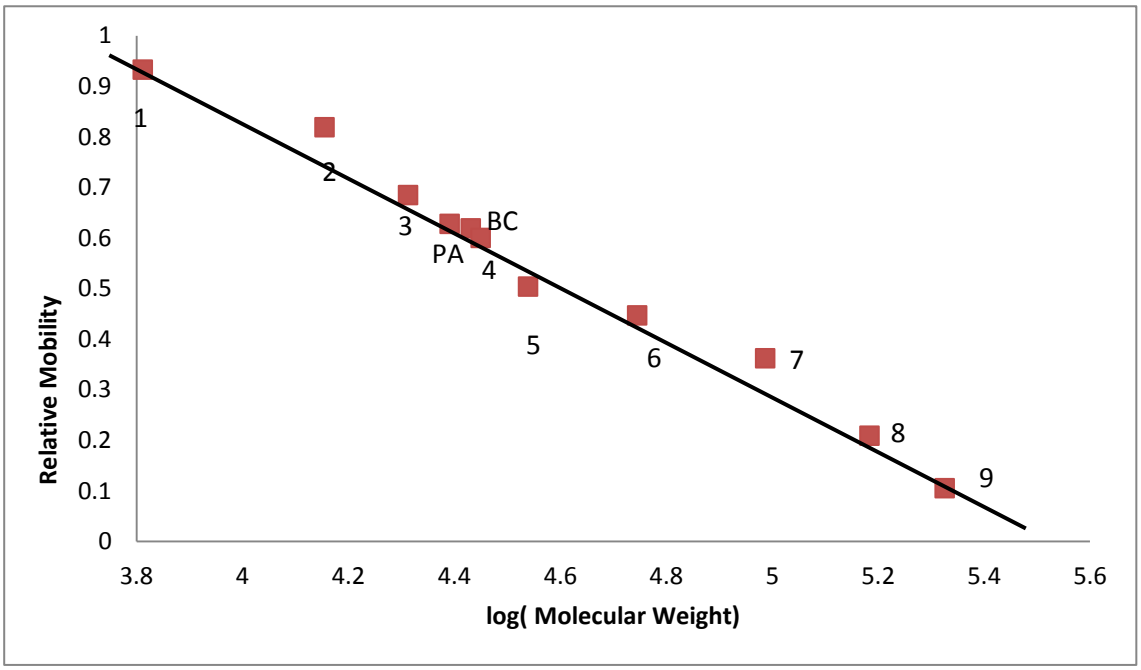


Figure 20 - Determination of molecular weight by SDS PAGE Electrophoresis

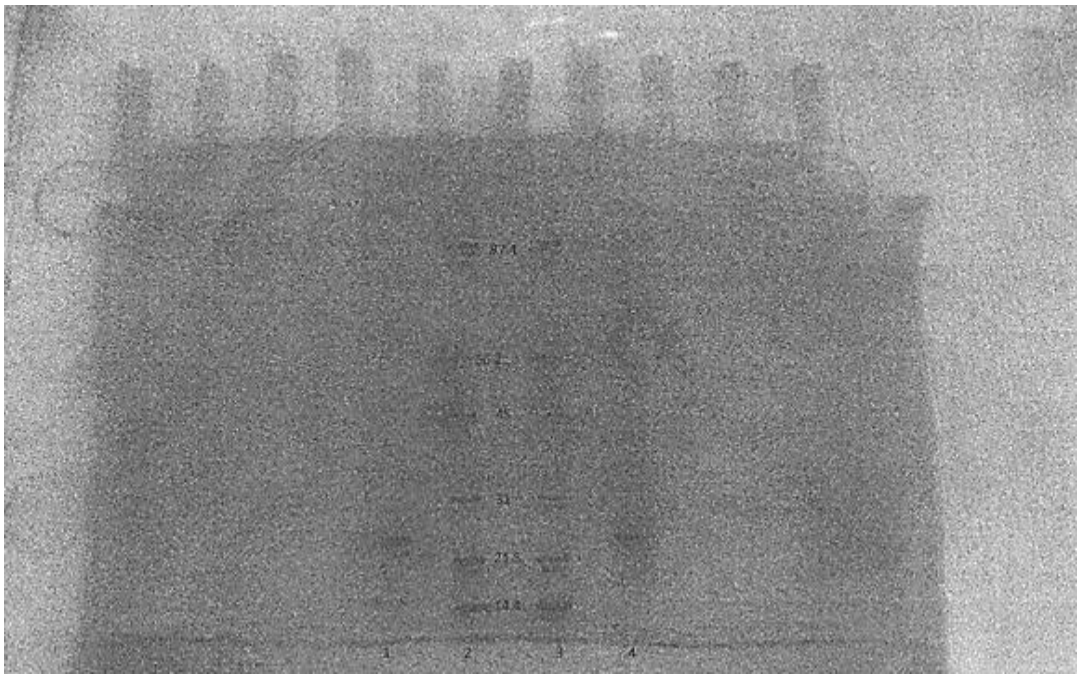


Figure 21 -: SDS PAGE electrophoresis after blue sepharose chromatography

Lane 1 and Lane4- Protease after blue sepharose chromatography

Lane 2 and Lane 3- Low range protein marker (Bio – Rad)

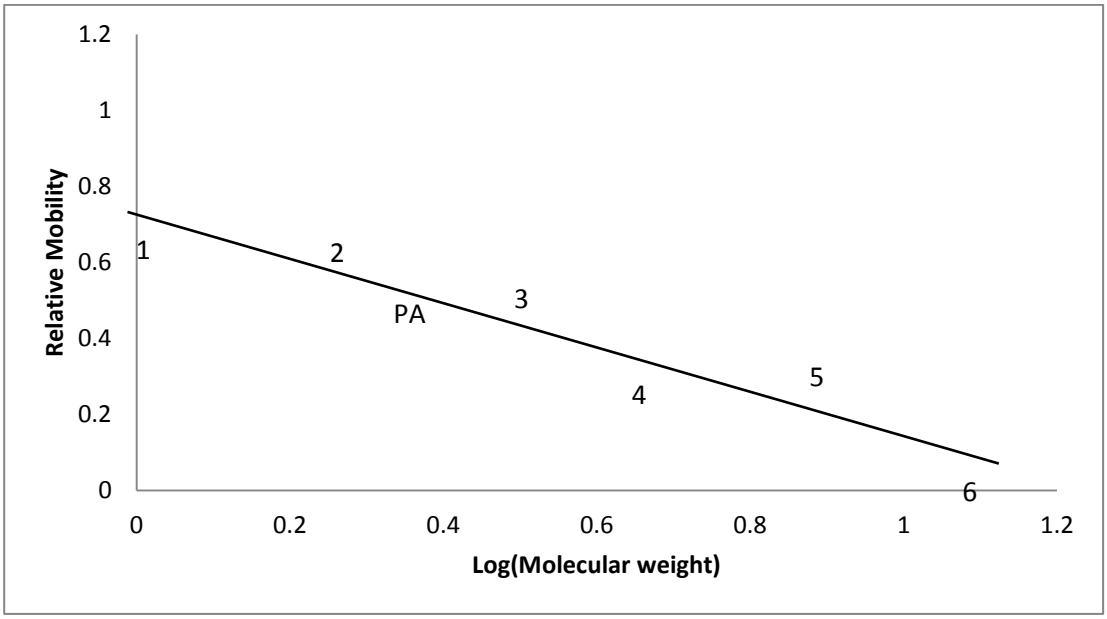


Figure 22 : Determination of molecular weight by SDS PAGE Electrophoresis after Blue sepharose chromatography

The apparent molecular mass of the purified elastase of *P. aeruginosa* was estimated to be around 25kDa. The molecular weight is in accordance with literature reports. Molecular mass of *P. aeruginosa* protease ranges from 20kDa to 50kDa when they are in the form of mature extracellular protease (47). Among the various identified proteases, all of which are in the form of pre proenzymes (with the exception of Las A) they all have a length of more than 400 Amino acids. Molecular weight of the proteases of *P. aeruginosa* has also shown wide variance with use of different strains (179). Molecular mass determination of proteases is difficult because of

the presence of a protease related processing intermediary protein, from which the enzyme is formed (180). The strain of *Pseudomonas aeruginosa* MTCC 3541 used in this study suggests that this strain had 2 proteases having elastase activity and chymotrypsin like proteolytic activity. Morihara et al (181) had isolated a protease from a strain of *P. aeruginosa* which appears homogenous on electrophoresis and having a broad proteolytic activity besides elastase activity. It had then been proposed, that no special properties of the enzyme other than proteolysis are needed to explain its elastase properties. There were no attempts to characterize the protease activity by use of other substrates. It was also postulated that strains having protease and elastase activity are more pathogenic than those which do not have either of the enzymes (182).

The use of Dye ligand chromatography using Blue Sepharose, showed that this dye could bind to the ATEE hydrolyzing enzyme in *P. aeruginosa* as binding with dye and enzyme caused co-elution. Blue Sepharose is one of the more common triazine dyes used for protein purification and has been used to purify albumin and some other enzymes. This study for the first time used this Dye ligand chromatography for purification of bacterial

enzymes. Though the ATEE hydrolyzing enzyme was bound elastase of *P. aeruginosa* was not bound by the dye. This method of purification indicated that there were 2 distinct enzymes produced by *Pseudomonas aeruginosa,* one being elastase and one having chymotrypsin like activity.

The protease from *Bacillus coagulans* consistently hydrolyzed ATEE in the crude form, the partially purified form as well as after DEAE and Gel chromatography. The reduced hydrolysis of ATEE substrate after being subjected to Gel chromatography was probably due to the removal of large amount of proteins with the various purification methods. The protease showed homogeneity with SDS PAGE electrophoresis with a single band corresponding to the molecular mass of around 28kDa.There is a large variation in the molecular masses of *Bacillus* species though it rarely more than 50kDa (183)

As a result of the screening studies in the previous chapter an interesting observation was made that *Acetobacter aceti* an organism not noted for the proteolytic activities was found to secrete proteases which hydrolyzed ATEE. So, it was thought worthwhile to partially purify this protease and study some of its characteristics. The results obtained after ammonium

sulfate precipitation of *Acetobacter aceti* indicated that the enzyme activity in this fraction showed caseinolytic activity (182 units/ml/min) and a preference to only ATEE (1600 units/dl/min) among synthetic substrates. The concentrated fraction also did not exhibit any hydrolytic activities on STANA and BAPNA. The protease of *A.aceti* after ammonium sulfate precipitation was also studied for its thermal and pH stability. The concentrated fraction after ammonium sulfate fractionation also provided similar data to that of crude preparation indicating that the major extra cellular protease produced by this organism is a serine protease with substrate specificity similar to chymotrypsin.

Thermal stability:

The thermal stability of the elastase from *Pseudomonas aeruginosa* showed that it was stable upto 60 ^{0}C with a significant loss of activity of more than 50% at 80^{0}C (Fig 23)

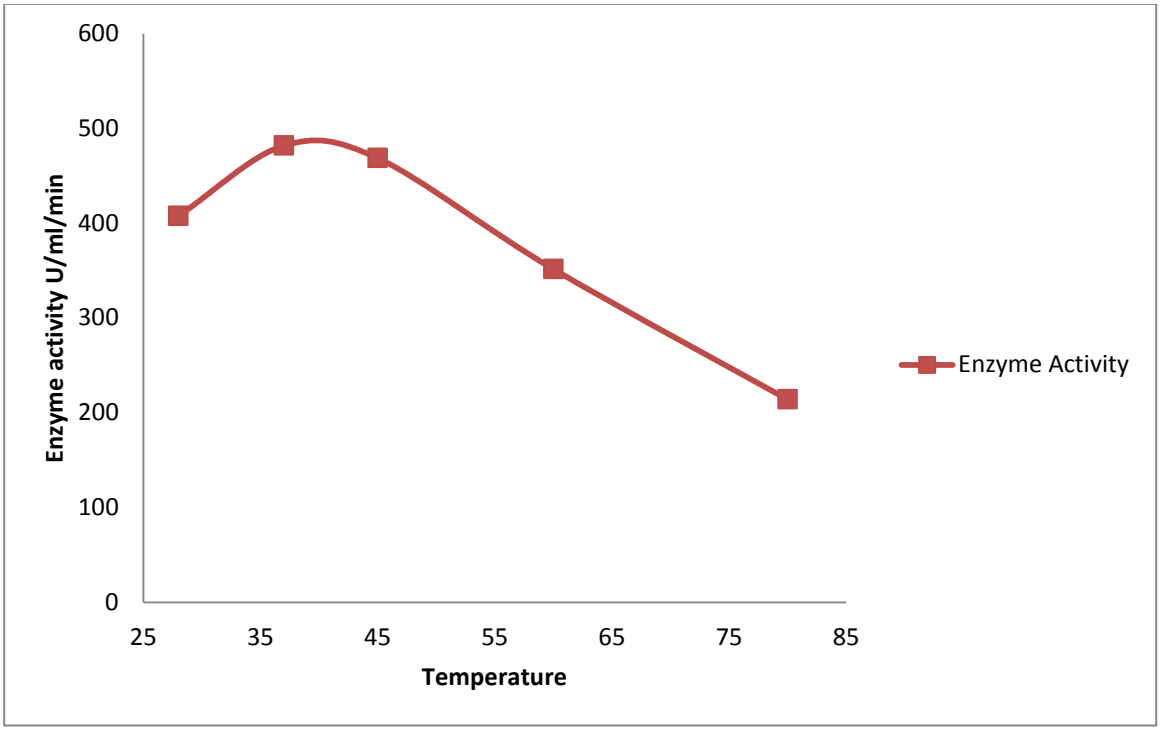


Fig - 23 Thermal stability of protease of P. aeruginosa using casein as a substrate

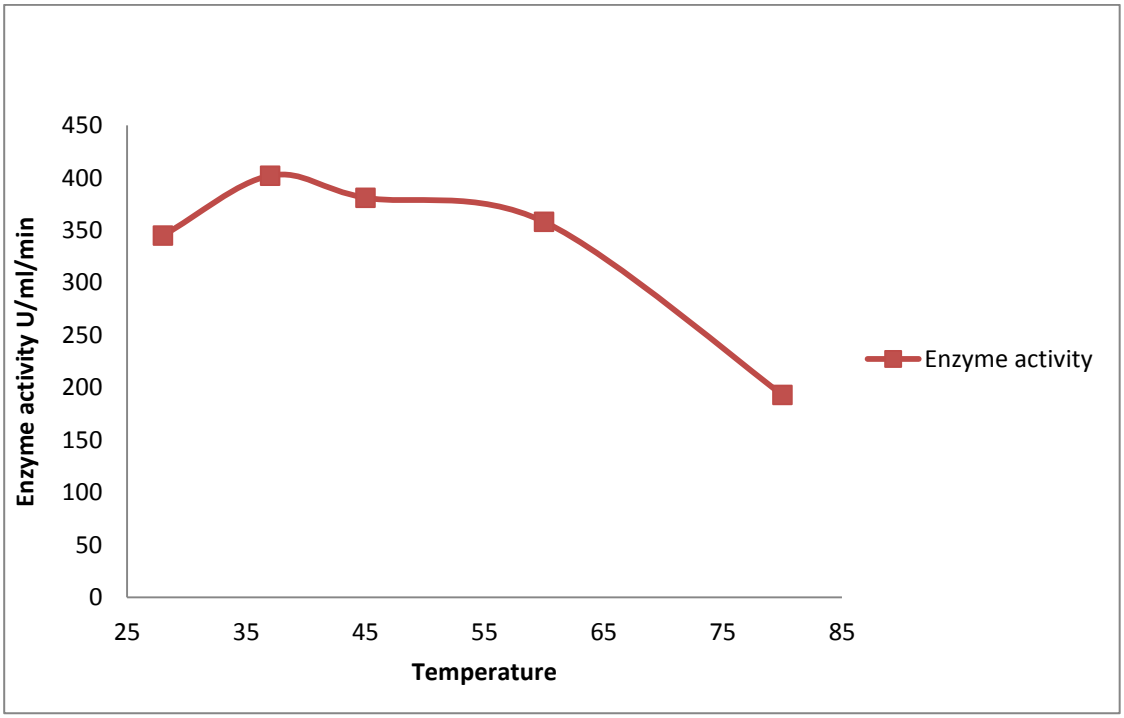


Fig -24 Thermal stability of protease of B. coagulans using casein as a substrate

The protease of *Bacillus coagulans* exhibited thermal stability upto 60^0C with significant loss of activity above 80^0 C which showed that the enzyme is denatured at this temperature (Fig 24)

Table –14 Thermal stability studies of *Acetobacter aceti* 3246

Temperature	28°C	37°C	45°C	60°C	80°C
Activity U/ml/min	111.2	144.5	95.3	49.1	02.3

The protease of *A.aceti* exhibited thermal stability upto 45°C.(Table -14)

pH stability

The data obtained on pH stability showed that the elasatse of *Pseudomonas aeruginosa* was stable over a range of pH from 5.0 to 9.0. Similar results were obtained for the enzyme of *Bacillus coagulans* (Fig - 25 and 26) .

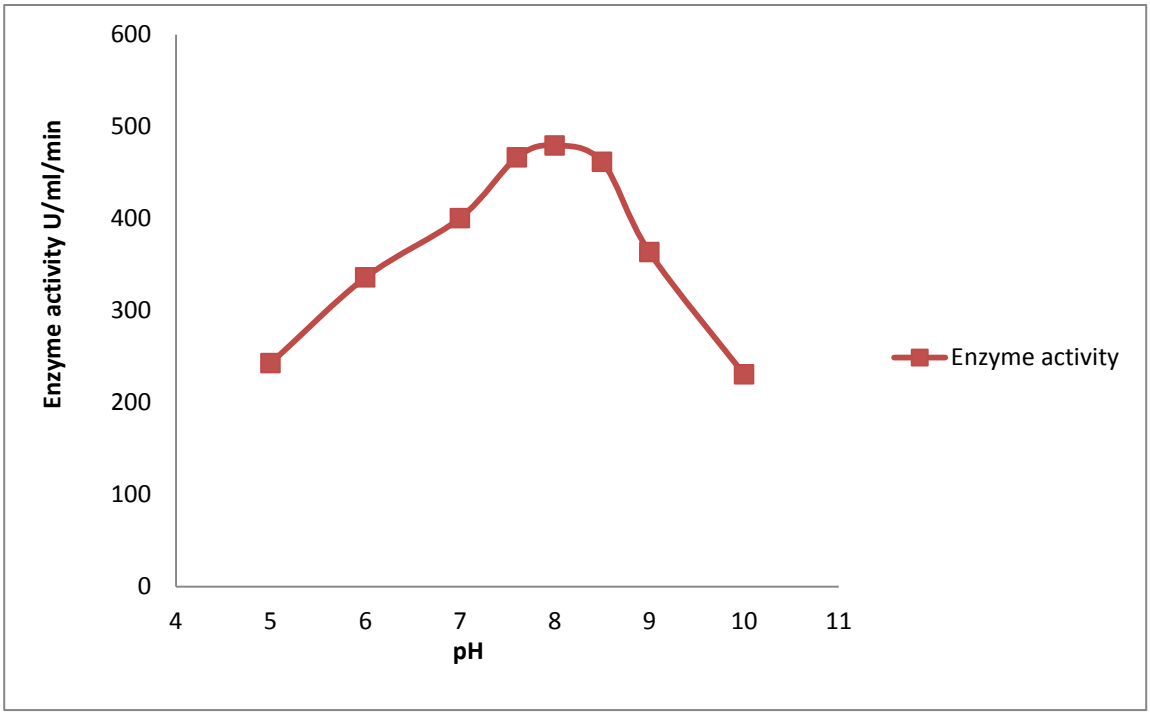


Fig -25 pH stability of protease of P. aeruginosa using casein as a substrate

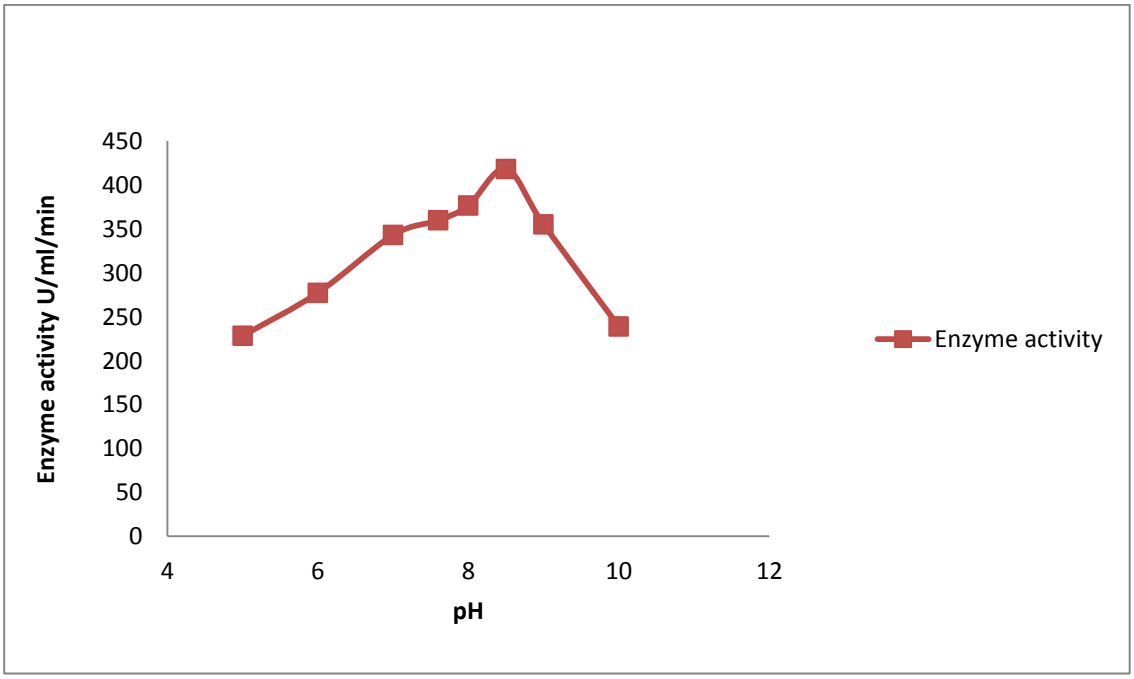


Fig -26 pH stability of protease of B. coagulans using casein as a substrate

Table 15 - pH stability studies of *Acetobacter aceti* 3246

pH	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Activity U/ml/min	72.2	83.6	96.8	132.1	148.3	128.2	70.1	20.4

The protease of *A.aceti* after ammonium sulfate precipitation showed that the protease was stable over a wide range of pH in the range of 3.0 – 8.0(Table 15)

The pH studies showed that the elastase of *Pseudomonas aeruginosa* had a range of pH stability from 5.0 to 9.0 and was most active in the pH range of 7.0-8.0. This pH stability is similar to that of a strain ME 4(184) and sp Dr 89(185). On the other hand it was different from the San Ani Strain which showed stability from pH 5.5- 11.5(186)

The temperature stability was determined by the purified enzyme in a range of temperatures for 30 minutes. The enzyme was stable upto 60^0C showing substantial loss of activity at 80^0C. Many of the *Pseudomonas aeruginosa* strains have shown thermal stability upto 60^0C (186, 187).

The protease of *Bacillus coagulans* showed a pH stability range of 5.0-9.0. . Many species of *Bacillus coagulans* have reported high thermostability retaining upto 90% of original activity at temperature of 60^0C (188). Protease of this species are also reported to be stable under alkaline conditions indicating their potential use in detergent and leather industry

(189). Most studies have reported pH optimum around 8.0, though activity falls sharply after a pH of 9.0 (190).

The protease of *A.aceti* after ammonium sulfate precipitation which was studied for its thermal and pH stability showed that the protease was stable over a wide range of pH in the range of 3.0 – 8.0 and exhibited thermal stability upto 45°C.(Tables 14,15) The studies on pH and temperature stability indicated that this enzyme exhibited closer relationship with chymotrypsin in respect of pH stability in the range of 3 to 8 and temperature stability upto 45^{o}C.

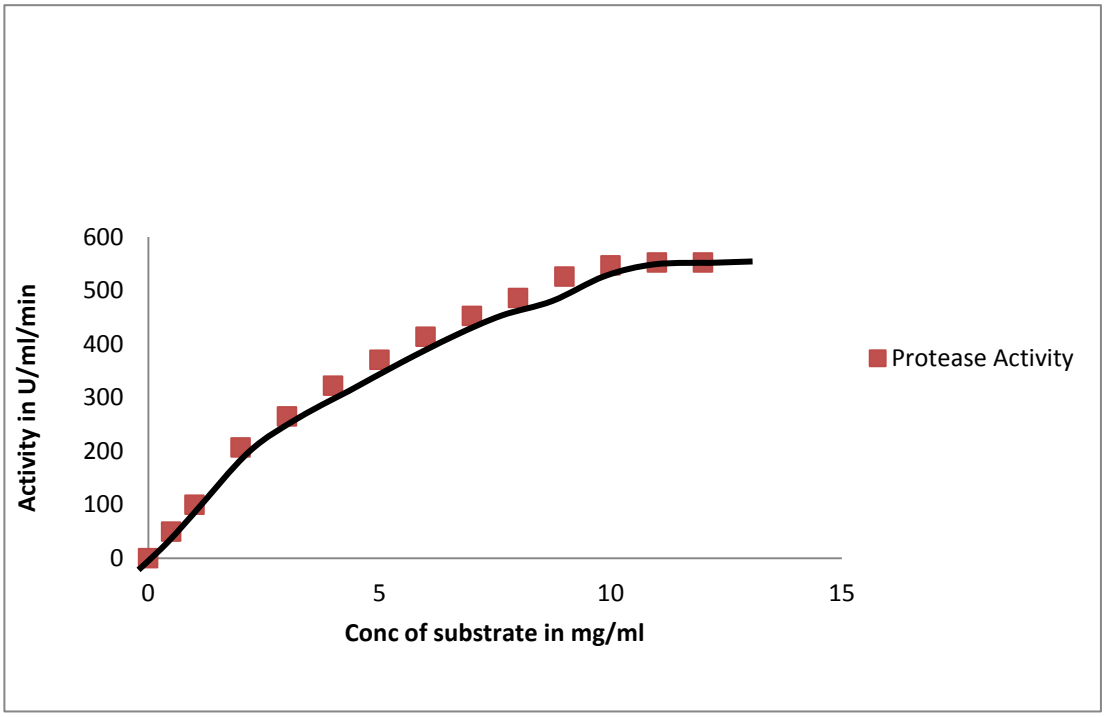


Fig -27 Substrate saturation curve of elastase *of P. aeruginosa* using casein as a substrate

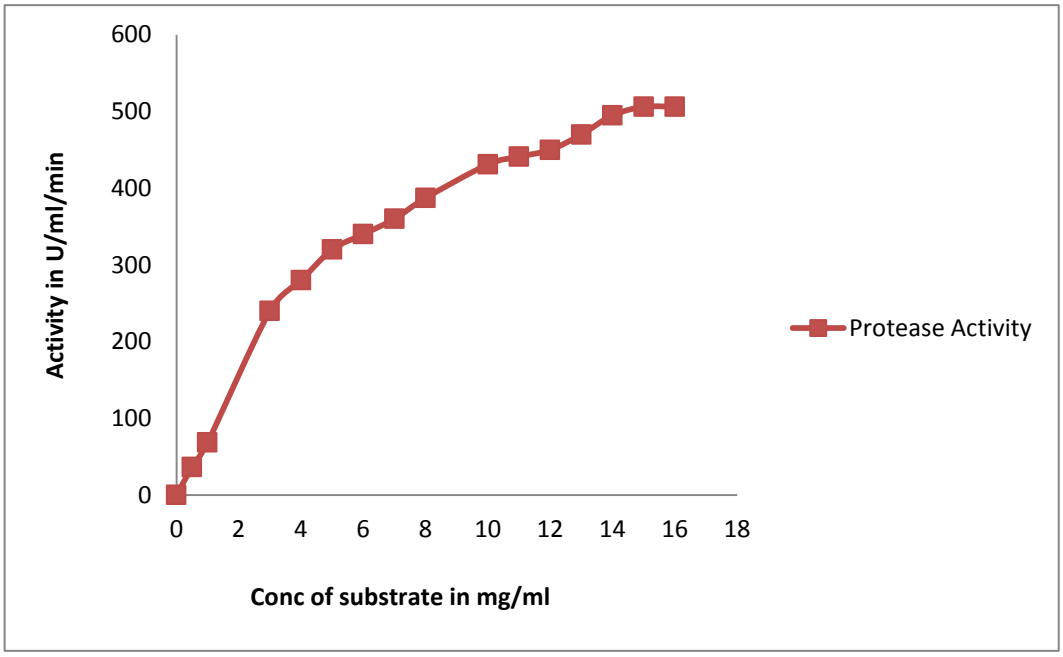


Fig -28 Substrate saturation curve of protease of *B. coagulans* using casein as a substrate

The values of the Kinetic parameters Km and Vmax were calculated from substrate saturation curves with Casein as the substrate (Fig 27) The elastase of *Pseudomonas aeruginosa* had a Km of 3.3mg/ml Vmax of 550 U/ml/min on Casein as the substrate at a concentration of 12 mg/ml after which its reached saturation. The assay was done at a temperature of 37^0C and pH 7.6.

Similarly, the protease of *Bacillus coagulans* showed a V max of 506 U/ml/min at a substrate concentration of 16 mg/ml after which it reached saturation. The Km of the protease was 4.4mg/ml as per substrate saturation curve (Fig 28).

A comparison of the kinetic properties of the proteases of a pathogenic organism, i.e. elastase of *Pseudomonas aeruginosa* and a non-pathogenic organism *Bacillus coagulans* were undertaken to see if there were marked differences in the kinetic parameters which could have given an insight into the activity of these enzymes. However, an analysis of the results of these two enzymes did not show any marked differences in their kinetic properties.

Conclusion:

In the case of *Acetobacter aceti* , the specificity on the substrates Casein and ATEE by the crude enzyme in the culture supernatant is identical with that of the more pure enzyme obtained after ammonium sulfate fractionation and suggests that, the extracellular enzyme produced by this strain appears to be predominantly a chymotrypsin like serine protease. However, the possibility of more than one protease produced by other strains or under different conditions cannot be excluded.

Protease of *B. coagulans* had molecular weight of around 28kDa and had chymotrypsin like activity while elastase of *Pseudomonas aeruginosa* had a molecular weight of 24.6kDa i.e. around 25kDa and the other chymotrypsin like enzyme also from *Pseudomonas aeruginosa* had a similar molecular weight of 25.2kDa. Most studies on *P. aeruginosa* focuses only on elastase and its purification. This study has shown that the strain used *P. aeruginosa* MTCC 3541 was having two proteases with almost identical molecular weight. While elastase was bound by DEAE cellulose resin, the other protease which hydrolyzed ATEE was bound by the dye ligand Blue Sepharose. SDS – PAGE gel showed that there were

two distinct proteases capable of hydrolyzing STANA and ATEE respectively.

The Bacillus species have been widely exploited for their ability to produce alkaline proteases on a commercial scale. The attempt made in this study, to characterize the protease by the use of synthetic substrates, showed that the enzyme of this strain had chymotrypsin like activity.

Since, the focus of this study is mainly on elastase, for further studies such as, inhibition by anti elastase factors in plant extracts the focus was more on elastase obtained and purified by *Pseudomonas aeruginosa*-3541.

Chapter V

Comparative Inhibition studies on elastase of Human and Bacterial origin

Introduction –

Enzymes are catalysts and they enhance the rates or velocities of chemical reactions in biological systems. Essentially an enzyme brings about a reaction after formation of enzyme substrate complex which is influenced by several factors which include the concentration of the enzyme and substrate in addition to temperature, pH, ionic strength of the medium of reaction as well as the presence of activators and inhibitors. The complex formations follow certain kinetic patterns which provide insights in to molecular characteristics of the enzymes. In general the enzymes (E) and the substrate (S) form a complex (ES) before the formation of the products. In majority of the cases, the enzymes follow reaction kinetics and exhibits a hyperbolic curve and in some cases it exhibits a sigmoidal pattern (191). The hyperbolic saturation curve is exhibited by enzymes with single polypeptide chain but the sigmoidal curve by the allosteric enzymes which possess more than one polypeptide chains (191).

The substrate saturation curve helps to know the affinity of the substrate to the enzymes and this leads to concepts of Km and V max in respect of

enzyme actions. These parameters are utilized effectively to identify the most appropriate substrates or inhibitors for a particular enzyme under given set of conditions. The kinetic parameters also provide information for the development of synthetic substrates and inhibitors and have a great role in the development of drugs where the enzymes are key targets. Diseases can involve alteration in enzymes and their activity. So, an understanding on how an enzyme works is critical for analyzing how drugs function by interaction with enzymes.(23,24)

There are several mechanisms to control enzyme catalyzed reactions and inhibition of enzymes is one of the major control mechanisms in biological systems. Inhibition studies give an insight into the mechanism of enzyme action and specific inhibition can be utilized to identify amino acid residues which are critical for an enzyme's catalytic activity. Knowledge on the inhibitor enzyme interactions is important for drug discovery and development as it helps in making quantitative comparisons of different compounds against an enzyme. Quantitative assessment of an inhibitor's potency can be obtained by measuring the concentration of an inhibitor

required to bring about 50% reduction in enzyme activity under a specific of conditions and is referred to as IC_{50}. (192).

Human neutrophil elastase which is released during the attack of an invading microorganism can be inhibited by anti proteolytic substances secreted by the microorganism which would try to combat the destructive nature of Human Neutrophil Elastase (HNE.) Uncontrolled secretion of HNE is restricted by the Serpins, principally, $\alpha_1$antitrypsin(α_1AT), α_2 macro globulin (α_2MG), elafin and secretory leukocyte inhibitor (193) They inhibit target proteases by forming a stable covalent complex. Of these serpins, α_1AT is a 52 kDa glycosylated protein which is the major physiological inhibitor of a range of serine proteases and maintains a protease- anti protease balance. The α_2MG is a large spectrum protease inhibitor whose main functions are to target endogenous or exogenous proteases (194).

Serpins, inactivates serine proteases especially those structurally related to trypsin.. These Serpins bring about inhibition by mimicking the three dimensional structure of the protease so that the protease binds to it. (195). α_1AT is synthesized in hepatic parenchymal cells and are also catabolized

in hepatic parenchymal cells. Inhibition begins by docking of the serpin and protease and formation of a complex. In one mechanism, α_1AT protease complexes are removed by Serpin enzyme complex receptors. In the other mechanism, desialylated α_1AT is removed by hepatic asialoglycoprotein receptors.

α_1AT is the most abundant proteinase inhibitor in plasma and physiologically the most important inhibitor of leukocyte elastase, released during phagocytosis. This enzyme also reacts with elastin in the tracheobronchial tree. α_1AT's small molecular size and facility of diffusion into these tissues is important for prevention of elastic recoil. Uninhibited elastase in bronchial tree is due to excess elastase or deficiency of α_1AT and lead to emphysema. α_1AT concentrations are increased by estrogens and low in neonatal respiratory distress, severe pancreatitis and proteins losing disorders. Decreased α_1AT is associated with high risk of pulmonary emphysema. A deficiency of α_1AT is associated with diseases of the liver. (196)

α_2 Macroglobulin (α2MG) is a major plasma proteinase inhibitor, unlike α_1AT , it is a very large molecule and does not diffuse from plasma in to

ECF. It inhibits different proteinases including those with serine, cysteine and metal ions in their proteolytic sites. It is synthesized in hepatic parenchymal cells . The levels in children and infants are 2-3 times higher than adults probably as a protective mechanism against exposure to intestinal proteases and bacterial or leukocytic proteases. Increased levels are seen in nephrotic syndrome because of renal loss of low molecular mass proteins and increased hepatic synthesis to compensate this. Decreased concentration is seen in acute pancreatitis (196). From this it can be seen that both α_1AT and α2MG play an important role in the control of proteases and might also have an important role in inhibiting the proteases released by invasive bacteria.

Elastase released by microorganisms is a virulent factor and elastolysis is important for pathogenesis as many of the tissues contain elastin. The multiple virulence components of *P.aeruginosa* work together to inactivate the hosts' immune response (197) *P. aeruginosa* elastase rapidly inactivates $\alpha_1$antitrypsin inhibitor by splitting its Pro-357 -Met 358 peptide bond. (132) Pure crystalline elastase was a potent inactivator of α_1 Anti Trypsin and the enzyme inactivated the inhibitor within an hour at 25^0C at molar ratio of E/1=1:100. Crystalline protease of *P. aeruginosa* also

inactivated the inhibitor but a 100 fold less (134) The affinity of α_1AT is however much higher for trypsin rather than elastase (134). Pseudomonal proteases can inactivate α_1AT inhibitor in vivo during Pseudomonal diseases, and the loss of this inhibitor activity may allow the endogenous serine proteases to cause tissue destruction.

The α_2MG also has been found to inactivate pseudomonal proteolytic enzyme (136). So, successful establishment of an infection depends on the ability of the bacteria to overcome the host defence or the efficiency of the host to destroy the invasive microorganism. Hence, it was felt worthwhile to study the inhibitory effect of these endogenous protease inhibitors on HNE and bacterial elastase to know the relative preferences or affinities.

Materials and Methods:

Materials:

Sources of the enzyme used were elastase of *P. aeruginosa,* protease of *B. coagulans*, (purified as per the protocol explained in Chapter –II.) and HNE (E8140 Sigma) . Inhibitors used α_1 Anti Trypsin, α_2 Macroglobulin (A9024, 63013 procuded from Sigma USA).

All other chemicals used were analytical grade and obtained locally.

Methods:

Procedure of inhibitory assay of *P. aeruginosa* elastase and HNE by α_1AT and α_2MG

The assay of elastase using STANA as the substrate has been described already in Chapter I. Varying concentrations of the inhibitors α_1AT(0.5-4.0µg/ml) and α_2MG (4.0-20.0µg/ml) were preincubated with HNE and *P.aeruginosa* elastase for 20 minutes. After 20 minutes preincubation, the reaction was initiated by fixed volume of the substrate. It was then kept for incubation for 15 minutes at 37^0C. After this, the reaction was stopped by

the addition of 1 ml of 30% Acetic Acid. The residual activity was determined by estimating the amount of p. nitroaniline liberated at 410nm.

Similarly, a fixed concentration of HNE was taken (concentration equivalent to 10U/ml). The same assay procedure was followed with varying concentration of the inhibitors ie. α_1AT (0.5-4.0µg/ml) and α_2MG (4.0-20.0µg/ml). The pre incubation period was 20 minutes and the reaction was initiated by addition of the substrate STANA. After stopping the reaction by the addition of 1 ml 0f 30% Acetic acid, the residual activity was calculated by measuring the p-nitroaniline released at 410nm. Suitable controls were also run without the inhibitor.

The inhibitory activity was determined by the difference between activity of the bacterial elastase, HNE and then the residual activity of the same solution after adding the inhibitor.The percentage of inhibition was calculated using Inhibition (%) = [1-(B/A)] X100 where A is the activity of the enzymes without inhibitor and B is the activity in presence of inhibitor .IC_{50} was determined for both elastase of *P. aeruginosa* and HNE with varying concentrations of the inhibitor required to bring about 50% inhibition.

Procedure of inhibitory assay of *B. coagulans* using casein as substrate.

The substrate used was casein and the assay was carried out by the method of Sumathi et al mentioned earlier (168) and the enzyme concentration was fixed at 10 U/ml.The inhibitor source was varied concentrations of α_1 AT (0.5-25.0µg/ml) and $\alpha_{2\ MG}$ (4.0-50.0 µg/ml). The inhibitors were pre incubated for 20minutes with the enzyme. The reaction was initiated with 1% Casein. The residual activity was determined by measuring the optical density at 540nm. Suitable controls and blanks were also included in the assay along with the test. The inhibitory activity was determined by the difference between the activity of the enzyme without inhibitor and the residual activity of the reaction system after addition of the inhibitor.

The percentage of inhibition was calculated using Inhibition (%) = [(1-(B/A)] x 100 where A is the activity of the enzymes without inhibitor and B is activity in presence of the inhibitor.

Results and Discussion:

The kinetics of the enzymes *P.aeruginosa* elastase and HNE was initially studied with the specific synthetic substrate STANA. . Km values had been determined with varying concentrations of STANA in the range of 20.0mM to 300mM. Km value was determined by Lineweaver Burke plot for HNE as 167mM and Vmax was 11.1 U/dl/min as represented in Fig 29.

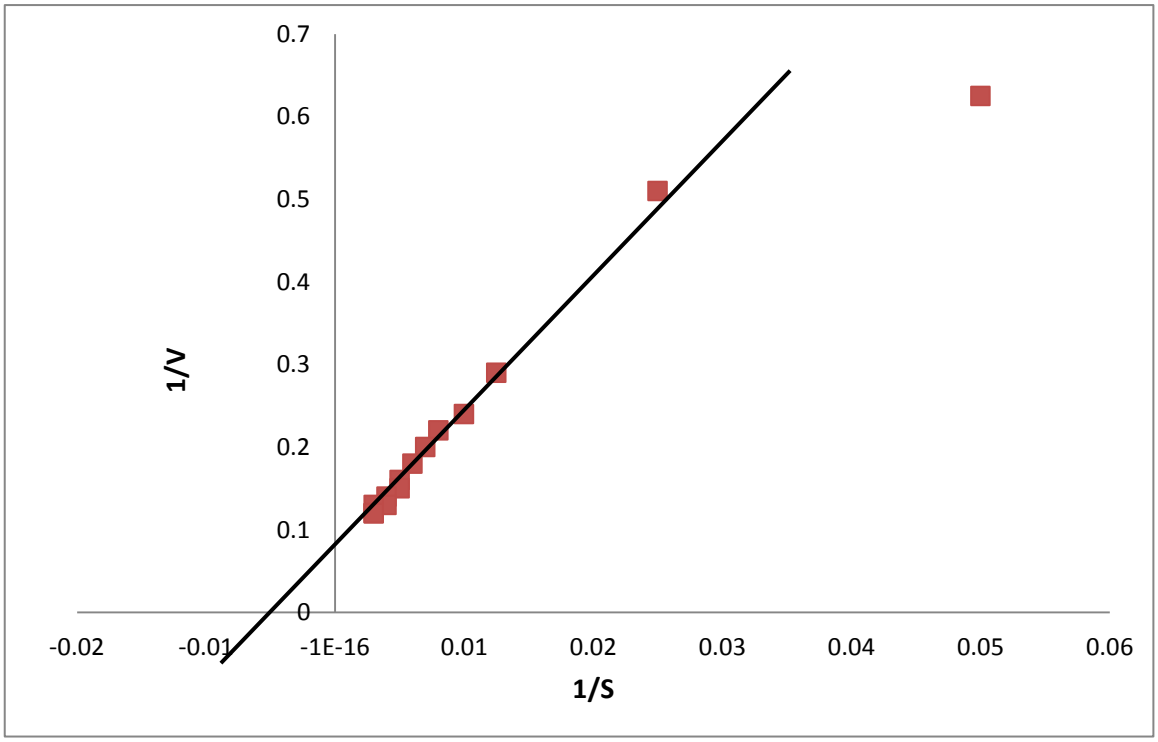


Fig – 29 Lineweaver Burke plot for HNE on STANA (Km = 167mM and Vmax = 11.1 U/dl/min)

Similarly, Km was calculated for elastase of *P. aeruginosa* using substrate STANA (concentration of STANA in the range of 20.0mM to 300mM) . Km was 153mM and Vmax was 12.5 U/dl/min.(Fig 30). The data indicate closer reaction kinetics for HNE and *P.aeruginosa* elastase under given set of conditions.

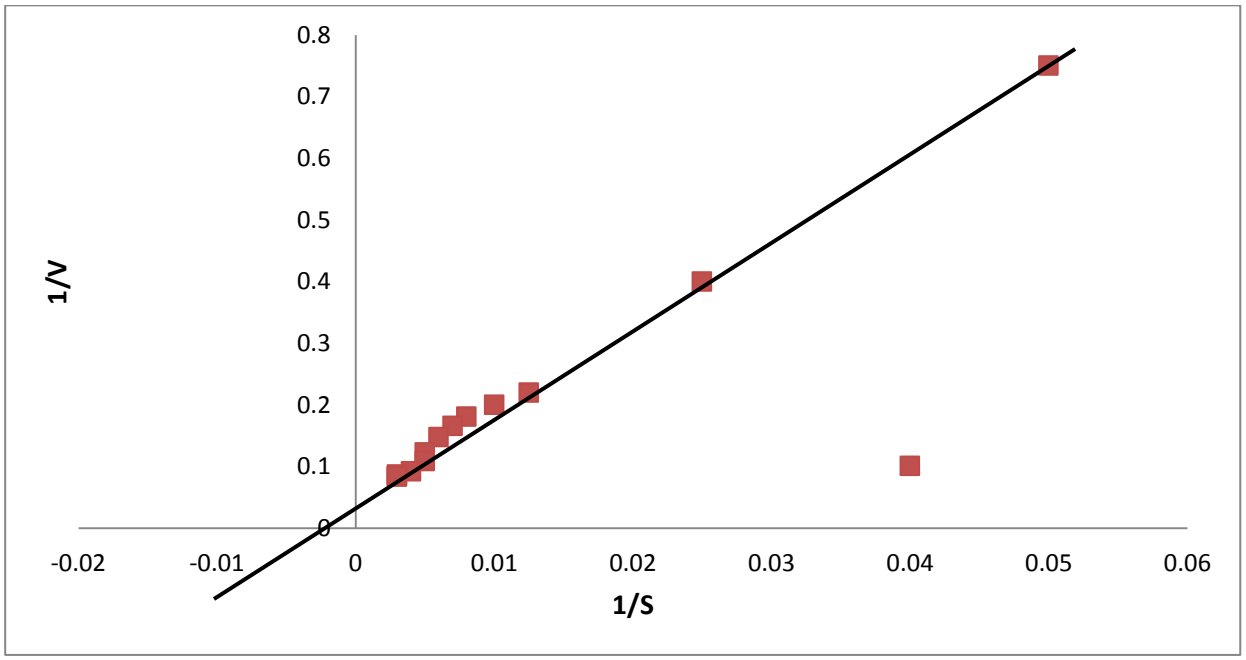


Fig – 30 Lineweaver Burke plot for P.aeruginosa elastase on STANA (Km = 153mM and Vmax = 12.5 U/dl/min)

Following the determination of the kinetic parameters ie. Km and Vmax for HNE the effect of varying concentrations of the inhibitors α_1AT and α_2MG on HNE with fixed concentration of the substrate were determined to study the type of inhibition. The reaction kinetics indicate that Vmax was changed with out affecting Km suggesting non competitive type of inhibition.The effect is represented in Fig 31 and 32 for HNE .

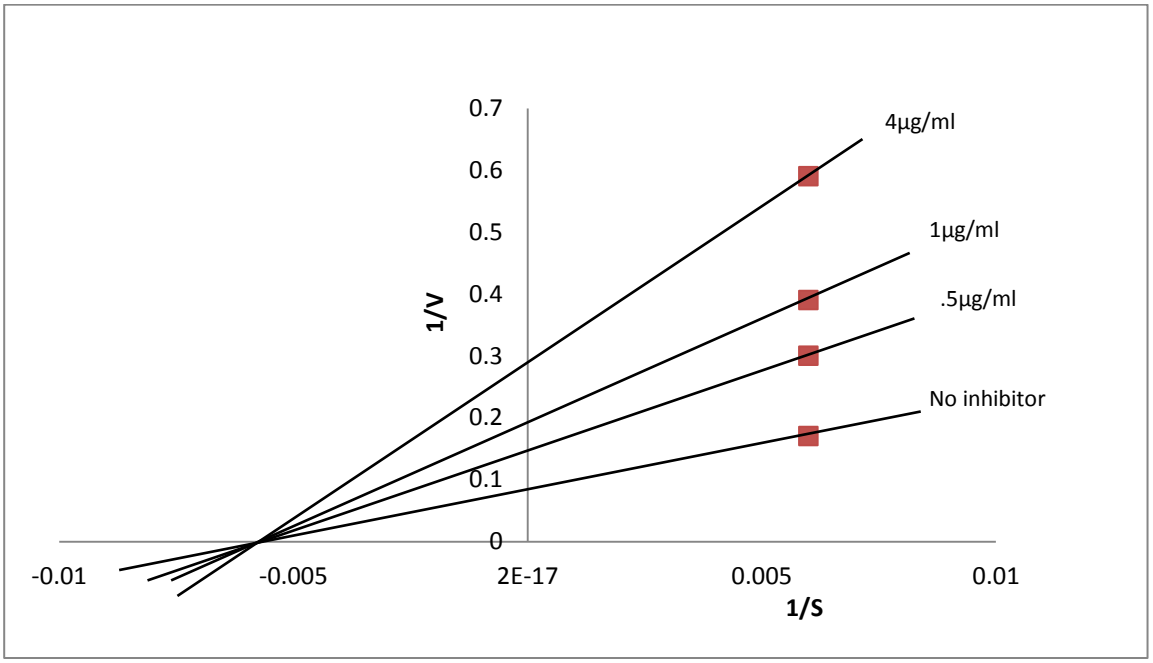


Fig – 31 Inhibition of HNE by α1 Anti Trypsin with STANA as substrate

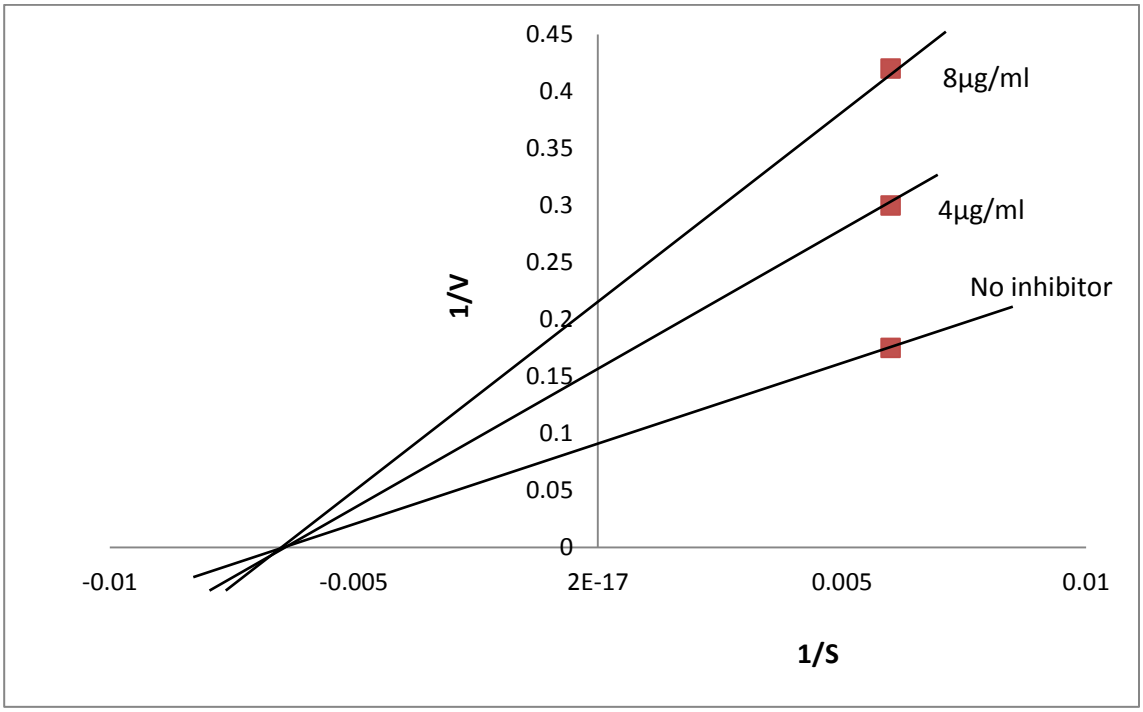


Fig – 32 Inhibition of HNE by α2 Macro Globulin with STANA as substrate

Similarly, the effect of varying concentrations of the inhibitors α_1AT and α_2MG on *P.aeruginosa* elastase with a fixed concentration of the enzyme and substrate were done and the results are depicted in Figs 33 and 34.

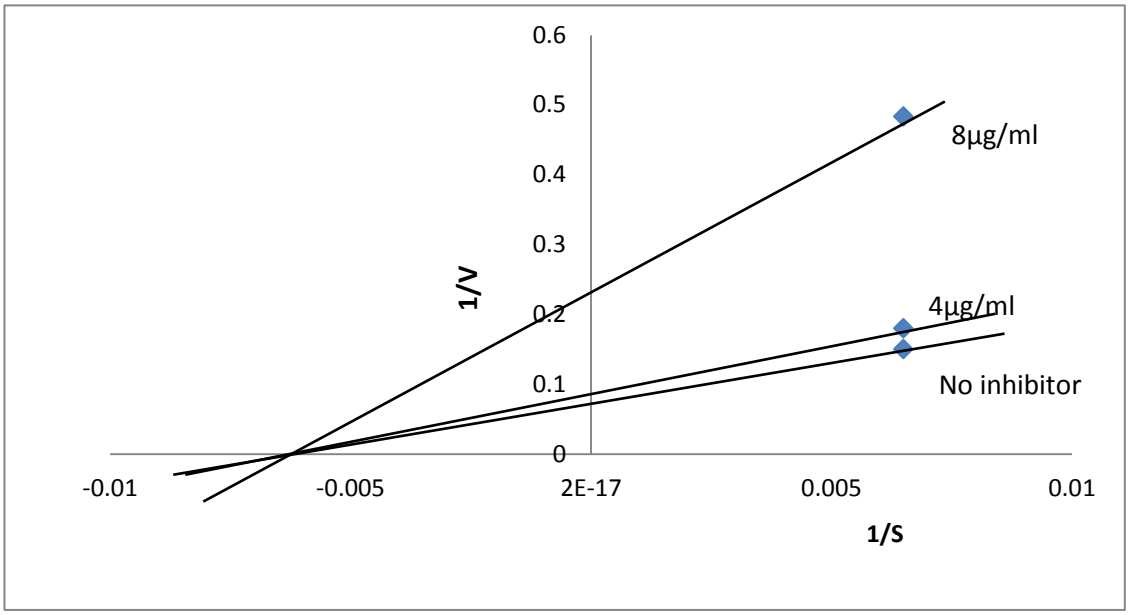


Fig – 33 Inhibition of *P. aeruginosa* elastase by α1 Anti Trypsin with STANA as substrate

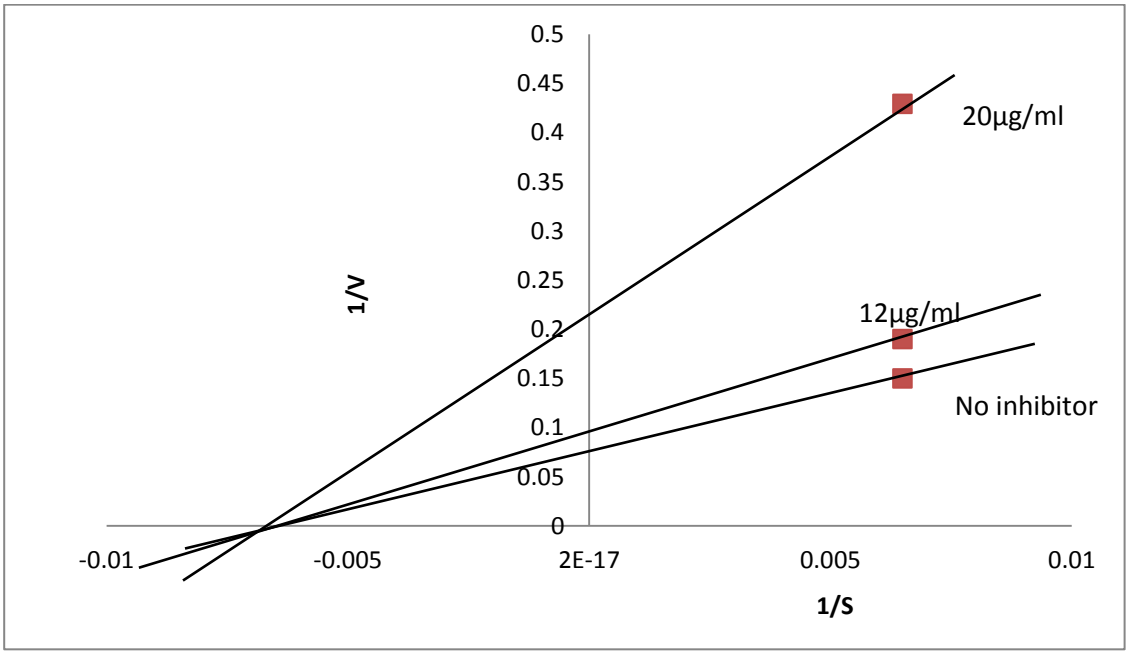


Fig – 34 Inhibition of *P. aeruginosa* elastase by α2 Macro Globulin with STANA as substrate

It can be seen that under the given set of conditions , these Serpins behaved as non competitive inhibitors as in the presence of varying concentrations of α_1AT and α_2MG as the Km values were unaltered but the Vmax was decreased. Literature survey shows that once an irreversible complex is formed it is physiologically destroyed. However, a review of literature did not seem to have much information as to the interaction between α_1AT, α_2MG and HNE and that of *P. aeruginosa* elastase and nature of inhibition. Km value was HNE as 167mM and Vmax was 11.1 U/dl/min while for *P.aeruginosa* elastase Km was 153mM and Vmax was 12.5 U/dl/min.

Though it has been shown that α_1AT exhibited hundred times more affinity to HNE than *P. aeruginosa* elastase by Morihara et al (134) the substrate used in that study was elastin, a natural substrate while a synthetic substrate has been used in this study.

In order to quantitatively explain the amount of α_1AT and α_2MG required to bring about 50% inhibition of HNE and *P. aeruginosa* the assay was performed with varying concentrations of the inhibitors (α_1AT and α_2MG). The results indicated a preferential inhibition of HNE by α_1AT and α_2MG

than *P. aeruginosa* elastase as reported earlier. The results are as depicted in Figs 35,36,37 and38..

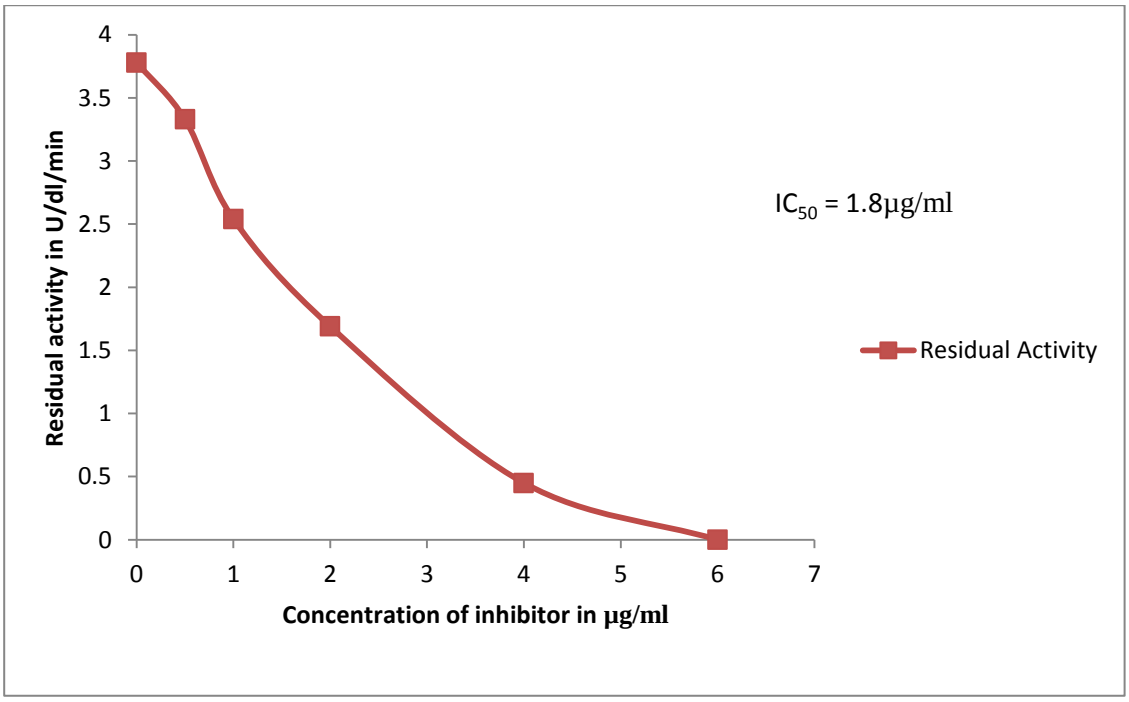


Fig -35 Inhibition of HNE by α1 anti trypsin with STANA as substrate

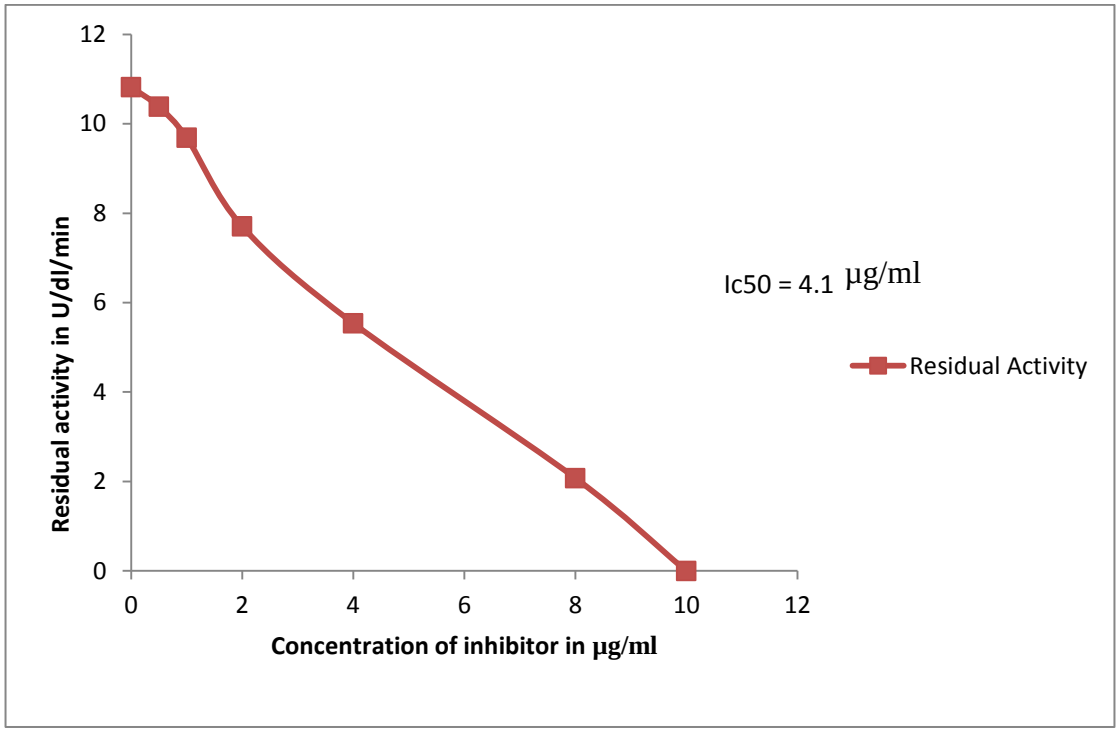


Fig -36 Inhibition of Elastase of *P.aeruginosa* by α1 anti trypsin

The I_{c50} for HNE Vs α_1AT was 1.8 µg/ml while for Elastase of *P. aeruginosa* it was $IC_{50} = 4.1$ µg/ml. The lower IC_{50} values for HNE showed that less concentrations of the inhibitor were required to inhibit HNE. Complete inhibition of HNE was brought about at a concentration of 6.0 µg/ml while 10.0 µg/ml was required for complete inhibition of elastase of *P. aeruginosa.*

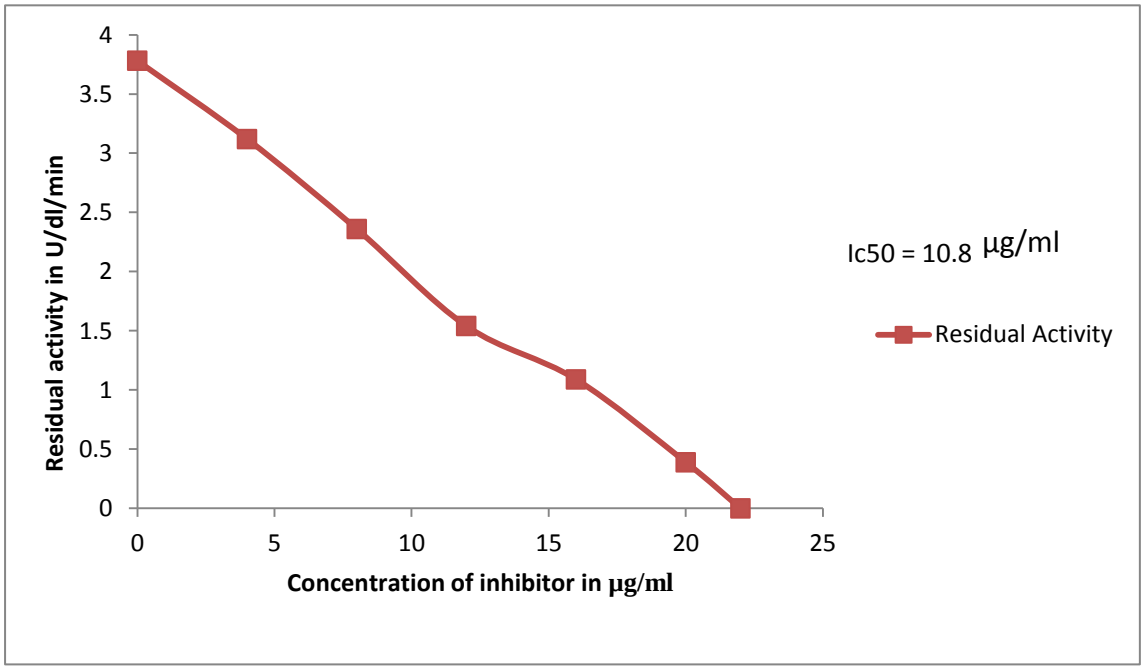


Fig -37 Inhibition of HNE by α2 macroglobulin with STANA as substrate

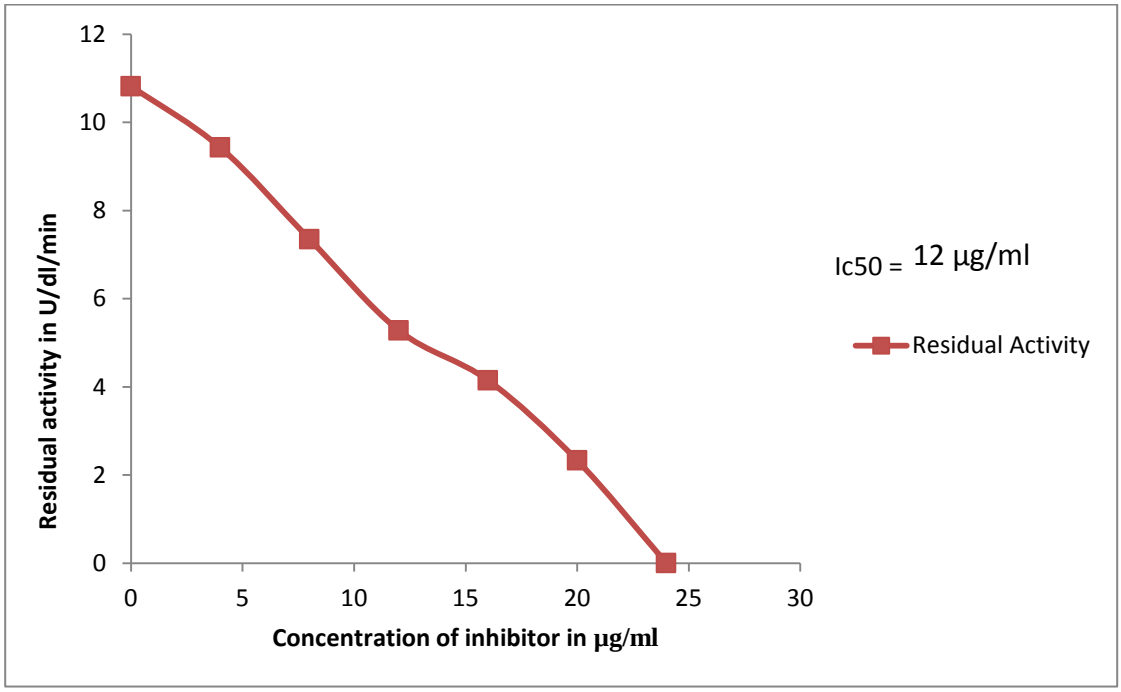


Fig -38 Inhibition of Elastase of *P. aeruginosa* by α2 macroglobulin with STANA as substrate

Similarly the IC_{50} for HNE Vs α_2MG were 10.8 µg/ml which was lower than the IC_{50} = 12 µg/ml of *P. aeruginosa* elastase. Complete inhibition of HNE was brought about by 22 µg/ml while 24 µg/ml was required for complete inhibition of *P. aeruginosa* elastase. (Fig 37 and 38).

The consolidated IC_{50} values are as represented in Table 16

Table -16 IC_{50} µg/ml of HNE and *P. aeruginosa* elastase with α_1AT and α_2MG

Enzyme	Inhibitor	IC_{50} µg/ml
HNE	α_1 AT	1.8
P. aeruginosa elastase	α_1 AT	4.1
HNE	α_2 MG	10.8
P. aeruginosa elastase	α_2 MG	12

The half maximal inhibitory concentration IC_{50} is a measure of the effectiveness of substances in inhibiting an enzyme. The results obtained indicate that when faced with its target protease ie HNE, α_1AT exhibited a greater affinity and required a lesser quantity of α_1AT in comparision to

P.aeruginosa elastase. Studies by Padrines et al (132) and Morihara (134) show that affinity of α_1AT is higher for trypsin and HNE rather than that of *P.aeruginosa* elastase.

As mentioned earlier, α_2MG a large spectrum protease inhibitor is known to target both endo and exogenase proteases and is known to inactivate Pseudomonal proteolytic enzymes (136,194). Recent studies have shown that *P. aeruginosa* synthesizes a structural homolog of human α_2 MG. The mechanism of a synthesized macroglobulin complex plays a protective role against a bacterial aggressor and is also known to undergo conformational modification on binding to HNE (198) The difference in IC_{50} values between HNE and P.aeruginosa by α_1AT is more marked when compared to α_2MG.

α_1AT preferentially inhibits HNE over *P. aeruginosa* elastase and has a greater affinity for binding to HNE as seen by the IC_{50} values of 1.8 µg/ml and 4.1 µg/ml. α_2MG known to inhibit *P. aeruginosa* elastase, also inhibited HNE but difference was not marked. So, results of the inhibitory assay showed that 50% inhibition of HNE required lower concentrations of α_1AT and α_2MG.

Though the present study was aimed at the inhibition of elastase the objective of the screening done initially was to obtain spectra of proteases pathogenic and non pathogenic with special emphasis to elastase. The studies indicated that none of the non pathogenic bacteria screened were capable of producing elastase under the given set of conditions. Further studies of these non pathogenic organisms relied on proteases which hydrolyzed casein and had a preference to a synthetic substrate ATEE. Since, the *B. coagulans* produced the largest amount of proteases, this protease which is capable of hydrolyzing both casein and ATEE was subjected to inhibition studies by α_1AT and α_2MG.

The result showed that inhibition by α_1AT was negligible initially at the concentrations of the inhibitor used in the assay system and 50% inhibition was finally brought about by 14 µg/ml while α_2MG brought about 50% inhibition at a concentration of 29 µg/ml (Fig 39,40). So large amounts of the inhibitors were required to inhibit the protease of *B. coagulans.*

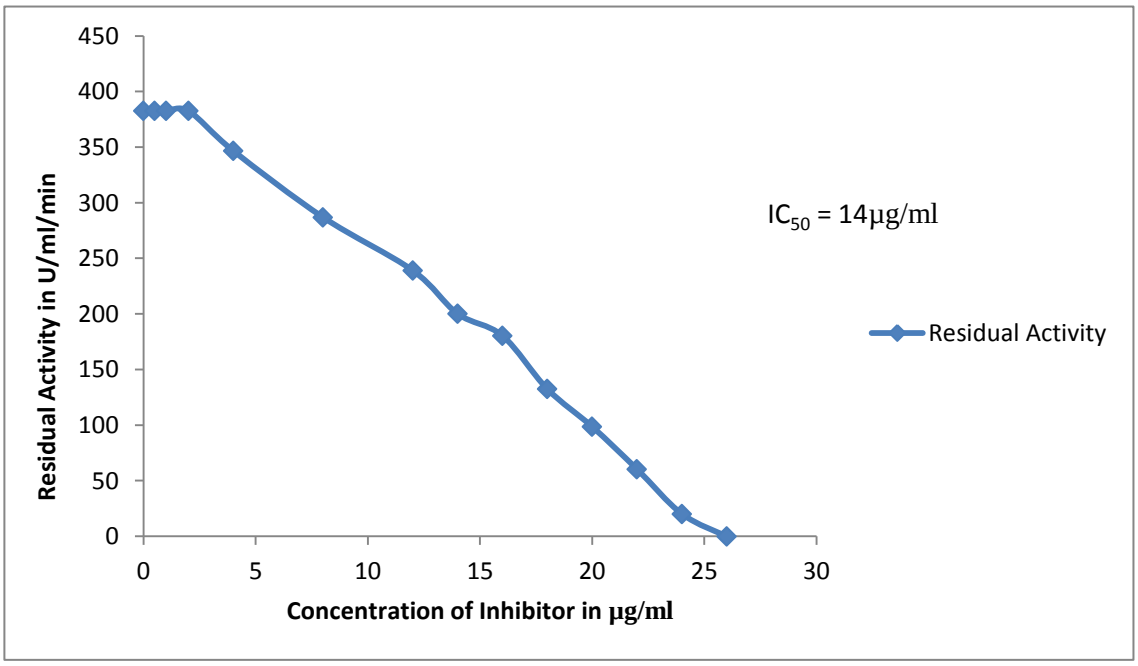


Fig -39 Inhibition of Protease of *B. coagulans* by α_1 Anti trypsin with casein as substrate

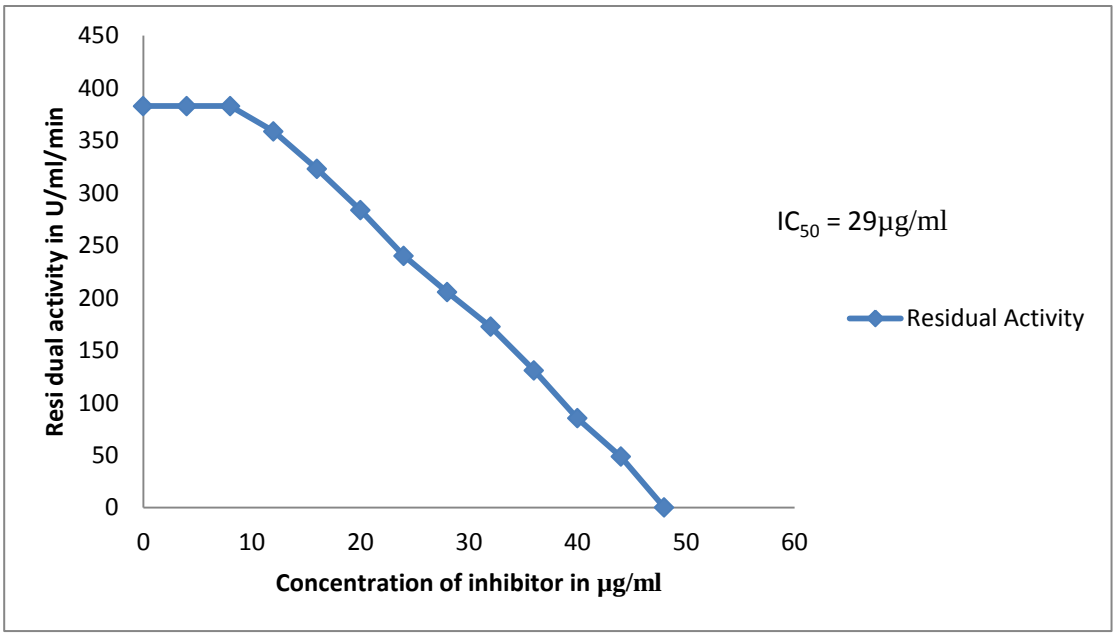


Fig – 40 Inhibition of Protease of *B. coagulans* by α_2 macroglobulin with casein as substrate

Conclusion:

The studies on the inhibitory effects of α_1AT and α_2MG on HNE and *P. aeruginosa* elastase indicated that α_1AT preferably inhibited HNE than *P. aeruginosa* elastase. However, the inhibition of HNE and *P. aeruginosa* elastase by α_2MG when compared with, not much difference was noted in their preferences. This study also indicates that all the proteases produced by pathogenic and non pathogenic organisms was inhibited by the endogenous inhibitors but to a lesser extent. The observation warrants the need for additional specific inhibitors for *P. aeruginosa* elastase to control the devastating effects on infection system.

Chapter VI

Effect of Aqueous and Methanolic Extract of *Psidium guajava* on Proteases

Introduction:

The presence of anti protease factors in plants is well established (199) . Several of them have been isolated and characterized and are shown to exhibit specificities towards group of proteases or a specific enzyme (199). The physiological function of these inhibitors are varied as they are shown to posses regulatory effects on endogenous proteases during dormancy of seeds and inhibitory effects on exogenous proteases of pathogenic organisms, insects which infect or attack the plants. These inhibitors apart from their physiological role have been tested for inhibition properties on enzymes of other sources. Many of these inhibitors have been shown to have numerous applications including therapeutic uses. Several of these factors have been studied extensively for their physio-chemical properties and are characterized as proteins and phenolic substances.

This chapter deals with the inhibitory activities of *Psidium guajava* leaf aqueous and methanolic extracts on elastase of *P. aeruginosa* and human neutrophils as this plant leaf extract is widely used for

wound healing. Though the curative power of leaves have been widely reported, a review of literature showed that most studies have limited the studies to Agar well diffusion or disc inhibitory methods merely detailing its antimicrobial effect (200).

During wound infection, bacteria at the wound site release proteolytic enzymes to improve their invasive powers. The bacteria present also stimulate protease production via activation of the immune system. Elastase of *P. aeruginosa* has also been shown to degrade proteins in the host and is commonly found in chronic ulcer infections (148) .

During bacterial infections, HNE is secreted against the invasive microorganisms and is the first line of defence as they kill bacteria. HNE is also found to be elevated in wounds that fail to progress towards healing (149) While proteases are important for organizing and remodeling new ECM (extra cellular matrix) high levels of proteases in wounds impair healing as they degrade the ECM (149) So, application of an external anti protease agent can control infection and facilitate healing by inhibiting excess protease present.

It is note worthy that a regulation of endogenous protease activity is critical to normal physiology which is achieved by endogenous anti proteases which are present in various tissues and body fluids (4,10). During infection there is an added burden of handling the exogenous proteases of the invading organism. Contrary to the expectation the innate anti proteases show more specificity to the endogenous enzymes than exogenous ones and this situation warrants for requirement of additional factors which could combat the devastating effects of the proteases of exogenous nature. So, preferred approach could be application of external inhibitors which have preferential inhibition on bacterial elastase. The anti bacterial property of the leaves of *Psidium guajava* could be due to various phenolic compounds present in the leaves or an anti protease inhibitor of proteinase nature. A study on the effect of leaf extracts on porcine pancreatic elastase has shown inhibition of the enzyme and provided a cue for assessing the effect of the extracts on HNE and *P. aeruginosa* elastase (146). The current knowledge available in literature and the observations recorded on the effect of α_1AT and α_2MG on *P.aeruginosa* elastase and HNE also suggest the

importance of exogenous anti proteases to control infection. This chapter deals with an aim to study the effect of an inhibitor from *Psidium guajava* on HNE, *P. aeruginosa* elastase and other proteases.

Materials and Methods:

Materials:

The purified bacterial elastase obtained from *Pseudomonas aeruginosa* (MTCC 3541) as mentioned in chapter II, HNE (E8140), Bovine pancreatic trypsin(T9935) proteinase K (P6556). STANA(S4760) and Bovine Casein (C7078) were from Sigma and Tannic acid(from Merck). All other chemicals used were analytical grade and obtained locally. Fresh mature leaves of *Psidium guajava* were collected in the month of August.

Methods:

Preparation of the leaf extract- The leaves were washed thoroughly with distilled water and dried at room temperature. The dried leaves were then ground uniformly in a mechanical grinder to get a fine powder. A methanolic extract and aqueous extract were prepared from the fine powder as under.

Five grams of the powder was mixed with 30ml of methanol in an Erlenmeyer flask. It was kept in a rotatory incubator at 120 rpm for 24 hours. The mixture was filtered using Whatmann filter paper No. 1 dried and stored at 4^0C. It was then dissolved when required for use.

The aqueous extract was prepared similarly using hot de ionized water (60^0C) and filtered. The total tannin content of the leaf extract was determined by using Folin Ciocalteu Reagent (201) and compared to known standards of commercially available tannic acid (Merck). The results obtained were expressed as micrograms of Tannic acid per millilitre of the extract as per the method of Makkar (201).

Aqueous extract without phenolic compounds- Five grams of fresh leaves was homogenized with 30ml of deionized water. This was filtered and the filtrate treated with equal volume of cold acetone. The precipitate obtained after centrifugation at 3000rpm at 4^0C was washed repeatedly to obtain a clear white precipitate which is expected to be devoid of any phenolic compounds. The precipitate obtained was then dissolved in 0.2M Tris Hcl buffer pH 8.0 and again centrifuged at 3000rpm for 10 minutes. The clear supernatant obtained was analyzed for protein content by Lowrys' Method (169) and used for assessment of inhibitory activity.

Elastase Assay: Assay of elastase of *P. aeruginosa* and HNE was performed as in Chapter III

Elastase Inhibition assay: Three sets of assay systems were run. The inhibitor source in each set were varied concentrations of the methanolic, aqueous, and aqueous fraction (without phenols) extracts of the leaves in the assay buffer i.e. 200mM Tris HCl buffer pH 8.0. The assay systems used as the source of the enzyme elastase obtained from *Pseudomonas aeruginosa* by the process mentioned

earlier. The leaf extracts were preincubated for 10 minutes with a fixed volume of the enzyme (concentration equivalent10U/ml) mentioned above. The reaction was initiated with a fixed volume of the substrate 200mM STANA .After 15 minutes incubation at 37^0C, the reaction was stopped by the addition of 1ml of 30% acetic acid and the residual activity was obtained by measurement of optical density of p- nitroaniline liberated at 410 nm. Similarly, the assays were also run using a fixed volume of HNE(concentration equivalent10U/ml) as an enzyme source. The concentration of both enzymes i.e. HNE and the bacterial elastase was fixed at 10U/ml for comparison purpose. All assays were done in triplicate and the average values were taken.

The inhibitory activity was determined by the difference between activity of the bacterial elastase, HNE and then the residual activity of the same solution after adding the inhibitor.

The percentage of inhibition was calculated using Inhibition (%) = [1-(B/A)] X100 where A is the activity of the enzymes without inhibitor and B is the activity in presence of inhibitor.

Ki and IC_{50} were determined for both elastase of *P. aeruginosa* and HNE with varying concentrations of the inhibitor in the assay systems.

Enzyme Assay of trypsin and proteinase K: Two sets of assay systems were run. In one, trypsin was the source of the enzyme and in the other Proteinase K. Here the substrate used was casein and the assay was carried out by the method of Sumathi et al mentioned earlier (168)The reaction was initiated by addition of the enzyme. The concentration of the enzymes were trypsin 15µg/ml and proteinase K was 40µg/ml. The enzyme activity was determined by measuring the optical density at 540nm.

Inhibition assay of trypsin and proteinase K:

Varied concentrations of the methanolic and aqueous extracts were used as the source of the inhibitor. They were prepared in the assay buffer i.e. 0.2 M Phosphate buffer pH 7.6. The leaf extracts were pre incubated for 10minutes with a fixed volume of the enzyme. The reaction was initiated with a fixed volume of the substrate i.e. 1% Casein. and the assay was carried out by the method of Sumathi et al

mentioned earlier (168). The residual activity was determined by measuring the optical density at 540nm. Suitable controls and blanks were included in the assay along with the test. The inhibitory effect was determined by the difference between the activity of the enzymes (trypsin and proteinase K) without inhibitor and the residual activity of the same solution after addition of the inhibitor.

The percentage of inhibition was calculated as previously mentioned using the formula Inhibition (%) = [(1-(B/A)] x 100 where A is the activity of the enzymes without inhibitor and B is activity in presence of the inhibitor.

Results and Discussion:

Km values for HNE and *P. aeruginosa* elastase had been determined earlier with STANA as the substrate using varying concentrations of STANA in the range of 20mM to 320mM and are shown in chapter V (Fig 29, 30). Km value for HNE on STANA as substrate was 167mM and Vmax was 11.1 U/dl/min. Km value for elastase of *P. aeruginosa* was 153mM and Vmax was 12.5 U/dl/min.

In the enzyme inhibition assay system the concentration of the inhibitor was expressed in Tannic Acid equivalents. In order to determine the type of inhibition varying concentrations of the inhibitor when introduced into the system while keeping the concentrations of the enzyme and substrates fixed (similar to the procedure followed earlier for the inhibitory effect of Serpins). The introduction of the methanolic and aqueous leaf extract in the assay system altered both the Km and Vmax so the type of inhibition was uncompetitive.

In the case of HNE after addition of the inhibitor (expressed in concentration of Tannic Acid equivalents) when 4µg/ml of aqueous extract was used the values were altered to 142mM and 10.0U/dl/min and when 8µg/ml of aqueous extract was used there was further alteration to 100mM and 6.6U/dl/min (Fig 41).

Similar values and pattern of inhibition was seen when the methanolic extract was used as seen in Fig 42 .Addition of higher concentrations of the leaf extracts further decreased the Km and Vmax.

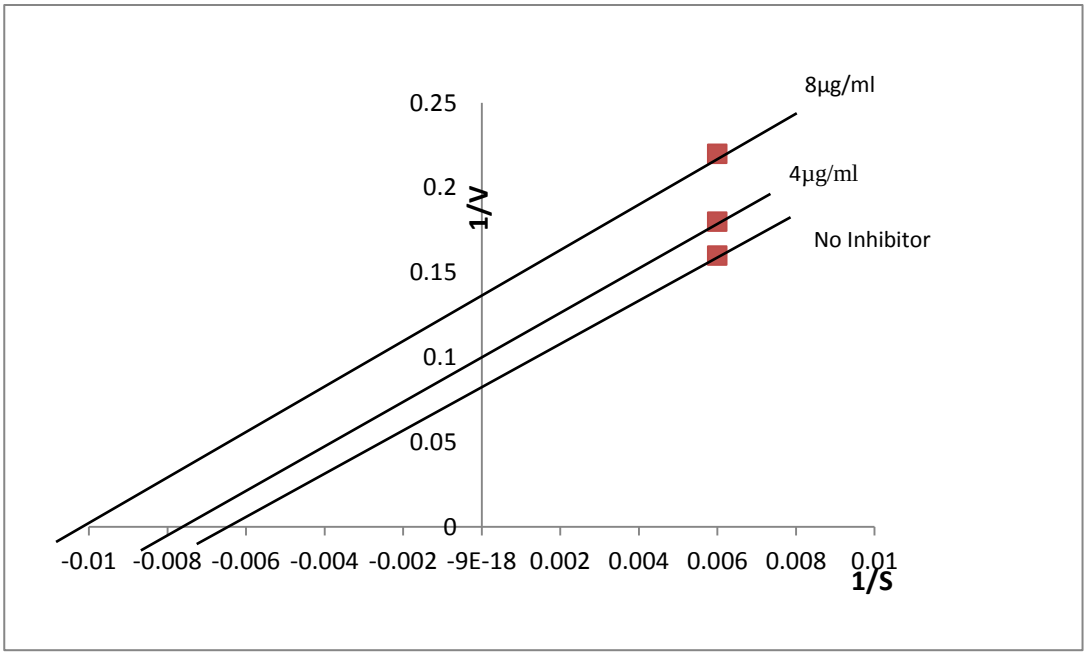


Fig 41 -Inhibition of HNE by aqueous extract of *P. guajava* with STANA as substrate

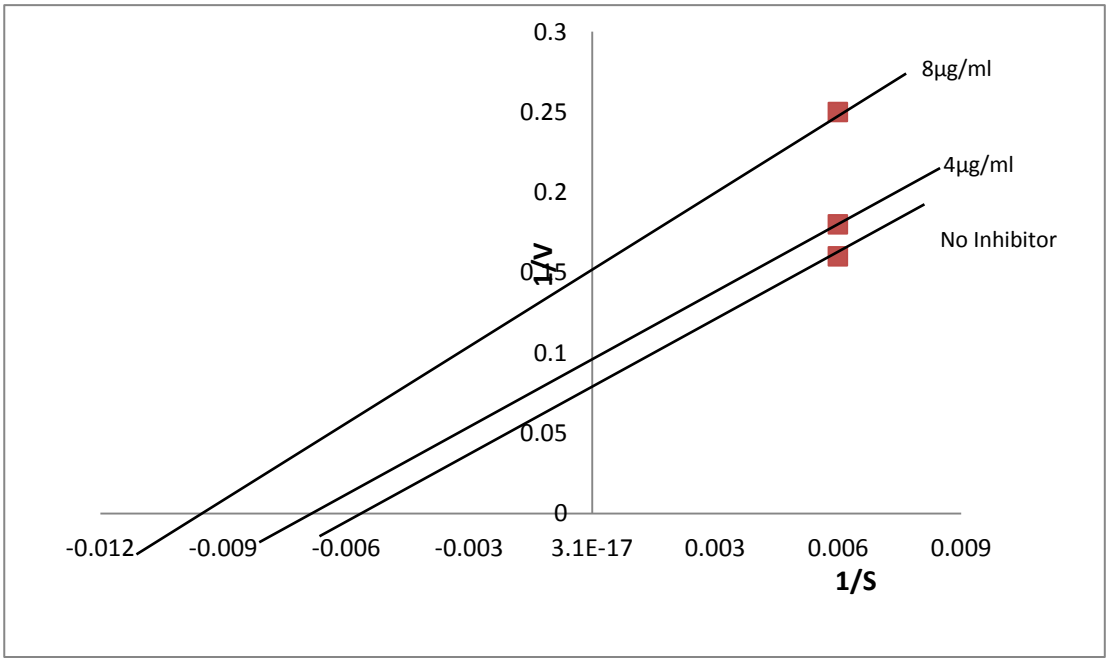


Fig 42-Inhibition of HNE by methanolic extract of *P. guajava* with STANA as substrate

In a similar manner the introduction of the aqueous and methanolic leaf extract into the assay system with P.aeruginosa elastase as the enzyme source brought about similar results as represented in Figs 43 and 44.

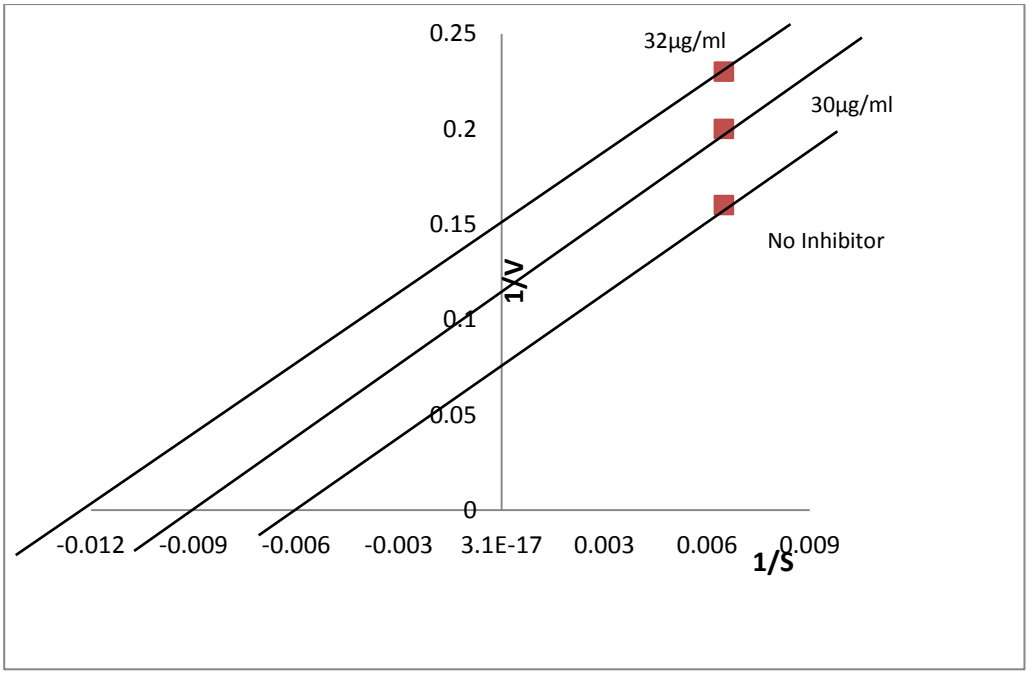


Fig 43-Inhibition of *P. aeruginosa* elastase by aqueous extract of *P. guajava* with STANA as substrate

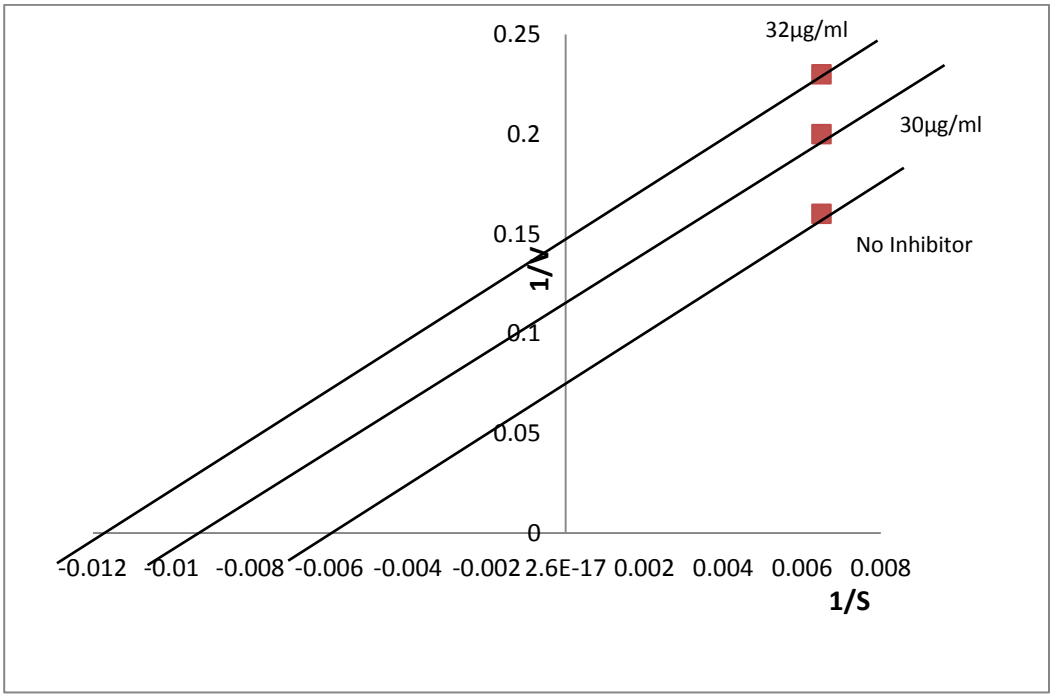


Fig 44-Inhibition of *P. aeruginosa* elastase by methanolic extract of *P. guajava* with STANA as substrate

An inhibitor concentration of 30µg/ml (aqueous) altered the Km value to 91mM and Vmax to 8.3U/dl/min and an inhibitor concentration of 32µg/ml further altered the values to 81mM and 6.6U/dl/min. Similar results were obtained when methanolic extract was used as represented in figures 44.

Pattern of inhibition was non specific and it is known that Tannins are a group of secondary metabolites and inhibition by these

compounds are complex and depend on a wide variety of factors (202, 203).

As the results obtained indicated that the type of inhibition appeared non competitive in order to know the percentage of inhibition and determination of IC_{50} an assay was run with varying concentrations of the methanolic and aqueous extract of the leaves and the degree of inhibition and were determined as shown in Table number 17,18 and Figs 45,46.

Table 17: The percentage of inhibition brought about by methanolic extract of the leaves on bacterial elastase and HNE.

Concentration of inhibitor in Tannic acid equivalents(µg/ml)	% of inhibition brought about on HNE by methanolic extract	% of inhibition brought about on elastase of *P.aeruginosa* by methanolic extract
4.0	5.5	18.0
8.0	11.1	26.6
16.0	26.7	32.8
32.0	39.9	54.0
40.0	46.7	74.4
52.0	64.0	86.0
60.0	86.6	100
72.0	100	----

Table 18:

The percentage of inhibition brought about by aqueous extract of the leaves on bacterial elastase and HNE.

Concentration of inhibitor in Tannic acid equivalents(µg/ml)	% of inhibition brought about on HNE by aqueous extract	% of inhibition brought about on elastase of *P.aeruginosa* by aqueous extract
4.0	5.5	18.4
8.0	11.1	28.6
16.0	28.7	32.8
32.0	39.9	56.0
40.0	46.7	74.4
52.0	64.0	86.0
60.0	86.6	100
72.0	100	-----

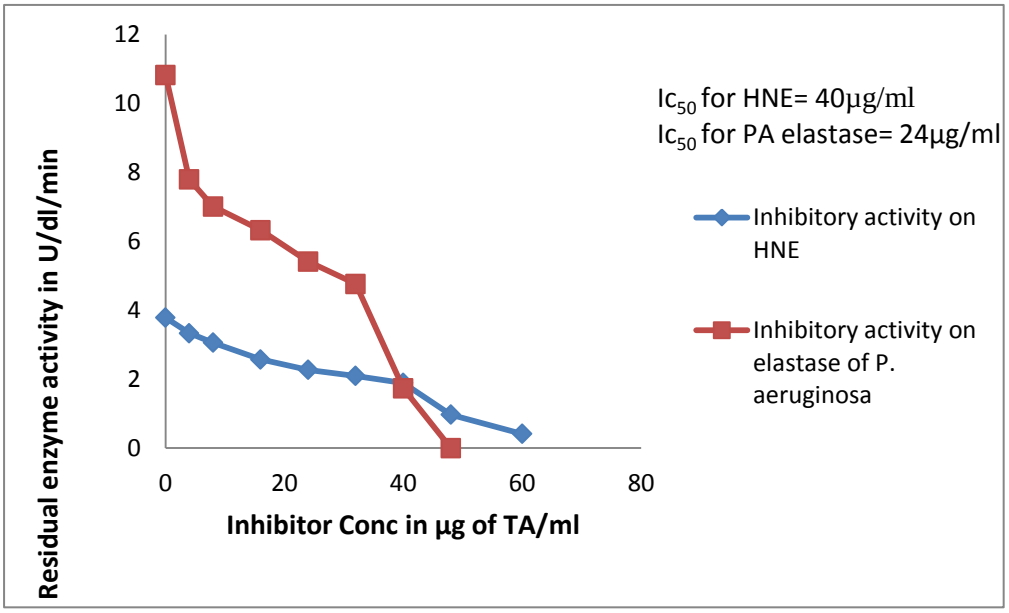


Fig 45– Inhibition of HNE and elastase of *P.aeruginosa* by methanolic extract of *Psidium guajava*

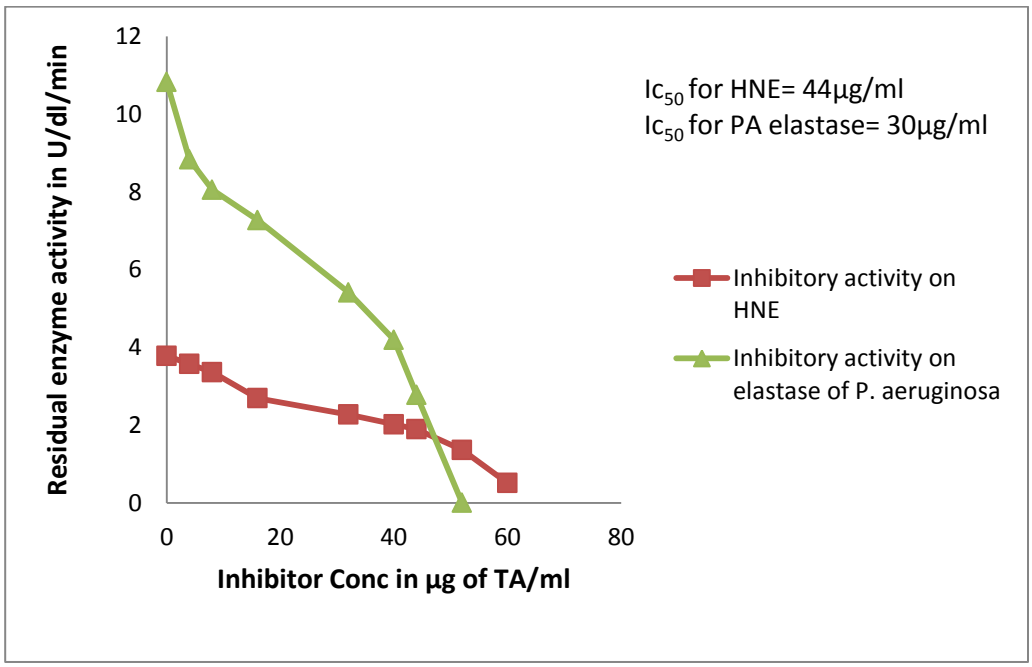


Fig 46– Inhibition of HNE and elastase of *P.aeruginosa* by aqueous extract of *Psidium guajava*

Methanol and water as solvents would extract all the active compounds present in the leaves such as tannins, triterpenes, flavonoids and other phenolic compounds. However an aqueous extract devoid of phenolic compounds could have inhibitors of protein nature.

The results showed that there is not much difference between the methanolic extract of the leaves and the aqueous extract in the degree of inhibition as represented in Tables 17 and 18.

The aqueous extract after removal of phenolic substances with acetone did not have any measurable amounts of protein or inhibitory activity clearly indicating the situation that the inhibitory activity observed in the aqueous and methanolic extracts are due to compounds other than proteins in nature.

After keeping the enzyme and substrate concentrations fixed and varying the inhibitor concentration as shown in Figures 45 and 46, the IC_{50} (concentration in µg/ml at which the inhibition of the enzyme is 50%) for HNE was 40 µg/ml and 44 µg/ml for the methanolic and aqueous extracts while for bacterial elastase it was

24µg/ml and 30µg/ml. Ki was calculated by application of Cheng Prusoff equation for uncompetitive inhibition.

The result analysis showed that Ki values with methanolic and aqueous extract was 18.3 µg/ml and 20.2 µg/ml for HNE and 10.4 µg/ml and 13.0 µg/ml for bacterial elastase. Though both elastase of *P. aeruginosa* and HNE are inhibited, data analysis showed that the amount of inhibitor required for inhibition of *P. aeruginosa* is about 40% lower than that of HNE. The lower Ki values for bacterial elastase in both methanolic and aqueous fractions suggest that it is more prone to the effect to the inhibitor in the leaves as the lower Ki indicates that less amounts of the inhibitor brings about greater degree of inhibition. As the leaf extract appears to preferably inhibit bacterial elastase over HNE, application of these leaves to a wound decreases the amount of bacterial elastase at a wound site. This was published as part of this study (Publications).

From the results of this study, there appears to be some scientific validation for the use of guava leaves to treat wounds as the active components in the leaves inhibit bacterial elastase and

HNE. Though, it appears to be more suitable for chronic non healing wounds, rather than minor cuts or small wounds.

The study was extended so as to know if the Leaf Extracts of *Psidium Guajava* inhibited other proteases such as bovine pancreatic trypsin and proteinase K of fungal origin from *Engyodontium album*. There have been several studies which confirm protease secretion by fungus as a determining factor of its pathogenicity (204) . Varying concentrations of the aqueous and methanolic leaf extracts were used, IC_{50} values were calculated and results are shown in Figures 47 and 48.

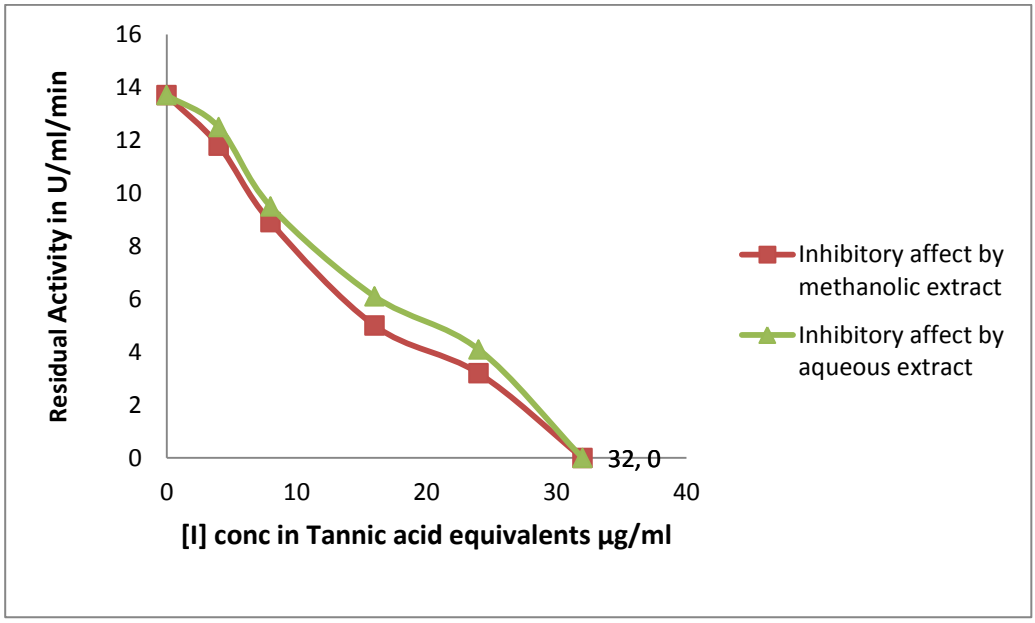


Fig 47-Inhibition of trypsin by methanolic and aqueous extract of *P. guajava*

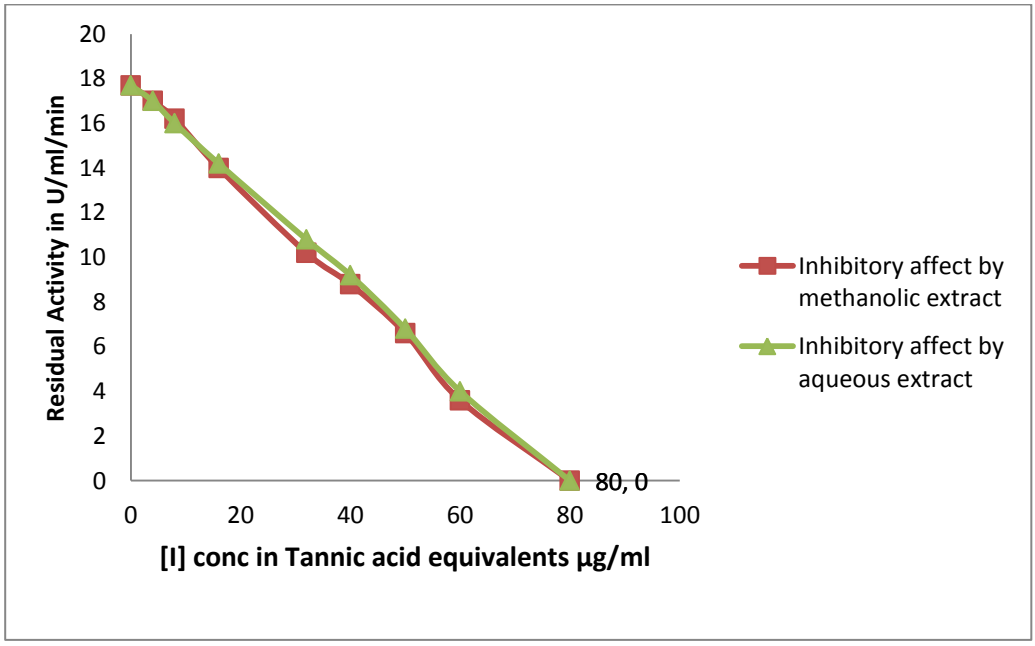


Fig 48 -Inhibition of proteinase K by methanolic and aqueous extract of *P. guajava*

The methanolic and aqueous extract of the leaves showed good inhibitory capacity against trypsin. IC_{50} for Trypsin was 13.6 µg/ml and 14.0 µg/ml for the methanolic and aqueous extracts while for proteinase K it was 47.0 µg/ml and 52.0 µg/ml for methanolic and aqueous extracts respectively as shown in Figs-47and 48. The inhibitors were able to inhibit both the enzymes completely and the pattern of inhibition was always similar.

The methanolic extract showed good inhibitory activity against trypsin and though it inhibited proteinase K, inhibitory effect was less when compared to its inhibitory effect on trypsin. The less inhibitory effect on proteinase K however does not imply that the degree of inhibition on fungal proteases are less. Proteinase K a broad range endolytic protease used in the study was a commercial preparation and is known to be a highly stable compound, used to digest unwanted proteins in nucleic acid purification methods, while trypsin is known to be inhibited by a wide variety of extraneous inhibitors. As seen earlier, α_1AT inhibits trypsin preferentially over other proteases(134)

The leaves of *Psidium guajava* are used for treating wounds and ulcers. As mentioned earlier, elastase release by bacteria is an important invasive factor. The aim of this study was to clarify whether the wound healing capacity of the leaves is due to a protease inhibitor present in the leaves. As both HNE and bacterial elastase would be present at wound sites this study aimed to determine whether the inhibitory effect of the leaves is more on the bacterial elastase than on HNE.

In wound healing the major proteases are the matrix metalloproteinases (MMPS) and the serine proteases such as elastase. The wound related proteases act on different proteins which include ECM, collagen, gelatin, proteoglycans and elastin (205). In normal wound healing, proteases break down damaged ECM so, new tissue can form and closure of a wound can take place. Thus, protease activity is an essential part of wound healing (206). But when protease levels are unchecked, they cause damage to the ECM and impairs healing besides destroying normal tissue. (205).

In the normal course, initially there is rapid increase in protease levels which then tapers off. When bacteria is present at the site, there would be prolonged high protease activity at the wound site (207). As mentioned previously, any prolonged high levels of proteases either HNE or bacterial elastase are detrimental to wound healing. However, in the initial few days, some level of proteases is required to break down the damaged ECM so as to facilitate formation of new tissue.

Result of this chapter also show, that though there is inhibition of both HNE and bacterial elastase, it is due to the large amounts of phenolic compounds. Though the type of inhibition was non specific and there was overall inhibition of proteases, the efficacy of the inhibitor showed that bacterial elastase was more susceptible to this inhibition when compared to HNE.

The extraction medium also has a role to play in the extraction of the active compounds from the leaf extract. Here, of the two solvents used for extraction, there was not much difference in the degree of inhibition brought about.

Tannins are compounds of interest with a potential for use in a diseased state due to its degree of reactivity with proteins and enzymes (208). Phenolic compounds extracted from plants have been used for medicinal purposes such as anti inflammatory agents from *Achillea millefolium* (209), anti elastase agents from Areca catechu used for anti aging creams (210) and free radical scavenging compounds from *Cornus kousa* (211). A pentagalloyl glucose (PGG) which is a condensed tannin has ben reported to bind with enzymes elastase and renders resistance to elastase inhibitors in aortic aneurysm (212).

Conclusion -The results obtained in this study, opens up further avenues to explore the most active ingredient which would behave as a more specific preferred inhibitor on exogenous proteases.

Chapter VII

Summary and conclusion

The current study was aimed at assessment of ability of pathogenic and nonpathogenic organisms for production of proteases in general and elastase in particular. On the basis of this both pathogenic and nonpathogenic organisms were cultured under defined conditions and the medium was analyzed for proteases using enzyme specific synthetic substrates. The data obtained indicated that all the organisms studied produced proteases under given set of conditions. However, the elastase enzyme production was restricted to only pathogenic organisms viz. *P. aeruginosa*. All organisms exhibited protease activity. One of the salient feature of this study is the production of protease by *Acetobacter aceti* an organism which is known for its ability to produce acetic acid and not explored for protease production. In addition, screening study also has yielded information on the ability of *P.aeruginosa* to produce an additional protease similar in substrate specificity of chymotrypsin.

The presence of the 2nd enzyme in *P.aeruginosa* culture evoked interest in the study as *P.aeruginosa* has been studied extensively for its ability to produce elastase and its virulence power during infections. It is clear from the screening studies that elastase enzymes is not present in all the

organisms studied under the conditions specified. The screening studies provided back ground for extension of studies with regard to purifications and characterization of the two enzymes in *P.aeruginosa* and enzymes of *B.coagulans* and *A. aceti*. The data obtained clearly indicate the production of two enzymes by *P. aeruginosa* with relatively close molecular weight but with distinct substrate specificities. A new method for purification of chymotrypsim like enzyme was also proposed in the study. The purification results of the enzyme from *B. coagulans* showed that the enzyme is different from *P. aeruginosa* in respect to its molecular weight, they however, had the same substrate specificity on ATEE. *B. coagulans* has been utilized widely for industrial application and these organisms are known for its ability to produce variety of proteases including alkaline protease depending up on the strain. In the present study, the proteases of *P.aeruginosa* and *B.coagulans* were all neutral ones with respect to their pH and thermolabile with regard to temperature studies. The significant outcome with regard to purification and characterization is the study on the 2^{nd} enzyme of *P.aeruginosa* and development of a protocol for its purification.

In respect of the inhibition of HNE and elastase of *P.aeruginosa* by endogenous protease inhibitors α_1 AT and α_2 MG, it was observed that α_1 AT exhibited preferential inhibition of HNE than *P.aeruginosa* elastase. However, it had no such preference when α_2 MG was the inhibitor. The data obtained indicated that α_1 AT and α_2 MG could not be dependable inhibitors for control of exogenous protease destruction activity which is a salient observation as it warrants that any proteases from exogenous sources could be a potential threat to the body's physiology as they could trigger inflammatory responses. Thus, this study opens up the need for a potent inhibitor to control such devastating activities.

The use of *P. guajava* extracts for wound healing has been documented well in literature. However, its mechanism of action was restricted to the levels of the ability to inhibit the growth of bacteria. In the attempt to obtain the specific inhibitor with ability to specifically inhibit bacterial enzyme, it was found that *P. guajava* extract had ability to preferentially inhibit *P.aeruginosa* elastase than HNE indicating its potential as a therapeutic agent capable of controlling the damaging activity of the *P.aeruginosa* elastase and thus justifying the age old practice with

scientific bases. The inhibitor has not been specifically characterized but is assayed as phenolic compound in terms of tannic acid equivalents. Further studies might provide insight to the active ingredient in the extract specific to the bacterial enzyme.

This study highlights that pathogenic organisms only had elastase while non pathogenic organisms have good proteolytic activity. The screening studies indicated the identification of two enzymes in *P. aeruginosa* and the protocol for the purification of the second enzyme was developed. This study also showed that endogenous inhibitors are not enough for the exogenous proteases and there is a need for exogenous inhibitors to inhibit bacterial proteases. This study also identified an inhibitor of phenolic nature which preferentially inhibited bacterial elastase over HNE.

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