

# *Introduction*

## INTRODUCTION

Proteases are enzymes that bring about the breakdown of proteins in all organisms. In animal and microbial cells, proteolysis plays a vital role in processes such as enzyme activation, membrane assembly, lysosomal functions, protein excretion, cellular differentiation and aging (1). Further, proteolysis also plays a regulatory role in animal cells with regard to other physiological processes, such as blood coagulation, fibrinolysis and the complement reaction. Thus proteolytic processing is the final step in the expression of activity of a great number of proteins (2). Proteolysis is also essential for many aspects of plant physiology and development including providing the supply of amino acids required for protein synthesis, zymogen and hormone maturation, cellular housekeeping, homeostasis and programmed cell death/ apoptosis of specific plant cells or organs (3).

The growing realization of the physiological importance of proteases together with the novel methodologies of protein chemistry, molecular biology and biotechnology has generated renewed interest in their study (4).

### Discovery of proteolytic enzymes

William Beaumont was the first to observe that "substances other than hydrochloric (muriatic) acid" played an important role in gastric digestion. This marked the first evidence of the existence of proteolytic enzymes. However, Kuhne coined the term 'enzyme' only in 1876, following his discovery of trypsin, which is a pancreatic protease (5). The first isolation and characterization of an enzyme, namely pepsin was performed in 1930 by Northrop (6), who proved that this enzyme was a pure protein (7).

### Classification of proteolytic enzymes

Originally, the molecular size and charge of enzymes formed the basis of the classification of proteases (8). The nature of the linkages hydrolyzed was observed to vary from one protease to another. Based on studies involving gastro-intestinal proteinases, Bergmann and Fruton observed that proteolytic attack always involved peptide bonds that possessed certain 'typical' side-chain amino acid residues. Thus, proteases exhibit specificity towards certain side-chain amino acid residues in the protein. Chymotrypsin cleaves the peptide bonds involving the carboxy-terminus of aromatic amino acids such as tyrosine or phenylalanine, while pepsin hydrolyses at the amino-terminus of linkages involving the same amino acids. Similarly, trypsin cleaves peptide bonds at the carboxy-terminus of basic amino acid residues such as lysine and arginine. Structural modifications in these 'typical' amino

acids rendered the substrate resistant to cleavage by the particular proteinase e.g. resistance of  $\alpha$ -hippuryl- $\epsilon$ -carbobenzoxylysineamide towards trypsin and of carbobenzoxy-l-glutamyl-l-diiodotyrosine towards pepsin.

Further, Bergmann and Fruton also observed that synthetic peptides of relatively simple structure could serve as substrates. Thus, substrate specificity also formed an important parameter in the classification of proteases (8).

The discovery of site-specific reagents capable of inhibiting peptidase and/or esterase activity of digestive enzymes like chymotrypsin, trypsin and carboxy-peptidase led to the identification of specific amino acid residues essential for enzyme activity. Thus, characterization of the active site and understanding the mechanism of action led to the emergence of these parameters as essential criteria for enzyme classification. With the advent of amino acid sequence analysis and X-ray crystallography, the comparison of proteases based on their linear and three-dimensional structures became possible (2). The International Union of Biochemistry has therefore categorized all the proteases of known amino acid sequence into distinct mechanistic classes, based on the following criteria: -

- 1) Catalytic amino acid residues at the active site: based on which the four categories- namely serine, cysteine, aspartic and metalloproteases are distinguished (2).
- 2) Mechanism of action
- 3) Three-dimensional protein structure.

#### **Mechanistic categories**

Four broad mechanistic classes of proteases are recognized namely the serine, cysteine, aspartate and metalloproteases. This classification is based on the amino acid residues that direct the catalytic process at the active site. Within these mechanistic classes, six families of proteases, are recognized to date as indicated in Table.1

One of the initial steps in the characterization of any newly isolated protease involves the determination of its mechanistic class, based on the mode of catalytic action.

The four mechanistic classes are briefly elaborated as follows: -

- 1) **Serine proteases:** Among the best-characterized and widely studied proteases, this class of proteases is distinguished by the presence of the catalytic triad of Asp, His, Ser, in the active site. These proteases have a pH optimum of 7-9 (9). X-ray crystallographic studies performed on  $\alpha$ -chymotrypsin indicated that the serine residue at position 195 (Ser 195) and histidine at position 57 (His 57) were directly involved in catalysis. Moreover the

distance between these two residues was sufficient to permit the formation of a hydrogen bond between them. Similarly, another hydrogen bond also existed between His 57 and the aspartate residue at position 102 (Asp102), thus facilitating the formation of a salt bridge between both these residues (10). Existence of identical triads was also observed in the other serine proteases such as trypsin, elastase and subtilisin (11).

**Table .1: Families of proteolytic enzymes (12)**

Family	Representative protease	Characteristic active site residues
Serine protease 1	Chymotrypsin Trypsin Elastase Pancreatic kallikrein	Asp <sup>102</sup> His <sup>57</sup> Ser <sup>195</sup>
Serine protease 2	Subtilisin	Asp <sup>33</sup> His <sup>64</sup> Ser <sup>221</sup>
Cysteine protease	Papain Actinidin Rat liver cathepsins B and H*	Cys <sup>25</sup> His <sup>159</sup> Asp <sup>158</sup>
Aspartic protease	Pencillopepsin Renin	Asp <sup>33</sup> Asp <sup>213</sup>
Metalloprotease 1	Bovine carboxypeptidase A	Zn, Glu <sup>270</sup> Tyr <sup>248</sup>
Metalloprotease 2	Thermolysin	Zn, Glu <sup>143</sup> His <sup>231</sup>

\* Three-dimensional structure surmised by analogy with papain.

The serine proteases of the pancreas (namely chymotrypsin, trypsin and kallikrein) display a global conformation consisting of two compact, similarly folded domains arranged about a two-fold axis of symmetry (2). Catalysis involves the formation of an ester between the oxygen of serine 195 (chymotrypsin numbering system followed for convenience) and the acyl portion of the substrate, with release of the 'amino' portion of the substrate as the first product (13). Hydrolysis of the peptide bond of the substrate is brought about by the nucleophilic attack of the oxygen belonging to the hydroxyl group of serine against the carbonyl carbon atom of the susceptible double bond, thereby making the carbon-oxygen bond of the C=O group a single bond. The four atoms bonded to the carbonyl carbon atom become arranged as a transient tetrahedral intermediate complex, as a result of hydrogen bond formation between the negatively charged carbonyl oxygen atom/oxyanion and the two main chain NH groups.

The mechanism of catalysis consists of two steps, namely a) acylation and b) deacylation. During acylation, a proton is transferred from serine 195 to histidine 57. First, Asp 102 forms a hydrogen bond with the imidazole group of histidine 57, thereby increasing the pKa (and thus the basicity) of this histidine residue. This step is absolutely essential for the His 57 to accept a proton from Ser 195; otherwise the His 57 would be buried by the substrate during the reaction, owing to a low pKa value. Thus, the acceptance of the proton by His 57 would be inhibited (11). The histidine 57 residue donates the proton to the nitrogen atom of the susceptible peptide bond, which is cleaved. Thus, the  $-NH_2$  component is hydrogen-bonded to histidine 57 whereas the  $-COO^-$  component becomes esterified with serine 195. The amine component diffuses away, completing the acylation.

During deacylation, histidine 57 accepts a proton from water. The resulting  $OH^-$  attacks the carbonyl carbon atom of the acyl group attached to serine 195. A transient tetrahedral intermediate is formed after which the histidine 57 donates the proton to the oxygen of serine 195, which releases the acid component of the substrate, making the enzyme available for another round of catalysis (9).

The binding template of the protease molecule consists of a number of elements that act together—namely a) antiparallel beta-binding site for the acylating polypeptide chain of the substrate, b) specific side-chain binding sites that vary with the particular enzyme, c) less specific leaving-group site; where hydrogen bonding to the tetrahedral oxyanion takes place and the d) reactive serine side-chain for covalent bonding to the substrate's carbonyl carbon atom. (14).

**2) Cysteine proteases:** The enzymes of this family show similarities with serine proteases in the formation of a covalent intermediate. The major catalytic residue is cysteine 25, which functions as a hydrogen donor. These enzymes have a pH optimum in the range of 4-7 (1). Catalysis proceeds via the formation of a thiol ester intermediate and is facilitated by the side chains of adjacent residues, namely histidine 159 and aspartate at position 158. The sulphur atom of the cysteine brings about a nucleophilic attack, with the histidine 159 involved in a hydrogen acceptor role as in serine proteases. The tetrahedral intermediate formation is enabled by two  $-NH$  groups from residues Gln 19 and Cys 25, which by hydrogen-bonding with the carbonyl carbon atom of the peptide bond undergoing attack, forms an 'oxyanion'-like analog (13). The best known among the thiol proteases is papain, which is a single folded polypeptide of 212 amino acid

residues, containing three disulfide bonds and one free -SH at the active site. The molecular mass of papain is 23.4 kDa (15).

**3) Aspartic proteases:** The characteristic active site residues consist of two aspartic acid residues, which are in close geometric proximity with one another. The active pH range is 2-3 where one of these residues is ionized and the other unionized. (2).

The catalytic site of the free enzyme contains Asp 218, a protonated Asp 35 and a water molecule that hydrogen bonds between them. Binding of the substrate to the active site of the enzyme causes a distortion in the peptide bond of the substrate, by making the bond non-planar. This distortion causes the trigonal nitrogen atom configuration to change, yielding a pyramidal geometry. These configurational changes result in a reduction of the double bond character as well as the strength of the peptide bond. Hydrogen bond formation occurs between the carbonyl oxygen and O $\delta$  1 of Asp 35 and O $\gamma$  of Ser 38 of the enzyme. The carboxylate of Asp 218 facilitates nucleophilic attack by the bound water on the carbonyl group by proton abstraction. Binding of substrate to enzyme is facilitated by interactions with Asp 35 and Ser 38. The peptide nitrogen leaving group accepts a proton (thought to be the same proton abstracted from the attacking water molecule) from the Asp 218. Diffusion of products restores the hydrated environment for another catalytic cycle (16).

The aspartic proteases include bacterial pencillopepsin, renin, chymosin and mammalian pepsin. The HIV protease, which brings about cleavage of the key viral proteins during the replication of the AIDS virus, is also an aspartic protease (17, 18). This protease is contained within a polyprotein complex, which also contains several vital proteins. The HIV protease first cleaves peptide bonds within the polyprotein, in order to release itself from the complex. It then hydrolyses peptide bonds in the polyprotein complex, thereby releasing the proteins required for replication. X-ray crystallographic studies have indicated that the HIV protease is a symmetric dimer of identical polypeptides, each of which contributes an aspartate residue, at the sub-unit interface. This aspartate pair is critical for activity, and mutation in either aspartate yields a totally inactive virus. Since the HIV protease is essential for the replication of the virus, approaches to AIDS therapy include the use of HIV-protease inhibitors, also (19).

**4) Metalloproteases:** Metalloproteases are characterized by a neutral to slightly alkaline pH optimum and possess Zn<sup>2+</sup> in the active site. The zinc ion in association with the adjacent

carboxylate of glutamate 270 activates a water molecule, the nucleophilic oxygen atom of which, attacks the C=O group of the sessile peptide bond. Glutamate 270 simultaneously accepts a proton from the water. A negatively charged tetrahedral intermediate is formed. The intermediate is stabilized by electrostatic interactions with  $Zn^{2+}$  and the positively charged side chain of arginine 127. Following proton transfer from the COOH group to the peptide NH group, cleavage of the peptide bond and diffusion of the reaction products from the active site takes place (9). Bovine carboxypeptidase A has been extensively investigated among the mammalian metalloproteases, while thermolysin is a bacterial metalloprotease.

#### **Methods for determination of the mechanistic class to which a protease belongs:**

In the elucidation of the mechanism of proteolytic action, factors such as kinetic data under different conditions, susceptibility to a wide range of inhibitors, chemical modification studies and cleavage-site specificity are taken into account (13). Some of the methods that are commonly used for assigning the protease to one of the four mechanistic classes include the following: -

- 1) Effect of inhibitors **specific for the active site residues**
- 2) Effect of chemical modification
- 3) Nature of reaction intermediates.

1) **Effect of inhibitors specific for the active site residues:** Testing the effects of class-specific protease inhibitors on proteolytic activity involves the incubation of the protease with each of the inhibitors for 30–60 min under the assay conditions. The experimental controls would consist of the free enzyme incubated without inhibitor, maintaining the same conditions as that for the test reactions. The protease inhibitors specific for each class of protease are discussed as follows: -

- a) **Serine proteases:** One of the inhibitors that is highly specific for this class of proteases is diisopropyl phosphofluoridate (Dip-F), which reacts with the serine residues at the active site. Inhibition of enzyme activity in the presence of Dip-F is therefore considered to be one of the surest tests for the enzyme being a serine protease. Since this is a highly toxic compound, relatively safer compounds like phenylmethyl sulfonyl fluoride (PMSF) are commonly used. Both PMSF and Dip-F are also capable of inhibiting cysteine proteases, but this inhibition is reversible in the presence of reduced thiols such as  $\beta$ -mercaptoethanol (11). Other compounds such as

TPCK (tosyl-phenylalanine-chloromethyl ketone) and TLCK (tosyl-lysine-chloromethyl ketone) inhibit the enzyme by modification of the histidine residues in chymotrypsin and trypsin-like serine proteases, respectively (12). Other inhibitors of serine proteases include 3, 4-dichloroisocoumarin (20) and boronic acid derivatives of peptide aldehydes (e.g. MeO-Suc-Ala-Ala-Pro-Boro-Val that inhibits neutrophil elastase) among the synthetic compounds. Naturally occurring, high molecular weight inhibitors such as the avian ovomucoids, Kunitz-type trypsin inhibitors (e.g. aprotinin) and the soyabean trypsin inhibitor form tight, but reversible complexes with several proteinases (21).

- b) **Cysteine proteases:** the class-specific inhibitors of cysteine proteases consist of the following: -
- i) **Peptide diazomethanes,** which are a group of oligopeptide derivatives wherein the OH of the terminal carboxylate is replaced with a diazomethyl moiety CH-N=N. These compounds produce irreversible inhibition via the formation of a thiohemiketal intermediate, thus resulting in the alkylation of the cysteine residue in the active site e.g. Z-Phe-Phe-CHN<sub>2</sub>.
  - ii) **Peptide epoxides:** cause irreversible inhibition by the trans-epoxide group alkylating the active-site cysteine e.g. E-64 [L-Trans-epoxysuccinyl-leucylamide- (4-guanidino)-butane], which was originally isolated from an extract of *Aspergillus japonicus* (22), is a highly specific active site titrant, whose action is unaffected by reducing agents. Several derivatives of this compound have also been synthesized e.g. Ep-479, Ep-460 (21).
  - iii) **Cystatins,** which are naturally occurring protein inhibitors that have a molecular size of about 12kDa. They are tight-binding, highly specific, reversible inhibitors of cysteine proteases. e.g. Human cystatins A, B and C, chicken ovocystatin(21,23).
- c) **Aspartic proteases:** Pepstatin A or isovaleryl-Val-Val-statine-Ala-statine (secreted by *Streptomyces* species) is the best-characterized inhibitor of aspartic proteases (21).
- d) **Metalloproteases:** 1, 10-phenanthroline acts by chelating the essential metal ion contained in metallopeptidases. However 1, 10-phenanthroline is also capable of inhibiting metal-activated enzymes that may belong to one of the other mechanistic categories. Mechanistic determination of a newly isolated protease as a

metallopeptidase should therefore be based on its insensitivity to the inhibitors that are specific for the other three categories, apart from inhibition by 1, 10 phenanthroline. Phosphoramidon is also an excellent inhibitor of thermolysin and several other metallopeptidases (13). Tissue inhibitor of metalloproteinase (TIMP) is an endogenous protein inhibitor, which inhibits several matrix metalloproteinases such as collagenase (MMP1), gelatinase (MMP 2) and stromelysin (MMP3).

2. **Chemical modification/identification:** Apart from inhibition of enzyme activity by active-site modification, modification of non-catalytic amino-acid residues also could alter the conformation of the enzyme molecule in a manner that results in loss of activity. This loss of activity may even exhibit linearity with respect to the concentration of the modifying agent, thereby resembling active-site modification in terms of stoichiometry, as well. For example, Dip-F could inhibit not merely the serine residue at position 195 in the active site of chymotrypsin but also any of the other 32 non-catalytic serine residues. In order to determine which residue has been modified, experimentally, the enzyme is incubated with the substrate/inhibitor at concentrations higher than the  $K_m$  or  $K_i$  value followed by exposure to the modifying agent. Chemical modification would occur independent of the presence of substrate/inhibitor if the residues being modified were outside the active site. If the modification involved amino acid residues at the active site, the presence of the substrate/inhibitor in the active-site cleft at concentrations higher than the  $K_m$  or  $K_i$  would cause a decline in the accessibility of the concerned amino acid residue to the modifying agent. Hence, under these conditions there would be a decline in the rate of chemical modification.

This method of analysis enables information on the position of the amino acid residues undergoing modification—namely, whether the same are situated at the active site or whether they constitute non-catalytic residues (21). e.g. parthenain is a serine protease, whose activity is unaffected by thiol inhibitors such as iodoacetic acid even at a  $10^6$  molar excess of the inhibitor. However,  $Hg^{2+}$  was found to inhibit the enzyme to the extent of 70%, when present in molar ratios of 1:  $10^5$  (enzyme: inhibitor) (24). This clearly indicates that mercuric ions are acting at sites (containing  $-SH$  groups) other than the active site. An integrated study of this kind, enables us to probe the reaction mechanism of the protease and assign it to its rightful mechanistic category.

**3) Mechanistic distinctions- intermediates:** Covalent intermediates are formed in proteolytic reactions involving serine and cysteine proteases, but not in the case of aspartic / metalloproteases. Through kinetic analysis (i.e. by performing the reaction under a variety of conditions of substrate and enzyme concentration, incubation and so on), the quantitation of the released product corresponding to the amount of enzyme utilized can be determined. Addition of nucleophilic agents such as alcohols, amines, hydroxylamines, hydrazines and other nucleophilic agents in a reaction mixture influences the release of a modified product, differently from what would be otherwise expected in the reaction. Thus, the presence of a reactive intermediate is indicated. Identification of a covalent enzyme-substrate complex is thus possible through this nucleophilic trapping technique (21) e.g. chymostatin and phenyl ethane boronic acid (PEBA) form distinct covalent adducts upon reaction with chymotrypsin. It is the side-chain hydroxyl of Ser 195 that reacts with the aldehyde group of chymostatin to form the tetrahedral hemiacetal intermediate. Similarly, covalent bonding between Ser 195 and PEBA causes the formation of the tetrahedral adducts. The mode of binding between the enzyme and these inhibitors is consistent with the mechanistic proposal for serine proteases (16). Thus, the elucidation of the enzyme mechanism becomes clear and this also helps in defining the mechanistic class of a new enzyme. Such studies have become possible, following the advancement in X-ray crystallography and related techniques.

### **Plant latex proteases**

Specialized elongated parenchyma cells termed as laticifers were discovered in 1877 by H.A de Bary. These cells, found mainly in xerophytes, produced a viscous white/yellowish or colourless fluid termed as latex. Latex consists of a suspension of various substances such as waxes, resins, proteins, essential oils, alkaloids and sugars. Apart from various active principles and metabolites, the latex of plants is also a rich source of several enzymes such as chitinases and proteolytic enzymes. The latex- producing families include Apocynaceae, Asclepiadaceae, Euphorbiaceae, Asteraceae, Caricaceae, Musaceae, Utricaceae, Moraceae and Papaveraceae. The main functions of plant latex include protection from drought, wound healing and defense against herbivores (25).

One of the earliest plant latex proteases to be isolated was papain, which is a widely used protease in the food and beverage industries today. Papain was extracted and crystallized from the undried latex of the green papaya (*Carica papaya*) fruit. The enzyme was found capable of digesting casein and hydrolyzing hippurylamide, in the presence of

cysteine (26). The modifications in the original isolation technique for papain (27) resulted in the increased yield of the crystals and determination of the enzyme's substrate specificity (28). Meanwhile, chymopapain, another acid-stable protease with a "higher ratio of protein-digesting activity to milk-clotting power" than crystalline papain was isolated from the latex of *Carica papaya* (29). This was followed by reports of proteolytic activity from the latex of the madar plant (*Calotropis gigantea*) leading to the isolation and purification of calotropain, a sulfhydryl protease, capable of digesting a wide range of protein substrates like casein, hemoglobin, egg-albumin, fibrin and edestin (30). Also, the discovery of proteolytic activity in the pineapple plant (*Ananas sativus*) led to the findings on stem bromelain, which consisted of five different proteolytic fractions (31). Besides separation of these fractions, their substrate specificity was also determined (32).

Ten proteolytic enzymes termed ficins A-I were isolated from the latex of *Ficus carica* var. kadota that were found capable of hydrolyzing casein and alpha-benzoyl-L-argininamide (33). Calotropains F-1 and F-2 were isolated from the latex of *Calotropis gigantea* by CM-cellulose chromatography. Both these proteases were thermostable, displayed a broad pH optimum and were fully active in 8 M urea. They also contained carbohydrate (34). *Asclepias syriaca* latex yielded thiol proteases termed as 'asclepains', which exhibited both esterase and amidase activity. These enzymes that were purified by CM-Sepharose chromatography, digested insulin B-chain and showed preference for peptide bonds adjacent to basic amino acid residues (35-36).

Latex proteinases are abundant in several genera of Euphorbiaceae such as *Euphorbia lathyris* (37), *E. pulcherimma* (38), *E. lactea*, *E. cyparissias*, *E. tirucalli* (39), *Hevea brasiliensis* (40) and *Elaeophorbium drupifera* (41). All these proteases were classified as serine proteases. Additionally, they also required the presence of an essential histidine residue for activity. They were also highly thermostable (37, 39, 41). The enzymes purified from *Parthenium argentatum* (24), a member of Asteraceae and from *Ficus elastica* of family Moraceae (42) are glycoproteins.

Since the production of latex by plants serves a protective function, it has been observed that many of the latex proteinases, exhibit good thermal stability and resistance to the action of synthetic inhibitors (34, 36-42). There is a good industrial potential for such proteases that are active even under harsh conditions like high temperature, extreme pH and high salt concentrations.

## Industrial applications of proteolytic enzymes

All proteolytic enzymes have characteristic properties with regard to temperature and pH, ion requirements, specificity, activity and stability. These biochemical parameters determine the application of a protease in industry apart from other factors, which include the costs of production and development, market and the economics of application. Some of the major industries in which proteases find application are given in Table.2.

Table.2. Proteases in industry (43)

Industry	Enzyme	Application
Detergents	Alkaline proteases	Extensive use in laundry detergents for protein stain removal
Dairy	Calf rennet and others-trypsin, chymotrypsin, ficin and papain.	Coagulation of milk protein(cheese production); production of enzyme-modified cheese; whey processing
	Fungal proteases	Replacement of calf rennet
	Chymosin	Active component of calf rennet; also production by genetically engineered microbes developed.
Leather	Trypsin	Bating of leather
	Other proteases	Dehairing and dewooling of skins
Beverages	Papain	Removal of turbidity in beverages.
Baking	Neutral protease	Dough conditioner
Meat and fish	Papain	Meat tenderizing
	Several proteases	Protein recovery from fish wastes
Food processing	Several proteases	Modification of protein-rich material, i.e soy protein or wheat gluten
Confectionery	Thermolysin	Reverse hydrolysis in aspartame synthesis
Pharmaceutical	Trypsin	Removal of dead tissue and dissolution of blood clots
	Chymopapain	Treatment of certain types of hernia
	Carboxypeptidase	Conversion of hog insulin
	Trypsin	Production of human insulin (recombinant)

The latex of several plants has constituted a valuable source of many of the proteases that are of commercial value e.g. papain from the latex of *Carica papaya*, which was found to have meat tenderizing properties (44), apart from being used in other industries indicated in

Table.no.2. Anainain, from the latex of *Ananas sativus* is found effective in the debridement of burns (45). Bromelain, another complex of enzymes from the latex of the same plant has the following properties that indicate considerable promise for the plant latex, from the pharmacological point of view. They include:-

(a) interference with growth of malignant cells; (b) inhibition of platelet aggregation; (c) fibrinolytic activity; (d) anti-inflammatory action; (e) skin debridement. These properties are likely to enable the medicinal use of bromelain in the treatment of third degree burns and malignancies, modulate blood coagulation in circulatory disorders and enable the enhanced absorption of drugs (46).

**Plant latex in industry and medicine:** The latex of several plants such as the para- rubber tree (*Hevea brasiliensis*), *Cryptostegia grandiflora* and the Indian rubber tree (*Ficus elastica*) are commercially tapped for rubber. Likewise, the latex of *Calotropis gigantea* and *C. procera* are also used in the tanning industry for deodorizing, removal of hair and imparting colour to hides (47).

Plant latex has found considerable application in ethnomedicine and ethnopharmacology. The dried latex of *Euphorbia resinifera* has been in medicinal use since the time of recorded history, as evident from the writings recorded during the period of the Roman Emperor Augustus. The dried latex, commonly termed Euphorbium was applied to nerves for mitigation of pain and also used as a dental analgesic in ancient times. Today, resiniferatoxin, an ultrapotent capsaicin analog (isolated in 1975) is identified as the active principle on which studies are being conducted to assess its efficacy in relieving pain associated with diabetic polyneuropathy and post herpetic neuralgia. Resiniferatoxin is a comparatively less pungent capsaicin analog, with promise of better therapeutic potential, that is undergoing clinical trials (48). The latex of *Calotropis procera*, a plant commonly used in Ayurveda has also been tested in guinea pigs and found to have a good efficacy in healing of dermal wounds (49). The latex from *Himatanthus sucuuba* is used in popular amazonian medicine as an anti-inflammatory remedy, which is chiefly contributed by the lupeol cinnamates present in it (50). The latex of *Ficus laurifolia* has been traditionally used as vermifuge in Central and South America. Termed as 'leche de higueron', the milky sap is reported to destroy *Ascaris* (roundworm) in vitro. The purified protein fraction containing the active antihelminthic principle isolated from 'leche de higueron' was termed as ficin (51). Ficin was subsequently characterized as a proteolytic enzyme, whose pharmacological

properties have also been studied (52). The utilization of the latex of *E. splendens* against the molluscs *Melanoides tuberculata* and *Biomphalaria glabrata* showed promise in reducing the *B. glabrata* population (53).

The latex of *Cerbera manghas* is the source of a pharmacologically potent drug cerberin, which is emetic and purgative. The dried latex of the roots of *Ferula asafoetida* is the source of commercial asafoetida, which is used as a condiment and also in medicine as a stimulant of the respiratory and intestinal tracts and also the nervous system (47).

*Synadenium grantii* is a widely distributed shrub, cultivated along hedges and fences along gardens. Three acetylcholinesterase isozymes have also been isolated from this latex. These glycoproteins were dimeric proteins, having a molecular mass of 70 kDa and a temperature optimum of 40 °C (54).

### SCOPE OF THE PRESENT WORK

In addition to their important role in the protein turnover process, proteases are intricately involved in the regulation of many physiological processes through limited but specific proteolytic action on protein precursor molecules. These vital functions are carried out by different classes of proteases.

Among the four well-recognized classes, the serine proteases are the most extensively studied. The biochemical characterization together with the computer analysis and X-ray crystallographic, mass spectroscopic and multidimensional NMR data have given us a much clearer insight into the structure and function of proteases in general and serine proteases in particular.

The investigation of proteases from diverse sources suggests that although some features of the serine proteases appear common, there could be structural differences between them. The most common feature is the presence of the catalytic triad- Asp/His/Ser in this class of proteases. The protease from the cytomegalovirus has a catalytic triad of His/His/Ser instead of the conventional Asp/His/Ser triad mentioned above (55). Furthermore, a single beta barrel structure per monomer was found in the viral enzyme instead of the two observed in the mammalian counterpart. Even larger structural variations were observed in the adenovirus cysteine protease, in comparison with the plant latex-derived papain-another well-studied cysteine protease (55).

It is therefore important to gather biochemical and structural information on proteases from diverse sources and to enlarge our understanding of the structure-functional relationship in this class of enzymes. This may prove useful in our efforts to design proteins of well-defined proteolytic activity and to realize the full application potential of proteases.

There are several reports of the isolation and characterization of proteases from plant latex. Some of these proteases appear larger in size and seemed thermostable. Preliminary investigation on the latex of *Synadenium grantii* indicated the presence of good proteolytic activity. The latex from *Synadenium grantii* therefore formed the source of the enzyme for the present study.

The latex material was clarified to give a clear water-soluble enzyme source. The enzyme was purified by resorting to ammonium sulphate precipitation, gel-filtration and ion-exchange chromatography. The purified enzyme was analyzed by SDS-PAGE and used for further studies.

This thesis has been organized as follows: -

- 1) Introduction
- 2) Chapter 1: Isolation and purification of the proteolytic enzymes from the latex of *Synadenium grantii*.
- 3) Chapter 2 : Structural and physico-chemical characterization of the proteases
- 4) Chapter 3: Biotechnological applications: possible utility of the *S. grantii* proteases.
- 5) Summary and conclusion.
- 6) Appendix

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