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Review

Papain-like proteases: Applications of their inhibitors

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Proteases are one of the most important classes of enzyme and expressed throughout the animal and plant kingdoms as well as in viruses and bacteria. The protease family has drawn special attention for drug target for cure of several diseases such as osteoporosis, arthritis and cancer. Many proteases from various sources are being studied extensively with respect to activity, inhibition and structure. In this review, we hope to bring together the information available about the proteases with particular emphasis on papain-like plant cysteine proteases. Besides, protease inhibitors and their potential utilities are also discussed.

Key words: Proteases, plant latex, reaction mechanism and protease inhibitors.

INTRODUCTION

Proteolytic enzymes are of widespread interest because of there industrial application and because they have been implicated in the design and synthesis of therapeutic agents (Neurath, 1989). With the advent of molecular biology, proteolytic enzymes have become a fertile and exciting field of basic as well as applied research. Identification of novel genes encoding proteases has considerably increased our knowledge of proteases and provided fresh imputes. The proteases have different cellular distribution and intracellular localization which may contribute to defining specific functional roles for some of these proteases.

Proteases have been divided into six mechanistic classes by the International Union of Biochemistry. These include the cysteine, serine, aspartic, metalloprotease, threonine and unknown type (Enzyme nomenclature, 1992). The threonine protease is the most recently discovered (Seemuller et al., 1995). Each class has a characteristic set of functional amino acid residues arranged in a particular configuration to form the active site. The different proteases class includes distinct families and the members from different family differ from each other in amino acid sequence despite a common active site geometry and enzymatic mechanism. Family of peptidases shows evidence of their evolutionary relationship by their similar tertiary structures, by the order of catalytic residues in their sequences, or by common sequence motifs around the catalytic residues. The proteases have been organized in to evolutionary families and clans by Rawlings and Barrett (1993, 1994), which led to development of MEROPS database of proteases. MEROPS database (http://merops.sanger.ac.uk) includes listing of all peptidase sequences from different families and clans. Each new updates adds new members and families. Some representative family and clans of cysteine, serine and threonine proteases are listed in Table 1. The related families are grouped into clans, which contains all the peptidase that arose from a single evolutionary origin. The designation of family follows the catalytic type, serine (S), cysteine (C), or threonine (T). However, some of the clans are mixed type and contains families with two catalytic types or more catalytic types and designated with the letter "P". The cysteine protease family comprises six major families: the papain family, calpains, clostripains, streptococcal cysteine proteases, viral cysteine proteases and most recently established, caspases (also called apopains). Overall, twenty families of cysteine peptidases have been recognized (Rawlings and Barrett, 1994). The order of cysteine and histidine residues (Cys/His or His/Cys) in the linear sequence differs between families. The families C1, C2 and C10 can be described as papain-like, C3, C4, C5, C6, C7, C8, C9, C16, C18 and C21 are represented in viruses while C11,

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C15 and C20 are from bacterial source.

CATALYTIC MECHANISM

Hydrolysis of a peptide bond is an energetically favorable reaction, but extremely slow (Wolfenden and Snider, 2001). The active site residues of serine, cysteine, and threonine proteases are shown in Figure 1A. The active site residues in all class of proteases have many mechanistic features in common. Each enzyme has an active site nucleophile and a basic residue, which can also function as a general acid in the catalytic mechanism. The transition states for serine, cysteine, and threonine proteases all involve formation of a tetrahedral intermediate shown in Figure 1B. The oxyanion of the tetrahedral intermediate is frequently stabilized by interaction with several hydrogen bond donors, which is commonly referred to as the oxyanion hole. The oxyanion hole of serine proteases is usually quite rigid and involves backbone peptide bond NH groups as hydrogen bond donors. Interaction with the oxyanion hole is usually essential for effective substrate hydrolysis. With cysteine proteases, the oxyanion hole does not seem to be as essential and is much more flexible at least in the case of the papain family.

Cysteine peptidases of the papain family catalyze the hydrolysis of peptide, amide, ester, thiol ester and thiono ester bonds (Brocklehurst et al., 1987). The basic features of the mechanism include the formation of a covalent intermediate, the acyl-enzyme, resulting from nucleophilic attack of the active site thiol group on the carbonyl carbon of the scissile amide or ester bond of the bound substrate. The first step in the reaction pathway corresponds to the association (or noncovalent binding) of the free enzyme and substrate to form the Michaelis complex. Acylation of the enzyme, with formation and release of a first product follow this step from the enzyme, the amine $R'NH_2$. In the following step, the acyl-enzyme reacts with a water molecule to form the second product (deacylation step). Release of this product results in the regeneration of the free enzyme.

STRUCTURAL FOLDS

The structural determination (x-ray of NMR) of proteases is lagging considerably behind the sequence determination. MEROPS database indicates that the structures of majority of proteases are not yet available and opens a wide area of studies. However, the available structures show high degree of variability. The proteases seem to be distributed into all of the major structural classes of proteins [α-proteins, β-proteins, α- and β- proteins (α/β or $\alpha + \beta$), multidomain proteins, membrane and cell surface proteins, and small proteins]. Prokaryotic and eukaryotic trypsin-like serine proteases, some viral serine proteases, and viral cysteine proteases with the trypsin-fold are classified as β-proteins. The proteasome subunits are $\alpha + \beta$ proteins composed mainly of antiparallel β-sheets with segregated α and β regions. The group of cysteine proteases with papain, cruzain, and cathepsin also has this structure. The subtilisins and caspases are members of the α/β group of proteins with parallel $\tilde{\beta}$sheets (β-α-β units). All known cysteine proteases can be grouped in at least 30 protein families. Each family contains proteins with similar amino acid sequences and evolutionarily conserved sequence motifs, which reflects the family members' similar 3D structures.

Three-dimensional structure has been elucidated for papain, a representative member of papain-like cysteine proteases (Drenth et al., 1971; Kamphuis et al., 1984), as well as other members like actinidin (Baker, 1980), calotropin (Heinemann et al., 1982), cathepsin B (Musil et al., 1991), caricain (Pickersgill et al., 1991), glycyl endopeptidase or papaya proteinase IV (O'Hara et al., 1995), chymopapain (Maes et al., 1996) and cruzain (McGrath et al., 1995) and all show bilobed molecules in which catalytic site is located in a cleft between the lobes. All papain-like cysteine proteases share similar sequences (Kamphuis et al., 1985; Kirschke et al., 1995; Berti and Storer, 1995) and have similar 3-dimensional structures. The structural data provides a strong evidence that all arose from a common ancestor. All known papain-like cysteine proteases, irrespective or origin, except cathepsin C, are monomers whose structure consists of two domains (referred to as the R- and L- domains) according to their right and left position in the standard view. The domains fold together in the form of a closed book. The interactions between the domains have hydrophobic as well as hydrophilic character and are specific for a particular enzyme. The structure of papain has been extensively studied. The two catalytic residues that is, Cys 25 and His 159 in papain each from N- and C-terminal domains respectively, are present in a 'V' like shaped active site cleft situated on the top of the enzyme structure. Recently structure of two new of papain-like cysteine proteases, ervatamin B and ervatamin C, purified in our laboratory, have been reported (Biswas et al., 2003; Thakurta et al., 2004), which also shows strong structural similarly with papain (Figure 2).

Papain has a large binding site and there are a number of interactions that exist between the enzyme and the substrate over an extended region. Coupling of these substrate binding interactions to the hydrolytic process occurring at the active site is an important aspect of catalysis. Schechter and Berger (1967) proposed that the active site of papain contained seven subsites each capable of accommodating a single amino acid residue of a peptide substrate. The subsites are located on both sides of the catalytic site, four on the N-terminal side and three on the C-terminal side. The amino acid residues on the amino-terminal side of the scissile bond are numbered P1, P2, P3,… counting outwards; the residues on

Table 1. Representative families and clans of cysteine, serine and threonine proteases.

Clan	Family	Examples
CYSTEINE PROTEASES CLANS		
CA	C1, C2	papain, cathepsins B, K,L,S,H
CD	C11, C13, C14, C25, C50	legumain, caspases. gingipain, separase
MIXED (CYSTEINE, SERINE, THREONINE) PROTEASES CLANS		
PA(C)	C3 (viral), C30 (viral)	picornain 3C, SARS virus 3C-like endopeptidase
PA(S)	S1	chymotripsin, trypsin, elastase, cathepsin G
THREONINE PROTEASES CLANS		
PA(T)	T1	archaean proteasome
SERINE PROTEASES CLANS		
SB	S8	subtilisin
SC	S9, S10	Prolyl oligopeptidase, carboxypeptidase Y
SE	S11, S12	D-Ala-D-Ala carboxypeptidases A and B

Rawlings and Barrett (1993, 1994).

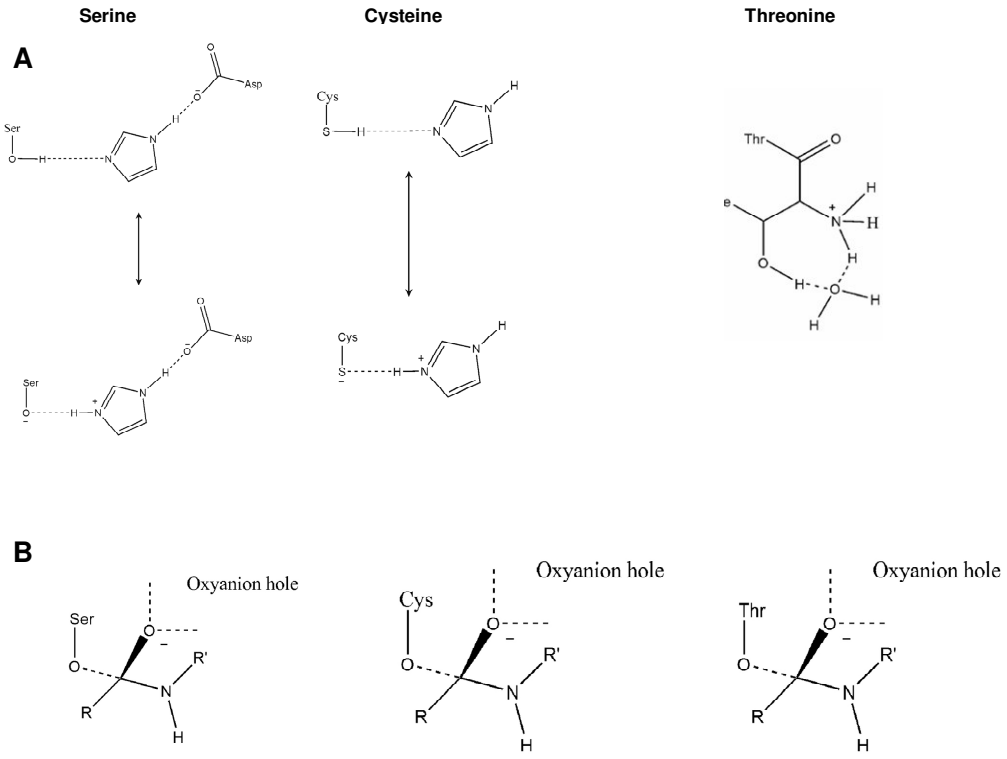

Figure 1. (A) Active site residue (B) transition states of protease hydrolysis of serine, cysteine, and threonine proteases. In serine proteases, three residues form the catalytic triad are essential in the catalytic process, that is, His 57, Asp 102 and Ser 195 (chymotrypsinogen numbering). In cysteine proteases, catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue. While active site of threonine proteases comprises of threonine, methionine and backbone amide.

the carboxy-terminal side of the scissile bond are numbered $P1^1$, $P2^1$, $P3^1$,... The subsites on the protease are termed S3, S2, S1, $S1^1$, $S2^1$, $S3^1$... to complement the substrate residues that interact with the enzyme. In papain one of the subsites, S2, specifically interacts with a phenylalanine side chain of peptides.

Thus, all members of the papain family inspite of their dissimilar origin, show considerable similarity in terms of

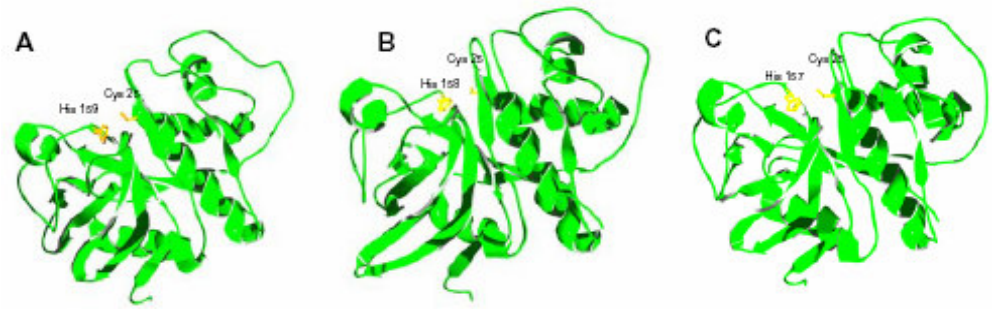

Figure 2. Ribbon diagram of (A) Papain (PDB accession: 9PAP), (B) Ervatamin B (PDB accession: 1IWD) and (C) Ervatamin C (PDB accession: 1O0E) showing catalytic residues i.e., Cys and His between cleft of two domain. All papain-like cysteine proteases have catalytic residue between domain cleft.

activity, pH optima, molecular mass, catalytic mechanism and the peptide regions near the active site cysteines are quite similar. However, despite the similarities, it has been suggested that all the cysteine proteases may have arisen by convergent evolution (Lowe, 1976). More structural information from sulfhydryl enzymes of more closely related genera of plants and microbes is needed to conclude that whether or not any evolutionary relationship exists. Structural determination of several proteases purified in our laboratory may provide additional insights (Dubey and Jagannadham, 2003a; Patel and Jagannadham, 2003; Nallamsetty et al., 2003).

PAPAIN SUPER FAMILY (C1 CYSTEINE PROTEASES FAMILY)

Cysteine proteases of the papain super family are widely distributed in nature. They can be found in both prokaryotes and eukaryotes e.g. bacteria, parasites, plant, invertebrates and vertebrates (Berti and Storer, 1995). Papain-like cysteine proteases are the most abundant among the cysteine proteases. The papain family contains peptidases with a wide variety of activities, including endopeptidases with broad specificity (such as papain), endopeptidases with very narrow specificity (such as glycyl endopeptidases), aminopeptidases, a dipeptidyl-peptidase, and peptidases with both endopeptidase and exopeptidase activities (such as cathepsins B and H). There are also family members that show no catalytic activity. Enzymes of papain family are found in a wide variety of life forms: baculovirus (Rawlings et al., 1992), eubacteria like *Porphyromonas* and *Lactococcus*, yeast (Enenkel and Wolf, 1993), and probably all protozoa, plants, and animals.

The family consists of papain and related plant proteases such as chymopapain, caricain, bromelain, actinidin, ficin, aleurain, etc. Lysosomal cysteine proteases, also known as cysteine cathepsins (Cats), include Cat B, Cat H, Cat S, Cat K, Cat O/2, Cat F, Cat W and Cat U (Chapman et al., 1997; Turk et al., 1997) and also belong to the papain family sharing similar protein structure and mechanism of action. However, slight structural differences make these enzymes distinct with respect to their substrate specificity and regulation. Cathepsins are synthesized as 30 - 50 kDa precursor proteins, which are glycosylated and phosphorylated in the Golgi apparatus. They are processed in the lysosomes to their active forms by one or more proteolytic cleavage. The optimum activity of cathepsins is pH 5.0 - 6.5, although they can hydrolyze large substrates also at neutral pH. The pH dependent activity of cathepsins is rather complex and depends not only on the microenvironment and the nature of the conformation of the substrate, but also on the presence or absence of stabilizing factors (Keppler and Sloane, 1996).

Most of these papain-like enzymes are relatively small proteins with M_r values in the range 20 - 35 kDa (Brocklehurst et al., 1987; Polgar et al., 1989; Rawlings and Barrett, 1994; Berti and Storer, 1995). However, cathepsin C is an oligomeric enzyme with M_r ~ 200 kDa (Metrione et al., 1970). All cysteine proteases except cathepsin C are endopeptidases (Kirschke et al., 1995). Cathepsin B is a dipeptidyl carboxypeptidase (Aronson and Barrett, 1978), cathepsin H is an aminopeptidase (Koga et al., 1992) and cathepsin C is a dipeptidyl aminopeptidase but at higher pH exhibits dipeptidyl transferse activity (Kirschke et al., 1995).

Disturbance of the normal balance of enzymatic activity of lysosomal cysteine proteases may lead to pathological conditions, and these proteases have been found to be involved in many such cases. The participation of these enzymes in various diseases seems to be restricted to their proteolytic function outside the lysosomes, after secretion from lysosomes or after translocation into different intracellular granules. The resulting uncontrolled proteolysis is a result of an imbalance between catalytically active proteases and their natural inhibitors, and can be observed in e.g. inflammation and tumor growth, although these processes are very complex.

Cysteine proteases of the papain family have been reported in bacteria as well. Proteolytic enzymes produ-

ced by *Porphyromonas gingivalis* are important virulence factors of this periodontopathogen. In the periodontal disease proteolytic enzymes are produced in large quantities. It has been shown that these proteases can directly or indirectly degrade constituents of the periodontal tissues, destroy host defense elements, dysregulate coagulation and complement kallikerinkinin cascades. Recently, proteases belonging to two catalytic classes and produced by *P. gingivalis* have been identified. One enzyme is described as an Arg-X specific proteinase (Chen et al., 1992) and another is Lys-X specific (Pike et al., 1994). Since the first purified enzyme shared some properties with clostripain, it was named as a gingipain. Following the recommendations by the IUB, these proteases are referred to as gingipain-R and gingipain-K to account for their unique specificity.

PAPAIN-LIKE CYSTEINE PROTEASES FROM ANIMAL

Medically interesting proteases in Family C1 (the papain family) include mammalian enzymes such as cathepsins B and L (involvement in cancer growth and metastasis) and cathepsin K (of importance for bone degradation an osteoporosis) as well as parasitic enzymes being essential for the parasite-host interaction (e.g. cruzipain from Trypanosoma cruzi - causing Chagas' disease, and falcipain from Plasmodium falciparum - causing malaria). Predominant expression of cathepsin K in osteoporosis and its well documented role in bone remodeling makes cathepsin K and interesting target for the pharmaceutical industry. Enzymes belonging to Family C13 (the legumain family) have been shown to play key roles in antigen presentation. Interleukin converting enzyme (ICE) and other enzymes belonging to Family C14 (the caspase family) have gained much interest recently, as key mediators of apoptosis. Significant activation of calpain, often associated with loss of calcium homeostasis, implicated in pathology of several diseases like muscular dystrophy, stroke, traumatic brain injury, alzheimer's disease, cancer and type 2 diabetes mellitus (Carragher, 2006). As a result of recent reports that animal papain-like proteases are involved in several pathological conditions, interest in the development of inhibitors has substantially increased. Many pharmaceutical companies are seeing it as big drug market and several clinical trails of inhibitors are under process (Table 2). To ensure selectivity of inhibition, which is major challenge, structure based drug design is becoming more desirable. In addition to animal sources, structural determination of papain-like proteases form plant and other sources would certainly provide better understanding of the structural feature of these proteases and help us in designing inhibitors for papain-like proteases for therapeutic application.

PAPAIN-LIKE PROTEASES FROM PLANT

Plant sources have yielded many useful endopeptidases, among them calotropins (Abraham and Joshi, 1979), bromelain (Takahasi et al., 1973), papain (Kimmel and Smith, 1954), and ficin (Englund et al., 1968) and have used extensively in food and medicine industry. Besides, some of these proteases have also been used as model systems for studies on structure-function relationships and protein folding problems (Kundu et al., 1999; Edwin and Jagannadham, 1998, 2000; Dubey and Jagannadham 2003b). Proteolytic enzymes from plant sources have received special attention in the pharmaceutical industry and biotechnology due to their property of being active over wide ranges of temperature and pH. All the plant cysteine proteases exhibit pH optima in the region 5.0 - 8.0, and almost all have a molecular mass in the range 25 - 30 kDa except a few in the range of 50 - 75 kDa. It is probable that all such sulfhydryl endopeptidases employ similar catalytic mechanisms to hydrolyze peptide bonds of proteins but, because of evolutionary diversity, their sizes, specificities and kinetic properties may vary considerably.

Papain from the latex of *Carica papaya* was the first sulfhydryl enzyme discovered and has been the subject of mechanism and structural studies for many years (Drenth et al., 1971; Glazer and Smith, 1971). The M_r of papain is 23.4 kDa and pH optimum is 5.5 - 7.0. The enzyme is very stable at neutral pH, even at elevated temperatures (Glazer and Smith, 1971). It contains six sulfhydryls and one free cysteine, which is part of the active site. The complete aminoacid sequence of the enzyme is known, and the three-dimensional structure has been determined by X-ray crystallography (Drenth et al., 1971). Schechter and Berger (1967) concluded that as many as seven sites for recognizing substrate amino acid residues exist on the enzyme, all contributing to substrate specificity. It hydrolyses amides of arginine, lysine readily and glutamine, histidine, glycine and tyrosine at reduced rates (Glazer and Smith, 1971). Besides papain, papaya latex also contains chymopapain, (Jansen and Balls, 1941) and papaya peptidase A now known as caricain (Schak et al., 1967). All the three endopeptidases differ in primary structure, have very similar substrate specificities and are generally assayed with synthetic substrates having Arg in P_1 position. Another fraction has been detected that demonstrated activity against the glycine ester but not against Bz-Arg-Pna, the ideal substrate for the three endopeptidases isolated from papaya latex. This fraction was named papaya peptidase B. This enzyme is now called proteinase IV or glycyl endopeptidase (Buttle et al., 1989).

The proteolytic enzymes of the pineapple plant *Ananas comosus* are known as bromelains, from stem called stem bromelain and from fruit called fruit bromelain. There has been a considerable confusion as to whether these enzymes are distinct proteins (Ota et al., 1972; Ota et al., 1985) or represent two forms of the same enzyme (Iida et al., 1973; Sasaki et al., 1973). The pineapple plant has been shown to contain at least 4 distinct endo-

Table 2. Pharmaceutical application of inhibitor of papain-like proteases.

Company	Proteases	Disease	Inhibitor/ clinical trial
[1]Vertex/Aventis	Caspase-1 (ICE)	osteoarthritis, Psoriasis	VX-740 / Phase II
[1]Vertex	Caspase-1 (ICE)	Inflammatory	VX-765/ Phase I
	Caspase (Broad Spectrum)	Liver diseases	IDN-6556/ Phase IIb
	Caspase	Acute myocardial infarction	IDN-6734/Phase-I
[2]Idun Pharmaceuticals	Caspase-1 (ICE)	Inflammatory (Asthma, arthritis)	IDN-9862, Preclinical
[3]GlaxoSmithKline	Cathepsin K	Osteoporosis	SB-462795/ Phase I
[4]Novartis	Cathepsin K	Osteoporosis	AAE581/ Phase IIb

[1]http://www.vrtx.com; [2]http://www.idun.com; [3]http://www.hgsi.com; http://dominoext.novartis.com.

peptidases (Rowan et al., 1988; Rowan et al., 1990). These include, besides stem and fruit bromelain, two other cysteine endopeptidases, ananain (Rowan et al., 1988) and comosain (Rowan et al., 1990). Similarly, fivecysteine proteases known as ficins have been purified to homogeneity from the latex of *Ficus glabrata* (Jones and Glazer, 1970). All the enzymes had a molecular mass of 25 - 26 kDa with an amino terminal residue leucine. They displayed similar specificity and kinetic properties towards the insulin B chain. Actinidin is an anionic protease isolated from the latex of *Actinidia chinensis* (Chinese gooseberry). The enzyme shows a molecular mass of 15.4 kDa and is inhibited by DTNB and iodoacetamide (McDowall, 1970). The latex of *Calotropis gigantea* also contain four cysteine proteases designated as calotropin FI, FII (Abraham and Joshi, 1979a, 1979b) and Calotropin DI, DII (Pal and Sinha, 1980). Two groups of cysteine proteases called asclepains have been isolated from the latex of *Asclepias syriaca* and a representative of each has been purified. Asclepains A3 and B5 are homogeneous proteins with molecular weights of 23 kDa and 21 kDa respectively. They are inhibited by thiol specific inhibitors and have a pH optimum of 7.0 - 7.5 (Brockbank and Lynn, 1979). Five forms of asclepains have been purified to homogeneity and the sequence of first 21 residues has been determined and compared to papain (Lynn et al., 1980a, 1980b).

A cysteine protease has been isolated from the ripe yellow fruits of the bead tree, *Melia azedarach* (Kaneda et al., 1988). Later, the pressed juice of greenish fruits of the tree showed very high caseinolytic activity leading to the isolation of another cysteine protease called melain G (Uchikoba et al., 1999) and the protease isolated from ripe juice was called melain R. From the sites cleaved in the oxidized insulin B-chain and synthetic oligopeptide substrates by melain G, the enzyme preferred small amino acid residues such as Gly or Ser at the P_2 position and negatively charged residues such as glutamic or cysteic acid at the P_3 position. This is clearly different from the specificity of papain, which prefers the large hydrophobic amino acid residues such as Phe, Val, and Leu at the P_2 position (Drenth et al., 1971; Asboth et al., 1988; Kaneda et al., 1995). Accordingly, it is presumed that the bottom of the S_2 pocket of melain G is shallow due to the presence of a Phe residue, and a bulky P_2 substrate (for example Phe residue) is not preferred by the enzyme. Negatively charged residues at the P_3 positions of substrates well suited the S_3 site of melain G for making a salt bridge. Thus, it seems that the sensitive binding pockets of melain G were consequently formed by S_2 and S_3. So far as is known, this is the first reported protease having substrate specificity like this (Uchikoba et al., 1999). Also, melain G was little affected by the inhibitor E-64. It seems that the conformation of E-64 is well suited for the formation of enzyme-inhibitor complex against the cysteine proteases such as papain, cathepsin B and calpain, but not for melain G. This also shows that the subsite of melain G is different from that of papain.

A cysteine protease of molecular mass 61 kDa has been identified in the juice of the stem of *Dieffenbachia maculata*. The pH optimum is 8.0 and the enzyme is inhibited by PCMB and iodoacetate (Chitre et al., 1998). However, protease activity in leaves, petiole and stem exhibited different pH optima, indicating a possibility that different molecular forms of protease exist in these parts. The enzyme in leaves could be a neutral protease, whereas alkaline proteases are apparently present in petiole and stem. The highest enzyme activity was present in the stem. Age of the plant affects the activity and the optimum pH of the enzyme. Highest protease activities were recorded in old (yellowing) leaves, mature petiole and mature stem. A cysteine protease with leucyl peptidase activity was isolated from stem. A similar cysteine protease has been recently isolated from the young stems of *Asparagus officinalis*, using cystatin affinity chromatography. The molecular mass was estimated to be 28 kDa and the pH optimum was 7.0 (Yonezawa et al., 1998).

Similarly, a number of cysteine proteases with novel properties have been isolated in our laboratory from the latex of *Ervatamia coronaria,* a flowering plant indigenous to India (Sundd et al., 1998; Kundu et al., 2000; Dubey and Jagannadham. 2003a).

Being secreted or lysosomal enzymes, peptidases of the papain family are synthesized with signal peptides,

and there are also propeptides at the N-terminus. Proteolytic cleavage of the propeptides is necessary for activation of the proenzymes. The majority of the propeptides are homologous to that of papain. The existence of enzymes in the preproform has some role in regulation of proteolytic activity. In order to prevent unwanted proteolysis by the proteases, their activity must be rigorously regulated (Bond and Butler, 1987; Neurath, 1989; Twining, 1994). After transcription, the synthesis of the enzymes as inactive precursors (Chan et al., 1986; Cohen et al., 1986), which are subject to several steps of post-translation modification, is the next regulatory mechanism for the papain like cysteine proteases. In the case of the lysosomal enzymes, the signal peptides are removed when the molecules pass into the lumen of the endoplasmic reticulum, and glycosylation, phosphorylation and formation of disulfides then take place in the golgi complex. In the lysosomes, the proenzymes are dephosphorylated and converted to the active enzymes by limited hydrolysis (Kirschke et al., 1995). The N-terminal proregions of the enzymes, which are removed during this final maturation step, act as a potent reversible inhibitor against the mature enzymes (Fox et al., 1992; Carmona et al., 1996).

The propeptides of the plant enzymes act as their inhibitors (Taylor et al., 1995). The crystal structure procaricain (Groves et al., 1996) have shown that the structure of the mature enzyme is already formed in its zymogens and the propeptides prevent the enzymatic activity by blocking the active site cleft using the inhibitory mechanism. Unusual in this respect seem to be the two aminopeptidases, cathepsin H and C. An octapeptide from the proregion remains bound to mature cathepsin H (Baudys et al., 1991) and an even larger portion of the proregion remains in the mature cathepsin C (Dolenc et al., 1995).

Localization of plant papain-like cysteine proteases

Papain homologs are usually lysosomal (vacuolar) or secreted proteins. Cathepsin B, C, H and L are ubiquitous in lysosomes of animals whereas cathepsin S has a more restricted localization (Barrett and Kirschke, 1981). In plants, they are primarily found in the latex and fruits of plants. In the latter, they are located in the vacuoles, which are plant counterpart of lysosomes, but are also extracellular as in the latices like papaya, figs and in arthopods such as lobsters (Chua et al., 1988; Laycock et al., 1991). Bleomycin hydrolase being a cytosolic enzyme in fungi and mammals is an exception (Sebti et al., 1987).

Functional diversity of plant papain-like proteases

Papain-like plant cystein protease show extensive functional diversity. Many proteases have been isolated from latices, fruits and seeds and most of them belong to papain super family (Boller, 1986). Cysteine proteases of plants play a major role in intracellular and extra cellular processes such as development and ripening of fruits (Brady, 1985); as nutritional reserve; degradation of storage protein in germinating seeds (Kembhavi et al., 1993); activation of proenzy-mes, and degradation of defective proteins (Rudenskaya et al., 1998). Besides, enzymes in the latex are also involved in protection of the plant against predator attack (Boller, 1986; Smith et al., 1955). The presence of bacte-riolytic action in the latices of *C. papaya* (Howard and Glazer, 1969), *F. glabrata* (Glazer et al., 1969) and *E. coronaria* (Kidwai and Murti, 1963, 1964) confirms the fact that bacteriolytic and proteolytic enzymes act in unison to degrade undesirable proteins. They are a class of enzymes that have been widely studied over the years. Cysteine proteases play an important role in seed germination. They have been observed during maturation of storage proteins in *Cannavalia ensiformis* (Abe et al., 1993), *Riccinus communis* (Hara-Nishimura et al., 1991), *Glycine max* (Scott et al., 1992). The name legumain (EC 3.4.22.34) in the 1992 edition of Enzyme Nomenclature (Nomenclature Committee of The International Union of Biochemistry and Molecular Biology) is adopted instead of 'proteinase B' as the general term for these aspara-ginyl bond-specific cysteine proteases found in legume seeds. A role of degradation of storage proteins during seed germination has been reported for these proteases (Baumgartner and Chrispeels, 1977; Csoma and Polgar, 1984). A rice cysteine endopeptidase has been purified and characterized that digests glutein (Kato and Minamikawa, 1996). A cysteine protease WCP-P3 has been purified from germinating wheat seeds and has a molecular mass of 25 kDa and pH optima around 5.5 - 6.0 (Kuroda et al., 1997). The latex enzymes have been suggested to be involved in protein degradation during the course of laticifer development, promoting the coagulation of the latex or protecting the plants against predator attack. The fruit enzymes have been suggested to have protective role (Boller, 1986). The main *in vivo* function of the lysosomal cysteine proteases is the degradation of proteins. Proteins are degraded in lysosomes non-selectively, and the resulting end products, dipeptides and aminoacids, diffuse through the lysosomal membrane and are reused in protein biosynthesis (Brocklehurst et al., 1987; Bohley and Seglen, 1992; Kirschke et al., 1995). In addition, lysosomal proteases have been shown to be able to specifically process other proteins e.g. hormones and are probably involved in tissue resorption (Wang et al., 1991; Dunn et al., 1991). Cathepsin B has been suggested to be the major cysteine protease involved in protein degradation for antigen presentation (Mizuochi et al., 1994).

Plant cysteine proteases inhibitors

Studies of plant cysteine proteases are of considerable importance. In addition to providing functional role in

plant, structural determination of plant cysteine proteases in complex with the inhibitor may provide clue for designing inhibitors for many cysteine proteases involved in human diseases. Inhibitors of cysteine proteases of the papain family are best characterized. The cysteine proteases inhibitors constitute a family phytocystatin in cystatin superfamily (Turk and Bode, 1991). The cystatin superfamily consists of tightly and reversibly binding inhibitors against cysteine proteases of papain family. Cystein protease inhibitors from different parts (seeds, root, fruits and leaves) of monocotyledonous and dicotyledonous plants were studied and reviewed by Mosolov and Valueva (2005). The majority of plant cystatins are proteins of 12 - 16 kDa, devoid of Cys residue with exception of cystatin from *C. papaya* (Song et al., 1995). Oryzacystatin, a cysteine protease inhibitor of the rice is first well defined phytocystein and has potential inhibitory activity against papain and several other cysteine proteases. Recently, Kudo et al. (2000) reported the solution structure of oryzacystatin-I. The structure of oryzacystatin-I (PDB code: 1EQK) shows a high degree of similarity with animal cystatin.

The main function of plant cystein proteases inhibitors is thought to be in plant defense and the regulation of endogenous proteases, but they may also function as storage proteins (Mosolov et al., 2001; Shewry, 2003). Hilder et al. (1987) expressed the gene of Baumann-Birk inhibitor gene from cowpea (*Vigna unguiculata*) in tobacco plant. The content of inhibitor in the leaves accounted for as little as 1% of total soluble protein. The studies show that such transgenic plant was affected by budworms (the larvae of the noctuid moth *Heliothis virescens*). The defensive role of plant cystatin may be due to their inhibitory activities towards the digestive enzymes of insect, their larvae and other pathogens' proteases involved in some vital processes. Several other transgenic plant expressing cystein proteases inhibitors were shown to be effecting against phytophagous insects. Thus, transgenic plant expressing protease inhibitors seems to be one of the prime candidates for development of pest resistant plant.

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