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Protease Inhibitors: Emphasizing Functional Aspects of Aspartic Protease Inhibitors

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ABSTRACT

Aspartic proteases are relatively a small group of proteolytic enzymes. Over the last decade, they have received tremendous research interest as potential targets for pharmaceutical intervention as many have been shown to play significant roles in physiological and pathological processes. Despite numerous efforts, however, the only inhibitors for aspartic proteases currently in the market are directed against the HIV protease of viral origin. Nevertheless, several inhibitors including those targeting renin-angiotensin system and β-secretase are in clinical or preclinical developments and few other aspartic proteases are discussed as potential drug targets. Currently the research strategies are focusing on the need for improved comprehension of protease-regulated cascades, along with precise selection of targets and improved inhibitor specificity. There is plethora of synthetic inhibitory compounds targeting aspartic proteases; however there are few reports documented in literature on biologic inhibitors from microorganisms. Protease inhibitors (PIs) are widely distributed in the plant kingdom. One of the important defense strategies that are found in plants to combat predators involves PIs which are particularly effective against phytophagous insects and microorganisms. In plants, these PIs act as anti-metabolic proteins, which interfere with the digestive process of insects. The defensive capabilities of PIs rely on inhibition of proteases present in insect guts or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development. The present chapter is a comprehensive state-of-the-art review describing the aspartic protease inhibitors from microbial and plant origin. In addition, the chapter highlights the therapeutic perspectives of aspartic protease inhibitors and biocontrol aspects of protease inhibitors with special emphasis on aspartic protease inhibitors.

Keywords: aspartic protease inhibitors, biocontrol aspects, physiological role, proteases, therapeutic perspectives
Abbreviations: **AIDS**, acquired immunodeficiency syndrome; **API**, aspartic protease inhibitor; **ATBI**, alkalothermophilic *Bacillus* inhibitor; **CDI**, cathepsin D inhibitor; **Chi A**, chitinase A from *Serratia marcescens*; **HIV**, human immunodeficiency virus; **HIV-1 PR**, HIV-1 protease; **OPTA**, o-pthaldehyde; **PI**, protease inhibitor; **SBBTI**, soybean bowman-birk trypsin inhibitor; **SQAPI**, squash aspartic proteinase inhibitor; **STI**, soyabean trypsin inhibitor; **TI**, trypsin inhibitor; **VrAPI**, *Vigna radiata* aspartic protease inhibitor; **WRK**, Woodward's reagent K; **Xyl I**, xylanase I

CONTENTS

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Invited Review

INTRODUCTION

The diversity and specificity of proteases constitutes the basis for their serendipitous nature and multifaceted physiological activities. Proteases participate in most aspects of cell nutrition, physiology, signaling cascades and microbial pathogenesis (Ward 2009). Their activity, if uncontrolled, would be destructive to the cell or organisms and therefore must be precisely regulated. The most significant aspect of protease action is the control of protease activity to limit cleavage to intended substrates without destruction of functional proteins. Many proteases are also essential for propagation of diseases, and hence inhibition of proteases is emerging as a promising approach in medicinal application for cancer, obesity, hepatitis, herpes, cardiovascular, inflammatory, neurodegenerative diseases, and various infectious and parasitic diseases (Rao *et al*. 1998). Aspartic proteases are relatively a small group of proteolytic enzyme that has received enormous interest because of their significant roles in human diseases like involvement of renin in hypertension, cathepsin D in metastasis of breast cancer, β-secretase in Alzheimer's disease, plasmepsins in malaria, HIV-1 peptidase in acquired immune deficiency syndrome, and secreted aspartic peptidases in candidal infections. There have been developments on clinically active inhibitors of HIV-1 peptidase, which have been licensed for the treatment of AIDS. The inhibitors of plasmepsins and renin are considered a viable therapeutic strategy for the treatment of malaria and hypertension (Dash *et al*. 2003). Cathepsin D inhibitors have broadened the knowledge of structure, mechanism and contribution of cathepsin D in therapy of diseases (Gacko *et al*. 2007). These inhibitors are mainly synthetic molecules; however there is paucity on biologic inhibitors from microbes. The application of biologic inhibitors will stimulate renewed interest in the therapeutic targeting of aspartic proteases.

Plants have elaborated protective mechanisms that allow them to successfully resist different kinds of unfavorable conditions including insects and phytopathogenic microorganisms. The most important components of all protective mechanisms are based on proteinaceous compounds. Protease inhibitors (PIs) are widely dispersed in plant tissues, often occurring in quite high concentrations (Murdock and Shade 2005). They are an important element of the plant defense response to insect predation, and may also act to restrict infection by some nematodes. They are able to suppress the enzymatic activity of phytopathogenic microorganisms. Along with inhibitors of trypsin and chymotrypsin, many plants have proteins that act predominantly as inhibitors of microbial proteinases. Production of these inhibitors is highly regulated by a signal transduction pathway that is initiated by predation and transduced as a wound response. Local and systemic extracellular inducers of the signal pathway are released by injury (Koiwa *et al*. 1997). PI induced in response to infection can sufficiently differ from similar inhibitors present in a healthy plant. Recent achievements in biotechnology resulted in creation of transgenic plants with an increased resistance towards different kinds of unfavorable conditions including effects of phytopathogenic microorganisms and viruses. This approach allows not only increasing productivity of many cultured plants, but also promotes the improvement of the ecologic situation through the decreased use of highly toxic plant protection agents. PI active against different mechanistic classes of proteases have been classified into different families on the basis of significant sequence similarities and structural relationships. The physiological significance of serine and cysteine PIs from plants and their role in plant protection is extensively studied. However, very few reports on plant protection based on aspartic protease inhibitors (APIs) are documented. The present chapter is a comprehensive state-of-the-art review describing the therapeutic perspectives of API and biocontrol aspects of PIs with special emphasis on API.

OCCURRENCE

Proteases

Proteases are ubiquitous in occurrence and constitute 1-5% of the gene content. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g., the blood clotting cascade, the complement system, apoptosis pathways, etc). Proteases can break either specific peptide bonds (limited proteolysis), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (unlimited proteolysis). The activity can be a destructive change abolishing a protein's function or digesting it to its principal components, it can be an activation of a function or it can be a signal in a signaling pathway. However the roles played by proteases, which participate in many essential general processes of cells and their regulation, are far more complex. Proteases are found in wide diversity of sources such as plants, animals and microorganisms. The use of plants as a source of proteases is governed by several factors such as availability of land for cultivation and suitability of climatic conditions for growth. Moreover, production of proteases from plants is a time-consuming process. Papain, bromelain, keretinases and ficin represents some of the well known proteases of plant origin. Aspartic proteases produced by plants are mostly confined to seeds and are involved in the processing of storage proteins during ripening and in their degradation during germination. In plant seeds they have been purified from barley, buckwheat, wheat, rice, common bean, cowpea, *Cynara* and *Arabidopsis* and their enzymatic properties have been investigated

(Doi *et al*. 1980; Belozersky *et al*. 1989; D'Hondt *et al*. 1993; Runeberg-Roos *et al*. 1994; Asakura *et al*. 1995; Zhang *et al*. 1999; Timotijevic *et al*. 2003). The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin and renins. Their production in pure form is depended on the availability of livestock for slaughter, which in turn is governed by political and agricultural policies. The inability of the plant and microbial proteases to meet current world demands has led to an increased interest in microbial proteases. Microbial proteases account for approximately 40% of the total worldwide enzyme sales. Proteases from microbial origin are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications. Aspartic proteases from microbial origin are typically sorted into two groups, (i) pepsin-like enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus* and *Neurospora* and (ii) rennin-like enzymes produced by *Endothia* and *Mucor* sp. (Rao *et al*. 1998).

Protease inhibitors

Multicellular organisms possess endogenous PIs to control proteolytic activity. Most of these inhibitory proteins are directed against serine protease, although some are known to target cysteine, aspartyl, or metalloproteases. Indeed, inhibitors of serine, cysteine, and metalloproteases are distributed ubiquitously throughout the biological world. Proteinaceous PIs are ubiquitously abundant in tubers and plant seeds (Ryan 1973). In higher plants, several gene families of these PIs have been characterized, particularly the serine protease inhibitors from *Leguminosae*, *Solanaceae* and *Graminae*. Protein inhibitors of aspartic proteases are relatively uncommon and are found in specialized locations. Few examples include proteins from the plants such as potato, squash, a pleuripotent inhibitor from sea anemone, an 8-KDa polypeptide inhibitor from yeast inhibiting saccharopepsin (Kreft *et al*. 1997; Christeller *et al*. 1998; Lenarcic and Turk 1999). Pepstatin, a natural peptide produced by various *Actinomyces* species, is a well known inhibitor of aspartic proteases and may be applied to inhibit these proteases where they cause undesirable degradation of

other proteins for example when heterologous proteins are produced by a cost which also produces aspartic proteases. Other microbial sources reported for proteinaceous API are from *Bacillus* sp. (Dash and Rao 2001) and *Bacillus licheniformis* (Kumar and Rao 2006).

CLASSIFICATION OF PROTEASES

Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteases are degradative enzymes which catalyze the total hydrolysis of proteins. Proteases execute a large variety of functions, extending from the cellular level to the organ and organism level, to produce cascade systems such as hemeostasis and inflammation. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions. Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms (Rao *et al*. 1998) In general the active sites of protease are flanked on one or both sides by one or more subsites capable of accommodate the side chain of specific single amino acid residue from the substrate. The degree of amino acid specificity delineated by the active site and the subsites ultimately defines the general biocatalytic properties and especially the specificity of the proteolytic reaction.

Currently, proteases are classified on the basis of three major criteria: (i) type of reaction catalyzed, (ii) chemical nature of the catalytic site, and (iii) evolutionary relationship with reference to structure. Depending on their site of action, proteases are categorized into two major groups, i.e., exoproteases and endoproteases. Exoproteases act only near the ends of the N or C termini of the polypeptide chains and are classified accordingly as amino- and carboxyproteases, respectively. Endoproteases attack peptide bonds in more central locations of the polypeptide chain more remote from the N and C termini and indeed free amino or carboxyl groups are known to inhibit or retard enzyme action (Ward 2009). Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases and two newly established families, i.e., glutamic acid proteases and threonine proteases. There are a few miscellaneous proteases which do not precisely fit into the standard classification, e.g., ATP-dependent proteases which require ATP for activity (Dash *et al*. 2003).

Serine protease

As the name implies, serine proteases contain a serine group in their active site, which is essential for substrate binding and cleavage. Generally, serine proteases are characterized by their broad substrate specificity, and their activity extends beyond purely peptidase to include esterase and amidase activities. Serine proteases exist among exoprotease, endoprotease, oligoprotease, and omega protease groups. Important representative enzyme groups include the chymotrypsins (SA), subtilisins (SB), carboxyprotease C (SC), and Escherichia D-Ala-D-Ala protease A (SE), and these have primary structures that are totally unrelated. Serine proteases are characterized by having a conserved glycine-containing peptide, Gly-Xaa-Ser-Yaa-Gly, associated with the catalytic serine. A common reaction mechanism in the form of a catalytic center containing serine as a nucleophile, aspartate as an electrophile, and histidine as a base, is exhibited by groups SA, SB, and SC, respectively. Interestingly, distinctive protein folding strategies among these groups accomplish similar geometric orientations of these residues, suggesting a convergent evolutionary background. Some groups may be differentiated from the latter groups in that they lack the serine-aspartate-histidine catalytic center (Kraut 1977).

Serine proteases generally exhibit pH optima in the range 7–11 and manifest isoelectric pH values in the range 4–6. Serine alkaline proteases, produced by certain bacteria including *Arthrobacter*, *Streptomyces*, and *Flavobacterium* species, filamentous fungi, including *Conidiobolus*, *Aspergillus*, and *Neurospora* species, and some yeasts represent the largest subgroup of serine proteases, and are active at the higher end of the above pH range (pH optimum ~10) with an unusually high isoelectic pH around 9. These enzymes are inhibited by diisopropylfluorophosphate (DFP) or a potato protease inhibitor, but not by tosyl-L-phenylalanine-chloromethyl ketone (TPCK) or tosyl-L-lysine-chloromethylketone (TLCK), which inhibit other serine proteases. These enzymes are characterized as having substrate specificities similar to but broader than that of chymotrypsin, where the carboxyl side of the peptide bond being attacked contains a tyrosine, phenylalanine, or leucine residue (Rao *et al*. 1998). The second largest family of serine proteases contains the subtilisins, which are best represented by subtilisin Carlsberg and subtilisin BPN, produced by *Bacillus licheniformis* and *B. amyloliquefaciens*, respectively. The active site conformations of both the enzymes are similar to trypsin and chymotrypsin despite their contrasting molecular structures (Kise 1990). While the subtilisin from *Conidiobolus coronatus* exhibits catalytic similarities with subtilisin Carlsberg, its protein structure is distinct (Phadtare *et al*. 1997).

Thiol/Cysteine proteases

Cysteine proteases generally may be assigned to one of the following four groups according to their side chain specificities: (1) papain-like (includes clostripain and streptopain), (2) trypsin-like with preference for cleavage at the arginine residue, (3) specific to glutamic acid, and (4) others. Most have neutral pH optima. All cysteine proteases have cysteine/histidine catalytic dyad, although the order of these residues, Cys-His or His-Cys, may vary (McGrath 1999). They generally need reducing agents such as sodium bisulphite, hydrogen cyanide, or cysteine for activity retention (Chapman *et al*. 1997). Sulfhydryl agents such as *p*-chloromercuribenzoate are inhibits or denaturants, whereas DFP and metal-chelating agents are not Clostripain (*Clostridium histolyticum*), which shows high specificity for arginyl residues contributing the carboxyl group to the peptide bond contrasts with papain in that it requires calcium for activity. Streptopain (*Streptococcus* sp.) manifests broad specificity toward synthetic substrates and oxidized insulin B chain (Rao *et al*. 1998). Biocatalysis is mediated by a double-displacement pathway involving general acid-base formation and hydrolysis of an acyl-thiol intermediate. These enzymes have broad specificity and also attack amide ester, thiol ester, and thiono ester bonds. The enzyme initially binds noncovalently to the substrate, after which acylation of the enzyme occurs together with release of the first product. Water reacts with the acyl enzyme releasing the second product through deacylation.

Metalloproteases

The divalent metal-requiring metalloproteases are a very diverse group of proteases, which include both endoproteases and exoproteases. They are inhibited by chelating agents such as ethylenediaminetetraacetic acid, but not by sulfhydryl agents or DFP (Holmes and Matthews 1981). Thermolysin, a neutral zinc protease produced by *B. stearothermophilus*, is one of the most thoroughly characterized metalloproteases where a histidine and glutamine participate in the active site, providing a ligand for zinc and a catalytic function, respectively. As its name suggests, thermolysin exhibits high thermostability and has a half-life of 1 h at 80°C (Reddy 1991). Four calcium atoms enhance the thermostability of the protein. The metalloprotease collagenase is produced by a variety of microorganisms, including *Achromobacter iophagus* and *Clostridium hystolyticum*.

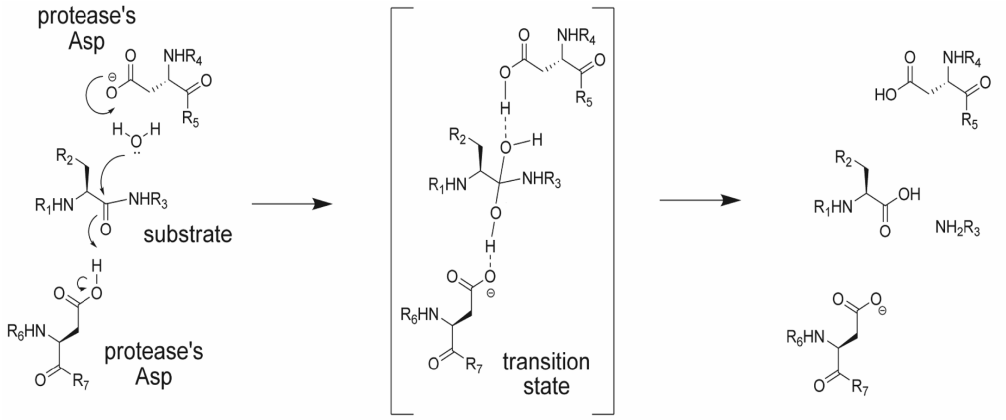

Fig. 1 Mechanism of action of aspartic proteases.

Pseudomonas aeruginosa produces the neutral metalloprotease elastase as well as alkaline metalloproteases (Rao *et al*. 1998). The alkaline protease I from *Myxobacter* lyses the cell walls of *Arthrobacter* crystallites, AU1 whereas *Myxobacter* protease II cannot lyse the bacterial cells. Generally, the metal-binding site motif includes the motif His-Glu-Xaa-Xaa-His. Biocatalysis requires bound divalent cations, and removal of the metal through dialysis or with chelating agents causes inactivation. In thermolysin, Glu143 participates in the nucleophilic attack of a water molecule on the carbonyl carbon of peptide bond being cleaved, which has been polarized by the Zn ion (Rao *et al*. 1998). Metalloproteases are important in many aspects of biology, ranging from cell proliferation, differentiation and remodeling of the extracellular matrix (ECM) to vascularization and cell migration (Chang and Werb 2001).

Glutamic acid and Threonine proteases

These new class of proteases, glutamic acid protease was derived from a former pepstatin-insensitive carboxyl protease and theronine proteases was discovered in 1995 as part of protoeasome complex. Glutamic acid proteases have been discovered from *Stylidium lignicola* (Fujinaga *et al*. 2004) and *Aspergillus niger var. macrosporus* (Sims *et al*. 2004). Although absent from the Saccharomycetales class, glutamic acid proteases appear to be present in all other ascomycetes species examined. A large number of coding regions for glutamic proteases were also found clustered together in the *Phanerochaete chrysosporium* genome, despite apparently being absent from three other species of Basidiomycota (Sims *et al*. 2004). The catalytic mechanism is based on the two enzymes from the aspergilloglutamic and scytalidoglutamic proteases. The active site diad glutamic acid and glutamine play a critical role in substrate binding and catalysis. These amino acids along with their associated water molecules act as nucleophiles to exhibit an acid-base mechanism distinct from that of the aspartic proteases. The glutamic acid acts as a general acid in the first phase of catalysis, donating a proton to the carbonyl oxygen of the scissile peptide bond of the substrate. Simultaneously, an OH– is donated by water associated with the active site of the enzyme to the carbonyl oxygen of the peptide bond of the substrate. Sometimes, two water molecules are involved in the reaction. The transition state of the substrate is thought to be stabilized by hydrogen bonding with the two catalytic residues. Then, glutamic acid donates a proton to the amide nitrogen atom of the scissile peptide bond triggering the breakdown of the tetrahedral intermediate and thus effecting peptide bond cleavage. The glutamine residue is then responsible for recovering the original state of the glutamic acid residue. Recently, Sriranganadane *et al*. (2011) reported a novel glutamic protease, homologous to *Aspergillus niger* aspergillopepsin II from *Aspergillus fumigatus*. Theronine proteases are part of a multicomponent proteasome complex in microbial cells. It assembles into a multimeric complex in order to position its substrates, and uses a Thr-Glu/Asp-Lys triad (Brannigan *et al*. 1995; Lowe *et al*. 1995; Baumeister and Lupas 1997). The archaebacterial proteasome has 14 active sites in the inner channel, one on each β subunit. The hydrolytic sites are spatially separated from the intracellular components. Recent reports have indicated that the active site nucleophile is the hydroxyl group on the threonine at the N-terminus of the β subunit. The replacements of the terminal threonine by serine in archaebacterial proteasomes allows complete proteolytic activity (Baird *et al*. 2006). Therefore, the conservation of the threonines in the active sites of all threonine proteases from bacteria to eukaryotes is unclear. Looking at the diverse functions of the threonine proteases in bacteria and mammals, it is evident that the phylogenetically ancient proteasome has undergone adaptations that favor different functions in different physiological situations.

Aspartic proteases

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3) (13), and have been placed in clan AA. The members of families A1 and A2 are known to be related to each other, while those of family A3 show some relatedness to A1 and A2. Most aspartic proteases show maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. Their molecular masses are in the range of 30 to 45 kDa. The members of the pepsin family have a bilobal structure with the active-site cleft located between the lobes. The active-site aspartic acid residue is situated within the motif Asp-Xaa-Gly, in which Xaa can be Ser or Thr. Retropepsins are monomeric, that is, they carry only one catalytic aspartate, and thus dimerization is required to form an active enzyme and the motif Xaa is Ala (Blundell *et al*. 1991; Sielecki *et al*. 1991). Penicillopepsin and endothiapepsin biocatalysis is mediated by a general base catalytic mechanism with a lytic water molecule participating in the reaction. The specificity of the catalysis has been explained on the basis of available crystal structures (James *et al*. 1977; Pearl and Taylor 1987). The structural and kinetic studies also have suggested that the mechanism involves general acid-base catalysis with lytic water molecule that directly participates in the reaction (**Fig. 1**). This is supported by the crystal structures of various aspartic protease-inhibitor complexes and by the thiol inhibitors mimicking a tetrahedral intermediate formed after the attack by the lytic water molecule (Rao *et al*. 1998). Aspartic protease-inhibitor crystal structures are currently available on the PDB database for viral proteases (HIV-1, HIV-2, SIV, FIV), Cathepsin D, renin, renin/chymosin, penicillopepsin, secreted aspartic protease, pepsin, mucoropepsin, retropepsin, saccharopepsin, rhizopuspepsin, and plasmapepsin II.

Table 1 Proteases commonly used as therapeutic drug targets.

Class	Protease	Disease indication	References
Aspartic proteases	Pepsin	Acidity	Dash *et al*. 2003; Eder *et al*. 2007; Gosh 2010
	HIV Protease	HIV infection	
	Cathepsin D	Cancer	
	Cathepsin E	Cancer	
	Protease A or Saccharopepsin	Yeast infections	
	Renin	Hypertension	
	Plasmepsin	Malaria	
	Secretory aspartic proteases (SAPS)	Candidial infections	
	β-secretase	Alzheimer's diseases	
Serine proteases	Neutrophil elastase	Cystic fibrosis, emphysema	Rosenblum and Kozarich. 2003; Turk 2006; Chien *et al*. 2009
	Plasma Kallikrein	Chronic inflammation and asthma	
	Tissue Kallikrein	Hypertension, cardiac ischemia, hypertrophy	
	Corin	Hypertension	
	Chymases	Cardiac hypertrophy, heart failure, atherosclerosis, and restenosis	
	Urokinase	Myocardial infarction and heart failure, hypoxia-induced pulmonary hypertension, abdominal aortic aneurysm	
Cysteine proteases	Cathepsin B and L	Cancer invasion, growth and angiogenesis and emphysema	Broemme 1999; Turk and Guncar 2003; Leung-Toung *et al*. 2006; Fricker 2010
	Cathepsin S	Cancer invasion, growth and angiogenesis and auto-immune disorders and artherosclerosis	
	Cathepsin K	Tumor bone metastates, osteoporosis	
	Cathepsin F	Atherosclerosis	
	Cathepsin C	Papillon-Lefevre syndrome	
	Cathepsin V	Autoimmune disease	
	Faclipains	Malaria	
Metallo-proteases	Angiotensin converting enzymes (ACE)	Hypertension	Overall and Kleifeld 2006; Morrison *et al*. 2009; Vanlaere and Libert 2009; Dejonckheere *et al*. 2011
	Matrix metalloproteases (MMP-2, 9, 14)	Myocardial infraction, cancer progression, angiogenesis	
	Tumor necrosis factor alpha activating enzyme (TACE)	Cancer growth and development	
Threonine proteases	Proteasome	Cancer growth and progression, malaria	Elliot *et al*. 2003; Adams 2004; Tschan *et al*. 2011

In contrast to serine and cysteine proteases, catalysis by aspartic proteases does not involve a covalent intermediate though a tetrahedral intermediate exists. The nucleophilic attack is achieved by two simultaneous proton transfers, one from a water molecule to the diad of the two-carboxyl groups and a second one from the diad to the carbonyl oxygen of the substrate with the concurrent CO-NH bond cleavage. This general acid-base catalysis, which may be called a "push-pull" mechanism, leads to the formation of a noncovalent neutral tetrahedral intermediate (Holm *et al*. 1984; Blundell *et al*. 1991; Veerapandian *et al*. 1992; Northrop 2001; Dunn 2002).

As an enzyme family, aspartic proteases are a relatively small group. Nevertheless, they have received enormous attention because of their significant roles in human diseases. The best-known examples are the involvement of renin in hypertension, cathepsin D in metastasis of breast cancer, and the protease of human immunodeficiency virus (HIV) in acquired immune deficiency syndrome (AIDS). Therefore, the new understanding of the structure and function relationships of these enzymes has a direct impact on the design of inhibitor drugs. Moreover, as structure and function are closely related among the aspartic proteases, model enzymes have been particularly informative. The aspartic proteases are inhibited by pepstatin. They are also sensitive to the active site-directed affinity labels such as diazoacetyl-DL-norleucine methyl ester (DAN) and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) in the presence of copper ions (Umezawa *et al*. 1970). Each of the latter reacts specifically with the side-chain carboxyl of a distinct aspartic acid residue to inactivate the enzyme. Together, these residues contribute to the catalytic mechanism and provide the basis for nomenclature for this class of enzyme. In general pepsins and other aspartic proteases exhibit broad based specificity towards cleavage in peptides consisting of at least six hydrophobic amino acids at specific substrate positions (Dash *et al*. 2003). Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin, but their action is less stringent than that of pepsin. **Table 1** summarizes the proteases widely used as therapeutic drug targets.

PROTEASE INHIBITORS

Proteases are responsible either directly or indirectly for all bodily functions, including cell growth, differentiation, and death (apoptosis), cell nutrition, intra- and extracellular protein turnover (house-keeping and repair), cell migration and invasion, and fertilization and implantation (Roa *et al*. 1998; Koblinski *et al*. 2000; Pandey *et al*. 2007). These functions extend from the cellular level to the organ and organism level to produce cascade systems such as homeostasis and inflammation, and complex processes at all levels of physiology and pathophysiology (Lopez-Otin and Matrisian 2007; Lopez-Otin and Bond 2008). Any system that encompasses normal and abnormal bodily functions must have effective regulatory counterparts, that is, PIs. Hence, the research interest in protease inhibitors has evoked tremendous attention in many disciplines (Friedl and Wolf 2008; Scott and Taggart 2010). Known proteinaceous aspartic acid proteinase inhibitors are rare and unevenly distributed among classes of organisms in contrast to proteinaceous inhibitors of serine and cysteine (Dash *et al*. 2003). PIs are generally classified into 5 groups (serine, threonine, cysteine, aspartyl and metalloprotease inhibitors) according to the mechanism employed at the active site of proteases they inhibit. Some PIs interfere with more than one type of protease. For example, the serine family of PIs (serpins) is generally thought of as active against serine proteases, yet contains several important inhibitors of cysteine proteases as well. PIs are also classified based on their mechanisms of

action as: (1) Suicide inhibitor; (2) Transition state inhibitor; (3) Protein protease inhibitor; (4) Chelating agents (Kulkarni 2007).

Aspartic protease inhibitors

The aspartic acid protease inhibitors are grouped into 7 families: the potato plant Kunitz inhibitors (Mares *et al*. 1989), the *Ascaris* inhibitors (Martzen *et al*. 1990), the yeast inhibitor IA3 (Schu *et al*. 1991), a domain of the sea anemone thyroglobulin type-1 inhibitor (Lenarcic and Turk 1999; Galesa *et al*. 2003), the pig serpin inhibitor (Mathialagan and Hansen 1996), the *Bacillus* sp peptide, ATBI (Dash and Rao 2001) and the squash aspartic acid proteinase inhibitor (SQAPI) (Christeller *et al*. 1998; Farley *et al*. 2002). The structures for some of these proteins have been solved and are to date, very different from each other and exhibit distinct, and in some cases, novel inhibitory mechanisms. Depending on their molecular nature, APIs can be classified in two categories (Dash *et al*. 2003).

1. Proteinaceous inhibitors

In a sharp contrast to the ubiquitous presence of multiple forms of proteinaceous inhibitors of other classes of proteases from different sources of plants, animals, and microorganisms, there is a paucity of proteinaceous inhibitors of aspartic proteases. With the exception of macroglobulins, which inhibit proteases of all classes, individual protein inhibitors inhibit only proteases belonging to a single mechanistic class. Protein inhibitors of aspartic proteases are relatively uncommon and are found in only a few specialized locations (Bennet *et al*. 2000). Few of the examples include renin-binding protein in mammalian kidney, which, intriguingly, has now been identified to be the enzyme, N-acetyl-D-glucosamine-2-epimerase (Kay *et al*. 1982; Phylip *et al*. 2001), a 17-kDa inhibitor of pepsin and cathepsin E from the parasite *Ascaris lumbicoides* (Kageyama 1998; Ng *et al*. 2000), proteins from plants such as potato, tomato, and squash (Kreft *et al*. 1997; Christeller *et al*. 1998), and a pluoripotent inhibitor from sea anemone of cysteine proteases as well as cathepsin D (Lenarcic and Turk 1999). There is a report of an 8-kDa polypeptide inhibitor from yeast, which inhibits the vacuolar aspartic proteases (proteases A or saccharopepsin).

2. Low-molecular-weight inhibitors

In contrast to the proteinaceous nature of the proteases inhibitors from plants and animals, the inhibitors produced by microorganisms are of smaller molecular nature. The presence of proteases inhibitors in microorganisms came into existence from the studies on antibiotics because they act as inhibitors of enzymes that are involved in growth and multiplication. Extracellular proteolytic enzymes hydrolyze organic nitrogen compounds in the medium and are thought to be harmful to cells. The production of inhibitors of the proteolytic enzymes by microorganisms has probably evolved as a mechanism to provide cell protection. Specific inhibitors of microbial origin have been used as useful tools in biochemical analysis of biological functions and diseases. Polysaccharide sulfates have been reported to be pepsin inhibitors; however, their antipepsin activity is weak, and the effect of such polyanionic compounds is not specific. Pepstatin, a low-molecular-weight aspartic proteases inhibitor, isolated from various species of *Streptomyces*, is a specific inhibitor of pepsin (Umezawa *et al*. 1970). Pepstatin also inhibits the activities of cathepsin D, cathepsin E, renin, pseudorenin, aspartyl proteases produced by microorganisms. Alkalo thermophilic *Bacillus* inhibitor (ATBI) is a low molecular weight hydrophilic peptidic API extracellulary produced by an alkalothermophilic *Bacillus* sp isolated from the soil sample of a hot spring at Vajreswari, Maharashtra, India (Dey *et al*. 1991). Another low molecular weight API was obtained from *Bacillus licheniformis* isolated from tomato (Kumar and Rao 2006).

Mechanism of inhibition

A strong inhibition of an active protease by a protein appears to be a paradox. Inhibitor structures, modes of inhibition, kinetic and thermodynamic parameters, and the nature of the enzyme–inhibitor complexes are surprisingly diversified. On the other hand, in many cases, convergence of structure and/or function can be observed, pointing to the fact that there are a limited number of inhibition modes (Otlewski *et al*. 2005). Proteolytic inhibition by PIs can occur via 2 mechanisms: irreversible trapping reactions and reversible tight binding reactions. Inhibitors which bind through a trapping mechanism change conformation after cleaving an internal peptide bond and "trap" the enzyme molecule covalently; neither the inhibitor nor protease can participate in further reactions. In tight-binding reactions, the inhibitor binds directly to the active site of the protease; these reactions are reversible and the inhibitor can dissociate from the enzyme in either the virgin state, or after modification by the protease (Fear *et al*. 2007). Aspartic proteases generally bind 6 to 10 amino acid regions of their polypeptide substrates, which are typically processed, with the aid of two catalytic aspartic acid residues in the active site (James and Sielecki 1987). Thus, there is usually considerable scope for building inhibitor specificity for a particular aspartic protease by taking advantage of the collective interactions between a putative inhibitor, on both sides of its scissile amide bond, and a substantial portion of the substrate-binding groove of the enzyme. Some aspartic protease also have one or more flaps that close down on top of the inhibitor, further adding to inhibitor protease interactions and increasing the basis for selectivity. The scissile amide bond undergoes nucleophilic attack by a water molecule, which is itself partially activated by deprotonated catalytic aspartic acid residue. The protonated aspartic acid donates a proton to the amide bond nitrogen, generating a zwitterionic intermediate, which collapses to the cleaved products. The water molecule that binds between the enzyme and inhibitor is thought to position a peptide substrate, stretching the peptide bond out of planarity toward a tetrahedral transition state that is stabilized by a second water molecule (Chatfield and Brooks 1995). A comprehensive list of 48 inhibitor families has been recently published (Rawlings *et al*. 2004).

Traditionally, PIs have been developed by natural product screening for lead compounds with subsequent optimization or by empirical substrate-based methods (West and Fairlie 1995). The optimization involves replacement of the hydrolysable amide bonds by a non hydrolyzable isostere and optimizing inhibitor potency through trial and- error structural modifications that progressively reduce the peptide nature of the molecule. This substrate/receptor-based drug design has been substantially improved in recent years with the availability of three dimensional structures. The structural information about the active site of the receptor (or protease) and selection of designed molecules with the aid of computers has helped to design receptor based inhibitors (Dash *et al*. 2003). Combinatorial chemistry also presents opportunities both to discover new molecular entities for assaying and to optimize lead structures for development of PIs.

MICROBIAL SOURCES

Streptomyces sp.

Pepstatin, a low molecular weight aspartic peptidase inhibitor, isolated from various species of *Streptomyces*, is a specific inhibitor of pepsin (Umezawa *et al*. 1970). *Streptomyces testacus* was reported to produce various pepstatins that differed from one another in the fatty acid moiety (C2-C10). A pepstatin containing an isovaleryl group has been most widely used for biological and biochemical studies. More-

over, as minor components, pepstanone containing (S)-3-amino-5-methylhexane-2-one instead of the C-terminal (3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid (AHMHA), and hydroxypepstatin containing L-serine instead of L-alanine, have also been isolated. Pepstatin containing an acetyl group and propanoyl or isobutyryl groups were isolated from *Streptomyces naniwaensis* and *Streptomyces* no. 2907 (Dash *et al*. 2003). A simplified analogs of pepstatin A representing 'tripeptides' with two valine residues which are C-terminated by an amino alcohol moiety was found to exhibit inhibition against pepsin (Kratzel *et al*. 2000).

Pepstatins, pepstanones, and hydroxypepstatins have almost identical activity against pepsin and cathepsin D. Pepstatin also binds to procathepsin D and inhibits its autoactivation. However, pepstatin is more effective against renin than are pepstanone or hydroxypepstatin, and its potency against renin increases with the increasing numbers of carbon atoms in the fatty acid moiety. The inhibition of aspartyl proteases by pepstatin depends to a large extent on the presence of acid residue in their structure. The inhibitory effect of pepstatin on cathepsin D does not depend on the carbon chain length of acid radicals (Gacko *et al*. 2007). Esters of pepstatin, pepstatinal and pepstatinol possess antipepsin activity similar to pepstatins. Several pepstatin analogs have also been synthesized to date. AHMHA and its N-acyl derivative exhibit no potency toward pepsin; however, N-acetyl-valyl-AHMHA is active, and the addition of another valine between the acetyl and valyl groups does not increase their activity. The addition of L-alanine to the C-terminal group increases the activity about 100 times. This suggests that the acetyl-valyl-AHMHA-L-alanine is the smallest molecular structure that exhibits inhibition against pepsin and cathepsin D similar to pepstatin. Acetyl-L-valyl-L-valyl-[(3S, 4R)-4-amino-3-hydroxy-6-methyl] heptanoic acid prepared by chemical synthesis shows absence of activity. This suggests that the 4S-configuration of AHMHA is essential for activity.

The bacterial enzyme that hydrolyzes the isovaleryl bond in pepstatin has been identified, and from the residual peptide benzoyl-L-valyl-AHMHAL-alanyl-AHMHA and L-lactyl-L-valyl-AHMHA-Lalanyl-AHMHA have been synthesized. These analogs are more water-soluble than pepstatin and have almost identical activity against pepsin and cathepsin D. However, these water-soluble analogs have much weaker activity against renin when compared with pepstatin. The addition of aspartic acid or arginine to the C-terminus of pepstatin increases its water solubility. Such water-soluble analogs have same activity against renin as does pepstatin and also have a hypotensive action (Rich 1985). Pepstatin also inhibits carageenin-induced edema and suppresses the generation of Shay rat ulcer. Therapeutic effects on stomach ulcers in man have also been observed. Pepstatin has been reported to be effective against experimental muscle dystrophy and enhances the effect of leupeptin. Pepstatin also inhibits leukokinin formation and ascites accumulation in ascites carcinoma of mice. Pepstatin inhibits the growth of *Plasmodium beghei* and inhibits focus formation in murine sarcoma virus (Yuasa *et al*. 1975).

Bacillus sp.

ATBI (Alkalo Thermophilic Bacillus Inhibitor) is a hydrophilic peptidic API extracellulary produced by an alkalothermophilic *Bacillus* sp isolated from the soil sample of a hot spring at Vajreswari, Maharashtra, India (Dey *et al*. 1991). The inhibitor was found to be a hydrophilic peptide with Mr of 1147, and an amino acid sequence of Ala-Gly-Lys-Lys-Asp-Asp-Asp-Asp-Pro-Pro-Glu. ATBI was found to inhibit pepsin with a two-step inhibition mechanism with K_i values for the first reversible complex (EI) of $(17 \pm 0.5) \times 10^{-9}$ M, whereas the overall inhibition constant, K_i* was $(55 \pm 0.5) \times 10^{-12}$ M. Comparative analysis of the kinetic parameters with pepstatin, the known inhibitor of pepsin, revealed a higher value of k_5/k_6 for ATBI (Dash *et al*. 2001a). ATBI was also found to inhibit HIV-1PR (HIV protease) and the kinetic parameters revealed ATBI as a noncompetitive and tight binding inhibitor with the IC_{50} and K_i values 18.0 and 17.8 nm, respectively. Fluorescence spectroscopic studies revealed that ATBI binds in the active site of the HIV-1 PR and is the first report of a noncompetitive inhibitor from an extremophilic microorganism. It is well established that the Trp-42 is present adjacent to the flaps, and the flap regions of HIV-1 PR are the only dynamically flexible portions of the enzyme. The enzyme inactivation is caused by the loss of the flexibility of the flaps restricting the entry and exit of the polypeptide substrate and products (Dash and Rao 2001). The bifunctional nature of ATBI was also established by its potency toward Xyl I, the xylanase purified from the *Thermomonospora* sp. The steady-state kinetics revealed time-dependent competitive inhibition of Xyl I by ATBI, consistent with two-step inhibition mechanism. The inhibition followed a rapid equilibrium step to form a reversible enzyme-inhibitor complex (EI), which isomerizes to the second enzyme-inhibitor complex (EI*), which dissociated at a very slow rate. The inactivation of Xyl I is due to the disruption of the hydrogen-bonding network between the essential histidine and other residues involved in catalysis as demonstrated by the abolished isoindole fluorescence of o-phthalaldehyde (OPTA)-labeled Xyl I (Dash *et al*. 2002). In some cases, especially from cereals, bifunctional protease/amylase inhibitors are reported. These inhibitors have specific action against mammalian and insect amylase and trypsin enzyme. Cereal inhibitors have generally low molecular weight (10,000–50,000). These inhibitors have natural role in the control of endogenous amylase activity or in the defense against pathogen and pests. Amylase/trypsin inhibitors are reported to be anti-nutritional factor and have therapeutic application. Apart from this defense mechanism these bifunctional inhibitors are potentially valuable 'two-in-one' affinity ligands for the purification of proteases and amylases (Saxena *et al*. 2010).

Another API was isolated from a thermotolerant *Bacillus licheniformis* and exhibited a slow tight binding mechanism of the aspartic protease, pepsin. The inhibitor showed a Mr (relative molecular mass) of 1363 as shown by MALDI-TOF spectra and 1358 as analyzed by SDS-PAGE. The inhibitor was found to be specific for pepsin, showed very weak inhibitory activity against other aspartic proteases and did not show any inhibitory activity against other classes of proteases. The amino acid analysis and CD-spectra analysis of API suggest the peptidic nature. The kinetic studies of pepsin–inhibitor interactions reveal that the inhibitor is a slow-tight binding competitive inhibitor with the IC_{50} and K_i values of 4.0 nM and (3.83–5.31 nM) respectively (Kumar and Rao 2006). API is also reported to exhibit a slow tight binding inhibition against ChiA (Chitinase from *Serratia marcescens*). The ChiA–inhibitor kinetic interactions reveal noncompetitive, irreversible and tight binding nature of inhibitor with $I_{50} = 600$ nM and $K_i = 510$ nM. CD-spectra and tryptophanyl fluorescence analysis of ChiA incubated with increasing API concentrations confirms conformational changes in enzyme structure which may be due to irreversible denaturation of enzyme upon binding of the inhibitor. Chemical modifications by WRK abolished the anti-chitinase activity of API and revealed the involvement of carboxyl groups in the enzyme inactivation. Abolished isoindole fluorescence of OPTA-labeled ChiA demonstrates the irreversible denaturation of ChiA upon incubation with API for prolonged time and distortion of active site of the enzyme (Kumar and Rao 2010).

An almost two-decade research effort by academic and pharmaceutical institutions resulted in the successful commercialization of seven drugs that are potent inhibitors of HIV-1 protease activity and which, if used correctly, are highly effective in managing viral load. However, identification of clinical viral isolates that are resistant to these drugs indicates that this is a significant problem and that new classes of inhibitors are continually needed. Screening of microbial extracts followed by bioassay-guided isolation

led to the discovery of a natural hinnuliquinone, a C2-symmetric bis-indolyl quinone natural product that inhibited the wild-type and a clinically resistant (A44) strain of HIV-1 protease with K_i values of 0.97 and 1.25 μM, respectively. Crystallographic analysis of the inhibitor-bound HIV-1 protease helped explain the importance of the C2-symmetry of hinnuliquinone for activity (Singha *et al*. 2004).

PLANT PROTEASE INHIBITORS

PIs are found in plant species belonging to diverse taxa (Richardson 1977). These proteins are abundant in the seeds of monocot and dicot angiosperms and in gymnosperms and comprise up to 5-10% of the total content of water-soluble proteins (Mutlu and Gal 1999). They form a major part of wound induced defense components of plants especially in leaf tissue. Plants react to wounding by activating a set of genes, most of them playing a role in wound healing and prevention of subsequent pathogen invasion. The gene activation takes place in both wounded and non-wounded plant parts. This systemic accumulation of defense-related products then serves a preventive function, checking the spread of the damaging agent to the healthy part of the plant. The tomato and potato PI families are the best-studied examples of genes, systemically expressed upon wounding. The detailed analysis of PI gene expression in different plant species has shown that it is regulated by a variety of developmental and environment factors. Almost all the PIs known till date belong to serine and cysteine classes with recent reports on aspartic and metalloproteases emerging in the past few decades (Kulkarni and Rao 2009) PIs were first isolated from seeds in the 1940s, with the characterization of Kunitz and Bowman-Birk type PIs (Kunitz 1945; Bowman 1946) from soybean and of an α-amylase inhibitor from cereal grains (Kneen and Sandstedt 1943). Seeds are by far the richest source of PIs and it is probable that all seeds contain one or more inhibitors, although the levels vary greatly and their detection is dependent on the selection of the appropriate target enzymes and assay conditions. The vast majority of the inhibitors characterized to date are active against endoproteases, with a smaller number of amylase inhibitors, some of which are bifunctional (i.e. inhibit both amylases and proteases. There are a multitude of reports on the occurrence of serine, cysteine and metalloprotease inhibitors in seeds whereas there is a distinct lacuna of literature on APIs from plants. The overall distribution of PPI reveals that majority of the inhibitors belong to the serine protease inhibitor families with few inhibitors from cysteine and API families. The serine protease inhibitor families includes soyabean-Kunitz inhibitor, soyabean Bowman-Brik inhibitor, Potato PI-1, Potato PI-2, Squash inhibitor, Barley α-antitrypsin inhibitor, cereal trypsin/α-amylase inhibitor and Metallocarboxypeptidase inhibitor. Recent developments into the PIs belonging to the cysteine, aspartic and metalloproteases families have brought into focus their significance and roles in plants. The knowledge of APIs in plant protection is very scanty. The physiological significance of the inhibitors in addition to their role in defense is under investigation.

Plant aspartic protease inhibitors

There is a plethora of reports on the serine, cysteine and metalloproteases inhibitors but a lacuna of literature on APIs from plants. Proteinaceous aspartic proteinase inhibitors are described only in few plant species. APIs which have been reported from plants are all proteinaceous in nature with reports from potato (Keilova *et al*. 1976), tomato (Werner and Wemmer 1991), wheat (Galleschi *et al*. 2005), *Vicia sativa* (Roszkowska-Jakimiec and Bankowska 1983), *Anchusa strigosa* (Abuereish 1998), squash (Christeller *et al*. 1998) and *Vigna radiata* (Kulkarni and Rao 2009). The biochemical characterization of the potato and *V. sativa* members showed that they were both cathepsin D inhibitors (CDIs), whereas the proteins from *A. strigosa* and squash inhibit pepsin, a digestive aspartic proteinase. In potato, a large family of CDIs has been described. The two first isoforms purified (PDI and NDI) were biochemically characterized, showing inhibitory activity against cathepsin D and Ser proteinases (Keilova and Tomasek 1976; Mares *et al*. 1989; Ritonja *et al*. 1990) and displaying sequence similarities with the STI (Soyabean trypsin inhibitor). Since then, up to 15 isoforms have been found in potato and they have been classified as CDIs exclusively on the basis of sequence analysis. Potato CDIs constitutively accumulate in tubers and flower buds and are also induced by wounding in potato leaves (Strukelj *et al*. 1990; Hildmann *et al*. 1992; Maganja *et al*. 1992; Hannapel 1993; Herbers *et al*. 1994; Ishikawa *et al*. 1994; Kreft *et al*. 1997). The inhibitor comprises 187 amino acid residues, and has a calculated Mr of 20,450. Kreft *et al*. 1997 have cloned cDNA coding for an aspartic proteinase inhibitor homologue isolated from a potato tuber cDNA library and revealed that the gene transcripts encoding aspartic proteinase inhibitors occur mainly in potato tubers. Crystallization and preliminary crystallographic study of cathepsin D inhibitor from potatoes is also reported (Baudys *et al*. 1991). Re-evaluation of the potato cathepsin D inhibitor revealed that it was more potent (>20-fold) towards yeast proteinase A than cathepsin D and so might be renamed the potato inhibitor of proteinase A. The potency towards yeast proteinase A may reflect a similarity between this fungal enzyme and aspartic proteinases produced by fungal pathogens which attack tomato and/or potatoes (Cater 2002).

The squash (*Cucurbita maxima*) phloem exudate-expressed aspartic proteinase inhibitor (SQAPI) is a novel aspartic acid proteinase inhibitor, constituting the family of aspartic proteinase inhibitors. SQAPI evolved recently from the older widely distributed cystatin family, but also it has also utilized the cystatin inhibitory mechanism. The cystatin mechanism, which relies on steric hindrance by insertion of its inhibitory contact residues into the active site crevice of cysteine proteinases, does not directly interact with the proteinase nucleophilic sulfydryl. It remains to be seen exactly how SQAPI interacts with aspartic proteases. The protein-protein interactions for two protein aspartic inhibitors, PI-3 and IA3, have been characterized (Li *et al*. 2000; Ng *et al*. 2000) and are quite distinct from that proposed for SQAPI (Christeller *et al*. 2006). Anandan *et al*. (2009) reported two highly homologous promoters of a SQAPI multigene family exhibiting differential expression in transgenic tobacco phloem and trichome cells. The solution structure of SQAPI was determined and shares the same fold with cystatin, suggesting the PI is derived from the ancestral cystatin preotein, a family of cysteine proteinase inhibitors that are widely distributed through plants and animals. The structure is characterized by a four-strand anti-parallel β-sheet gripping an α-helix in an analogous manner to fingers of a hand gripping a tennis racquet. Truncation and site-specific mutagenesis revealed that the unstructured N terminus and the loop connecting β-strands 1 and 2 are important for pepsin inhibition, but the loop connecting strands 3 and 4 is not. Using ambiguous restraints based on the mutagenesis results, SQAPI was then docked computationally to pepsin. The resulting model places the N-terminal strand of SQAPI in the S' side of the substrate binding cleft, whereas the first SQAPI loop binds on the S side of the cleft. The backbone of SQAPI does not interact with the pepsin catalytic Asp(32)-Asp(215) diad, thus avoiding cleavage. The data show that SQAPI does share homologous structural elements with cystatin and appears to retain a similar protease inhibitory mechanism despite its different target. This strongly supports our hypothesis that SQAPI evolved from an ancestral cystatin (Headey *et al*. 2010).

Kulkarni and Rao (2009) have reported the purification and biochemical characterization of an API from the seeds of *Vigna radiata*. The inhibitor was a linear, hydrophobic, pH stable and thermostable peptide with molecular weight of 1660 Da. The purified inhibitor showed a pI of 4.36 with the sequence as AEIYN KDGNK LDLYG. The inhibitor

was found to be stable in a broad range of pH from 2 to 10 with an optimum of 3.0. The half-life of VrAPI at 100.8°C was 30 min whereas the maximum activity was observed at 37.8°C. The initial kinetic analysis of the inhibitor against the endogenous protease showed an IC_{50} value of 11 nM while the value of the inhibition rate constant K_i was 34×10^{-9} M.

PHYSIOLOGICAL ROLE OF PIs

PPI amount to a considerable part of seed protein: in legumes, this amount is about 6% (Weder 1981), whereas, in cereal crops, it is as high as 10% of the total water-soluble protein (Pusztai 1972). Because of their high content in seeds, they play the role of storage proteins. By their amino acid composition, some of PIs constitute an important source of amino acids for developing plants. PIs in legume seeds irreversibly inhibit the action of the animal digestive enzymes and hence they are considered antinutritional (Ryan 1981; Pearce *et al*. 1982; Ryan 1989; Jouanin *et al*. 1998). PIs regulate endogenous protease levels before and during seed germination for storage protein digestion and to control protein turnover (Baumgartner and Chrispeels 1976; Hilder *et al*. 1987). The involvement of PIs in the protection of seed reserves from premature hydrolysis has been established (Collins and Sanders 1976; McGurl *et al*. 1995). The concentration of inhibitors is reduced during germination, facilitating the hydrolysis of protein for utilization in the germination process. PI gene expression has been detected in leaves of several species following wounding, suggesting their role in protecting plants from insect attack and microbial infection. PIs in legumes are one of the most promising weapons that confer resistance against insects by inhibiting proteases present in the gut of insect larvae. After the identification of PI as a valuable trait suitable for developing insect-resistant transgenic plants, there was intense interest to identify the PI gene from different plant species. PI gene has been identified and cloned from a wide array of plant sources, including alfalfa, tomato, potato, maize, mustard, poplar, tobacco, rice, sweet potato, soybean, amaranthus, cowpea and barley (Ussuf 2001).

During seed development, PIs accumulate relatively later, and rapidly increase in the desiccation phase, implying a role in protein stabilization. Dehydration-related stresses such as drought, salinity and abscisic acid induce the expression of trypsin inhibitors in developing seeds of moong bean and lettuce (Ussuf 2001). Accumulation of TIs closely resembles that of late embryogenesis abundant protein, which has a specific function in stress dehydration. The role of the API and the enzyme in initial stages of germination of *V. radiata* has been tracked by differential timed expression and germination assays. The expression pattern revealed maximum expression of the inhibitor in the dormant seeds while the enzyme was predominant in the germinating seeds. Their expression patterns and interactions indicate their significance in initiation of germination. The expression of other classes of proteases was monitored during germination and a model predicting the events occurring during proteolysis of the storage protein in germination is hypothesized (Kulkarni and Rao 2009). A new role for cysteine protease inhibitor in the modulation of apoptosis or programmed cell death has been identified in soybean. Cysteine protease plays an important role in the regulation of programmed cell death leading to hypersensitive (HR) reaction, following pathogen attack. It has been shown that the ectopic expression of cystatin inhibits the induced cysteine protease activity, which in turn blocks programmed cell death (Ussuf 2001). It is suggested that in plants balancing between the cysteine protease and cysteine protease inhibitor activity regulates the programmed cell death. Thus a new role for PI in modulating the programmed cell death in plants has been identified.

PIs IN PLANT PROTECTION

The major biotic stressors responsible for huge crop losses are weeds, pests, viruses and fungi (Agrios 1997). There are serious losses due to diseases especially in cereals, legumes and ornamentals. The environmental impact caused by the continuous application of chemical agents have initiated the need for alternative, safe, effective and eco-friendly biocontrol agents. Extensive research on pest control has led to the discovery and use of biocontrol agents against pest infestation. The research on pathogen control has led to the development of *Trichoderma*, *Gliocladium*, *Bacillus* and *Pseudomonas* on a commercial scale. These agents are however used in the form of spores or as live organisms in the field. This results in alteration of the natural flora and fauna. Therefore alternative bio-based strategies wherein the active biological principle can be developed as biocontrol agent are required. The success of PI based strategy depends upon the selection of appropriate PIs and their proper expression. The discussion of this review will be restricted to PPIs in plant defense. PIs are the most studied class of plant defense proteins which can be classified as biochemicals. They are abundantly present in seed storage tissues and represent up to 10% of total protein (Casaretto and Corcuera 1995). PIs have been studied in plants for various roles which among others also include their importance in pathogen and insect attack (Brzin and Kidric 1995). In many cases their accumulation in quantities far more than required for inhibiting endogenous proteases, underline their role as defense proteins. In others an absence of inhibitory activity against endogenous plant proteases with presence of activity against proteases of pests and pathogens confirms their role in defense.

The development of recombinant PIs with strong inhibitory activity against specific herbivores is a worthwhile, but challenging task. Protein engineering approaches based on structure/function models have been used to improve the inhibitory potency of PIs against herbivore pest and parasitic nematode digestive proteases (Schluter *et al*. 2010), including site-directed mutagenesis of key amino acids (Kiggundu *et al*. 2006; Goulet *et al*. 2008) and molecular phage display procedures involving random mutagenesis in functionally significant regions of the inhibitor sequence (Koiwa *et al*. 2001; Ceci *et al*. 2003; Melo *et al*. 2003). Fusion proteins integrating complete or partial inhibitor sequences have also been designed to broaden the activity range and improve the overall efficiency of PIs against target herbivores (Inanaga *et al*. 2001; Zhu-Salzman *et al*. 2003; Outchkourov *et al*. 2004; Brunelle *et al*. 2005; Benchabane *et al*. 2008). Such protein engineering strategies, together with 'transgene stacking' (or gene pyramiding) in planta involving PI combinations (Abdeen *et al*. 2005; Senthilkumar *et al*. 2010; Dunse *et al*. 2010) or PIs combined with other pesticidal proteins (Maqbool *et al*. 2001; Han *et al*. 2007), have clearly confirmed the practical potential of these proteins in plant protection.

Serine protease inhibitors

The serine PIs are the most abundant and extensively studied proteins. They have antinutritional effects against several lepidopteran insects. These inhibitors inhibit enzymes from species of *Tribolium*, *Callosobruchus*, *Manduca*, *Haematobia*, *Stomoxys*, *Spodoptera*, etc. A direct test of the roles of the inhibitors in plant leaves to defend against insects was first demonstrated by Hilder *et al*. 1987. SBBTI (Soybean Bowman-Birk trypsin inhibitor) isoinhibitors have been reported to inhibit completely the activities of mealworm (*Tenebrio molitor*), red flour beetle (*Tribolium castaneum*) and the migratory locust (*Locusta migratoria*). It has been shown that these are associated with resistance to insect and fungal attack and that the inhibitors can inhibit the proteases of microbes including pathogens. The levels of trypsin inhibitors increase more in leaves in varieties of tomato that were resistant to *Phytophthora infestans* than in

susceptible varieties. An increased proteinase inhibitory activity in melon plants infected with *Colletotrichum* was noted by Roby *et al*. (1987). Reduced protease activity of proteases from *Fusarium solani* and *Colletotrichum* species was observed with PIs from healthy bean and tomato plants. Many protein inhibitors of trypsin and chymotrypsin isolated from seeds suppress microbial serine proteases like subtilisin and the proteases from molds like *Aspergillus*. Such inhibitors have been reported from families *Gramineae*, *Cucurbitaceae*, *Amaranthaceae* and *Polygonaceae*. These proteins are structurally similar to the potato proteinase inhibitor I which itself is active against many microbial enzymes (Ryan 1979; Valueva and Mosolov 1999). There are few reports of the effects of seed proteinase inhibitors on the enzymes of phytopathogenic microorganisms.

Soyabean and kidney bean inhibitors from the SBBTI family were shown to suppress proteases of phytopathogenic fungi from the genus *Fusarium*. The buckwheat (*Fagopyrum sculentum*) trypsin/chymotrypsin inhibitor interferes with spore germination and mycelium growth of the tobacco brown-spot fungus *Alternaria alternata* (Dunaevskii *et al*. 1997) while those from sunflower seeds suppressed the activity of the proteases of the causative agent of gray mold. In several cases, plant resistance to microorganisms was found to correlate with the content of PIs as seen in wheat caryopses and lupine and soyabean trypsin inhibitors. Serine protease inhibitors have been reported from potato, barley, tomato, pumpkin, chick pea, cowpea, black beans, mung beans, adzuki beans, broad beans, jack beans (Ryan 1990). The serine protease inhibitors having antifungal activity have the interesting property of inhibiting the α-amylase activity from insects but not from bacterial/mammalian sources. These bifunctional inhibitors which inhibit fungal and insect growth have been reported from corn, ragi, wheat, barley, potato and daisy. The trypsin inhibitors present in soybean were shown to be toxic to the larvae of the flour beetle (*Tribolium confusum*) (Lawrence and Koundal 2002). Following these early studies, there have been many examples of PIs that are active against certain insect species. Studies involved both *in vitro* assays against insect gut proteases (Pannetier *et al*. 1997; Koiwa *et al*. 1998) and *in vivo* artificial diet bioassays (Urwin *et al*. 1997; Vain *et al*. 1998). PIs also exhibit a very broad spectrum of activity against pathogenic nematodes. Cowpea trypsin inhibitor (CpTi) inhibits the growth of nematodes, *Globodera tabacum*, *Globodera pallida* and *Meloidogyne incognita* (Williamson and Hussey 1996). The expression of rice BBI from *Oryza sativa* is upregulated and induced by pathogens or insects during germination of rice seeds (Lin *et al*. 2006). Serine protease inhibitors have been used to produce transgenic plants. These transgenic studies were instrumental in establishing the importance of PIs in plant protection. One of the best demonstrated examples in this case was the transfer of the gene for the cowpea inhibitor. This inhibitor was proved to be a strong antimetabolite against insects from the genera *Heliothis*, *Spodoptera*, *Diabrotica* and *Tribolium*. Hence the modified gene for this inhibitor was transferred to tobacco plants. The transgenic plants manifested higher resistance to insects from the genus *Heliothis* and *Manduca* as compared to the wild type plants. Dunse *et al*. (2010) reported a new approach of crop protection using combination of inhibitors [*Nicotiana alata* proteinase inhibitor (NaPI) and *Solanum tuberosum* potato type I inhibitor (StPin1A)] in which one class of proteinase inhibitor is used to match the genetic capacity of an insect (*Helicoverpa armigera*) to adapt to a second class of proteinase inhibitor. Thus the serine protease inhibitors class remains the best studied class of inhibitors in the plant kingdom for their participation in plant defense.

Cysteine protease inhibitors

The presence of this class of PIs in plants has been known for years. Their role in the plant defense against pest attack has been more recognized. These inhibitors are now referred to as cystatins and there are at least three distinct families (Barrett 1987; Turk and Bode 1991). The amino acid sequences of the pineapple and rice cysteine protease inhibitor have now been determined. Cysteine protease inhibitors have been reported from pineapple (Reddy *et al*. 1975), potato (Rodis and Hoff 1984), rice (Abe *et al*. 1987), cowpea (Rele *et al*. 1980), mung bean (Baumgartner and Chrispeels 1976), tomato, wheat, barley and rye (Fossom 1970). Cysteine proteases from insect larvae can be inhibited by both synthetic and naturally occurring cysteine protease inhibitors (Gatehouse *et al*. 1985; Kitch and Murdock 1986; Campos *et al*. 1989). A specific cysteine protease inhibitor produced by *Aspergilllus japonicus*, called E-64 (trans-epoxysuccinyl-leucyl-augmentine), was shown to inhibit the digestive enzymes of several insect species including the Colorado potato beetle (Wolfson and Murdock 1987), the common bean beetle (Wieman and Nielsen 1985), the Mexican bean beetle, the red flour beetle and the cowpea weevil (Murdock 1987, 1988; Campos *et al*. 1989). E-64 caused a delay of development and larval death, supporting the importance of cysteine protease inhibitors in the control of insect larvae on plants. An interesting observation was that the protease complex from the red flour beetle was inhibited by the same protein component from wheat endosperm, which inhibits the cysteine protease from wheat caryopses. The oryzocystatins from rice suppressed the activities of proteases from the intestines of rice weevil and the red flour beetles. Transgenic rice plants expressing the phytocystatine gene show wider specificity to the different cysteine proteases than rice oryzocystatin (Irie 1996). The cysteine protease inhibitor oryzacystatin I (OC-I) induced moderate but significant growth inhibition on the pea aphid (*Acyrthosiphon pisum* Harris), the cotton/melon aphid (*Aphis Gossypii* Glover) and the peach potato aphid (*Myzus persicae* Sulzer). OC-I affects *M. persicae* through digestive tract targets, but also by reaching the haemolymph, thereby inhibiting extra-digestive proteolytic activities and interacting with functions related to aphid reproduction. Overall, it appears that PIs can display deleterious effects against phloem-feeding insects in addition to their activity on leaf-feeding insects (Rahbe *et al*. 2003). Engineered multidomain cysteine protease inhibitors provide a novel way of controlling western flower thrips in greenhouse and field crops, and open up possibilities for novel insect resistance applications in transgenic crops (Outchkourov *et al*. 2004). Phytocystatin isolated from the transgenic tobacco plants inhibited papain and Cathepsin H as well as the cysteine proteases from weevil digestive complex. Many cysteine protease inhibitors have been demonstrated to show potentiating effects on the toxins responsible for insecticidal activity. The combined activities of both serine and cysteine proteases show a higher protective effect against insect larvae than either of them alone.

Aspartic protease inhibitors

The knowledge of APIs in plant protection is very scanty. There have been reports of aspartic proteases in insect guts, but conclusive evidence showing the role of the inhibitors in protection has not been reported. Pepstatin, a powerful and specific inhibitor strongly inhibited the gut enzymes of the Colorado potato beetle (Wolfson and Murdock 1987). Potato tubers have shown the presence of a cathepsin D inhibitor that exhibits considerable amino acid homology with the soyabean trypsin inhibitor (Mares *et al*. 1989). ATBI, an API described earlier was found to exhibit activity against phytopathogenic fungi, including *Alternaria*, *Aspergillus*, *Curvularia*, *Colletotricum*, *Fusarium*, and *Phomopsis* species, and the saprophytic fungus *Trichoderma* sp. has been investigated. The negative charge and the absence of periodic secondary structure in ATBI suggested an alternative mechanism for fungal growth inhibition. Rescue of fungal growth inhibition by the hydrolytic products of xylanase and aspartic protease indicated the involvement of these enzymes in cellular growth (Dash *et al*. 2001b). The inhibi-

tion of molting fluid enzymes from *Helicoverpa armigera* by ATBI is reported. The in vitro experiments showed 80% inhibition of hemoglobin hydrolyzing and 95% inhibition of chitin hydrolyzing activity from molting fluid by ATBI. The treatment of *H. armigera* larvae with 400 mM ATBI recorded 20% larval mortality, 27.77% deformed pupae and 12.22% deformed adults. The results provide the basis for the selection of non-host inhibitors to develop a *H. armigera* insecticide formulation (Kumar *et al*. 2010). The effect of APIs on disease management of pigeon pea wilt caused by *Fusarium udum* has been reported (Kulkarni 2007). A bifunctional inhibitor ATBI and API from mung bean were used at the pot and field levels for the control of wilt on pigeon pea caused by *F. udum*. A powder formulation of API was developed and was used for coating the seeds of pigeon pea varieties ICP 2376 and ICP 87. A maximum control of 65-71% was reported with seeds coating and additional drenching. These results were at par in their efficiency with the chemical agent, carbendazim. The reasoning behind the control of the wilt by API is probably due to their ability to inhibit extracellular xylanase and aspartic protease produced by the fungus. These enzymes are crucial for the germination of fungal spores and proliferation of the mycelium.

INHIBITOR DESIGN AND FUTURE PROSPECTS

The family of aspartic proteases, although rather small, contains a number of validated and potential drug targets making drug discovery efforts in this area very fruitful and exciting. There have been substantive advances in our understanding of the use of PIs as therapeutic agents. Several synthetic PIs have been approved by the FDA for therapy of HIV and hypertension. These drugs represent prime examples of structure based drug design. Moreover, the inhibitory principles and compounds, which have been established and discovered, now enable mechanism-based drug discovery across the whole family (Gulnik and Micheal 2008). The sequencing of the human genome and the resulting knowledge on all human aspartic proteases allows an exhaustive profiling of inhibitors for specificity towards all family members thereby reducing the risk of unwanted side effects.

A number of natural and peptidomimetic inhibitors performed well in different phases of clinical testing to treat other human disorders, including cancer, inflammation, cardiovascular, neurodegenerative, and various infectious diseases. Despite this impressive progress, there is much to learn about the cross talk between signal transduction pathways and protease activation cascades. Additionally, development of successful PIs for clinical use is reliant on maximizing bioavailability, specificity, and potency of inhibition of the target enzyme. Ideally, localizing PIs to a single target area of the body may also help minimize the potential for complications and detrimental side effects. There is the further issue of the development of drug resistance to PIs in the face of a build up of substrate pressure, and selection of catalytically active mutant or other salvage proteases that do not have complementarities for carefully designed inhibitors of wild type proteases. The future appears to still hold considerable promise for PIs. We can anticipate new, overexpressed proteases from genomic/biochemical comparisons made between normal/diseased cells, host/ pathogen, healthy/unhealthy subjects leading to more effective and efficient validation of proteases as drug targets. New advances in protein chemistry will lead to faster production and greater quantities of pure recombinant proteases and advances in structural biology (crystallography, NMR spectroscopy) will produce faster and more accurate inhibitor-protease structures. Inhibitors (naturally occurring and synthetic) have permitted detailed biochemical and crystallographic investigations to be made, but an understanding of the selectivity of such inhibitors may be of just as much importance for the design and synthesis of specific inhibitors for use therapeutically in controlling individual aspartic proteases.

Discovery of novel selective inhibitors can proceed only through combination of screening of chemical libraries, rational design, computational technology, and exploration of natural compounds. The exploitation of vast microbial diversity will also generate large amount of biologic APIs. The application of biologic inhibitors will stimulate renewed interest in the therapeutic targeting of aspartic proteases. The mechanism by which these inhibitors modulate the proteases anticipates the development of biologic inhibitors as lead molecules for clinical approval in the near future. Furthermore, future research into the synergistic capabilities of inhibitors will help elucidate the most effective combination therapies. These advances, together with more careful attention to inhibitor conformation, mechanism of action, and drug-like composition are expected to result in more potent, more selective, more bioavailable inhibitors with a higher probability of success in the clinic.

PPIs have been isolated and characterized from a large number of sources, and that the natural inhibitors have been made available through transgenic plants overexpressing specific inhibitors with therapeutic significance. The potential for the natural inhibitors in agriculture is enormous, awaiting full-scale exploration. Recombinant PIs have proved to be of particular interest as promising models for studying the ecological impacts of insect-resistant transgenic plants, co-evolutionary processes in plant–insect systems, and recombinant protein-mediated pleiotropic effects in transgenic plants. Recent developments towards the successful implementation of transgenic plants expressing inhibitors in agricultural fields, along with the numerous and complex questions raised by such promising developments, should ensure the status of these metabolic effectors as useful models.

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