

Protease Inhibitors: Defensive Proteins in Plants

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ABSTRACT

One of the biggest and most varied groups of enzymes that specifically catalyze the hydrolysis of peptide bonds in proteins is the protease family (E.C. 3.4). They can be divided into serine, cysteine, aspartic, threonine, glutamic acid and metalloproteases based on the catalytic kinds they possess. Serine PIs predominate among them, followed by metalloproteases and cysteine PIs; aspartic and glutamic PIs are uncommon and distributed throughout several groups. The most researched, well-characterized and widely distributed class of plant PIs are serine PIs. Protease Inhibitors (PIs) are molecules that thwart the proteases proteolytic activities and are essential for controlling the body's protein catabolism. Fermi and Pernossi were the first to report on the presence of PIs in nature.

Keywords: Aspartic; Threonine; Glutamic acid; Metalloproteases

INTRODUCTION

15% of global productivity is lost before to harvest as a result of insect infestations, even with the usage of insecticides. The issue of insect pest competition is made more difficult in emerging nations by the annual rise in human population.

Therefore, crop protection is essential to modern agricultural production in order to reduce output losses and feed the world's ever-growing population. Numerous bug families are harming crops and having an adverse effect on the economy. Common insect pests that cause significant losses to economically significant crops include aphids, cabbage maggot, colorado potato beetle, corn earworm, autumn armyworm and others. Natural substances called protease inhibitors are prevalent in the seeds and tubers of *Graminea*, *Solanacea* and *Leguminosae* families. PIs typically accumulate 1%-10% of the total soluble proteins in storage tissues and are found in the seeds and other storage organs of plants. On the other hand, it has also been shown that they occur in the aerial portion of plants in response to various stimuli [1].

Plant protease inhibitors are essential for protecting plants from herbivores, particularly in-sects. The majority of PPIs bind with protease's active site to produce a stable inhibitor-protease complex that lacks enzymatic activity. The essential amino acid levels required for insect growth and development are reduced as a result of PPIs' suppression of protease activity. Plant PIs are

tiny regulatory proteins that are widely distributed and typically found in storage tissues at high concentrations (5%-15% of total protein). With an estimated 32.35 lakh hectares under cultivation, pigeon pea (*Cajanus cajan* (L.) Millisp.) is the second most popular crop in India [2].

Due to its low input requirements, strong agronomic adaptability for growth and good supply of firewood, it is expected to overtake other pulse crops as the primary crop in the near future. Protease Inhibitor (PI) buildup is one of the natural defense strategies used by most plants to fend off insect attack. When PIs are applied to insects that are vulnerable, the consequences are often observed as increased mortality, decreased growth rate and extended larval development time. These harmful effects are achieved by inhibiting insect midgut proteinases, which impedes or at least delays (in the case of mild inhibitors) protein digestion. The dissolution of amino acids and peptides from dietary protein. When an inhibitor is present, nutrients are lost, especially amino acids that include sulfur, which results in weak, stunted growth and eventually death [3].

LITERATURE REVIEW

Protease

A large class of enzymes known as proteases hydrolyzes or breaks down proteins or peptides. According to Razzaq et al., proteases

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cleave the peptide bonds that hold nearby amino acid residues in a protein molecule, resulting in the creation of shorter peptides and amino acids. These hydrolytic enzymes are widely distributed in nature and can be found in all living species, including prokaryotic domains such as bacteria and archaea and eukaryotes such as plants, animals, fungi and protists. According to Bernardo et al., a number of viruses are even known to encode their own proteases.

Proteases are classified as hydrolases in class 3 of the Enzyme Commission's classification scheme and they are allocated the unique number EC 3.4.x.x, which corresponds to each proteolytic enzyme [4].

These enzymes have been classified according to a number of factors, including the active pH range, substrate type, mechanism of action involving a specific amino acid present in the active site and site of action. These enzymes can be generically categorized as endopeptidase or exopeptidase, depending on the place of action. While the latter operate on peptide bonds at the substrate's termini, the former have a tendency to hydrolyze nonterminal peptide bonds, resulting in the creation of shorter peptides. Additionally, exopeptidases vary in the termini on which they preferentially operate [5].

Based on whether they act on the N or C terminal, respectively, are further divided into aminopeptidases and carboxypeptidases. Exopeptidases release shortened peptides that are dipeptides, tripeptides or amino acids. In every living thing, the physiology and metabolism are greatly influenced by the proteases. By regulating different stages involved in protein synthesis, protein activation-inactivation, signaling, protein turnover and gene expression, these enzymes contribute significantly to the regulation of a wide range of physiological processes in addition to their evident role in the digestion of proteins and peptides. Proteases are also highly important in a variety of industries. Proteases are traditionally used in industry as cleaning agents, such as detergent additives and components of contact lens cleaning solutions. Proteases are used by the textile industry for a number of tasks, such as degumming silk and biopolishing wool in a very sustainable and environmentally friendly manner. In a similar vein, the leather industry is using fewer chemicals and placing an increasing emphasis on the use of proteolytic enzymes to complete various stages of the leather processing process. Chicken farms, slaughterhouses and other similar establishments produce a lot of keratinous wastes, which are hard to manage and lead to a lot of issues like water and soil pollution, aesthetic issues, clogged drains, disease spread, etc. Collagen, which is produced by the seafood and fish processing industries as well as slaughterhouses, is another proteinaceous waste that should be taken seriously. Aside from the immediate health risk to humans and animals (because of the potential for the spread of pathogenic microbes), improper disposal of such wastes also poses a significant threat to pollution. By dissolving these troublesome components, proteases-particularly keratinases and collagenases-also play a critical role in the fields of waste management and pollution control. In order to aid in waste management, keratinases-proteases that can break down keratin-have been employed to break down keratin in waste materials. Additionally, the residues treated with keratinase can

be utilized as nitrogenous fertilizers and animal feed. Utilizing collagenase to extract collagen from fish and animal carcasses can reduce waste production while also aiding in the reclamation of collagen [6].

DISCUSSION

Classification of proteases

Proteases are divided into four classes by the International Union of biochemistry and molecular biology: Metalloproteases, aspartic proteases, cysteine proteases and serine proteases.

The cysteine proteases: The significance of a cysteine thiol group as the primary nucleophile in the enzyme's active site gave rise to the moniker "cysteine proteases." In the early stages of the peptide bond's catalytic cleavage, the thiol group functions as a nucleophile. Papain, which was extracted from *Carica papaya*, was the first Cysteine Protease (CP) to be isolated and characterized. It was also the second enzyme with a well-characterized structure, following pepsin. The catalytic residues found in the active sites of CPs are essentially made up of a histidine and a cysteine residue. The thiol group of the Cys-His-Asn triad is deprotonated by an amino acid with a basic side chain to begin the catalysis of proteolysis. The anionic sulfur of the deprotonated cysteine residue acts as a nucleophile to attack the scissile peptide bond's carbonyl carbon atom. Moreover, the release of a substrate fragment with an amino-terminus causes the histidine residue to revert to its deprotonated conformation. This leads to the creation of a thioester linked intermediate, which connects the substrate's newly formed carboxy-terminus to the cysteine thiol; for this reason, CPs and thiol proteases are used interchangeably. Following the hydrolysis of the thioester bond, the free enzyme is restored and a carboxylic acid moiety and substrate fragment are produced [7].

The aspartic proteases: Plant APs, which are primarily found in family A1, are primarily active at acid pH levels (pH 2-6), require two aspartic acid residues to be catalytically active and are specifically inhibited by pepstatin A. Plant aspartic proteases belonging to the A1 family typically possess the catalytic motifs Asp-Thr-Gly (DTG) or Asp-Ser-Gly (DSG). Plant APs have a Plant-Specific Insert (PSI) in the C-terminal region, despite having a general structure that is similar to that of microorganisms and mammals. A prosegment, a PSI, a signal peptide and hydrophobic-hydrophobic-DTG-Ser-Ser residues make up the catalytic site of typical APs.

The serine proteases: Although serine residues in the active site of serine protease (EC 3.4.21), an endopeptidase, cleave peptide bonds like any other protease; however, serine acts as a nucleophile and can coordinate numerous other essential functions through protein hydrolysis. Numerous vital processes are involved, including blood coagulation, digestion, apoptosis, immunity, development regulation and fertilization. The initial stage of proteolysis is the cleavage of Protease-Activated Receptors (PARs), which are G-protein-coupled receptors found on epithelial, vascular, neural and immune cells. Its function is too complex and widespread [8].

The metalloproteases: Any protease enzyme involving a metal in its catalytic mechanism is called a metalloproteinase or metalloprotease. While some metalloproteases use cobalt, most require zinc. Three ligands work together to coordinate the metal ion with the protein. Proteases of this type are the most diverse; over 50 families have been classified. Three ligands-histidine, glutamate, aspartate, lysine and arginine-interact with the protein to facilitate the metal ion. The unstable water molecules take up a different binding site in the metalloproteinase. Two classes of well-known metalloproteases are exopeptidases and endopeptidases, which include matrix metalloproteinases and ADAM proteins. Total inactivated metalloproteases can be obtained by chelating agents such as orthophenanthroline and EDTA (metal chelator removing zinc). There is growing evidence that metalloproteases are involved in numerous physiological processes, such as muscle damage, chronic venous disease, tumor initiation and progression.

Plant protease inhibitors

A family of tiny proteins known as proteinase inhibitors plays a crucial role in the defense mechanisms of plants against herbivory by insects or microorganisms that could jeopardize the structural integrity of the plant. The plant material is not able to be properly digested by the attacker because the proteinase inhibitors interfere with the digestive or microbial enzymes' ability to function. Recent research has also shown that some proteinase inhibitors offer defense for plants by inhibiting the growth of pathogens due to their antimicrobial qualities.

PIs are involved in numerous physiological processes in plants as well. Storage protein mobilization, endogenous enzyme activity regulation, apoptosis and programmed cell death modulation and stabilization of defense proteins or compounds against animals, insects and microbes have all been linked to them. Numerous plant PIs have been described due to their great adaptability and range of biotechnological uses [9].

Mechanisms of inhibition of protease inhibitors

Numerous authors thoroughly revised the mechanisms underlying the interaction between protease and inhibitor. Although there are two widely recognized mechanisms of interaction between inhibitors and proteases in nature, there are other ways in which they can interact. The irreversible trapping reaction is one of them and the best-characterized families of protease inhibitors that demonstrated this mechanism are the inhibitors of baculovirus protein p35, $\alpha 2$ macroglobulins and serpins. This kind of inhibition mechanism occurs when an internal peptide bond in the inhibitor structure is cleaved by the protease-inhibitor interaction, which results in a conformational shift. The inhibitor never regains its original structure and this reaction is irreversible. Because of this, the inhibitors involved in trapping reactions are also referred to as suicide inhibitors. A tight binding reaction is the alternative mechanism of protease-inhibitor interaction that is typically observed.

Another name for this mechanism is a standard mechanism and it was extensively explained by Laskowski, Qasim and most recently, Farady, Craik and associates. This mechanism is

canonical for all inhibitors and was shown to work with serine protease inhibitors. The standard mechanism of inhibition is employed by most plant Serine Protease Inhibitors (SPI). The inhibitors' interactions with the protease active site (P1) during tight-binding reactions are comparable to those between an enzyme and its substrate. The inhibitor's intact form and its modified forms, in which the reactive site's peptide bond is broken, coexist in a stable equilibrium with the protease inhibitor complex. As a result, the complex's inhibitor dissociates into its original or altered form [10].

The P1 specificities of serine proteinases that are inhibited by the conventional inhibitors vary. Multiple proteinases can be targeted simultaneously by the Bowman Birk, Potato II and Kunitz families, frequently with varying specificities. By using this tactic, plant SPIs enable plants to protect themselves from harmful proteolytic activity, which can be used to regulate growth or fend off insect invasions (Figure 1).

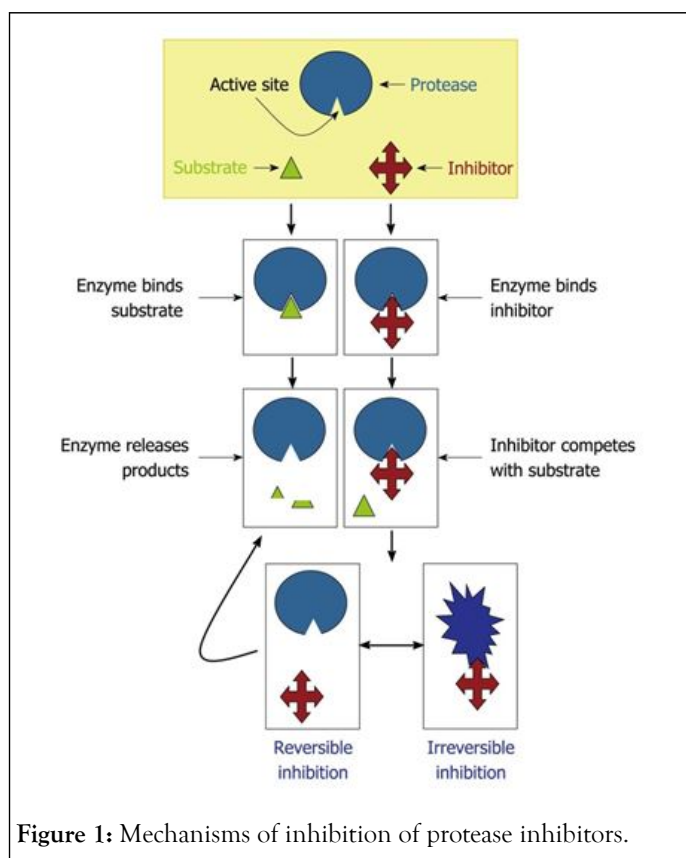


Figure 1: Mechanisms of inhibition of protease inhibitors.

Families of protease inhibitors

Classification: Based on their specificities and amino acid sequences, plant protease inhibitors are generally grouped into families.

Based on the reactive site's position, topological interactions between disulfide bridges and high levels of homology among its members, Laskowski and Kato divided the "Proteinase inhibitors" into multiple families. According to Laskowski and Kato, Birk, Schirra et al., soybean (Kunitz), potato I and II, Bowman-Birk and squash families are the groups of plant serine PIs that follow the conventional mechanism. There have also

been suggestions for several other inhibitor families, including barley, ragi 1 and 2, thaumatin and serpin.

Serine protease inhibitors: Serine PIs are found in all kingdoms of plants. They are the most researched class of PIs, with reports coming from a wide range of plant sources. Their physiological functions include mobilizing reserve proteins, controlling endogenous proteinases during seed dormancy and providing defense against parasitic and insect proteolytic enzymes. They might also serve as reserve or storage proteins. The Kunitz-type and Bowman-Birk types of plant serine PIs are the two families with the strongest characterizations. Kunitz-type inhibitors consist of one or two polypeptide chains, a molecular mass of 18-22 kDa, one reactive site, low cystine content (often four Cys residues in two disulfide bridges). Trypsin and chymotrypsin are two examples of the enzyme molecules to which they attach concurrently and independently. The reaction center structure and mechanism of action are well conserved in serine PIs despite variations in primary structure and topology. Certain plant serine PIs have dual functions, as they can inhibit both α -amylase and trypsins.

Cysteine protease inhibitors: Phytocystatins, also known as cysteine protease inhibitors, are the second most researched class of inhibitors. Numerous monocot and dicot species, including maize, rice, potatoes, soybeans and apples, have been found to contain. The majority of phytocystatins belong to one group, which has a single domain, while others, like the multicystatins found in sunflower seeds, potato tubers and tomato leaves, have multiple domains.

The phytocystatins exhibit strong inhibitory activity against insect gut proteinases, which sets them apart from animal cystatins. This characteristic makes the phytocystatins appealing as biological control agents of insect pests. According to Gatehouse et al. and Amirhusin et al., tomato and potato plants possess cysteine PIs, which provide resistance and shield the plants from cowpea weevils and Colorado potato beetles. These insects use cysteine proteinases as vital digesting enzymes. Studies conducted by Anadana et al. and Outchkourov et al. have demonstrated that the larvae are toxically affected by the inclusion of cysteine PIs in fake diets and transgenic plants.

Metallo-protease inhibitors: The metallo-carboxypeptidase inhibitor family in potato and tomato plants is a representative group of metallo-protease inhibitors in plants. On the other hand, *Ferula persica* produced two matrix metalloproteinase protease inhibitors that were isolated by Shahverdi et al. and showed a specific inhibitory impact on tumor cell invasion.

Aspartic protease inhibitors: Because aspartic PIs are uncommon, they are a class that has received relatively little research. An aspartic proteinase (cathepsin D) inhibitor found in potato tubers is substantially similar in amino acid sequence to the Soybean Trypsin Inhibitor (SBTI). Nonetheless, an aspartic protease inhibitor has been isolated and described by Christeller et al. from the phloem exudates of squash (*Cucurbita maxima*).

The family of Bowman Birk Inhibitors (BBIs): The PI from soybean (*Glycine max*) that was first identified and characterized by D.E. Bowman and Y. Birk is honored in the name of this

family. The soybean inhibitor, sometimes known as the "classic BBI," is now the most researched member of this family and has the capacity to block both trypsin and chymotrypsin. Legumes, cereals and the *Poaceae* family of grasses contain these inhibitors. Widely present in both monocot and dicot species, BBIs are cysteine-rich proteins that have inhibitory effect against proteases. Based on their inhibitor properties and structural attributes, BBIs have been categorized. The dicotyledonous plant inhibitors are made up of a single polypeptide chain with an 8 kDa molecular mass. With two homologous domains that each have a unique reactive site for the corresponding proteases, these are double-headed. These inhibitors interact with two proteases-which may be the same or different-independently and concurrently. Two forms of BBIs are found in monocotyledonous plants. A single polypeptide chain with a molecular mass of roughly 8 kDa makes up one group. They only have one responsive website. Two reactive sites and a molecular mass of 16 kDa characterize another group.

Cereal trypsin/ α -amylase inhibitors: Members of this family exhibit inhibitory effect against serine proteinase and/or α -amylase. Many members of this family of inhibitors exclusively exhibit α -amylase-inhibitory action; on the other hand, Odani et al. found that inhibitors derived from tall fescue (*Festuca arundinacea*), rye (*Secale cereale*) and barley (*Hordeum vulgare*) are effective against trypsin. Inhibitors of maize (*Zea mays*) and ragi (*Elusine coracana*) have dual activity and have the ability to inhibit both α -amylase and serine proteinases. A single polypeptide chain with five disulfide bridges and a molecular mass of roughly 13 kDa makes up the cereal trypsin/ α -amylase inhibitors.

Mustard (*Sinapis*) trypsin Inhibitor (MSI): These tiny, single-chain polypeptide chain inhibitors, which have a molecular weight of roughly 7 kDa, belong to the *Cruciferae* family and constitute a different subfamily of serine PIs. Several species, such as tape (*Brassica napus*) and white mustard (*Sinapis alba*), have been used to isolate and describe these inhibitors.

Potato type I PIs (PI 1): This family of inhibitors is widely distributed in plants and has been identified in numerous species, such as tomato fruit, squash phloem exudates, potato tubers and tomato leaves in response to wounding. These inhibitors are typically monomeric and have a molecular mass of 8 kDa.

Potato type II PIs (PI 2): Only members of the *Solanaceae* family have been identified as belonging to this group. These inhibitors, which were first identified from potato tubers, have also been discovered in the phloem, leaves, flowers and fruit of other solanaceous plants. According to Antcheva et al., inhibitors of this class have been shown to inhibit subtilisin, chymotrypsin, trypsin, elastase, oryzin and Pronase E.

Squash inhibitors: Numerous cucurbit families have characterized the members of this family. According to Heitz et al., the members of this family are made up of a tiny single peptide chain with a molecular mass of 3.0-3.5 kDa and 28-30 amino acids. These inhibitors fold in a unique knottin structure and contain three disulfide bridges. Because of their tiny size and possible activity against significant biological molecules such

cathepsin G, human leucocyte elastase and Hageman factor, these inhibitors are especially appealing for research on the interaction between proteinase and inhibitor.

CONCLUSION

As seed storage proteins, plant PIs are often concentrated (approximately 10%) in the seeds. Protease inhibitors function by impeding the digestive or microbiological enzymes in the attacker's stomach, making it difficult for the plant material to be properly broken down. This prevents the plant from growing normally and deters the attacker from injuring it further.

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