International Journal of Advance and Applied Research
www.ijaar.co.in
ISSN – 2347-7075 Impact Factor – 8.141
Peer Reviewed Bi-Monthly
Vol. 11 No. 6 July-Aug 2024

Protease Inhibitors in Plants: A Comprehensive Review of their Classification, Distribution and Functional Roles

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DOI- 10.5281/zenodo.13193617

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. 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.

Keywords: Protease, Protease Inhibitors, Serine Protease Inhibitors, Proteolytic activity, Microbiological enzymes etc.

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. (Sharma, Kanika, 2015).Natural substances called protease inhibitors are prevalent in the seeds and tubers of Graminea, Solanacea and Leguminosae families (Connors et al 2002).

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 (De Leo et al 2001).

Plant protease inhibitors are essential for protecting plants from herbivores, particularly insects (Koiwa et al. 1997). The majority of PPIs bind with protease's active site to produce a stable inhibitor-protease complex that lacks enzymatic activity (Norton 1991).The essential amino acid levels required for insect growth and development are reduced as a result of PPIs' suppression of protease activity (De Leo et al. 2002; Nanasahe et al. 2008). Plant PIs are tiny regulatory proteins that are widely distributed and typically found in storage tissues at high concentrations (5–15% of total protein) (Garciaoimedo et al., 1987).With an estimated 32.35 lakh hectares under cultivation, pigeon pea (Cajanus Cajan (L.)Millisp.) Is the second most popular crop in India (Gupta et al.,1991).

Due to its low input requirements, strong ag ronomic adaptability for growth, and good supply of firewood, it is expected to overtake other pulse crop s as the primary crop in the near future (Dwivedi, 19 86).Protease inhibitor (PI) buildup is one of the natu ral defense strategies used by most plants to fend off insect attack (Ryan 1973).When PIs are applied to i nsects that are vulnerable, the consequences are ofte n observed as increased mortality, decreased growth rate, and extended larval development time.These h armful effects are achieved by inhibiting insect midg ut proteinases, which impedes or at least delays (in t he 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 (Gatehouse et al 1992).

Protease

A large class of enzymes known as proteases hydrolyzes or breaks down proteins or peptides.

According to Razzaq et al. (2019), proteases cleave the peptide bonds that hold nearby amino acid residues ina 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. (2018), a number of viruses are even known to encode their own proteases.

Proreases 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 (Contesini et al. 2017).

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 (Guleria et al. 2016a).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.

Based on whether they act on the N or C terminal, respectively, are further divided into aminopeptidases and carboxypeptidases (Navee d et al. 2021).Exopeptidases release shortened pepti des 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 (Bond 2019).

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 (Salwan and Sharma 2019; Singh and Bajaj 2017; Lam et al. 2018). 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 (Chatha et al. 2017; Mamo and Assefa 2018). 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 (Fang et al. 2017). 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 (Kamarudin et al. 2017). 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 (Bhagwat and Dange 2018; Razzaq et al. 2019; Yusuf et al. 2019).

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 (Kumawat et al. 2018). Utilizing collagenase to extract collagen from fish and animal carcasses can reduce waste production while also aiding in the reclamation of collagen (Pal and Suresh 2016).

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.

1. 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. (2009, Erez). Papain, which was extracted from Carica papaya (Roy, 1874; Walsh, 2015), 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. (Coulombe et al., 1996; Verma et al., 2016).

2. 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) (Simões 2004). 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 (Mutlu 1999). A prosegment, a PSI, a signal peptide, and hydrophobic-hydrophobic-DTG-Ser-Ser residues make up the catalytic site of typical APs.

3. 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 (Gasteiger 2003). Numerous vital processes are involved, including blood coagulation, digestion, apoptosis, immunity, development regulation, and fertilization. (Gohara, 2013). 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.

4. 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.

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, α2 macroglobulins, and serpins.

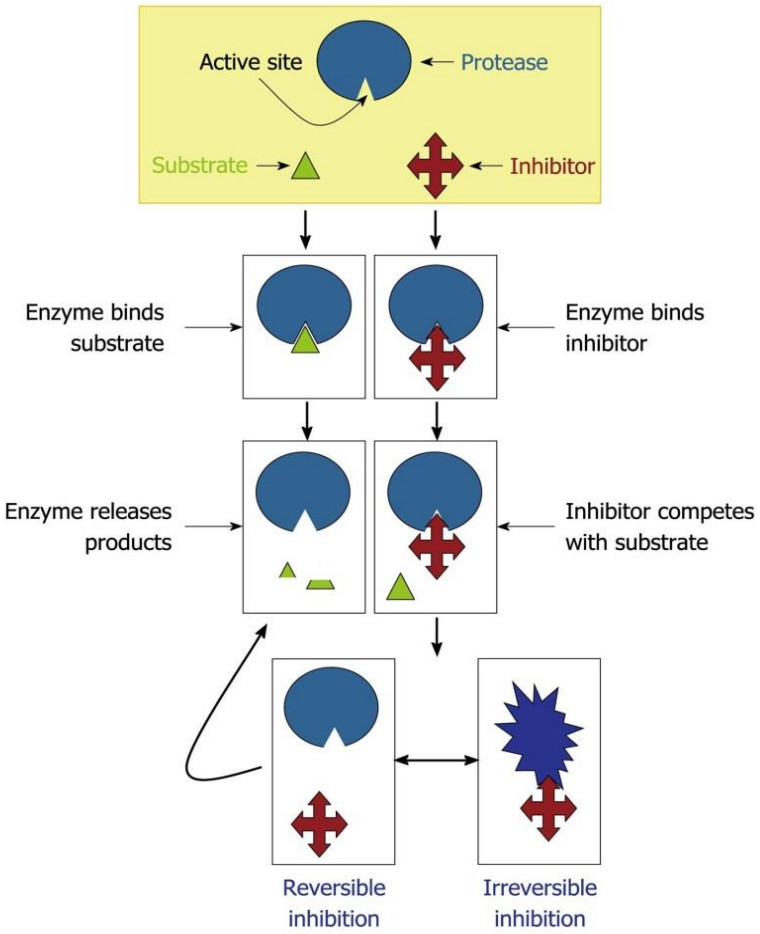
This kind of inhibition mechanism occurs when an internal peptide bond in the inhibitor structure is cleaved by the protease–inhibitor interaction, which results in a conformational shift. The inhibitor never regains its original structure, and this reaction is irreversible. Because of this, the inhibitors 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 stand ard 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 em ployed by most plant serine protease inhibitors (SPI) .The inhibitors' interactions with the protease active site (P1) during tightbinding reactions are comparab le to those between an enzyme and its substrate.The inhibitor's intact form and its modified forms, in whi ch the reactive site's peptide bond is broken, coexist in a stable equilibrium with he protease inhibitor

complex. As a result, the complex's inhibitor dissociates into its original or altered form.

The P1 specificities of serine proteinases th at 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 ebanle plants to protect themselves from harmful proteolytic activity, which can be used to regulate growth or fend off insect invasions.



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 (1980) divided the "Proteinase inhibitors" into multiple families. According to Laskowski and Kato (1980), Birk (2003), Schirra et al. (2008), 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 (Ryan, 1990; Dahl et al., 1996; Ascenzi and al., 1999).

A. 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 (Birk, 2003). 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 (Birk, 1985, 2003; Bode and Huber, 1992; Mc Bride et al., 2002; Qi et al., 2005). The reaction center structure and mechanism of action are well conserved in serine PIs despite variations in primary structure and topology (Qi et al., 2005). Certain plant serine PIs have dual functions, as they can inhibit both α-amylase and trypsins (Strobl et al., 1995; Haq et al., 2005).

B. 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 phytocystatins (Kondo et al., 1990; Abe et al., 1991, 1996; Botella et al., 1996; Gruden et al., 1997; Ryan et al., 1998; Tian et al., 2009). The majority of phytocystatins belong to one group, which has a single domain (Pernas et al., 1998), while others, like the multicystatins found in sunflower seeds, potato tubers, and tomato leaves, have multiple domains (Walsh and Strickland, 1993; Wu and Haard, 2000; Kouzuma et al., 2000).

The phytocystatins exhibit strong inhibitory activity against insect gut proteinases (Bode and Huber, 1992; Koiwa et al., 1997; Martinez et al., 2007), which sets them apart from animal cystatins (Kondo et al., 1991; Brown et al., 1997; Arai et al., 2002). This characteristic makes the phytocystatins appealing as biological control agents of insect pests (Gatehouse and Gatehouse, 1998; Ussuf et al., 2001; Benchabane et al., 2008). According to Gatehouse et al. (1986) and Amirhusin et al. (2004), tomato and potato plants possess cysteine PIs, which provide resistance and shield the plants from cowpea weevils and Colorado potato beetles (Wolfson and Murdock, 1987). These insects use cysteine proteinases as vital digesting enzymes. Studies conducted by Anadana et al. (2002) and Outchkourov et al. (2004) have demonstrated that the larvae are toxically affected by the inclusion of cysteine PIs in fake diets and transgenic plants.

C. Metallo-protease inhibitors:

The metallo-carboxypeptidase inhibitor family in potato and tomato plants (Hass et al., 1975; Graham and Ryan, 1981) is a representative group of metallo-protease inhibitors in plants (Rancour and Ryan, 1968). On the other hand, Ferula persica produced two matrix metalloproteinase protease inhibitors that were isolated by Shahverdi et al. (2006) and showed a specific inhibitory impact on tumor cell invasion.

D. 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 (Mares et al., 1989) 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. (1998, 2006) 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 (Bowman, 1946; Birk et al., 1963). 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 (Laing and McManus, 2002; Odani et al., 1986). Widely present in both monocot and dicot species, BBIs are cysteine-rich proteins that have inhibitory effect against proteases (Lin et al., 2006). 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 (Raj et al., 2002). 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 (Prakash et al., 1996).

E. Cereal trypsin/α-amylase inhibitors:

Members of this family exhibit inhibitory effect against serine proteinase and/or α-amylase (Gourinath et al., 2000). Many members of this family of inhibitors exclusively exhibit α-amylase-inhibitory action; on the other hand, Odani et al. (1983) 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 (Mahoney et al., 1984). A single polypeptide chain with five disulfide bridges and a molecular mass of roughly 13 kDa makes up the cereal trypsin/α-amylase inhibitors (Christeller and Liang, 2005).

F. 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 (Laing and McManus, 2002). Several species, such as tape (Brassica napus) and white mustard (Sinapis alba), have been used to isolate and describe these inhibitors (Ascenzi et al., 1999; Volpicella et al., 2000).

G. 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 (Wingate et al., 1989), squash phloem exudates (Murray and Christeller, 1995), potato tubers (Ryan and Balls, 1962), and tomato leaves in response to wounding (Lee et al., 1986). These inhibitors are typically monomeric and have a molecular mass of 8 kDa.

H. 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 (Christeller and Liang, 2005), have also been discovered in the phloem, leaves, flowers, and fruit of other solanaceous plants (Pearce et al., 1993). According to Antcheva et al. (1996), inhibitors of this class have been shown to inhibit subtilisin, chymotrypsin, trypsin, elastase, oryzin, and Pronase E.

I.Squash inhibitors:

Numerous cucurbit families have characterized the members of this family (Felizmenio et al., 2001). According to Heitz et al. (2001), 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 (Hara et al., 1989). Because of their tiny size and possible activity against significant biological molecules such cathepsin G, human leucocyte elastase, and Hageman factor (McWherter et al., 1989), these inhibitors are especially appealing for research on the interaction between proteinase and inhibitor.

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