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Plant serine protease inhibitor (SPI): A potent player with bactericidal, fungicidal, nematocidal and antiviral properties

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Abstract

This review describes plant originated serine protease inhibitors (SPIs) that target or inhibit different families of serine proteases (SPs), protein-digesting enzymes of various pathogens. SPs play a crucial task in many biological events by catalyzing proteolysis that serves as mediators of signal initiation, transmission and termination of the cellular events leading to regulation of an organism's life cycle. The activity of proteases has to be closely regulated by proteinase inhibitors (PIs) to avoid the damage that proteases might cause in vivo, in the host organism. Based on the selectivity and inhibitory activity PIs are categorized into four mechanistic class viz. aspartic PI (pepstatins), serine PI (serpins), cysteine PI (cystatins) and metallocoxy PI. Among these SPI family is the largest. Many organisms including bacteria, fungus, viruses, protists, insects and vertebrates derive their nutritional requirement from various life forms by finding a suitable host. Proteases constitute 1-5% of genomes of these infectious organisms among which SPs regulate protein synthesis, turnover and physiological functions viz. fertilization, growth and development, digestion, cell signaling or migration, immune defense, wound healing and disease propagation. SPs mediate the process of pathogenesis and/or host tissue penetration for a number of diseases in the host organisms. SPs are generally found either as constitutive components in plant storage tissues like seeds and tubers or expressed in response to pest and pathogen attack besides acting as a defense system against a wide variety of pathogens. These expressed or endogenously present SPIs can attenuate SPs with cross-protection against a wide array of protease families of pathogens like bacteria, fungus, nematodes and viruses.

Keywords: Serine protease inhibitors (SPIs), Serine proteases (SPs), Proteolysis, Attenuate

Introduction

Proteases also termed as peptidases or proteinases are enzymes involved in protein digestion [1]. These enzymes are ubiquitously present in plants, animals and also, most microorganisms. Proteases constitute about 2% of the human genome, and 1-5% of genomes of the infectious organism [2]. These enzymes catalyze various proteolytic events that serve as mediators of signal initiation, transmission and termination of the cellular events such as inflammatory response, cellular apoptosis, coagulation of blood and hormone processing pathways [3]. Proteases play an important role in the regulation of the life cycle of insects, agricultural pests, animal health, plants and marine food sources [2]. Many processes such as food digestion, metamorphosis, tissue modeling, blood coagulation, melanization, in animal use proteinase associated cascade of events to activate and augment the processes. These processes are regulated by PIs [4, 5, 6, 7, 8, 9, 10, 11, 12]. The activity of proteases has to be closely regulated and controlled to avoid the damage that it might cause in vivo, in the host organism and is invariably mediated by Protease inhibitors (PIs) [13]. Numerous compounds produced by plants are being screened by the pharmaceutical industry, which utilizes the molecules to produce drugs with greater efficacy and less toxicity. Among the various phytoextracts with drug potential, PIs are the one which is capable of inhibiting proteolytic activity associated with many diseases [2].

PIs are considered as one of the most abundant defensive classes of proteins in various life forms. For example, high concentration of plant originated PIs occurs in storage organs such

as seeds and tubers (1 to 10% of their total proteins comprise of PIs), which inhibit enzymes as well as play an important role in plant defense against insect herbivory^[14, 15, 16]. PIs bind to the digestive enzymes in the insect gut and inhibit their activity, thereby reduce protein digestion, resulting in the limitation of amino acids and slow development of the insects^[17]. Based on the selectivity and inhibitory activity, PIs are categorized into four mechanistic classes. Aspartic PIs (pepstatins), serine PIs (serpins), cysteine PIs (cystatins) and metalloprotease PIs. Among these, the serine PI (SPI) family is the largest^[18]. They have multiple roles in the plant defense systems against biotic and abiotic stresses. SPIs have regulatory roles in cancer metastasis, programmed cell death (PCD), maintenance of various intracellular ionic concentration, self-incompatibility, blood coagulation, fibrinolysis, inflammation, and immunity^[19, 20].

According to the MEROPS database, there are 79 families of PIs which are assigned to 39 clans (<http://merops.sanger.ac.uk>). A single evolutionary line of inhibitors defined by a single type of protein fold is termed as "clan". The total number of peptidase-inhibitor interactions of protein in origin is about 1,428^[21]. They have shown that the substrate cleavage specificity for a particular peptidase can be of either physiological (substrate and peptidase are from the same organism) or pathological (substrate and peptidase are from different origin i.e. from pathogen and host) origin. Based on the selectivity and inhibitory activity PIs are categorized into four mechanistic classes such as aspartic PIs (pepstatins), serine PIs (serpins), cysteine PIs (cystatins) and metalloprotease PIs. Among this serine protease inhibitor (SPI) family is the largest^[18].

Serine proteases and serine protease inhibitors (SPIs)

Serine proteases are receiving increased attention due to their diverse array of functions. They control protein synthesis and turnover, and physiological functions such as fertilization, growth and development, digestion, cell signaling or migration, immune defense, wound healing and disease propagation^[19]. They play crucial role in the pathogenesis and/or host tissue penetration of a number of diseases, such as cardiopulmonary disease and emphysema^[22]. The vast majority of serine proteases are digestive enzymes involved in food metabolism. These Serine proteases impart active functions in the development of seeds and sprouts, besides acting as defense system against predators and pathogens^[13, 23, 24]. Members of the Kunitz family of PIs inhibit specifically serine proteinases, mainly trypsin and chymotrypsin and to certain extent Kunitz other proteinases, such as aspartic and cysteine proteinases^[25, 26]. SPIs are the largest family of inhibitors distributed throughout nature. Most SPIs have low-molecular mass (3-25 kDa), inhibit trypsin and/or chymotrypsin. SPIs are classified into different categories based on their substrate specificity particularly by the type of residue found in PI viz. Trypsin-like (positively charged amino acid residue), Elastase-like (small hydrophobic amino acid residue) or Chymotrypsin-like (large hydrophobic residue)^[27]. SPI family differs from each other in mass, cysteine content and number of reactive groups^[28] and belong to diverse group of family from both animal and plant origin having their respective target proteases (Table 1).

The rational design of synthetic SPI requires an understanding of catalytic mechanisms and substrate specificity of the enzyme under investigation. The active site of a typical serine proteinase is made up of two domains: (1) the catalytic site and (2) substrate-binding site. SPIs interact with both these

regions. Catalytic residues of a serine proteinase are Ser-195, His-57 and Asp 102. These three residues form a hydrogen-bonding system often referred to as "Catalytic Triad". The γ -hydroxyl group of Ser-195 in the substrate-binding domain attacks the carbonyl carbon of the scissile amide bond of the substrate to give a tetrahedral adduct. The imidazole side chain of the adjacent histidine residues facilitates this process. The tetrahedral adduct is stabilized by hydrogen bonding with the backbone NH- group of Gly-195, which make up the oxyanion hole (Fig. 1). The decomposition of the tetrahedral adducts results in the release of the amino position of the substrate and the formation of the acyl serine derivative. The departing amino group receives a proton from the His-57 imidazole ring. Subsequently, hydrolysis of acyl-enzyme to an active enzyme and carboxylic products occur through a reaction sequence that is analogous to formation of acyl-enzyme^[29] (Fig. 1).

Source and production of SPIs

PIs are small molecules that inhibit the activity of a pathogen-derived proteinase by binding and blocking its active site, suppressing protein metabolism in phytopathogenic microorganisms. In plants, PIs are generally found either as constitutive components in storage tissues like seeds and tubers or expressed in response to pest and pathogen attack^[2]. PIs are synthesized constitutively during normal plant development or in response to a pathogen attack^[30]. Insect damage, mechanical wounding and/or elicitors in insect oral secretions (OS) stimulate the local and systemic release of signaling intermediates like systemin and/or jasmonic acid trigger defense cascade throughout the plant^[31, 32, 33]. Insect-mediated damage or mechanical wounding results in the accumulation of trypsin and chymotrypsin-like PIs throughout the aerial tissues of tomato and potato plants^[34].

Mechanical wounding or insects results in local and systemic accumulation of defensive PIs within few hours^[26, 35] which in turn activates defense response signaling by increasing the endogenous level of the jasmonate family of compounds including Jasmonate (JA), Methyl Jasmonate (MeJA) and their metabolic precursor, 12-oxo-phytodienoic acid (12-OPDA)^[36, 37, 38]. Kunitz inhibitors are stored in number of plant tissues, including seeds^[39], tubers^[30, 40, 41], rhizome^[31, 42] fruits^[43], reproductive organs^[33] and leaves^[25, 44, 45]. The process of sub-expression and location of these inhibitors in certain tissues depend on the genes that code for the production of these proteins, and their expression as constitutive or as inducible fashion^[32].

Bactericidal activity of SPI

Kunitz-type protease inhibitors (KPIs) are induced during pathogen infection, suggesting that they play a key role in defense against lytic enzymes involved in insect and pathogen attack^[46]. In general, SPI activities are regulated by controlling the excessive proteolytic activity of the corresponding serine proteases. SPIs are categorized into several families based on structural and functional characteristics^[47]. Some families of SPIs, Kunitz and Serpin, have been well documented from various organisms^[47, 48, 49]. Serine PIs (Serpins) are one of the well-characterized PI family. Serpins are widely distributed proteins with similar structures that use conformational change to inhibit serine proteases^[50].

Kazal type of serine proteinase inhibitors (KSPIs) shows inhibitory activity against bacterial and fungal proteinases such as those isolated from *Galleria mellonella*, *Bombyx mori*

[51] and *Fenneropenaeus chinensis* [52]. KPI gene products are involved in the pathogen defense system as it possesses antimicrobial activity [38] with gene up-regulation upon pathogen challenges [53, 54, 55] were reported to be up-regulated. KSPI has been abundantly present in many organisms, including animals, plants, and microbes. As per the MEROPS database, various subfamilies of serine protease inhibitors (SPI) have been identified from animal and plant origin [56].

SPIs from both animal and plant origin have been reported to have an inhibitory effect against various strains of bacterial pathogens. KSPI isolated from the starchy corms, a storage tissue of *Xanthosoma blandum* shown to inhibit bacterial pathogens viz. *Salmonella typhimurium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* [57]. Antimicrobial peptides have been isolated from a wide variety of organisms,

Table 1: Families of SPIs and their target proteases

Family of serine PI (SPI)	Target proteases	References
Hirudin *	Thrombin	Rydel <i>et al.</i> , 1990
Bovine pancreatic trypsin inhibitor (BPTI) *	Trypsin	Yu <i>et al.</i> , 1995
Kazal *	Trypsin , chymotrypsin	Mistry <i>et al.</i> , 1997
Chelonianin *	Trypsin , elastase	Stergios Doumas <i>et al.</i> , 2005
Streptomyces subtilisin inhibitor (SSI) *	Subtilisin , trypsin and chymotrypsin	Mitsui <i>et al.</i> , 1979
Serpin *	Trypsin , chymotrypsin	Janciauskiene, 2001
Cucurbit family θ	Trypsin	Zeng <i>et al.</i> , 1998
Bowman-birk (BBI) θ	Trypsin, Chymotrypsin, cathepsin G , Matriptase	Qi <i>et al.</i> , 2005
Cereal super family θ	Trypsin , α -amylase	Campos and Richarson ,1983
Potato serine PI family θ	Trypsin , α -chymotrypsin , elastase	Benken <i>et al.</i> , 1976
Thaumatococcus θ	Elastase , partially trypsin	Franco <i>et al.</i> , 2002
Kunitz-type θ	Trypsin , chymotrypsin , Plasma Kallikrein	Macedo <i>et al.</i> , 2004

*Mammalian or microbial origin

θ Plant origin

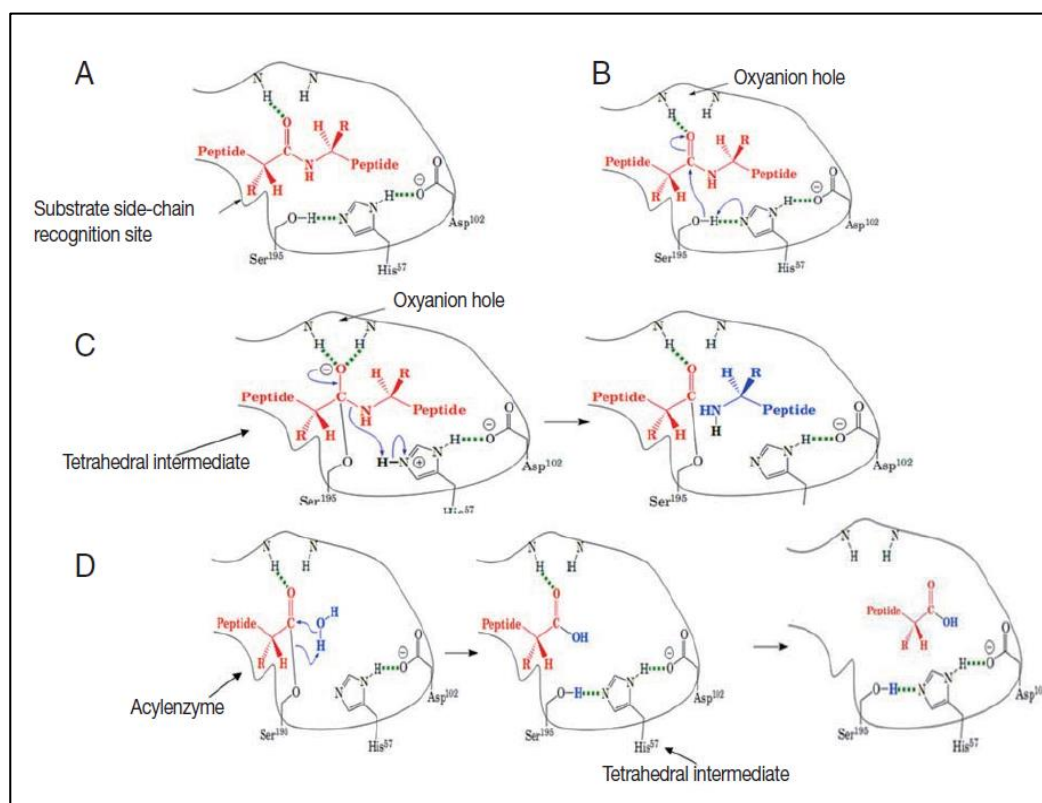


Fig 1: A schematic illustration of general catalytic mechanism for serine proteases (Yang *et al.*, 2015).

Fig 1: A schematic illustration of general catalytic mechanism for serine proteases

including animals, bacteria, insects and plants [58, 59]. Such peptides provide protection against bacteria, fungi and viruses by acting on the cell membranes of pathogens [60, 61]. These proteins have been divided into the following subfamilies: chitinases, β -1,3-glucanases, thaumatin-like (TL) proteins, PIs, endoproteinases, peroxidases, ribonuclease-like proteins,

γ -thionin and plant defensins, oxalate oxidases, oxalate oxidase-like proteins and other proteins of unknown biological properties [62, 63, 64, 65, 66]. Among these defense proteins, PIs inhibit the growth of microorganisms [67] and soapnut trypsin inhibitor from *Sapindus trifoliatus* exhibited

antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus vulgaris* and *Escherichia coli* [68].

PIs inhibit exogenous protease secreted by pathogenic microorganisms that use protease to penetrate the host cell wall and invade new tissues [52]. Proteases are inevitable component in all organisms ranging from microbes to mammals including fishes that act as a catalyst in cleavage of peptide bond [69]. Action of proteases is tightly regulated with endogenous inhibitors. Crustacean serine PI gene from freshwater crayfish *Procambarus clarkia* showed antibacterial activity against gram negative bacteria *E. coli* and *K. pneumoniae*; gram-positive bacteria viz. *B. subtilis*, *B. thuringiensis* and *S. aureus* [38]. Serine PI (Ranaserpin) from the eggs of the odour frog (*Rana graham*) showed antibacterial activity against gram positive bacteria *Bacillus subtilis* [70]. Serine PIs (serpins) are the well-characterized PI family that uses conformational change to inhibit serine proteases [71]. The recombinant construct of serine protease inhibitor 1 from kuruma shrimp (*Marsupenaeus japonicas*) showed bacteriostatic effect against gram positive bacteria (*S. aureus*, *B. subtilis*, and *B. megaterium*) and gram negative bacteria (*E. coli*, *K. pneumoniae*, and *V. anguillarum*) [72].

Transgenic plants with good expression of protein PIs could be a better approach for the molecular improvement of plants against biotic stress resistance. While doing so it needs to be taken care of PI variants with lower Kd (or Ki) dissociation constants for (and increased activity against) the target herbivore proteases, but with higher Kd values for (and weaker activity against) proteases of the same functional class in the host plant and non-target arthropods of the biological system considered [73]. Studies on recombinant CsKSPI isolated from freshwater striped murrel fish (*Channa striatus*), showed that CsKSPI inhibited the growth of gram-positive bacteria *Bacillus subtilis* as well as gram-negative bacteria *Aeromonas hydrophila* [74].

Many phytopathogenic bacteria are known to produce extracellular proteinases [75] which play an active role in the development of diseases in their respective hosts [76]. Various lines of evidence suggest that the major function of PI is to combat the proteinases of pests and pathogens [77]. PIs are also involved in defense mechanisms against insects and other pathogenic microorganisms [78]. Trypsin inhibitors from the seeds of chinese white cabbage and bottle gourd are reported to possess antibacterial activities [79].

Protein PIs are important defense molecules expressed in various plants, animals and microbes [80]. PIs form complexes with proteolytic enzymes and promote inhibition of their activity by competing for the catalytic site. Trypsin inhibitor, AMTI-II from seeds of *Abelmoschus moschatus* strongly affected the growth of *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, *Bacillus subtilis*, *Streptococcus pneumoniae*, *Bacillus cereus* followed by moderate effect on *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas syringae* and *Streptococcus pyogenes* [81]. Kazal type serine proteinase inhibitor, SPIPm2, from a marine crustacean, *Penaeus monodon* was reported to possess bacteriostatic activity against *Bacillus subtilis* [82].

Several skin-associated bacterial strains, including *E. coli*, *Staphylococcus aureus*, *P. aeruginosa* [83], shown to be affected by secretory leukocyte protease inhibitor (SLPI) *in vitro*, suggesting that SLPI controls microbial population on the skin surface. SLPI exhibited remarkable bactericidal activity against extracellular and intracellular bacteria, such as *Salmonella typhimurium* [84], *Streptococcus spp* [85], *Mycobacterium bovis* and *Mycobacterium tuberculosis* [86]

and *Neisseria gonorrhoea* [87]. Further intracellular expression of SLPI in *Escherichia coli* led to growth arrest, accompanied by reduced RNA and protein synthesis in bacteria [88].

Fungicidal activity of SPI

PIs in plants are able to suppress enzymatic activity of phytopathogenic microorganisms. Trypsin and chymotrypsin inhibitors of plant origin were shown to suppress activity of proteinases secreted by *Fusarium solani* [89]. Bean BBI is reported to suppress hyphal growth and conidial germination of *F. solani*, *F. culmorum*, and *B. cinerea* [90]. Maize trypsin inhibitor was reported to block hyphal growth and conidial germination of phytopathogenic fungi *A. flavus*, *A. parasiticus*, and *F. moniliforme* [91]. Buckwheat seed trypsin inhibitor found to suppress proteinase activity of *Alternaria alternata* and *F. oxysporum* [92, 93]. It has been demonstrated that potato tuber chymotrypsin inhibitors suppress the growth and development of the oomycete, *P. infestans* [94, 95]. Seed PI from barley and maize was shown to suppress proteinase activity of *A. tenuissima*, *A. oryzae*, *B. subtilis*, and *S. griseus* [96, 97].

Heat stable antimicrobial peptide, Potide G, completely suppresses the proteolytic activity of trypsin, chymotrypsin and papain, in addition to growth inhibition of variety of fungal strains [98]. In tomato and potato tubers infected with the oomycete fungus, *P. infestans*, increase in trypsin and chymotrypsin inhibitor content was observed, which correlated with resistance to pathogen [94, 95, 99]. Reports of trypsin inhibitor inhibiting the spore germination of fungal pathogen *Sclerotinia sclerotiorum* are also evident [100]. However, PIs induced in response to infection differed from inhibitors present in healthy plant [101]. Such induction in response to infection by pathogenic microorganisms is not limited to serine PIs, synthesis of cystatin like inhibitor was observed in chestnut leaves in response *B. cinerea* infection [102].

Nematicidal activity of SPI

Essential roles of parasite serine proteases and their diverse activities make them attractive targets for the development of novel immunotherapeutic, chemotherapeutic, and serodiagnostic agents for the next generation of antiparasite interventions. Overall existence and role of serine protease found in different types of parasitic helminth classes reveal the potency of a serine PI to attenuate these proteases thereby controlling them from invasion into the host [103]. Parasitic helminths are one of the most important pathogens worldwide and are classified into nematodes, trematodes, and cestodes. Sedentary plant endoparasitic nematodes are responsible for estimated crop damage of 125 billion Euros annually worldwide. Among them, the root-knot nematode (RKN) *Meloidogyne incognita* is able to infect the roots of almost all cultivated plants, which possibly renders this species to be the most damaging plant root pathogen in the world [104]. *Meloidogyne incognita* has evolved an intimate interaction with its hosts. Second-stage juveniles (J2) invade the root in the zone of elongation and migrate inter-cellular mode to the vascular cylinder, where permanent feeding sites are established.

Serine proteases that are involved in reproduction, evasion of the host immune system and developmental processes such as the cuticle structure and its biogenesis have been most extensively studied in the free-living model nematode *Caenorhabditis elegans* [105, 106, 107, 108]. Serine proteases play a major role in the processing of cuticular proteins during

moulting and development stage. SPIs regulate or control this class of proteases [107]. Many plants do produce defense proteins like PIs, which control a broad range of pests [109, 110]. PIs function as a specific pseudosubstrate for the digestive serine proteinase and reduce proteolysis in nematodes [111, 112]. In nematodes, proteases have important roles in a variety of physiological processes like moulting or cuticle remodeling [113] and embryogenesis [114, 115]. In addition, proteases are involved in several aspects of the parasitic lifestyle in parasitic helminths, among which tissue penetration, digestion of host tissues for nutrition and evasion of host immune responses [116, 117]. In plant-parasitic nematodes, studies have been focused mainly on cysteine and serine digestive proteases [118, 119]. Whole-genome analysis of the *Meloidogyne incognita* has revealed the presence of 10 serine protease families encoded by 52 genes [120] and chymotrypsin-like serine protease were also reported in other nematodes [121]. There are about 7 genes encoding for serine proteases in *Meloidogyne incognita* in egg stage whereas there are no genes for serine proteases in the pre-parasitic and parasitic stage. So SPIs can target those particular serine proteases at the egg stage and kill it before coming into the parasitic stage [122]. Therefore, the potential of disrupting proteases for plant nematode control, via expression of PIs in transgenic plants seem to be promising outlook [123].

Antiviral activity of SPI

Novel antiviral strategies include targeting either host or viral accessory protein to ultimately block viral replication or inhibit cellular proteins necessary for the virus life cycle [124]. Proteolytic cleavage of the precursor hemagglutinin (HA0) into HA1 and HA2 subunits by host proteases is essential for fusion of HA with the endosomal membrane and thus represents an essential step for viral infection [125, 126]. Human cytomegalovirus (HCMV) protease, a member of the serine protease is essential for capsid formation during viral replication [127]. The active site of HCMV contains a Ser-His-His catalytic triad (Ser-132, His-63, His-157) that basically overlays the Ser-His-Asp catalytic triad of α -chymotrypsin. Serine protease reported from the Human hepatitis C virus (HCV) also constitutes the domain of the chymotrypsin-like HCV NS3 protein and is responsible for processing the HCV polyprotein [127, 128]. The active site of HCV NS3 protease contains a catalytic triad comprised of Ser-139, His-57, and Asp-81 [129]. These serine proteases are compelling targets for both the development of anti-HCMV and anti-HCV drugs and SPIs.

Intracellular lipids, such as cholesterol-rich membrane and lipid droplets, play important roles in dengue virus penetration [130] and localization of viral capsid proteins [131]. These intracellular lipid levels are in a strict regulation under sterol regulatory elements-binding proteins, which are activated by serine proteinases [132, 133, 134]. Trypsin PI from the leaf extract of *Capsicum baccatum* var. *pendulum* inoculated with PepYMV (Pepper yellow mosaic virus) showed a reduction in yellow mosaic viral infection [135]. *Cucumis metuliferus* serine protease inhibitor (CmSPI) gene, isolated from *Cucumis metuliferus* which encodes for a serine-type PI (potato I type family). Overexpressed and silenced CmSPI in *Nicotiana benthamiana* and *Cucumis metuliferus* showed potyvirus resistance and synchronous development of potato ring spot viral symptoms respectively. Secretory leukocyte protease inhibitor (SLPI), a serine proteases inhibitor shows inhibitory activity against elastase and cathepsin G from human neutrophils, and of mast cell-derived chymase and

trypsin [136, 137, 138, 139]. SLPI was reported to inhibit human immunodeficiency virus type 1 (HIV-1) infection of monocytes and T cells in vitro [140, 141]. Studies on the antiviral function of the gene SERPINE1, encoding plasminogen activator inhibitor 1 (PAI-1), was reported to inhibit influenza A virus glycoprotein cleavage, thereby reducing the infectivity of progeny viruses [142].

Conclusion

In this review, we have summarized PIs while focusing mainly on the classifications, characterization, biological roles and practical potential of SPIs. Continuous efforts in the field of structure, functions, mode of action and biophysical characterization open up opportunities for their role in medicine, biotechnology and agriculture. Pathological situations arise due to the imbalance of proteolytic events and improper protease signaling pathways. With these increasing pieces of evidence of protease involvement in a large number of diseases, there has been an outburst of novel drug development involving the PIs.

It is established that SPIs play a major role in balancing and regulating proteases, are involved in the endogenous defense system. In plants, these SPIs also play a role in exogenous defense. Many transgenic plants with over-expressing different SPIs show resistance against various pathogenic organisms which opens new gateways in the field of modern agricultural techniques. Although SPIs have been isolated and characterized from a lot of sources, natural inhibitors have been made using gene therapy and transgenic plant expression, there is still a long way to go for a full-scale exploration of SPI potential in medicine and agriculture. Several transgenic plants expressing SPIs have been produced and tested in order to increase the resistance against various pathogenic organisms. Since proteases are the inevitable components in all organisms ranging from microbes to mammals, coordinated efforts to develop eco-friendly strategies for protection against pests and pathogens by exploring protease-protease inhibitor interactions with the added value of improving the overall well being of host organisms.

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