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Standard Review

Plant protease inhibitors: a defense strategy in plants

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Proteases, though essentially indispensable to the maintenance and survival of their host organisms, can be potentially damaging when overexpressed or present in higher concentrations, and their activities need to be correctly regulated. An important means of regulation involves modulation of their activities through interaction with substances, mostly proteins, called protease inhibitors. Some insects and many of the phytopathogenic microorganisms secrete extracellular enzymes and, in particular, enzymes causing proteolytic digestion of proteins, which play important roles in pathogenesis. Plants, however, have also developed mechanisms to fight these pathogenic organisms. One important line of defense that plants have to fight these pathogens is through various inhibitors that act against these proteolytic enzymes. These inhibitors are thus active in endogenous as well as exogenous defense systems. Protease inhibitors active against different mechanistic classes of proteases have been classified into different families on the basis of significant sequence similarities and structural relationships. Specific protease inhibitors are currently being overexpressed in certain transgenic plants to protect them against invaders. The current knowledge about plant protease inhibitors, their structure and their role in plant defense is briefly reviewed.

Key words: Proteases, enzymes, protease inhibitors, serpins, cystatins, pathogens, defense.

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INTRODUCTION

Proteolytic enzymes, also called proteases, are the enzymes that catalyse the hydrolytic cleavage of specific peptide bonds in their target proteins. These enzymes are widely distributed in nearly all plants, animals and microorganisms (Joanitti et al., 2006; Neurath, 1989; Valueva and Mosolov, 2004; Christeller, 2005; Haq et al., 2004; Supuran et al., 2002; Mosolov and Valueva, 2005; Mosolov et al., 2001; Lawrence and Koundal, 2002; Ryan, 1990). In higher organisms, nearly 2% of the genes code for these enzymes (Barrett et al., 2001). Being essentially indispensable to the maintenance and

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Abreviations: BBI, Bowman-Birk inhibitor; BTI, Barley trypsin inhibitor; CPI, Cysteine protease inhibitor; CpTi, Cowpea trypsin inhibitor; Cmps, Cucurbita maxima phloem serpin; MSI, Mustard trypsin inhibitor; PI, Protease inhibitor; PPI, Plant PI; PI1, Potato type1PI; PI2, Potato type2 PI; PVy, Potato virus Y; SFTI, Sunflower trypsin inhibitor; TEV, Tobaco etch virus.

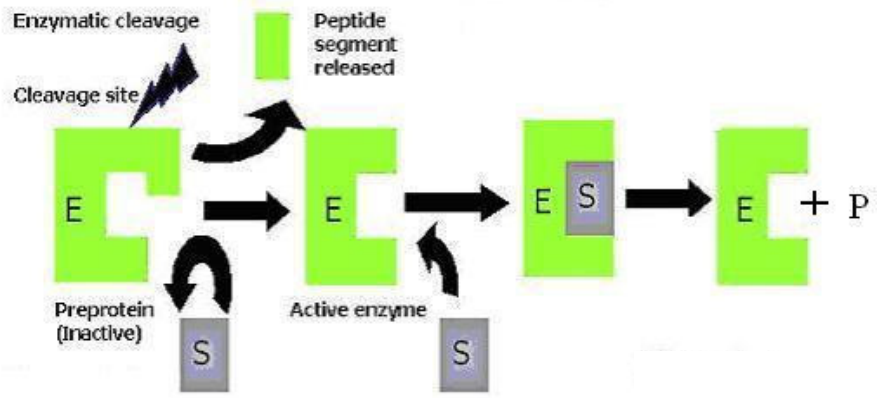

Figure 1A. A diagrammatic model for control of enzyme activity at the level of synthesis. Enzymes may be synthesized as inactive preproteins and are processed to be activated. E represents enzyme, S represents substrate and P represents the products.

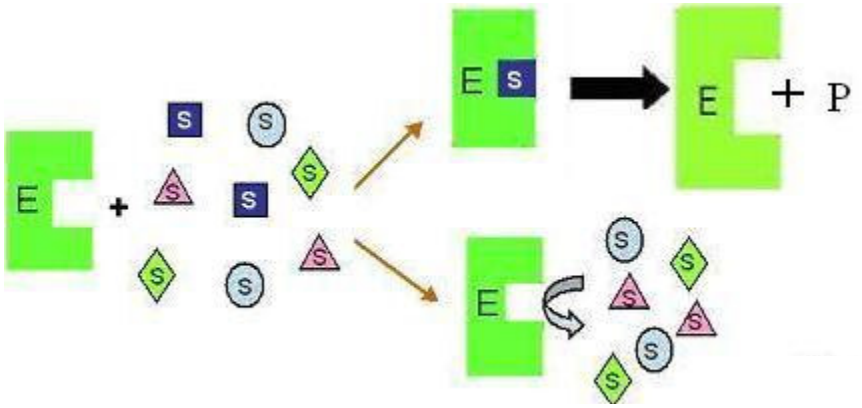

Figure 1B. Model showing enzymes activity regulation by virtue of substrate specificity. E,S and P have the same meaning as in Figure 1A.

survival of their host organism, proteases play key roles in many biological processes. The proteolytic events catalysed by these enzymes serve as mediators of signal initiation, transmission and termination in many of the cellular events such as inflammation, apoptosis, blood clotting and hormone processing pathways (Ivanov et al., 2006). Despite the fact that these enzymes are indispensable to the cells and organisms that host them, they may be potentially damaging when overexpressed or present in higher concentrations. For this reason the activities of these enzymes need to be strictly regulated and controlled (Rawlings et al. 2004a). The synthesis of these enzymes as inactive pre-proteins (Figure 1A), and their substrate specificity (Figure 1B) keeps a control on their activities, but it does not fulfill the desired level of regulation, and the fact remains, that cells and organisms require additional means of control. One important control mechanism involves interaction of the active enzymes with proteins that inhibit their activities (Figure 2). These inhibitors form less active or fully inactive complexes with their cognate enzymes, and are called protease inhibitors (PIs). Trypsin can be considered as a prototype of the class of enzymes synthesized as inactive precursors. Synthesised as trypsinogen, it requires proteolytic processing to be activated. Once activated trysin acts specifically only on peptide bonds whose carboxyl functions are contributed by lysine or arginine residues. Further check on the activity of trypsin is due to its interaction with antitrypsin, the protein inhibitor of the activated form (Laskowski and Qasim, 1999).

PIs are of very common occurrence. They have been isolated and characterized from a large number of organisms, including plants, animals and microorganisms (Valueva and Mosolov, 2004; Christeller, 2005; Haq et al., 2004; Supuran et al., 2002; Mosolov and Valueva, 2005; Mosolov et al., 2001). Naturally occurring PIs are essential for regulating the activity of their corresponding proteases and play key regulatory roles in many biological processes. For a few PIs, functions other than blocking protease action have also been found, such as

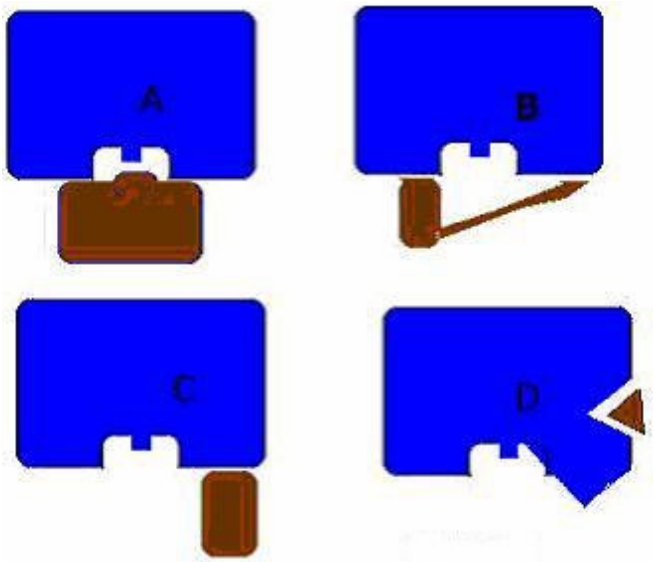

Figure 2. Diagrammatic representation of the different modes of protease inhibition (A) Direct blockage of the active center canonical inhibition of trysin like proteases (B) Indirect blockage of the active center (C) Adjacent or and exocite binding (D) Allosteric interaction.

growth factor activities, receptor clearance signaling or involvement in carcinogenesis (Qi et al., 2005). A number of inherited diseases such as emphysema, and epilepsy result from abnormalities in PIs (Lomas et al., 2002; Ritchie, 2003; Lehesjoki, 2003; Bitoun et al., 2002)

PIs are of common occurrence in the plant kingdom. Plant PIs (PPIs) are generally small proteins that have mainly been described as occurring in storage tissues, such as tubers and seeds, but they have also been found in the aerial parts of plants (De Leo et al., 2002). They are also induced in plants in response to injury or attack by insects or pathogens (Ryan, 1990). In plants, these PIs act as anti-metabolic proteins, which interfere with the digestive process of insects. One of the important defense strategies that are found in plants to combat predators involves PIs which are in particular effective against phytophagous insects and microorganisms. The defensive capabilities of PPIs rely on inhibition of proteases present in insect guts or secreted by micro-organisms, causing a reduction in the availability of amino acids necessary for their growth and development (Lawrence and Koundal, 2002)

With this background and the attached medical and therapeutic significance of PIs, the current review is an attempt to give a comprehensive presentation of the different families of PPIs. The structure of each class of PIs is briefly given with a description of their role in plant defence. The progress in the development of transgenic plants carrying PI genes I also reviewed briefly. Having enormous potential to intervene in a large number of human disorders, the scope for exploration of the natural PIs remains wide open.

Families of PIs

PIs have been grouped into families and subfamilies and into different clans on the basis of sequence relationship and the relationship of protein folds of the inhibitory domains or units. An inhibitor domain is defined as the segment of the amino acid sequence containing a single reactive site after removal of any parts that are not directly involved in the inhibitor activity. On the basis of sequence homologies of their inhibitor domains, PIs have been classified into 48 families (Rawlings et al., 2004b). Proteins containing a single inhibitor unit are termed simple inhibitors, and those that contain multiple inhibitor units are termed complex inhibitors. A total of 11 families belong to the latter category and contain between 2 and 15 inhibitory domains. Most of these are homotypic, containing inhibitor units from a single family, some are however heterotypic, and contain inhibitor units from different families (Richardson et al., 2001; Trexler et al., 2001, 2002). On the basis of tertiary structure, 31 of the 48 families have been assigned to 26 clans, indicating that a large proportion of families show no relationships in their three dimensional structures.

Peptidases have been classified on the basis of structural and evolutionary relationships into different families and clans (Rawlings and Barrett, 1993). These families have been divided into five groups based on chemistry of the catalytic site of enzymes (Barrett, 1986, 1994; Grudkowska and Zagdanska, 2004). The families of PIs could not, however, be grouped on the basis of the catalytic type of enzymes inhibited, since a number of families contain cross-class inhibitors (Rawlings et al., 2004b). The proteins in family 13 Kunitz-type PPIs generally inhibit serine peptidases, but they also include inhibitors of cysteine and aspartate proteases (Heibges et al., 2003). Family 14, the serpin family, mostly contain inhibitors of serine proteases, but it also contains inhibitors of some cysteine proteases. In the past, however, PIs have been classified into serine, cysteine, aspartate and metallocarboxy PIs. (De Leo et al., 2002; Laskowski et al., 2003; Koiwa et al., 1997). PIs that are active against all the mechanistic classes of proteases have been described in plants. A database for PPIs, co-ordinated by Luigi R Ceci at Centro di studio sui Mitocondri and Metabolismo Energetico-C.N.K, (via Amendola 165/A, Bari, Italy), contains information about 495 inhibitors with several isoinhibitors identified in 129 different plants (De Leo et al., 2002). The database describes nine families of PPIs based on sequence similarities. The families along with some representative examples are given in Table 1.

All these families except those of the cysteine-PI family and the metallocarboxypeptidase inhibitor family contain PIs of serine proteases. These may, however, also contain inhibitors active against other mechanistic classes of proteases. For example the plant Kunitz family (family 13) contains inhibitors of serine proteases (family S1), but also includes inhibitors of cysteine proteases (C1) and

Table 1. Families of plant protease inhibitors with some type examples.

Common name	MEROPS Family/subfamily	Type example	Source	Target Protease	Referencces
Kunitz (plant)	13A	soybean Kunitz trypsin inhibitor	Glycine max	Trypsin, Chymotrypsin	Laskowski and Kato (1980)
		barley subtilisin inhibitor	*Hordeum vulgare*	Subtilisin,Alpha-amylase	Vallee et al. (1998)
		winged-bean chymotrypsin inhibitor	*Psophocarpus tetragonolobus*	Alpha-chymotrypsin	Habu et al. (1992)
		Kunitz cysteine peptidase inhibitor 1	*Solanum tuberosum*	Cysteine proteases	Gruden et al. (1997)
Kunitz (plant)	I3B	proteinase inhibitor A inhibitor unit	Sagittaria sagittifolia	Trypsin, Chymotrypsin, Kallikerin	Laskowski and Kato (1980)
		Kunitz subtilisin inhibitor	*Canavalia lineata*	Subtilisin-type microbial serine proteases	Terada et al. (1994)
		cathepsin D inhibitor	*Solanum tuberosum*	Cathepsin D, Trypsin	Strukelj et al. (1992)
		trypsin inhibitor	*Acacia confusa*	Trypsin and alpha-chymotrypsin	Lin et al. (1991)
cereal	I6	ragi seed trypsin/α-amylase inhibitor	Eleusine coracana	Alpha amylase	Hojima et al. (1980)
		barley trypsin/factor XIIa inhibitor	*Hordeum vulgare*	Alpha-amylase, Trypsin	Lazaro et al. (1988)
		wheat trypsin/alpha-amylase inhibitor	*Triticum aestivum*	Alpha-amylase, Trypsin	Shewry et al. (1984)
		maize trypsin/factor XIIa inhibitor	*Zea mays*	Mammalian trypsin, activated hageman factor	Mahoney et al. (1984)
squash	I7	trypsin inhibitor MCTI-1	Momordica charantia	Pancreatic elastase	Wiezorek et al. (1985)
		trypsin inhibitor MCTI-II	*Momordica charantia*	Trypsin	Huang et al. (1992)
		macrocyclic squash trypsin inhibitor	*Momordica cochinchinensis*	Trypsin	Hernandez et al. (2000)
		trypsin inhibitor CSTI-IV	*Cucumis sativus*	Trypsin	Wieczorek et al. (1985)
Potato type I	I13	chymotrypsin inhibitor I	Solanum tuberosum	Chymotrypsin, Trypsin	Richardson (1974)
		glutamyl peptidase II inhibitor	*Momordica charantia*	Glu S.griseus protease , Subtilisin	Ogata et al. (1991)
		subtilisin-chymotrypsin inhibitor CI-1A	*Hordeum vulgare*	Subtilisin , Chymotrypsin	Greagg et al. (1994)
		wheat subtilisin/chymotrypsin inhibitor	*Triticum aestivum*	B.lichenoformis subtilisin, Alpha-chymotrypsin	Poerio et al. (2003)

Table 1. Contd.

mustard	I18	mustard trypsin inhibitor	Sinapis alba	Beta-trypsin	Menegatti et al. (1992)
		mustard trypsin inhibitor-2	*Brassica hirta*	Bovine beta-trypsin, Alpha-chymotrypsin	Ceci et al. (1995)
		rape trypsin inhibitor	*Brassica napus*	Trypsin, Chymotrypsin.	Ceciliani et al. (1994)
cystatin	I25B	onchocystatin	Onchocerca volvulus	Cysteine proteinase	Lustigman et al. (1992)
		ovocystatin	Gallus gallus	Thiol proteases	Laber et al. (1989)
		oryzacystatin II	Oryza sativa	Cysteine proteinases	Ohtsubo et al. (2005)
Kininogen	I25C	metalloprotease inhibitor	Bothrops jararaca	Atrolysin C, Jararhagin.	Cornwall et al. (2003)
		sarcocystatin	*Sarcophaga peregrina*	Cysteine proteinase	Saito et al. (1989)
Bowman-Birk	I12	Bowman–Birk plant trypsin inhibitor unit 1	Glycine max	Trypsin, Chymotrypsin	Odani and Ikenaka (1976)
		Bowman-Birk trypsin/chymotrypsin inhibitor	*Arachis hypogaea*	Trypsin, Chymotrypsin	Suzuki et al. (1987)
		sunflower cyclic trypsin inhibitor	*Helianthus annuus*	Trypsin, Cathepsin G, Elastase, Chymotrypsin and thrombin	Mulvenna et al. (2005)
Potato type II	I20	proteinase inhibitor II	Solanum tuberosum	Trypsin, Chymotrypsin.	Greenblatt et al. (1989)
		potato peptidase inhibitor II inhibitor unit 1	*Solanum tuberosum*	Trypsin, Chymotrypsin.	Keil et al. (1986)
		tomato peptidase inhibitor II inhibitor unit 1	*Solanum lycopersicum*	Trypsin, Chymotrypsin	Graham et al. (1985)
		tomato peptidase inhibitor II inhibitor unit 2	Solanum lycopersicum	Trypsin, Chymotrypsin	Barrette-Ng et al. (2003)

the aspartic protease cathepsin D. Similarly, the serpin family of PIs active against serine proteases also contains inhibitors of cysteine proteases (Heibges et al., 2003; Laskowski et al., 2003). Serine PIs belonging to arious families have been reported either in storage organs or in the vegetative cells of a wide variety of plants (Garcia et al., 1987). The inhibitors from at least four families belonging to serine PIs are induced sequentially in various plants. These families include potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*) inhibitors I and II in solanaceous plants (Melville and Ryan, 1972; Bryant et al., 1976; Plunkett et al., 1982; Valueva et al., 2001; Farran et al., 2002), Bowman-Birk inhibitors in alfa alfa (Brown and Ryan, 1984) and a Kunitz inhibitor in poplar trees (Bradshaw et al., 1989; Ledoigt et al., 2006).

Serpin (Serine PI) family

The serpin family is the largest and the most widespread superfamily of PIs. Serpin-like genes have been identified in nearly all types of organisms, including viruses, bacteria, plants and animals (Irving et al., 2000; Rawlings et al., 2004b; Christeller and Liang, 2005; Law et al., 2006; Gettins, 2002). Whereas prokaryotes generally have a single serpin gene (Irving et al., 2002a), large multicellular eukaryotic organisms, on the other hand, have several to many genes. Analysis of the thale cress (*Arabidopsis thaliana*) genome shows 29 serpin genes (Silverman at al., 2001).

Plant serpins have been purified and characterized from cereal seeds (Laskowski and Kato, 1980; Yoo et al., 2000; Tsybina et al., 2004), pollens and from the phloem exudates of *Cucurbita maxima* (Wieczorek et al., 1985). Plant serpins have been shown to inhibit model trypsin like proteins (Roberts et al., 2003), but there are no obvious targets for these inhibitors in plants, which may, apparently be involved in inhibiting proteases of plant pathogens (Hejgaard, 2005). Wieczorek and his coworkers have shown an inverse correlation between the up-regulation of squash phloem serpin-1 (cmps) and aphid survival. On the other hand Yoo and his coworkers have reported that feeding of purified serpin to aphids had no impact on insect survival. These data suggest a more complex role for plant serpins in defense (Wieczorek et al., 1985; Yoo et al., 2000).

The serpin 1 of Arabidopsis has been shown to act on metacaspase-like proteins *in vivo* and play a role in the plant immune response (Vercammen et al., 2006). It has been suggested that rather than directly interacting with pathogens, plant serpins may have a role in the complex pathways involved in up-regulating the host immune response (Law et al., 2006).

Plant serpins have molecular mass of 39 - 43 kDa, with amino acid and nucleotide homology with other well-characterized serpins. The majority of serpins inhibit serine proteases, but serpins that inhibit caspases (Ray et al., 1992) and papain like cysteine proteases (Schick et al., 1998; Irving et al., 2002b, c) have also been reported. Plant serpins exhibit differing and mixed specificities towards proteases (Al-Khunaizi et al., 2002). Barley (*Hordeum vulgae*) serpin is a potent inhibitor of trypsin and chymotrypsin at overlapping reactive sites (Dahl et al., 1996a). This inhibitor also inhibits thrombin, plasma kallikrein, Factor VIIa and Factor Xa (Dahl et al., 1996b). Wheat (*Triticum aestivum*) serpins inhibit chymotrypsin and cathepsin G and have glutamic acid, lysine or arginine at P1 site (Roberts et al., 2003). Two oat (*Avena sativa*) serpins show specificity for chymotrypsin and / or elastase, and another one has specificity for trypsin and chymotrypsin at overlapping loop sites (Irving et al., 2002b, c). Squash serpin Cmps-1 also inhibits elastase at two overlapping sites (Ligoxygakis et al., 2003).

Serpins are irreversible 'suicide' inhibitors. The cleavage of an appropriate peptide bond in the reactive centre loop of the inhibitor triggers a rapid conformational change so that catalysis does not proceed beyond the formation of an acyl-enzyme complex (Huntington et al., 2000).

Bowman Birk inhibitors (BBIs) family

On the basis of sequence homology, this forms another family of serine PIs. The family is named after D.E. Bowman and Y. Birk, who were the first to identify and characterise a member of this family from soybean (*Glycine max*) (Bowman 1946; Birk et al., 1963). The soybean inhibitor is now the most-well-studied member of this family and is often referred as the classic BBI. The inhibitors have been found in legumes and cereals (Laing and McManus, 2002; Tanaka et al., 1997; Norioka and Ikenaka, 1983) and in the grass family Poaceae (Odani et al. 1986). The inhibitors of this family are generally found in seeds, but are also wound-inducible in leaves (Eckelkamp, 1993). A small cyclic inhibitor has been identified in sunflower (*Helianthus annuus*) called sunflower trypsin inhibitor 1 (SFTI-1) (Korsinezky, 2001; Luckett et al., 1999).

BBIs have been classified on the basis of their structural features and inhibitor characteristics. The inhibitors from dicotyledonous plants consist of a single polypeptide chain with the molecular mass of 8 kDa. These are double-headed, with two homologous domains each bearing a separate reactive site for the cognate proteases. These inhibitors interact independently, but simultaneously, with two proteases, which may be same or different (Raj et al., 2002; Birk, 1985). The first reactive site in these inhibitors is usually specific for trypsin, chymotrypsin and elastase (Qi et al., 2005). The active-site configuration in these inhibitors is stabilized by the presence of seven conserved disulfide bonds (Chen et al., 1992; Lin et al., 1993).

BBIs from monocotyledonous plants are of two types. One group consists of a single polypeptide chain with a molecular mass of about 8 kDa. They have a single reactive site. Another group has a molecular mass of 16 kDa with two reactive sites (Tashiro et al., 1987, 1990; Prakash et al., 1996). It has been suggested that larger inhibitors have arisen from smaller ones by gene duplication (Odani et al., 1986).

In the case of double-headed BBIs, it has been found that the relative affinity of binding of proteases is altered when one site is already occupied. Peanut (*Arachis hypogoea*) inhibitor has been found to exhibit no activity against chymotrypsin when preoccupied with trypsin and vice versa (Tur et al., 1972). In the same way, the activity of soybean BBIs decreases 100-fold when trypsin is bound at the other site (Gladysheva et al., 1999).

The BBI family of protease inhibitors contains a unique disulfide-linked nine-residue loop that adopts a characteristic canonical conformation (Bode and Hubr, 1992). The loop is called protease-binding loop and binds the protease in a substrate-like manner (Lee and Lin, 1995).

SFTI-1 is a very strong naturally occurring BBI. It consists of a cyclic 14-amino-acid residue-long peptide structure and contains a disulfide-linked loop of nine-amino-acid residues that shares the sequence homology with the first residue loop of BBIs (Korsinezky et al., 2001; Luckett et al., 1999). Analysis of the three-dimensional structure of SFTI-1 in solution, and that of its crystal structure in complex with trypsin, has shown the structures are quite similar (Korsinezky et al., 2001). This has lead to the suggestion that the structure of SFTI-1 is rigid and pre-organized for potent binding, making it a stronger and potent inhibitor than other naturally occurring BBIs.

BBIs are cysteine-rich proteins with inhibitory activity against proteases that are widely distributed in monocot and dicot species (Lin et al., 2006). They have been shown to act as anticarcinogenic compounds. The soybean derived BBI with a well-characterized ability to inhibit trypsin and chymotrypsin is particularly effective in suppressing carcinogenesis in a variety of *in vivo* and *in vitro* systems (Kennedy 1998). BBI has been shown to reduce the proliferation of MCF7 breast cancer cells through accumulation of MAP kinase phosphatase-1 (Wen et al., 2005).

Kunitz family

On the basis of sequence homologies, Kunitz-type inhibitors form a separate family. The members of this family are mostly active against serine proteases, but may also inhibit other proteases (Laing and McManus, 2002; Ritonja et al., 1990). The inhibitors in this family are widespread in plants and have been described in legumes, cereals and in solanaceous species (Ishikawa et al., 1994; Laskowski and Kato, 1980). A 20.5 kDa Kunitz-type trypsin inhibitor with antifungal activity has been reported from the roots of punce ginseng (*Pseudostellaria heterophylla*) (Wang and Ng 2006).

Kunitz-type PIs are also produced under stress, as has been found in potato tubers (*S. tuberosum*) (Park et al., 2005; Ledoigt et al., 2006; Plunkett et al., 1982). The inhibitors usually have molecular mass of 18 - 22 kDa; have two disulfide bridges and one reactive site.

The members of this family are mostly active against serine proteases and have been shown to inhibit trypsin, chymotrypsin and subtilisin (Laing and McManus, 2002; Park et al., 2005), but they also inhibit other proteases, including the aspartic protease cathepsin D and the cysteine proteinase papain. These inhibitors are canonical and form a tight complex with the target protease, which dissociates very slowly (Ritonja et al., 1990).

Squash inhibitors

Squash-family inhibitors have been described only in plants and form yet another family active against serine proteases. The members of this family have been described from many cucurbit families (Lee and Lin, 1995; Hamato et al., 1995; Felizmenio et al., 2001). Seven serine PIs belonging to this family have been isolated and characterized from the seeds of wild cucumber (*Cyclanthera pedata*) (Kuroda et al., 2001). Recently two different but inter-convertible (cis-trans isomers) inhibitors have been isolated and characterized from seeds of wax gourd [*Benincasa hispida* (Thumb) cogn] (Atiwetin et al., 2006). The members of this family consist of a small single peptide chain containing between 28 and 30 amino acids with molecular mass of 3.0 - 3.5 kDa (Heitz et al., 2001; Le Nguyen et al., 1990). These inhibitors have three disulfide bridges and fold in a novel knottin structure (Hara et al., 1989). The small size of these inhibitors, combined with potential activity against important biological molecules such as Hageman factor, human leucocyte elastase and cathepsin G (Hojima et al., 1982; McWherter et al., 1989), has made them particularly attractive for studying proteinase and inhibitor interactions. Chemical synthesis of these inhibitors has created powerful tools for investigating their structure and function relationships (Kupryszewski et al., 1985, 1986; Rolka et al., 1992). The structures of squash inhibitors, and inhibitor and proteinase complexes have been determined by X-ray crystallography and NMR spectroscopy (Holak et al., 1989 a, b; Nilges et al., 1991; Thaimattam et al., 2002). These inhibitors have been shown to follow the standard mechanism for inhibition.

Cereal trypsin/α-amylase inhibitors

The members of this family have serine proteinase inhibitory activity and/or α-amylase- inhibitory activity (Gourinath et al., 2000). A large number of inhibitors in this family have only α-amylase-inhibitory activity; how-

ever inhibitors from barley (*Hordeum vulgare*), rye (*Secale cereale*) and tall fescue (*Festuca arundinacea*) are active against trypsin (Odani et al., 1983). Maize (*Zea mays*) and ragi (*Elusine coracana*) inhibitors show dual activities and can inhibit serine proteinases as well as α-amylase (Mahoney et al., 1984; Shivraj and Pattabiraman, 1981). The cereal trypsin/α-amylase inhibitors consist of a single polypeptide chain containing five disulfide bonds with a molecular mass of about 13 kDa (Christeller and Liang, 2005). The structure of the ragi inhibitor solved by NMR spectroscopy and that of its complex with yellow-mealworm (*Tenebrio molitor*) α-amylase by x-ray crystallography has shown that the proteinase-binding loop adopts a canonical conformation (Strobl et al., 1998).

Mustard (Sinapis) trypsin inhibitor (MSI)

These are small single polypeptide chain inhibitors with the molecular mass of about 7 kDa, found in the family Cruciferae and form yet another family of serine PIs (Laing and McManus, 2002; Menengatti et al., 1992). These inhibitors have been isolated and characterized from a number of species including white mustard (*Sinapis alba*) and tape (*Brassica napus*) (Ascenzi et al., 1999; Volpicella et al., 2000). These inhibitors are expressed in seeds during their development and are also wound-inducible (Ceci et al., 1995; De Leo et al., 2001). The inhibitors form a tight binding complex with trypsin and apparently follow the standard mechanism (Ceciliani et al., 1994).

Potato type I PIs (PI 1)

The inhibitors of this family are widespread in plants and have been described in many species, including potato tubers (Ryan and Balls, 1962), tomato fruit (Margossian et al., 1988, Wingate et al., 1989), squash phloem exudates (Murray and Christeller, 1995) and in tomato leaves in response to wounding (Lee et al., 1986). These inhibitors have the molecular mass of 8 kDa and are generally monomeric. While the inhibitors from cucurbit and potato tubers contain a single disulphide bond, the inhibitors in this family in general lack any disulphide bridges (Cai et al., 1995). The inhibitory mechanism in this family is considered to fit the standard model.

Potato type II PIs (PI 2)

The members of this group have been reportedonly from the members of Solanaceae family. Initially characterized from potato tubers (Christeller and Liang, 2005), these inhibitors have been found in leaves, flowers, fruit and phloem of other solanaceaous species (Iwasaki et al., 1971; Pearce et al., 1993). A low molecular-mass inhibitor of this family has been found to be constitutively present in Jasmme tobacco (*Nicotiana alata*) flowers (Atkinson et al., 1993). Six small wound-inducible proteinase inhibitors of this family have been reported from tobacco leaves (Pearce et al., 1993). An analysis of these inhibitors and genes has shown that they are composed of multiple repeat units varying between one and eight (Antcheva et al., 2001; Miller et al., 2000; Choi et al., 2000). Inhibitors in this family have been reported to inhibit chymotrypsin, trypsin, elastase, oryzin, Pronase E and subtilisin (Antcheva et al., 1996).

Cysteine PIs (CYS), the cystatin superfamily

The cystatin superfamily is composed of several families and includes proteins that are related in structure and function to an inhibitor of cysteine proteinase, first described in egg white and referred to as 'chicken egg-white cystatin' (Colella et al., 1989). The members of these families inhibit the activity of cysteine proteases and are called cysteine PIs or cystatins. They are widely distributed in plants, animals and microorganisms (Oliveira et al., 2003). These inhibitors are grouped into four families based on sequence relationships, molecular mass and disulfide-bond numbers and arrangements (Turk and Bode, 1991; Barrett, 1987).

Family-1 cystatins (stefin family)

The members of this group have a molecular mass of about 11 kDa. They are generally present in the cytosol and are devoid of any carbohydrate groups and disulfide bonds (Stato et al., 1990; Machleidt et al., 1983)

Family-2 cystatins (cystatin family)

These inhibitors consist of proteins having 120 – 126 amino acids and the molecular mass of 13.4 - 14.4 kDa. These inhibitors contain two disulphide bonds but are devoid of any carbohydrate groups (Grzonka et al., 2001). They also contain a signal sequence and are known to be secreted (Abrahanson et al., 1987). All the family-2 cystatin inhibitors contain a conserved tripeptide of sequence Phe-Ala-Val near the C-terminus and a conserved dipeptide, Phe-Tyr, near the N- terminus. These conserved sequences are important in binding to the target proteases (Machleidt et al., 1983; Turk et al., 1997)

Family-3 cystatins (kininogen family)

These inhibitors are glycoproteins and are of three different types. High Molecular Weight kininogens (HMW) with a molecular mass of 120 kDa and Low Molecular Weight kininogens (LMW) with molecular mass ranging between

60 and 80 kDa are known. A third type T kininogens with molecular mass of 68 kDa has also been reported. These proteins contain tandem domains that result from gene duplication of the family–2 cystatins. These proteins are also secreted and play key roles in blood coagulation (Otto and Schirmeister, 1997; Salvesen et al., 1986).

Family 1 and 3 cystatins contain a conserved pentapeptide sequence Gln-Val-Val-Ala-Gly and the family-2 members have the homologous peptide Gln-X-Val-Y-Gly, in which X and Y represent any amino acid.

Family–4 cystatins (phytocystatins)

This family includes nearly all the cysteine PIs described in plants. They have been identified in rice (Abe et al., 1987 a, b), maize (Abe et al., 1992), soybean (Hines et al., 1991; Botella et al., 1996), apple (*Malus*) fruit (Ryan et al., 1998), carnation (*Dianthus caryophyllus*) leaves (Kim et al., 1999) and several other monocotyledonous and dicotyledonous plants (Brown and Dziegielewska, 1997; Pernas et al., 1998; Sakuta et al., 2001). Celostatin, a cysteine PI from crested cock's comb (*Celosia cristata*) has recently been cloned and characterized (Gholizadeh et al., 2005).

Phytocystatins have sequence similarity to stefins and cystatins, but do not contain free cysteine residues (Fernandes et al., 1993; Zhao et al., 1996). The unique feature of this superfamily, however, is a highly conserved region of the G58 residue, the glu-val-val-ala-gly (QVVAG) motif and a pro-trp (PW) motif. The studies on the papain inhibitory activity of oryzacystatin and its various truncated forms have identified the conserved QVVAG motif as a primary region of interaction between the inhibitor and its cognate enzyme The PW motif is believed to act like a cofactor (Arai et al., 1991; Abe et al., 1988).

Phytocystatins, on the basis of protein structure, can be divided into two groups. One group consists of single-domain proteins and includes a majority of these inhibitors (Abe et al., 1987 a, b; Pernas et al., 1998). Another group contains multiple-domains and includes the cysteine PIs isolated from potato tubers and tomato leaves (Walsh TA and, Strictland JA 1993; Bolter, 1993).

Plant cysteine PIs are encoded by gene families (Fernandes et al., 1993) and show different expression patterns during development and defense response to biotic and abiotic environmental stress (Felton and Korth, 2000). Expression is usually limited to specific organs or to specific phases during development, such as germination (Botella et al., 1996), early leaf senescence (Huang et al., 2001) cold and salt stress (Van der- Vyver et al., 2003; Pernas et al., 2000).

Aspartyl and metallocarboxypeptidase inhibitors

Plants contain two families of metalloproteinase inhibitors, the metallocarboxypeptidase inhibitor family in potato and tomato plants (Graham and Ryan, 1997; Rancour and Ryan, 1968) and a cathepsin D inhibitor family in potatoes (Keilova and Tomasek, 1976).

The inhibitors that bind to metallocarboxypeptidases have been identified in solanaceaous plants, in the medicinal leech (*Hirudo medicinalis*), in the intestinal parasite roundworm *Ascaris suum*, in the blood tick *Rhipicephalus bursa* and in rat and human tissues (Arolas et al., 2005; Homandberg et al., 1989; Reverter et al., 1998; Normant et al., 1995; Liu et al., 2000). The plant inhibitors have been described in tomato and potato. These inhibitors are small peptide inhibitors consisting of 38 - 39 amino acid residues and have the molecular mass of about 4.2 kDa (Hass et al., 1975; Hass and Hermodson, 1981). These inhibitors inhibit strongly, but competitively, a broad spectrum of carboxypeptidases from both animals and microorganisms, but do not inhibit serine carboxypeptidases from yeast and plants (Havkioja and Neuvonen, 1985). A metallocarboxypeptidase inhibitor is found to accumulate in potato tuber tissues during development, along with the potato inhibitor I and II families of serine PIs. The inhibitor also accumulates in potato leaf tissues, along with the inhibitors of other families, as a response to wounding (Ryan, 1990).

Aspartyl PIs have been described in sunflower, barley and cardoon (*Cynara cardunculus*) flowers and in potato tubers (Park et al., 2000; Kervinen et al., 1999; Lawrence and Koundal, 2002; Marres et al., 1989; Wolfson and Murdock, 1987). The cathepsin D inhibitor, an aspartyl PI described in potato tubers shares considerable amino acid sequence homology with soybean trypsin inhibitor. It is a 27 kDa protein and inhibits serine proteases trypsin and chymotrypsin in addition to the aspartyl protease cathepsin D, but does not inhibit pepsin, cathepsin E and rennin, which are all aspartyl proteases (Lawrence and Koundal, 2002). Pepstatin, a powerful and strong inhibitor of aspartyl proteases has been shown to inhibit proteolysis of the midgut enzymes of Colorado potato beetle (*Lptinotarsa decemlineata*) (Wolfson and Murdock 1987).

Plant protection

Plant PIs are small proteins generally present in high concentration in storage tissues, contributing upto 10% of the total protein content, they are also detectable in leaves in response to the attack of insects and pathogenic microorganisms (Ryan, 1990). PPIs have been shown to play a potent defensive role against predators and pathogens. Many PIs have been shown to act as defensive compounds against pests by direct assay or by expression in transgenic crop plants, and a body of evidence for their role in plant defense has continued to accumulate (Krattiger, 1997).

The possible role of PIs in plant protection was initially noticed when it was observed that the larvae of certain insects were unable to develop normally on soybean pro-

ducts. Subsequently 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).

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). Cysteine PIs from pearl millet (*Pennisetum glaucum*) inhibit growth of many pathogenic fungi, including *Trichoderma reesei* (Joshi et al., 1998). These advantages make protease inhibitors an ideal choice to be used in developing transgenic crops resistant to insect pests.

Expression of the PI genes is usually limited to specific organs or to particular phases during plant growth, such as germination (Botella et al., 1996), early leaf senescence (Huang et al., 2001) and drought (Van der- Vyver et al., 2003; Pernas et al., 2000). Wounding or treatment with methyl jasmonate evokes a similar pattern of gene expression. Further, the cytosolic localization of the inhibitors also suggests that they are involved in plant defense against insects (Zhao et al., 1996).

Kazal-type inhibitors play important roles in maintenance of normal cellular processes in animals (Magert et al., 2002; Kreutzmann et al., 2004) and the pathogenesis of mammalian parasitic apicomplexan protozoa and plant pathogenic oomycete fungi (Pszenny et al., 2002; Tian et al., 2004). Typical Kazal domains contain six cysteine residues forming a 1-5, 2-4, 3-6-disulfide bond pattern (Lee and Lin, 1995). However, a novel class of Kazal domains in which the third and sixth cysteine residues are missing has been described. These typical Kazal domains are ubiquitous in serine PIs of plant pathogenic oomycetes. Two of the Kazal like inhibitors namely EP11 and EP110 of the potato late-blight fungus *Phytophora infestans* target the defense-related protease P69B of the host plant tomato (Magert et al., 1999; Tian and Kamoun, 2005).

It has been found that potato tubers treated with elicittors, jasmonic, salicylic or arachidonic acids are able to excrete potatin and three chymotrypsin inhibitors.

Wounding and water stress prompts the secretion of two kinds of Kunitz-type PIs by potato tubers. These inhibitors are closely associated with other secreted polypeptides and would protect them against degradation by extracellular chymotrypsin like protease. The secreted inhibitors could therefore interact with plant defense system (Valueva et al., 2001; Ledoigt et al., 2006).

Oryzacystatin is found to prevent the growth of rice weevil (*Sitophilus oryzae*) by inhibiting the cysteine proteases in the gut of this organism (Hosoyama et al., 1994).

Two extracellular cysteine protease inhibitors (ECIP-1 and ECIP-2) isolated from species of the unicellular green alga *Chlorella* seem to have a role in protecting the cells from attacks by viruses and insects (Ishihara et al., 1999, 2000).

Phytostatins are involved in the control of endogenous cysteine proteinases during maturation and germination of seeds (Abe and Arai, 1991) and play a role in the apoptosis required in plant development and senescence (Solomon et al., 1999). Oryzacystatins have been shown to inhibit the α,β and γ cysteine proteinases that are produced during seed germination (Watanabe et al., 1991) Zeins and maize proteinases are inhibited by maize cystatins, suggesting a role for these inhibitors in the endogenous defense mechanism (Steller, 1995; Hoorn and Jones, 2004). Phytostatins from various plants inhibit the activity of gut cysteine proteinases involed in protein digestion in the gut of various members of the Coleoptera (beetles) attacking these plants, and thus play a role in the exogenous defense system of these plants (Oliveira et al., 2003)

The overexpression of cystatin in soybean cell suspensions blocked programmed cell death (PCD) (Solomon et al., 1999). The overexpression of a cystatin that inhibits papain activity in *Arabidopsis* cell cultures blocked cell death in response to avirulent bacteria and nitric oxide. The overexpression of this inhibitor in tobacco plants blocked the hypersensitive response induced by avirulent bacteria (Hoorn and Jones, 2004; Belenghi et al., 2003).

The use of cystatins has served as a specific approach to control insect predation and diseases in plants (Sakuta et al., 2001). Transgenic rice expressing maize cystatin has been shown to exhibit enhanced resistance against insect predation (Irie, 1996). The rice cystatins have been reported to confer resistance against polyviruses in transgenic tobacco and sweet-potato (*Ipomoea batatas*) plants (Campos et al., 1999).

Transgenic plants

Several transgenic plants expressing PIs have been produced in the last 15 years and tested for enhanced defensive capabilities, with particular efforts directed against insect pests (Valueva et al., 2001). Since the economically important orders of insect pests namely Lepidoptera, Diptera and Coleoptera, use serine and cysteine proteinases in their digestive system to degrade proteins in the ingested food, efforts have generally been directed at genes encoding PIs active against these mechanistic classes of proteases for developing transge-

nic plants. The PI genes have been particularly useful in developing transgenic plants resistant to insect pests, as they require the transfer of a single defensive gene, and can be expressed from the wound-inducible or constitutive promoters of the host (Boulter, 1993).

The first PI gene to be successfully transferred was that coding for CpTi and produced transgenic tobacco with significant resistance against tobacco hornworm (*Manduca sexta*) (Hilder et al., 1987). The efficiency of transgenic tobacco plants expressing CpTi was tested against armyworm (*Spodoptera litura*) in feeding trials under laboratory conditions. Reduction to the extent of 50% was observed in the biomass of army worm larvae fed on transgenic leaves expressing 3 - 5 µg of CpTi/g of fresh leaves (Sane et al., 1997).

Potato PI-II gene from potato was introduced into several japonica rice varieties to produce transgenic rice plants shown to be insect resistant in greenhouse trials. Wound-inducible PI-II promoter with the first intron of rice actin I gene was able to give high-level expression of PI-II gene in transgenic rice plants. These transgenic plants were resistant to pink stem borer (*Sesamia inferens*) (Duan et al., 1996).

The transformation of plant genomes with PI-encoding cDNA clones appears attractive not only for the control of plant pests and pathogens, but also as a means to produce PIs useful in alternative systems, and the use of plants as factories for the production of heterologous proteins (Sardana et al., 1998). The plant derived PI genes have been used for developing insect-resistant transgenic crops. Several transgenic plants expressing PIs have been produced, and these plants are found to be more resistant against insect pests. Recently, protease inhibitors have also been used to engineer resistance against viruses in transgenic plants (Ussuf et al., 2001). Bean α-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions (Roger et al., 2000).

When both soybean BBI and Kunitz inhibitors were introduced and expressed in sugar-cane (*Saccharum officinarum*), the growth of neonate larvae of sugar-cane borer (*Diatraea saccharalis*) feeding the leaf tissues was significantly retarded as compared to larvae feeding on leaf tissues from untransformed plants (Falco and Silva, 2003).

Transgenic potato expressing two cystatin genes developed resistance to a nematode, coleopteran insects (Cowgill et al., 2002) and transgenic rape plants expressing rice cystatin 1 were resistant to aphid (Rahbe et al., 2003).

Pearl millet cysteine protease inhibitor (CPI) has been found to possess anti-fungal activity in addition to its anti-feedent (protease inhibitory) activity (Joshi et al., 1998). The presence of anti-fungal and anti-feedent activity on a single protein opens up a new possibility of raising a transgenic plant resistant to pathogens, as well as pests, by transfer of a single CPI gene.

The transgenic wheat (*Triticum aestivum*) carrying barley trypsin inhibitor gene (BTI) showed a significant reduction of infection with Angoumois grain moth (*Sitotroga cerealella*). However, only early-instar larvae were inhibited in transgenic seeds, and expression of BTI protein in transgenic leaves did not have a significant protective effect against leaf-feeding insects (Altpeter et al., 1999). Expression of oryzacystatin, the rice cysteine-proteinase inhibitor, into the tobacco plant induced significant resistance against two important polyviruses, tobacco etch virus (TEV) and potato virus Y (PVY). A good correlation existed between the level of oryzacystatin protein and resistance to TEV and PVY at all levels tested. These results suggest that plant cystatins can be used against different potyviruses and potentially also against other viruses whose replication involves cysteine proteinase activity (Campos et al., 1999).

Resistance to inhibitors

PIs are highly specific for a particular class of digestive enzymes. However, insects have shown enough flexibility to switch the proteinase composition of their guts to overcome the particular PI expressed in the transgenic plants (Jongsma et al., 1995). It has been observed that insects can adapt to the ingestion of PIs. Insects belonging to both the Lepidoptera and Coleoptera can overexpress existing gut proteinases or induce the production of new types that are insensitive to the introduced PIs to overcome the deleterious effect of PI ingestion. This might be a contributing factor to the decreased effectiveness of the PIs expressed in transgenic plants. In a recent study it was shown that high level expression of soybean-trypsin-inhibitor gene in transgenic tobacco plants failed to confer resistance against *Helicoverpa armigera*. It is known that gut digestive enzymes are not the only targets affected by PI they can also affect water balance, molting and enzyme regulation of insects (Boulter, 1993).

A number of phytophagous insects including *Helicoverpa zea* a common pest of many solanaceous plants such as potato have adapted to the protease inhibitors of their host plants. Their survival and larval development is not affected by the presence of such molecules in their diet. They seem to have the ability to express specific proteases that are insensitive to the inhibitors, depending upon the specific inhibitor repertoire of the host plant (Jongsma et al., 1995). It has been found that trypsin that is sensitive to the inhibitor differs only marginally in amino acid sequence and substrate specificity from the insensitive form. They do, however, differ in their response to the trypsin inhibitor. It has been shown in the case of the tomato fruitworm that a B-type carboxypeptidase developes resistance to the potato carboxypeptidase inhibitor as a result of rearrangement of two small

regions that otherwise stabilizes the enzyme-inhibitor complex. This leads to a displacement of the active-site entrance, which impairs a proper interaction between the protease and its inhibitor (Bayes et al., 2005).

Conclusion

Proteases play key roles in pathogenesis. A large number of human disorders result from an imbalance in proteolytic activity. In this context, PIs are key players in the endogenous defense system, as they help regulate and balance protease activities. In plants these inhibitors are also important participants in the exogenous defense. The importance of PIs has been realized for some time now, and many transgenic plants overexpressing different PIs have been produced with resistance against different pathogenic organisms. This is, however, yet to be fully appreciated, and it can have important consequences beyond their recognized scope. PIs have been generally thought to counter tumour progression and metastasis. They also have the potential to counteract many of the inherited disorders such as emphysema and epilepsy. These inhibitors can also interfere with the life cycle of many viruses and may help prevent many viral disorders. Synthetic PIs currently form a part of the combinational therapy against AIDS, and have potential to be used as drugs against many other diseases. Although plant PIs have been isolated and characterized from a large number of sources, and that the natural inhibitors have been made available by gene therapy and through transgenic plants overexpressing specific inhibitors with therapeutic significance, the potential for the natural inhibitors in medicine and agriculture is enormous, awaiting full-scale exploration.

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