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## Role of Inhibitors of Proteolytic Enzymes in Plant Defense against Phytopathogenic Microorganisms

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**Abstract**—This review analyzes the literature on various mechanisms of proteolytic enzyme inhibitors involved in plant defense against attack by phytopathogenic microorganisms. The action of proteinase inhibitors from plants upon the enzymes from pathogenic microorganisms and viruses is reviewed. Considerable attention is given to the induction of proteinase inhibitors in plants in response to the invasion of pathogens. Some aspects of application of proteinase inhibitors in biotechnology for production of transgenic plants with enhanced resistance to diseases are discussed.

**Key words:** extracellular proteinases, proteinase inhibitors, phytopathogenic microorganisms, viruses

In the course of evolution, plants have elaborated protective mechanisms that allow them to successfully resist different kinds of unfavorable conditions including insects and phytopathogenic microorganisms [1-3]. The most important components of all protective mechanisms are proteinaceous compounds. These include enzymes such as  $\beta$ -1,3-glucanases and chitinases, inhibitors of proteases and  $\alpha$ -amylases, lectins, and also other proteins and peptides which have antimicrobial activity [4-7]. For instance, damaging of tomato leaves (*Lycopersicon esculentum* [Mill.]) by insects and microorganisms induced the synthesis of more than 20 different proteins including inhibitors of serine, cysteine, and aspartic proteinases and also a metallocarboxypeptidase [8].

The question about the participation of proteinase inhibitors in protective reactions in plants has been discussed in a number of reviews [7, 9-12]. However, new experimental data are constantly emerging and widening the current knowledge in this field, as well as giving an opportunity for a new look on the entire problem. Questions discussed in this review are not only of theoretical interest; they are also gaining important practical significance, especially during recent years in connection with achievements of biotechnology in creation of transgenic plants with increased resistance towards pathogenic microorganisms [7, 13, 14].

Many phytopathogenic microorganisms produce active extracellular proteinases that along with other enzymes play an important role in pathogenesis, e.g.,

polygalacturonases, pectolyases, and xylanases. Already in 1973, it was demonstrated that the phytopathogenic fungus *Colletotrichum lindemithianum* when grown on plant cell walls or on artificial nutrient medium secretes an active protease of 25 kD molecular weight and pH optimum at 8.6 [15]. This was the first extracellular proteinase of a plant pathogen obtained in its pure form. In recent years, many extracellular proteases produced by phytopathogenic microorganisms have been isolated and characterized to some extent. Among these serine proteinases prevail, but there are enzymes belonging to other mechanistic classes. All known serine proteinases of phytopathogens can be divided into trypsin-like and subtilisin-like enzymes. The first group contains proteinases that are produced by *Cochliobolus carbonum* [16], *Verticillium dahliae* [17, 18], *Stagonospora* (*Septoria*) *nodorum* [19], and *Phytophthora infestans* [20] microorganisms. Subtilisin-like enzymes are secreted by *C. carbonum* [16], *P. infestans* [20], *Acremonium typhium* [21], *Magnaporthe poae* [22], *Trichoderma harzianum* [23], and *Fusarium oxysporum* [24]. Among extracellular proteinases of phytopathogens, the aspartic proteinases are fairly widespread. These include the enzymes produced by *Botrytis cinerea* [25], *Cryphonectria parasitica* (endothiasepsin) [26], and *Glomerella cingulata* [27]. Cysteine proteinase is secreted by the fungus *Pyrenopeziza brassicae* [28]. Metalloproteinases include a family of Zn-dependent bacterial enzymes belonging to the genus *Erwinia* [29-31]. One of these proteinases, extracted from *Erwinia carotovora* subsp. *carotovora*, is similar in its properties to thermolysin from *Bacillus thermoproteolyticus* [32].

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Based on present data it can be concluded that extracellular proteinases apparently play an active role in the process of pathogenesis [25, 28, 33]. For instance, it has been revealed that in *P. brassicae* (a leaf pathogen damaging plants of the mustard family, Cruciferae) non-pathogenic mutants are unable to produce extracellular cysteine proteinase. Recovery of pathogenesis in these mutants was accompanied with the recovery of their ability to produce the proteinase [28]. An important role in disease progression is also played by aspartic proteinase of the fungus *B. cinerea*, which is a wide profile pathogen. Proteinase secretion by this microorganism was observed already at early stages of infection progression (before the formation of pectolytic enzymes begins) and was accompanied by the death of plant cells [25]. Development of infection was significantly retarded by the initial treatment of *B. cinerea* spores with the aspartic proteinase inhibitor pepstatin [25]. However, pepstatin did not affect spore germination [25]. It has been recently revealed that a cell-free sample obtained from spore suspension and germination cysts of late blight causing agent (the oomycete *P. infestans*) causes plant tissue necrosis when injected into potato leaves [33]. A correlation between the level of proteolytic activity in the sample and its necrotic action could be observed [33].

Contrary to earlier presented examples, in a number of cases dependency between extracellular proteinase activity and pathogenicity of the microorganism was not found. For instance, decrease in pathogenicity of trypsin-deficient mutants of *C. carbonum* graminoid pathogen with deficiency of trypsin-like proteinases was not observed [16]. Directed inactivation of subtilisin-like extracellular Prt1 proteinase of the fungus *F. oxysporum* did not affect its pathogenicity towards tomatoes [24]. These and other similar data [27, 34] suggest that in certain cases the role of the extracellular proteinases is limited to providing phytopathogenic microorganisms with amino acids essential for their growth and development [35].

In those cases when extracellular proteinases are actively involved in pathogenesis, their functions can be widely diversified including participation in microorganism intrusion into the plant, irreversible inactivation of the protective proteins, and participation in transformations of the pathogen's own proteins. Despite the fact that plant cell walls are mainly formed by polysaccharides, they also contain proteins and even certain enzymes [36]. Recent study reveals that metalloproteinase of the bacterium *Xanthomonas campestris* pv. *campestris* (black rot causative agent in crucials) is able to cleave glycoproteins of extracellular matrix in turnip petals (*Brassica campestris* L.) [37]. The fact is that this kind of proteins, which are characterized by high content of proline and oxyproline residues, play an important role in plant protection from the pathogenic microorganisms [38]. Trypsin-like serine proteinase SNP1 of another microorganism (*Stagonospora nodorum*) released oxyproline upon

its action on wheat cell walls. This activity in combination with early expression within pathogenesis suggests that SNP1 proteinase plays an active role in destruction of plant cell walls [19].

Proteinases found in pathogens can also play an active role in the degradation of other proteins involved in plant protection, for instance, such enzymes as chitinase and  $\beta$ -1,3-glucanase [39]. Purified extracellular metalloproteinase from the bacterium *E. carotovora* subsp. *carotovora* cleaved potato lectin [30] and also acted upon extensin, which is an extracellular matrix protein with high content of oxyproline residues [40]. Apparently, proteinases of phytopathogenic microorganisms can also perform other specific functions. For instance, in the bacterium *E. chrysanthemi* extracellular metalloproteinase catalyzed the transformation of pectate lyase into the mature form of this enzyme, which is crucial for plant tissue maceration [41]. It has been assumed that certain peptides released upon the action of extracellular proteinases of phytopathogenic microorganisms can act as elicitors, activating plant protection reactions [42].

Proteinase inhibitors in plants are able to suppress enzymatic activity of phytopathogenic microorganisms. Already in 1976 scientists revealed, that trypsin and chymotrypsin inhibitors from soy and bean seeds and also from potato tubers are able to suppress activity of proteinases secreted by phytopathogenic fungus *Fusarium solani* [43]. Furthermore, inhibitors from beans belonging to the Bowman–Birk inhibitor family suppressed the growth of hyphae and conidium germination of *F. solani*, *F. culmorum*, and *B. cinerea* fungi [44]. Similar results were later obtained from the study of the action of other proteinase inhibitors from plants upon extracellular enzymes and also upon the growth and development of phytopathogenic microorganisms. In such way, trypsin inhibitor from maize seeds blocked hyphal growth and conidium germination for a number of phytopathogenic fungi including *Aspergillus flavus*, *Asp. parasiticus*, and *F. moniliforme* [45]. Trypsin inhibitor from buckwheat seeds (*Fagopyrum esculentum* L. Moench) suppressed proteinase activity of the fungus *Alternaria alternata* affecting different cultivated and wild plants [46]. Inhibitor from buckwheat also suppressed spore germination and mycelium growth of phytopathogenic fungi *A. alternata* and *F. oxysporum* [47]. It has been demonstrated that chymotrypsin inhibitors from potato tubers suppress the growth and development of the oomycete *P. infestans* Mont de Bary, which is potato late blight causative agent [48, 49].

Along with inhibitors of trypsin and chymotrypsin, many plants have proteins that act predominantly as inhibitors of microbial proteinases [50, 51]. Besides acting upon microbial enzymes, some of these proteins are also able to inhibit trypsin; others were not active at all towards proteinases of animal origin. The first specific inhibitor of microbial proteinases was extracted from barley seeds (*Hordeum vulgare* L.) [52]. The inhibitor was present in

multiple forms and suppressed proteinase activity of *Asp. oryzae*, *Bacillus subtilis*, *Streptomyces griseus*, and *Alternaria tenuissima* microorganisms [52]. A protein with similar properties was later extracted from maize seeds [53]. Both the barley and maize proteins exhibited a complete absence of inhibitory activity towards trypsin, but acted as relatively weak and non-stoichiometric chymotrypsin inhibitors [52, 53]. Afterwards, their affiliation to the family of potato inhibitor I was established [54]. Inhibitor I from potato tubers differs from the abovementioned proteins by high inhibitory activity towards chymotrypsin and lack of activity towards subtilisin and some other proteolytic enzymes from microorganisms [55].

As we already mentioned, some inhibitors of microbial proteinases exhibit lack of activity towards proteinases of animal origin [56]. One of the most specific inhibitors of proteinases from phytopathogenic microorganisms was extracted from bean seeds (*Phaseolus vulgaris* L.). The inhibitor suppressed activity of serine proteinase of *C. lindemithianum* (the causative agent of anthracnose) but neither affected trypsin nor chymotrypsin [57]. In turn, trypsin and chymotrypsin inhibitors from beans were not active towards *C. lindemithianum* [57].

Not only plant inhibitors of serine proteinases are able to suppress enzymatic activity of phytopathogenic microorganisms. A protein with molecular weight of 10 kD has been recently extracted from pumpkin fruit phloem exudation (*Cucurbita maxima* L.); it acted as an aspartic proteinase inhibitor. Besides pepsin, it also suppressed activity of extracellular aspartic proteinase of the fungus *Glomerella cingulata* (the causative agent of anthracnose) [58]. Cystatin extracted from chestnut fruits (*Castanea sativa* L.) displayed high antifungal activity and suppressed the growth of certain pathogens such as *B. cinerea* [59]. Unfortunately, data regarding its ability to act upon microorganism proteinases is still unavailable. Another inhibitor of cysteine proteinase from millet seeds (*Pennisetum glaucum* L.), different in its properties from usual cystatins, also inhibited high antifungal activity [60]. However, doubts were later expressed regarding whether the antifungal activity of millet protein is associated with its ability to inhibit fungal proteolytic enzymes [61].

Apparently, the inhibitors of cysteine proteinases can play a vital role in relations between plants and viruses. The explanation is that cysteine proteinases play an active role in protein processing in many viruses. In this respect, cysteine proteinases become an attractive target for the development of efficient control means of plant and animal viral diseases [62]. Initial experiments have revealed that plant inhibitors (oryzacystatins I and II) are able to suppress replication of animal viruses belonging to the picornaviruses family [63]. Later, transgenic tobacco plants containing the oryzacystatin I gene were obtained. They exhibited increased resistance towards tobacco etch virus and potato Y virus [64]. Both viruses are potyviruses, which use cysteine proteinase for protein processing.

Just as in the case of other PR (pathogenesis related) plant proteins [65], the synthesis of proteinase inhibitors is induced in response to phytopathogenic microorganism infection. This phenomenon was first observed in tomatoes infected with the oomycete *P. infestans*. In that case, correlation between an increased content of trypsin and chymotrypsin inhibitors and plant resistance to the pathogen was observed [66]. Increase in activity of serine proteinase inhibitors was also noticed in potato tubers infected with *P. infestans* [48, 49]. The same phenomenon took place not only in solanaceous plant family, but also in other plant families. It was demonstrated that melon infection with *Colletotrichum lagenarium* causes an increase in the activity of inhibitor acting upon pathogen proteinase [67]. A similar pattern was observed in monocotyledonous plants. Affecting of maize germinants by the fungus *F. moniliforme* resulted in both local and systematic induction of serine proteinase inhibitor belonging to structural family of potato inhibitor I [68]. Induction in response to infection by pathogenic microorganisms is not limited to serine proteinase inhibitors. For instance, production of cystatin took place in chestnut leaves in response to infection with the fungus *B. cinerea* [69].

It is worth mentioning that proteinase inhibitors induced in response to infection can sufficiently differ from similar inhibitors present in a healthy plant. In tobacco leaves (*Nicotiana tabacum* L.) in response to tobacco mosaic virus (TMV) a protein was produced, which by its properties belonged to the structural family of potato inhibitor I; however, it differs from the other inhibitors in this family by its action upon enzymes. For instance, the induced inhibitor has high activity towards fungal and bacterial proteinases, but it acts weakly towards trypsin and chymotrypsin [70]. This property differentiates it from inhibitor I in healthy tobacco leaves, which is an efficient chymotrypsin inhibitor [71]. Further studies demonstrated that inhibitor from tobacco leaves infected with TMV contains Glu residue in P1 position of the reactive site [51]; at the same time, affinity inhibitors extracted from healthy tobacco and potato plants have Leu or Met residues in this position [72, 73]. The induction of protein with unusual properties was also observed in leaves of another tobacco species (*Nicotiana glutinosa* L.) infected with TMV. Based on its structural features, the protein was classified to the structural family of Kunitz trypsin soybean inhibitor, but it also has certain features similar with cysteine proteinase inhibitors of cystatin family. The action of this protein upon enzymes is still uninvestigated [74].

A common and characteristic feature for PR-proteins is their localization in intercellular space [65]. Already in early histochemical studies, it was established that most of the proteinase inhibitors in soy seeds are located in the cell membrane region [75]. When seeds of soy and other pea family members (Leguminosae) are swelling, trypsin and chymotrypsin inhibitors (both of

Bowman—Birk and Kunitz type) along with lectins quickly diffuse into the surrounding solution [76, 77], which apparently improves their ability to easily exude into the intercellular space. The ability for secretion was not limited to serine proteinase inhibitors. It was revealed that cysteine proteinase inhibitor contained in carrot cells (*Daucus carota* L.) also was easily exuding into the surroundings [78]. In tomatoes, serine proteinase inhibitors I and II accumulate in endosperm cell walls and in secretory cells of root cap, and are secreted into the milieu. Hence, it was assumed that proteinase inhibitors can protect the growing root meristem from pathogenic microorganisms and other pests [79]. This hypothesis correlated well with the data demonstrating that certain soil microorganisms (even those not pathogenic for plants, such as *Pseudomonas putida*) are able to induce the synthesis of proteinase inhibitors in plant roots [80].

Recent achievements in biotechnology resulted in creation of transgenic plants with an increased resistance towards different kinds of unfavorable conditions including affects of phytopathogenic microorganisms and viruses. This approach allows not only increasing productivity of many cultured plants, but also promotes the improvement of the ecologic situation through the decreased use of highly toxic plant protection agents [81, 82]. At the moment, fairly widespread are transgenic plants containing  $\delta$ -endotoxin genes from the bacterium *Bacillus thuringiensis* (Bt) and exhibiting the increased resistance towards insects [83]. In the coming years we can expect agricultural use of plants containing the genes of other proteins increasing their resistance to pests and diseases. Among those proteins, proteolytic enzymes inhibitors play an important role [13]. Currently, the genes of more than 14 proteins, proteinase inhibitors, are expressed in various cultured plants [14]. The majority of transgenic plants containing proteinase inhibitor genes are characterized by increased resistance to insects and some other pests. At the same time, these transgenic plants display lower stability than plants containing Bt toxin genes [35]. Apparently, the most promising are plants containing the genes of proteinase inhibitor in combination with genes of other proteins. Batatas plant (*Ipomoea batatas* Lam.) can be used as an example; it contains simultaneously genes of three proteins— $\beta$ -glucuronidase, trypsin inhibitor from *Vigna unguiculata* [L.] Walp seeds, and lectin from snowdrop (*Galanthus nivalis* L.) [84]. Tobacco plants containing Bt toxin genes and inhibitor from *Vigna unguiculata* have also been obtained. These plants have higher insecticide activity compared to plants containing only Bt toxin genes [85, 86]. It can be assumed that in these cases proteinase inhibitors not only act by themselves, but also protect other recombinant proteins from the destructive action of plant proteinases.

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