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Minireview

Proteinases from pollen and pests

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An examination of the proteinases present in two very different systems is described, in order to illustrate the diversity in function of this class of enzymes. In the first case we have noted the importance of gut proteinases from the fire ant *Solenopsis invicta* in relation to the nutritional requirements of the entire colony. In the second we have investigated the properties of endoproteases from both ragweed and mesquite pollen, relative to their role in the development of allergies and asthma. If the function of each type of enzyme(s) is correct, then it is clear that addition of exogenous inhibitors might be useful in a) controlling the infestation associated with the fire ant, and b) reducing the deleterious effects associated with the development of asthma.

Investigations into the role of proteolytic enzymes in the development of diseases in humans have primarily focused on dysregulation of host proteinase/proteinase inhibitor equilibrium. This can clearly occur through increased production of endogenous proteinases and/or decreased synthesis of host inhibitors, with the most illustrative example involving the development of familial emphysema due to the lack of sufficient inhibitory activity in the lung [1]. Here, it is obvious that there is no longer control of neutrophil proteinase activity, and the resulting effect is the uncontrolled proteolysis of connective tissue within the alveoli of this organ. Other studies, involving investigations of the endogenous metalloproteinase/metalloproteinase inhibitor equilibrium, support the groundbreaking results for the development of emphysema and indicate that this particular dysregulation may result in the development of various types of arthritis and tumor invasion and metastasis [2]. While investigations of host proteinase/inhibitor interactions have given strong indications of the deleterious effect of increasing the proteinase load, only recently have studies turned to possible functions of proteolytic enzymes from exogenous sources. In particular, it should be pointed out that nearly all sources of these enzymes are unaffected by host proteinase inhibitors. This is of significant importance since it indicates that such enzymes can act with impunity, degrading host proteins (including proteinase inhibitors) and activating the normally tightly controlled coagulation, complement, fibrinolytic, and kallikrein/kinin cascade pathways. This has been

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Abbreviations: AEBSF, 4-(2-aminoethyl) benzene sulfonyl fluoride; DFP, diisopropyl fluorophosphate; LBTI, lima trypsin inhibitor; PMSF, phenylmethylsulfonyl fluoride; pNA, *p*-nitroanilide residue; SBTI, soya bean trypsin inhibitor; TLCK, *N*-α *p*-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone; VIP, vasoactive intestinal peptide.

particularly well documented in the development of periodontitis, where it has been found that proteinases from the infective organism *Porphyromonas gingivalis* play a major role in the pathogenesis associated with this disease [3]. This model system which pinpoints a major role for exogenous proteinases in infection will likely be extended to other diseases involving foreign organisms, including gonorrhea, group A *Streptococcus* infections, and *Staphylococcal* infections [4].

It is significant that studies on the role of exogenous proteinases in various disease states has been primarily limited to those secreted by fungal and bacterial organisms. In addition, few investigations have involved any attempt to determine whether proteinases from insects might be important for their development, survival, and/or infestation. In this report we will describe results obtained in our laboratory which illustrate the importance of investigating the proteolytic activities of foreign organisms from diverse sources, whose functions may be critical to both human and agricultural diseases.

DESTRUCTIVE ACTIVITIES OF THE FIRE ANT

The imported fire ant, *Solenopsis invicta*, is an uninvited and unwelcome species accidentally introduced into North America during the 1930's by ships arriving from Paraguay [5]. Due to suitable climate conditions, as well as its aggressive behavior, this species has easily outcompeted the indigenous species and quickly spread over the southern United States. This infestation has caused (i) massive loss of cultivable land, (ii) destruction to farm equipment and (iii) due to its venomous sting, bodily injury to man and other animals.

The venom of this organism is mostly composed of alkaloids, but also contains 0.1% protein [6]. It is these proteins which are responsible for the immune response, and in certain individuals this may result in induced sensitization and possible anaphylaxis, even in nanogram doses [7]. Clearly, a mechanism for eradicating this insect, while not harming nontarget organisms, would prove useful since native ants are the best defense against invasion [8].

DEVELOPMENT OF THE ADULT FIRE ANT

The stages of development of *Solenopsis invicta* are as follows: eggs — 1st, 2nd, 3rd, and 4th instar larvae — prepupae-pupae-adult. The 4th instar larvae plays an important role in the survival of the colony in that it is totally responsible for the digestion of solid foods. Thus, it is the source of nutrients for the queen and adult workers. The mechanisms utilized within the nest for proper feeding involves placement of food on the anteroventral opening of the 4th instar larvae, secretion of proteinaceous fluids which digest the food, and sharing of the fragmented materials, trophalactically, by the entire colony. Examination of proteolytic enzymes from such larvae could thus provide useful information not only as to how digestion of proteins occurs but also for the development of inhibitors to control this pest.

PROTEOLYTIC ENZYMES IN FIRE ANT LARVAE

In our studies we have been successful in purifying and characterizing three serine proteinases from the 4th instar larvae. Based on substrate specificity they appear to represent two elastase-like and one chymotrypsin-like proteinase(s). These are referred to as Soli E1, Soli E2, and Soli C. Soli E1, having elastase-like activity based on its specificity towards MeO-succinyl-L-Ala-L-Ala-L-Pro-L-Val-pNA, was purified from a crude extract of larvae in two steps, primary isolation involving binding and subsequent release from a lima bean trypsin inhibitor (LBTI)-Sepharose column. The purified enzyme behaved as a single protein on SDS/PAGE of molecular mass 23 kDa, had a pH optimum between 7.0 and 9.0, and had a typical N-terminal sequence Ile-Val-Gly-Gly- which is found in any serine proteinases. The enzyme was inhibited by DFP, but not by pepstatin A, iodoactic acid or EDTA, clearly indicating that it was a serine proteinase. In addition, it was also inhibited by a variety of chloromethyl ketones and phosphonates, especially those with either valine or isoleucine in the P_1-position. Finally, it was also inhibited by

typical protein-derived inhibitors of serine proteinases, including trasylol, eglin C, LBTI, soybean trypsin inhibitor (SBTI), and alpha-1-proteinase inhibitor (alpha-1-PI).

The second elastase-like proteinase, referred to as Soli E2, possessed similar properties to that of Soli E1 but was more easily purified by chromatography on a Mono Q FPLC column, followed by gel filtration. The isolated enzyme had a molecular mass of 24 kDa, a more narrow pH range of activity than Soli E1 (pH optimum 9.5), but an identical N-terminal sequence. Again, it was found to be a typical serine proteinase with similar inhibition characteristics to Soli E1. However, it was not inhibited by trasylol and only weakly by LBTI. Utilizing a degenerative probe designed from the N-terminal sequence and a cDNA library, Soli E2 has been cloned and sequenced. A summary of the data obtained indicates that the enzyme is expressed as a proform and is activated after the putative 23 amino-acid prosequence is removed by proteolytic cleavage. A BLAST search showed a 60% and a 71% similarity in primary structure to human neutrophil elastase and European hornet chymotrypsin II, respectively, with the putative active site residues being highly conserved in Soli E2.

The third proteinase isolated and characterized, referred to as Soli C, was obtained by identical steps utilized for Soli E2. The purified enzyme had a specificity towards chymotrypsin-like substrates, based on hydrolysis of Suc-L-Ala-L-Ala-Pro-L-Phe-pNA. As with the two other previously described enzymes, Soli C had a molecular mass near 25 kDa, an alkaline pH range of activity (8.5–11.5), and an inhibition profile typical of serine proteinases. In particular, it was rapidly inhibited by chloromethyl ketone and phosphate compounds containing phenylalanine in the P_1-position, although the latter were not as active, presumably because of the charge carried by phosphonates. With protein-derived inhibitors the only one which worked poorly was SBTI. The N-terminal sequence was the same as found for Soli E1 and Soli E2, and attempts are currently being made to determine its sequence and that of Soli E1 by recombinant DNA technology. In summary for this section, we have isolated three distinct proteinases from the 4th instar larvae of the insect *Solenopsis invicta* which are responsible for the digestion of food for the colony. Inhibition studies suggest the strong possibility that development of inhibitors against one or all of these enzymes could be useful as pesticides to slowly starve the colony. Such a program is currently underway in our laboratory.

THE ROLE OF POLLEN PROTEINASES IN DISEASE

Hay fever (allergic rhinitis) and asthma, a chronic inflammation of the upper airways of the lungs, are two of the most widespread and clinically important of all allergic diseases [9]. Although the causes of each are not completely understood, both can easily be triggered by inhalation of airborne pollutants such as dust and pollen. Each of these agents is known to initiate a) the production of immunoglobulins (IgE), b) the degranulation of mast cells with the release of histamine, tryptase, and chemoattractants, c) the invasion of inflammatory cells into the lungs, and d) the release of hydrolytic enzymes, inflammatory mediators, and neuropeptides which cause inflammation and affect airway function [10, 11]. Dust mites, the causative agents of allergenicity of house dust, contain allergens which are also proteinases. These enzymes are capable of releasing bradykinin from kininogen primarily through the activation of prekallikrein, thereby exacerbating the inflammatory reactions associated with allergenic and asthmatic responses [12–14]. On this basis, an assumption was made by us that perhaps some pollen allergens may also influence the development of allergies and asthma by virtue of also having proteolytic activity.

PROTEOLYTIC ACTIVITY IN MESQUITE POLLEN

Mesquite (*Prosopis velutina*) pollen is a major hay fever-associated allergen primarily located in the south central and south western regions of the United States [15]. Extraction of this pollen in neutral buffer indicated the presence of at least two endoproteases, one with the ability to cleave peptide bonds after basic residues and the other after aromatic and hydrophobic residues [16]. Each proteinase was purified to homogeneity and shown to have similar but

Table 1
Properties of mesquite pollen arginyl-lysyl and isoleucyl-phenylalanyl endopeptidases

Properties	Arginyl-lysyl endopeptidase	Isoleucyl-phenylalanyl endopeptidase
Molecular mass	84 kDa (94 kDa)	94 kDa
Calcium requirement	+	-
Stability	48 h at 37°C	48 h at 37°C
pH optimum	pH 7.5–9.0	pH 7.5–9.5

not identical properties (Table 1). Although each enzyme appeared to be related to trypsin and chymotrypsin, respectively, inhibition studies indicated that neither could be readily inhibited by protein-derived proteinase inhibitors, despite the fact that both were inactivated by DFP and specific chloromethyl ketones. A summary of inhibition studies on each enzyme is given in Table 2 and Table 3. Significantly, neither enzyme could degrade proteins, their activities being restricted to the fragmentation of polypeptides. Indeed, each enzyme could readily digest neuropeptides and vasoactive peptides known to be present and active in the lungs. For example, angiotensin II and atrial natriuretic peptide were almost completely cleaved by the arginyl/lysyl endopeptidase at extremely low E:S ratios (1:3000 to 1:7000) in less than one hour, while other peptides, including neurotensin and bradykinin, were cleaved more slowly, and substance P and vasoactive intestinal peptide (VIP) almost not at all. In all cases cleavage occurred rapidly after arginine residues and more slowly after lysine residues. Studies on the endopeptidase which

Table 2
Effect of inhibitors on the amidolytic activity of mesquite pollen proteinase

Pre-incubation time	Inhibitor	Concentration	Residual activity (%)
10 min	DFP	0.01 mM	55.2
		0.1 mM	0.0
24 h	PMSF	2.0 mM	71.3
10 min	AEBSF	1.0 mM	14.7
24 h	3,4-Dichloroisocoumarin	0.1 mM	51.9
		0.4 mM	21.8
10 min	Iodoacetamide	10 mM	103.0
10 min	EDTA	4 mM	115.0
10 min	Benzamidine	10 mM	41.0
10 min	TLCK	2.8 µM	27.5
		28.0 µM	0.0
10 min	TPCK	1.0 mM	102.9
5 min	Antipain	8.3 nM	32.7
		82.7 nM	0.0
5 min	Leupeptin	0.4 µM	13.8
		4.2 µM	0.9
10 min	SBTI	0.5 mg/ml	118.5
10 min	LBTI	0.5 mg/ml	113.3
10 min	Aprotinin	0.5 mg/ml	104.3
10 min	Ovomucoid	0.5 mg/ml	114.5
10 min	D-Phe-Pro-Arg-CH_3Cl	0.2 µM	65.6
		2.0 µM	23.2
		20.0 µM	0.0

Table 3
Effects of inhibitors on the amidolytic activity of mesquite pollen alanyl endopeptidase

Inhibitor	Concentration	Residual activity (%)
DFP	200 μM	56
AEBSF	1 mM	75
3,4-Dichloroisocoumarin	2 mM	9
EDTA	5 mM	100
TPCK	50 μM	73
Z-Phe-CH_3Cl	20 μM	45
Z-Pro-Phe^P $(OPh)_2$	25 μM	35
Z-Gly-Leu-Phe^P $(OPh)_2$	25 μM	35
Z-Phe-Leu-Phe^P $(OPh)_2$	25 μM	54
Z-Gly-Gly-Phe-CH_3Cl	20 μM	58
Z-Val-Val $(OPh)_2$	25 μM	80
Z-Ala-Val $(OPh)_2$	25 μM	55
Z-Pro-Val $(OPh)_2$	25 μM	56
Boc-Val-Pro-Val $(OPh)_2$	25 μM	31
Z-Gly-Leu-Ala-CH_3Cl	25 μM	49
Ac-Ala-Ala-Phe-Ala-CH_3Cl	25 μM	14
Ac-Ala-Ala-Pro-Ile-CH_3Cl	25 μM	53

cleaved polypeptide substrates after aromatic and hydrophobic residues gave similar results, although hydrolysis was far more rapid with angiotensin I and II and more slowly with VIP, bradykinin, atrial natriuretic peptide, and substance P [16].

PROTEOLYTIC ENZYMES IN RAGWEED POLLEN

Ambrosia artemisiifolia (common/short ragweed) is the most important cause of hay fever and related diseases than all other plants combined. The complex mixture of proteins that are released from ragweed pollen grains have been shown to be some of the most powerful antigens/allergens known [15]. However, none of the major allergens characterized to date has been found to have any biological activity, other than the activation of mast cells [17].

As in the case of mesquite pollen, two endopeptidases with specificity directed towards either basic residues or aromatic and hydrophobic residues have been isolated, and most of our studies to date have involved only the latter enzyme. This enzyme had a molecular mass of 82 kDa, and it could be readily inhibited by both DFP and TPCK, indicating that despite its high molecular mass it was related to members of the chymotrypsin family. Comparison of the relative rates of hydrolysis of a number of substrates indicates that phenylalanyl residues are preferred in the P_1-position. As noted with the mesquite proteinases, neither of the two enzymes found in ragweed pollen could degrade proteins. However, the chymotrypsin-related enzyme readily degraded both VIP and substance P. In addition, it was found to inactivate alpha-1-PI through cleavage within the reactive site loop.

PROTEINASES AND ALLERGIES

Pollens are significant triggering agents in the development of allergenic responses and asthma. Mainly they have been studied from the aspect of stimulating the production of IgE antibodies which stimulate mast cells to release histamine. However, many have been shown to be hydrolytic in nature, having either lyase,

esterase, or galacturonidase activity [18–20]. As pointed out earlier, it has been clearly shown that a major dust mite allergen, Der I, is a serine proteinase which generates bradykinin through activation of the kallikrein/kinin system. It was our premise that this might also be occurring with pollen proteinases, but all of the enzymes we have so far isolated have only peptidase activity.

Despite this negative implication, it is clear that the release of such enzymes within the upper airways can be deleterious to bronchial homeostasis by virtue of the fact that they can readily degrade vasoactive peptides which regulate vasoconstriction, bronchial constriction, and bronchial dilation. For example, VIP and substance P have been proposed to have major roles serving as neurotransmitters modulating airway caliber [21], and it is their widespread distribution and numerous physiological effects that makes their uncontrolled inactivation excellent candidates for important roles in asthma [22, 23]. Any disruption in the balance between these competing polypeptides would clearly be deleterious.

In addition, it is obvious that the ability of these endopeptidases to cleave within the reactive site loop of plasma proteinase inhibitors, despite their lack of true endoprotease activity, would also affect the balance between inhibitory activity and proteinases from a variety of tissue sources. In particular, and as noted here, the inactivation of alpha-1-PI by pollen proteinases within the upper airways would put an increased neutrophil elastase within this area and possibly contribute to uncontrolled degradation of bronchial tissue.

CONCLUSIONS

The data summarized in the above sections indicates that there are many fruitful areas of research yet to be examined where proteinases may play major, yet subtle roles. Insects, fungi, bacteria, pollen, as well as animal tissues are replete with such enzymes, and their functions may directly or indirectly affect human life. We have only touched on two such examples. Obviously, one only needs to have the proper imagination to find other, similarly important systems, which need examination for their importance relative to health, nutrition, or agricultural development.

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