

Coevolution between pathogen-derived proteinases and proteinase inhibitors of host insects

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Virulence is thought to coevolve as a result of reciprocal selection between pathogens and their hosts. This paper focuses on coevolution between microbial proteinases operating as virulence factors and host defense molecules of insects. Owing to shorter generation times and smaller genomes, microbes exhibit a high evolutionary adaptability in comparison with their hosts. Indeed, the latter can only compete with pathogens if they evolve mechanisms providing a comparable genetic plasticity. Gene or domain duplication and shuffling by recombination is the driving force behind the countermeasures in host defense effectors. Recent literature provides evidence for both diversifications of fungal proteinases involved in pathogenesis and expansion host proteinase inhibitors subsets contributing to insect innate immunity. For example, the pathogen-associated spectrum of proteolytic enzymes encompasses thermolysin-like metalloproteinases that putatively promoted the evolution of corresponding host inhibitors of these virulence factors which complement the insect repertoire of antimicrobial defense molecules. Beyond mutual diversification of effector molecules coevolution resulted also in sophisticated molecular adaptations of host insects such as sensing and feedback-loop regulation of microbial metalloproteinases and corresponding countermeasures of pathogens providing evasion of host immunity induced by these virulence factors.

Introduction

Coevolution can be defined as a series of synchronous mutual evolutionary changes in interacting species which act as agents of natural selection for each other.¹ Progress in understanding molecular mechanisms behind coevolution is limited by the availability of suited models because reciprocal adaptations during evolutionary processes are difficult to trace and to reconstruct at the genetic level. Pathogens and their hosts provide powerful models to investigate coevolution characterized by reciprocal

changes in genetic composition of interacting species which have a close ecological relationship.² Insects are the most successful group of organisms on earth regarding species diversity, and their abilities in managing symbiotic bacteria or fungi and defending against pathogenic ones play a predominant role in the outcome of the evolutionary competition with microbes.³ In this paper I focus on parasitic fungi and their insect hosts as a model system for exploring coevolutionary mechanisms because it provides informative examples for interactions between molecules mediating either virulence of fungal pathogens or resistance of the host, which are characterized by reciprocal adaptations.⁴

The greater wax *Galleria mellonella* has emerged as powerful model hosts both for studying co-evolution between entomopathogenic bacteria⁵⁻⁷ and fungi host with their host insects,⁴ and as heterologous hosts for human pathogenic bacteria and fungi.⁸⁻¹⁰ *G. mellonella* combines a number of advantages when used as an alternative host for human pathogens. Firstly, the low overall costs of breeding large numbers facilitate its use as an inexpensive whole-animal high throughput infection system. Secondly, *G. mellonella* can be adapted in the laboratory to 37°C which is important because human pathogens are adapted to the physiological temperature of its host. Thirdly, *G. mellonella* can be used as an insect model to mimic oral infections both with human or insect pathogens.¹¹ *G. mellonella* provides also a model to study host-pathogen co-evolution, particularly in regard of entomopathogenic fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* which are world-wide used to control pest and vector insects. These fungi produce a defined spectrum of molecules considered as virulence factors such as proteolytic enzymes and secondary metabolites which have been designated as fungal toxins. The antifungal immunity of *G. mellonella* has been intensively studied during past decade and resulted in identification of an array of defense molecules which can either directly kill parasitic fungi (antifungal peptides) or inactivate their virulence factors (inhibitors of fungal proteinases or proteins detoxifying fungal toxins).^{4,12}

Analysis of the interactions between fungal virulence factors and *G. mellonella* defense molecules provides novel insights into mechanisms behind host-pathogen coevolution, particularly in those driving evolution of virulence or immune defense strategies. Host innate immunity relies on both cellular and humoral mechanisms. The latter is based on a variety of molecules used

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to arrest development of and kill invading pathogens among which antimicrobial peptides and peptide families such as the defensins are evolutionarily conserved.¹³⁻¹⁵ The diversity of constitutively expressed or induced host peptides exhibiting antibacterial and/or antifungal activity determines the resistance of a particular host against a broad spectrum of potentially pathogenic microbes. Owing to their short generation times and small genomes, pathogens exhibit a high capacity to genetically generate virulence factors. Consequently, the ability of higher organisms to successfully compete with pathogens in an evolutionary arms race depends on their sophisticated mechanisms allowing reciprocal diversification of defense molecules. This paradigm has recently attracted many researches to investigate the molecular mechanisms providing evolutionary diversification of antimicrobial peptides conferring immunity in a variety of host model organisms including, for example, insects such as termites and the dipteran species *Drosophila* and *Anopheles*, as well as in nematodes and mammals.¹⁶⁻²¹

In this paper, I will first briefly outline the current knowledge about antifungal peptides in insects, emphasizing those of the *G. mellonella*. In response to diversifying host defense molecules, fungal pathogens have evolved mechanisms mediating either suppression or avoidance of host immune responses.^{12,22} Another strategy of pathogens to overcome the host immune system targets its defense molecules directly. Pathogen-associated proteinases capable of digesting host defense proteins and peptides are essential during pathogenesis and operate as virulence factors.²³ Consequently, a subsequent chapter addresses the role of fungal proteinases during pathogenesis, particularly, in degradation of host defense molecules in insects. Host counter-adaptation to proteinases produced by pathogens or parasites to inactivate host defense molecules has led to the evolution of corresponding proteinase inhibitors which have become constituents of the host innate immune system. Microbial proteinases and immunity-related proteinase inhibitors can be considered as favorite models to analyze evolutionary arms races in an antagonist system at molecular level because their intimate interactions at the front-line between pathogens and their hosts are characterized by rapid reciprocal adaptations.²⁴ Emphasizing coevolutionary insights, I will briefly outline in the last chapter biological functions of proteinases associated with fungal pathogens and immunity-related proteinase inhibitors of their host insects.

Antifungal Peptides and Proteins of Insects

The antifungal defense of insects against fungal pathogens relies on both cellular and humoral components.²² The cellular defense encompasses phagocytosis of fungal cells entering the insect body by immune-competent hemocytes circulating in the hemocoel, and, if the number of fungal cells is too large or fungal mycelia are too large to be engulfed, multicellular encapsulation. The latter is a complex process in which pathogens or parasites are separated from the body by a multilayered sheet of different hemocytes types whose orchestrated action results in formation of a black capsule.²⁵ Phenoloxidase-mediated formation of chemically inert melanin around the entrapped microbes or parasites

arrests exchange of molecules between the host and the pathogen.^{27,28} However, the humoral part of insect immunity against parasitic fungi is based on peptides or proteins which can either kill fungi directly or which neutralize toxic molecules released thereof.^{4,12} Accordingly, I will first address insect antifungal proteins and then peptides with less than 10 kDa before I focus on insect-derived molecules involved in defense against fungal virulence factors such as toxic proteinases.

Innate immune response of *G. mellonella* encompasses the expression of lysozyme. Its activity against gram-positive bacteria has been attributed to its ability to degrade cell wall peptidoglycan by hydrolysis of the β -1-4 linkages between N-acetylglucosamine and N-acetylmuramic acid residues.²⁸ Besides moderate activity against gram-negative bacteria,²⁹ *G. mellonella* lysozyme was also shown to exhibit antifungal activity *in vitro*,⁴ similar to that of human lysozyme against the pathogenic yeasts *Candida albicans* and *Coccidioides immitis*.^{30,31} The effect of commercially available hen-egg-white lysozyme on virulent or non-virulent strains of *M. anisopliae* is illustrated in **Figure 1**. A recent analysis of the *G. mellonella* immunity-related transcriptome resulted in identification of four lysozyme homologues and an additional i-type lysozyme whose precise functions in antifungal immunity remain to be elucidated (Vogel, et al. unpublished).

To date, more than a thousand peptides and proteins exhibiting antimicrobial activity have been found in living organisms ranging from bacteria to humans.³³ The gene-encoded antifungal peptides and proteins of insects share unifying themes with those of other animals and plants, for example, they have retained their membrane-active efficacy despite the presence of highly mutable target microorganisms. The spanning of conserved motifs among particular polypeptides involved in antimicrobial defense across the phylogenetic continuum affirms their ancient role in coevolutionary relationships between hosts and pathogens.¹⁵ However, analysis of immunity-related genes and pathways in mosquitoes shows that evolutionary dynamics differs among functional gene categories. Genes involved in immunity-related recognition and signal transduction are rather conservative when compared with rapidly evolving genes encoding effectors involved in killing of pathogens. Antimicrobial peptides (AMPs) diversify not by sequence divergence but rather by gene duplication and creation of new families.³³ Naturally occurring polymorphisms of AMPs seems to drive the variability in immune-competence among natural insect populations.³⁴

AMPs are typically cationic and consist often of less than 100 and mostly between 12 and 50 amino acid residues.³⁵ Despite the low similarity at the amino acid sequence level the great majority of AMPs from insects can be categorized into one of three structural classes: (1) linear alpha-helical peptides free of cysteine residues, (2) peptides adopting a beta-sheet globular structure stabilised by intramolecular disulfide bridges, (3) peptides with unusual bias in certain amino acids such as proline and/or glycine. Insect AMPs such as those found in the greater wax moth *G. mellonella* can exhibit antibacterial and/or antifungal activity. The number of strictly antifungal peptides is rather low when compared with antibacterial ones. Two cysteine-rich peptides which exclusively inhibit growth of filamentous fungi have been isolated

from *G. mellonella*, the defensin-like antifungal peptide³⁶ and gallerimycin.³⁷ Confirming the postulated contribution of gallerimycin to antifungal defense in insects, its transgenic expression has been determined to confer resistance to fungal diseases even in crops.³⁸ Comparison of defensin sequences from arthropods and mollusks revealed that all exons and introns, aside from the exon encoding the mature peptide, differ widely in number size and sequence. This variability implicates that the exon encoding the mature peptide was modified by exon-shuffling and integrated down-stream of unrelated leader sequences during evolution.³⁹

The first linear and amphipathic α -helical antimicrobial peptide from insects was discovered and isolated from the hemolymph of the silk moth *Hyalophora cecropia* and has therefore been named cecropin.⁴⁰ The cecropin-like peptide from *G. mellonella* is synthesized as a propeptide, with a putative 22-residue signal peptide, a 4-residue propeptide, and a 39-residue mature peptide with a mass of 4.3 kDa. Like cecropins from other insects, it exhibits potent activity against both gram-positive and gram-negative bacteria,⁴¹ and against fungi.^{4,12} Moricins represent another family of amphipathic α -helical antimicrobial peptides that have been discovered in the silk moth *Bombyx mori*.⁴² Eight moricin homologues that may originate by gene duplication have recently been found in *G. mellonella*. They exert in vitro activity against both gram-negative and gram-positive bacteria, as well as against yeast and filamentous fungi.⁴³ The moricins belong to the immunity-related gene families whose occurrence seems to be restricted to Lepidoptera. Their recent diversification by gene duplication was plausibly driven by the coevolution with parasitic fungi. Multiple copies of genes encoding AMPs are common in insect genomes and may reduce the probability of pathogenic microorganisms to develop resistance.¹⁷ The impressive antimicrobial peptide arsenal of *G. mellonella* includes also proline-rich and anionic peptides.^{44,45}

The outlined diversity of antifungal peptides and proteins in *G. mellonella* is indicative for a strong antifungal immunity which is somehow surprising in regard to its prospering use as a surrogate model host for human and insect pathogenic fungi, but fungal species adapted to a parasitic life style have evolved mechanisms to avoid, to suppress or to overcome the antifungal defense of susceptible host insects. Entomopathogenic fungi such as the ascomycetes *B. bassiana* and *M. anisopliae* are capable to dampen induced synthesis of antifungal proteins such as lysozyme during pathogenesis in *G. mellonella*.⁴⁶ The ability to inhibit immune responses of the infected host represents a counter-adaptation of parasitic fungi to hinder antifungal peptides and proteins from reaching concentrations which can arrest fungal development or even kill the fungus within the host. Suppression of cellular and humoral immune responses in infected hosts by parasitic fungi has been attributed to their secondary metabolites, among which cyclic peptides such as cyclosporins and destruxins are prominent. The latter can impair host defense, for example, by induction of apoptosis in immune-competent hemocytes.^{47,48} In agreement, destruxin produced by *M. anisopliae* has been reported to be capable of suppressing immune responses in the fruit fly *Drosophila melanogaster* including expression of immunity-related molecules.⁴⁹ The latter encompass metchnikowin, a

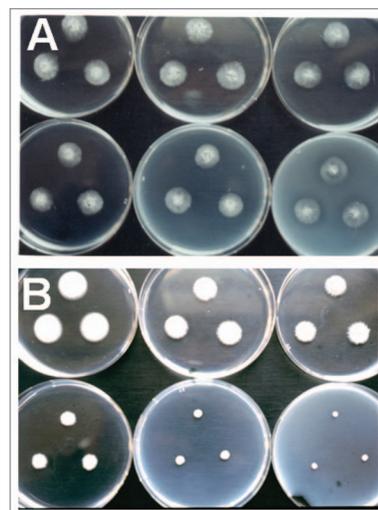


Figure 1. Growth of a virulent strain of the entomopathogenic fungus *M. anisopliae* (A) and a non-virulent strain of *B. bassiana* (B) on agar plates with hen-egg-white lysozyme in increasing concentrations. From the top left to down right: 0 (control without lysozyme), 0.5, 1, 2, 5 or 10 mg per ml agar. The presence of increasing lysozyme concentration caused dimming of the agar which disappeared around the growing mycelia of the virulent strain, indicating the release of proteinases, while growth of mycelia of the non-virulent strain was increasingly inhibited along with rising lysozyme concentrations in the agar, suggesting the inability to secrete proteinases capable of degrading lysozyme. Note that the non-virulent strain is not able to grow on agar with high lysozyme concentrations.

26-amino acid residue proline-rich antifungal peptide which is, in turn, particularly active against ascomycetes to which *M. anisopliae* belongs.⁵⁰ These examples illustrate adaptations of parasitic fungi that enable them to cope with host antifungal peptides either by inhibition of their synthesis or by their degradation.

Fungal Proteinases as Virulence Factors

The proteinaceous exoskeleton of insects forms an efficient primary physical barrier against most microbes. Viruses and bacteria infect their host insects usually upon up-take with the food via the alimentary channel while only parasitic fungi can directly penetrate the cuticle using a set of enzymes among which proteinases have been recognized as virulence factors.⁵¹ The substrate specificity and the controlled expression of invasive proteinases predict that adaptation to varying host ranges drives diversification and functional shifts of these enzymes. *M. anisopliae* produces at least three distinct types of proteinases during growth on insect cuticle: the subtilisin-like serine proteinase Pr1 (EC3.4), the trypsin-like serine proteinase Pr2 and a metalloproteinase.⁵² Trypsins and the subtilisins belong to distinct super-families of serine proteinases which independently evolved similar catalytic mechanisms. The function of these fungal proteinases during pathogenesis can be expanded beyond facilitating penetration of the exoskeleton to include utilization of host proteins for nutrition, suppression of host cellular defense,⁵³ and degradation of host defense molecules.^{4,12}

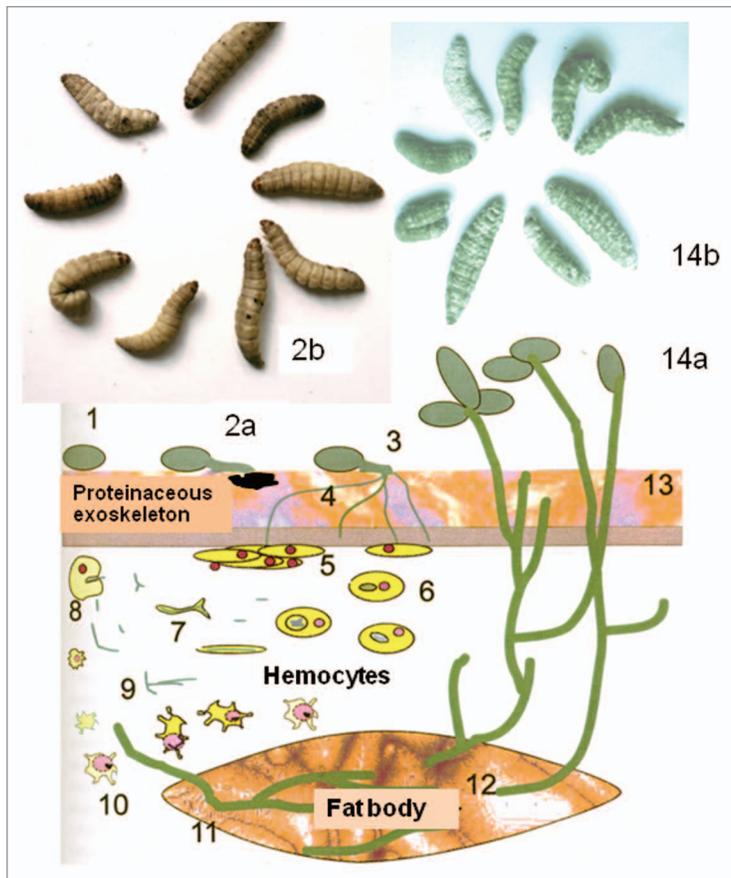


Figure 2. Parasitic life cycle of the parasitic fungus *Metarhizium anisopliae* in the insect model host *Galleria mellonella*. (1) Attachment of conidia on the host cuticle. (2) Germination and appressoria formation is accompanied by secretion of proteolytic enzymes which locally activate phenoloxidase-mediated melanin formation resulting in black spots (2a). This symptom is visible in infected larvae shown above (2b). (3) Penetration of the proteinaceous exoskeleton requires secreted proteinases. (4) Lateral growth and penetration of the epidermis which produces antimicrobial peptides that are subjected to proteolytic degradation. (5) Aggregation of host hemocytes at sites where the fungus penetrates the cuticle. (6) Phagocytosis of hyphal bodies by immune competent hemocytes. (7) Ingested hyphal bodies are not killed within the endocytic vacuole, but grow and multiply within. (8) Simultaneous occurrence of free circulating hyphal bodies and hemocytes exhibiting suppressed ability to ingest fungal cells. Fungal proteases are thought to contribute to evasion of host cellular responses. (9) Fungal cells produce toxins among which destruxin have been reported to induced apoptosis in host hemocytes. (10) Hemocytes undergoing programmed cell death exhibit swollen nuclei, clumped chromatin and formation of blebs at the cell membrane. (11) Infiltration of host tissue requires proteolytic enzymes such as thermolysin-like metalloproteinases capable of degrading extracellular matrix components. (12) Vegetative growth of the fungus in tissues causes death of the infected host. (13) The biomass of the host is totally converted into fungal biomass. (14) Penetration of the host cuticle by hyphae that produce air-borne conidia which can completely cover the cadavers and which permit both long-term persistence in the environment and dispersal (14a). The latter become green powdered due to the green color of the conidia as shown by the picture above (14b).

The functions of proteinases operating as virulence factors of *M. anisopliae* is illustrated in Figure 2 which schematically draws its pathogenesis in caterpillars of the greater wax moth *G. mellonella* as a model host.

Virulent strains of entomopathogenic fungi can grow on culture medium with antifungal lysozyme as the only source of nutrients whereas growth of strains which lost the ability to produce particular proteinases is inhibited by increasing lysozyme concentrations (Fig. 1). Such simple in vitro assays illustrate the essential roles of proteinases during pathogenesis. Mutation or inhibition of such virulence factors hinders the fungus to degrade antifungal peptides and proteins of the infected host and to nourish from its cells, tissues or body fluids. Particularly, fungal metalloproteinases seem to play an important role in overcoming of host defense.⁵³

Most, if not all, pathogens capable of infecting insects or humans produce and secrete metalloproteinases belonging to the M4 family with thermolysin as the prototype. Thermolysin preferentially cleaves at the N-terminal side of hydrophobic or bulky amino side chains such as Leu, Phe, Ile and Val. The vast majority of host proteins and peptides including components of the extracellular matrix exhibiting resistance to proteolytic cleavage are sensitive to degradation by thermolysin. Thermolysin-like metalloproteinases produced by human pathogens such as aureolysin, bacillolysin, pseudolysin and vibriolysin are responsible for many symptoms associated with pathogenesis among which increase of vascular permeability, hemorrhagic edema, sepsis and necrotic tissue destruction are fatal.^{54,55} Thermolysin-like metalloproteinases are also key factors of bacterial and fungal entomopathogens. For example, a novel member of M4 family of metalloproteinases has been found in gram-negative *Photorhabdus luminescens* bacteria which live in symbiosis with parasitic nematodes, and which can kill infected insects when released within the host hemocoel.⁵⁶ *M. anisopliae* produces a thermolysin-like metalloproteinase which has been suggested as a back-up enzyme used in the case that other proteolytic enzymes released by this fungus are inhibited by host proteinase inhibitors.⁵²

Host Inhibitors of Microbial Proteinases

Insect hemolymph contains relatively high concentrations of serine proteinase inhibitors belonging to Kunitz, Kazal, Serpin and α -macroglobulin families among which some have been recognized to function as effectors in innate immunity by inhibition of pathogen-associated proteinases.⁵⁷ Our previous efforts in identification and characterization of immunity-related peptides from *G. mellonella* larvae resulted in the discovery of a number of novel peptidic inhibitors of pathogen-associated proteinases which are simultaneously induced and secreted within the hemolymph during innate immune responses along with antimicrobial peptides. Three heat-stable serine proteinase inhibitors (ISPI-, ISPI-2 and ISPI-3) have been purified from hemolymph whose molecular masses ranged between 6.3 and 9.2 kDa. The determined N-terminal amino-acid sequences provide evidence that one belongs to the Kunitz and another to the Kazal

family of proteinase inhibitors while the third shared no sequence similarity with known peptides.⁵⁸ Inhibitors of the Kunitz and Kazal families are widespread in the hemolymph of arthropods and are likely involved in protecting host from microbial proteinases while also functioning in regulation of endogenous proteinases. Kazal and Kunitz type inhibitors share intra-domain disulfide cross-links determined by six conserved cysteine residues.^{59,60} Interestingly, all three serine proteinase inhibitors purified from immunized *G. mellonella* larvae were found to inhibit the trypsin-like proteinase (Pr2) produced by *M. anisopliae*, and ISPI-3 was also active against the chymoelastase secreted by this fungus.⁵⁸ These findings and our observation that inducible proteinase inhibitors in the hemolymph of *G. mellonella* inhibit both fungal proteases and fungal development in the host lend some credit to our hypothesis that low molecular mass proteinase inhibitors participate in antifungal defense in *G. mellonella* by inactivation of secreted fungal proteinases thereby preventing host defense molecules from degradation.⁶¹

Another inhibitor purified from hemolymph of *G. mellonella* larvae, which have been preinjected with microbial elicitors of innate immune responses to induce expression of defense molecules, turned out to represent the first peptide reported from animals which specifically inhibits microbial metalloproteinases.⁶² The insect metalloproteinase inhibitor (IMPI) is capable of inhibiting thermolysin-like metalloproteinases produced by human pathogenic bacteria making it a promising template for the rational design of a novel second generation antibiotic.⁶³ The determined amino acid sequence of the IMPI revealed no significant similarities with known proteins in NCBI database whereas searching the Pfam database for conserved protein domains implicates a significant similarity to a TIL domain (trypsin inhibitor-like cysteine-rich domain). Therefore, IMPI has been assigned to protease inhibitor family I8, a cysteine-rich trypsin inhibitor-like family in the MEROPS database.⁶⁴ The presence of a conserved domain typical for trypsin inhibitors implicates that the IMPI may originate from serine proteinase inhibitors and the coevolution with pathogens producing metalloproteinases has promoted selection for a corresponding shift in its inhibitor profile.⁶⁵ Because serine proteinase inhibitors are found in a diverse range of organisms and are involved in many physiological processes it is likely that they represent the ancestral form. Recruitment of one class of proteinase inhibitors to regulate another class of proteinases is an evolutionary mechanism that can be readily identified and for which several examples are known.⁶⁵

Another conceivable scenario for the recruitment of host proteinase inhibitors fulfilling immunity-related function is the co-option of molecules whose ancestral role focused on the regulation of endogenous enzymes. For example, Kunitz-type inhibitors from insects seem to exert pleiotropic roles associated either with tissue remodeling during metamorphosis or with antimicrobial defense.⁵⁹ The IMPI mentioned above represents a unique example for an inhibitor with pleiotropic roles in immunity and development. Its gene is expressed during innate immune responses and during pupation when histolysis of larval tissues occurs in *G. mellonella*.⁶⁶ The IMPI gene encodes two distinct inhibitors resulting from posttranslational cleavage by

furin-like proteinases. IMPI-1, encoded by the N-terminal part of its gene, corresponds with that purified from hemolymph of immunized *G. mellonella* larvae and inhibits thermolysin-like microbial metalloproteinases, whereas IMPI-2, encoded by the C-terminal part of its gene, is inactive against thermolysin, but exhibits activity against matrix metalloproteinases. The sequence similarity between IMPI-1 and IMPI-2 implicates their ancestral origin from a common gene or domain.⁶⁷ Based on our findings, I postulated an endogenous matrix metalloproteinase (MMP) as a putative target for IMPI-2 which has subsequently been identified and characterized.⁶⁸ Strikingly, this MMP turned-out to fulfill also pleiotropic functions in development and immunity. Similar to microbial thermolysin, the endogenous *G. mellonella* MMP has been shown to be capable to degrade collagen type IV resulting in formation of peptidic fragments which, in turn, induce expression of the IMPI and other antimicrobial peptides.⁶⁹ Sensing and feedback-loop regulation of microbial metalloproteinases has become a constituent of insect innate immunity. However, because the insect immune system can obviously not discriminate whether microbial or endogenous metalloproteinases degrade host proteins such as collagen IV, it makes sense to produce simultaneously two inhibitors with distinct activities.⁶⁷ Most interestingly, *M. anisopliae* has been discovered to produce a collagenous coat which makes it unattractive for host hemocytes and which has, therefore, been hypothesized to participate in evasion of host immune responses.⁷⁰ Whether the collagenous fibers extending from *M. anisopliae* cells can be degraded by the MMP of activated *G. mellonella* hemocytes, or whether they may also serve to block this hemocyte-associated host proteinase to limit the synthesis of danger signals which, in turn, elicit antifungal immune responses remains to be elucidated. However, these findings provide a new framework to reassess the coevolution between pathogen-associated proteinases and host proteinase inhibitors because their interactions are more complex than previously thought and must be interpreted in the context with host proteinases and pathogen-associated substrates which are also involved in the molecular arms race.

Coevolution Between Fungal Proteinases and Host Proteinase Inhibitors

The parasitic life has arisen and also lost multiple times in many independent lines of fungal evolution. Interestingly, there is also evidence for interkingdom host-jumping by parasitic fungi from plants to insects.⁷¹ Adaptation of fungal populations to different hosts has been suggested to drive sympatric divergence of parasitic fungi. In parasite-host associations, speciation in the host can lead to speciation of the parasite,⁷² but this is obviously not the case in entomopathogenic fungi infecting a broad host range such as *B. bassiana* and *M. anisopliae*. The evolution of a parasitic life style depends on the availability of enzymatic virulence factors which mediate utilization of the host as a source of nutrients and which can degrade its defense molecules. Particularly, genes encoding fungal proteinases should provide a good model to study adaptive evolution of multigene families and its impact on speciation.⁷³ For example, evidence has been elaborated that

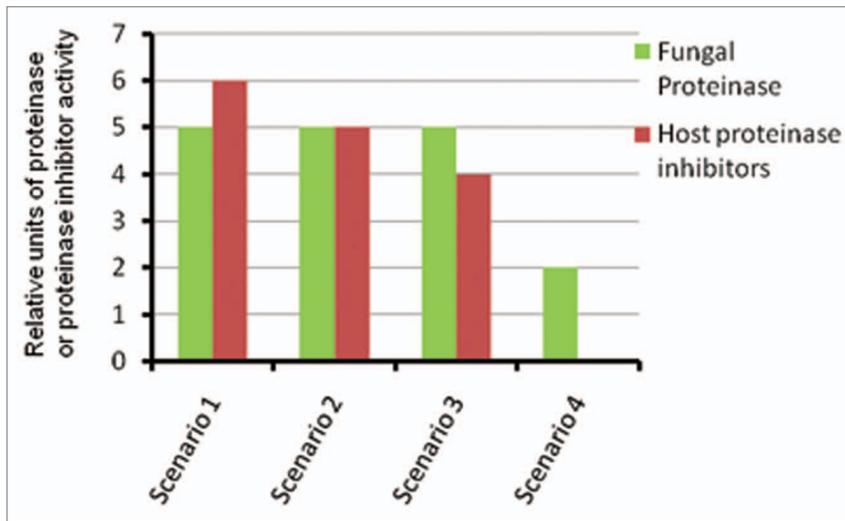


Figure 3. Interdependencies between fungal proteinases and insect host proteinase inhibitors. Parasitic fungi produce and secrete an array of proteinases, but only those that are either partially (scenario 3) or not inactivated (scenario 4) by corresponding proteinase inhibitors present in host cuticle, tissues or hemolymph can operate as virulence factors and complement each other in degradation of host peptides and proteins including defense molecules. As a result, there is a negative selection for proteinases for which a surplus of corresponding inhibitors is present in the hosts (scenario 1 and 2) because secretion of such enzymes would waste resources. On the other hand there is a positive selection for proteinases for which no inhibitors are present in host insects. Their secretion, even in low quantities (scenario 4), is sufficient to fulfill virulence-associated functions.

multiplication of an ancestral proteinase gene seems to precede species differentiation in parasitic fungi.⁷⁴ Because pathogen virulence is thought to coevolve as a result of reciprocal selection with its host, it can be postulated that positive selection exists for the evolution of novel proteinases or proteinase isoforms which are not inactivated by proteinase inhibitors of the host. This hypothesis regarding coevolution between proteinases and proteinase inhibitors in an antagonist system can be described as possible scenarios illustrated by the bars in **Figure 3**.

Being aware that relationships between proteinases and their inhibitors can be both more or less specific and determined by different types of kinetics, the graph has been simplified by showing proteinase or proteinase inhibitor activities in virtual but equivalent units. In the case of scenario 1 a proteinase which is produced in high quantities like, for example, the trypsin-like Pr2 of *M. anisopliae*, the activity of inhibitors of trypsin-like proteinases determined in high concentrations in the cuticle and hemolymph of many insects including *G. mellonella* can be higher.^{57,58} Consequently, secreted proteinase 1 cannot execute essential functions during pathogenesis. This is also the case when the activity of secreted microbial proteinases and that of host proteinase inhibitors is equivalent under in vivo conditions, as illustrated by scenario 2. But when the activity of the secreted fungal proteinase is not completely inhibited by host proteinase inhibitors, only the small portion of activity which is not regulated within the host (see scenario 3) permits limited nourishment from its peptides and proteins. If the host lacks inhibitors of a particular pathogen-associated proteinase such as in scenario

4, the latter will become the most important virulence factor although its absolute concentration or activity, respectively, can be remarkably lower than that of the other proteinases (1–3). Thermolysin-like metalloproteinase may represent microbial enzymes for which inhibitors are lacking in many insects since only the IMPI from *G. mellonella* has been reported to specifically inhibit members of the M4 family of proteinases.^{61,67} It can be expected that the activities of partially inhibited (scenario 3) and non-inhibited microbial proteinases (scenario 4) will complement each other in fulfilling the above mentioned multifaceted roles during pathogenesis. If the outlined scenarios reflect the complex host-pathogen relationships under natural conditions, we can postulate a strong diversifying selection on pathogen-associated proteinases. Indeed, all three proteinase types produced by *M. anisopliae*, the subtilisin-like serine proteinase Pr1, the trypsin-like serine proteinase Pr2, and the thermolysin-like metalloproteinase, occur in a variable number of isoforms which determine host range of different strains.^{73,75,76}

On the other hand we can predict loss or at least controlled expression of genes encoding secreted proteinases that are mostly inhibited by host molecules because the resources of bacterial or fungal pathogens are smaller than those of the infected host which has usually a remarkably higher biomass. Secretion of proteinases whose activities match that of host proteinase inhibitors would waste resources. Indeed, loss of proteinase encoding genes has recently been determined in host-specific strains of *M. anisopliae*.⁷⁷ Furthermore, differential expression of proteinases by *M. anisopliae* growing on different substrates such as either host cuticle or hemolymph may also be interpreted as physiological adaptation to host proteinase inhibitors.⁷⁸ In conclusion, hosts drive expansion of pathogen-derived proteinase diversity which is then subjected to selection.

A phylogenomic approach to reconstruct the diversification of serine proteinases in fungi revealed evolutionary selection of one gene family of proteinases over another in ascomycete fungi to which *M. anisopliae* belongs. Tandem duplication of Pr1 and expansion in subsets of subtilisin-like enzymes in pathogenic fungi may reflect adaptations to host ranges⁷⁵ while no comparable diversification of trypsin-like proteinases was found. The latter may confer only in those fungi selective functions that express them in high levels and possibly pre-adapt them to a parasitic life style.⁷³ A recent study provides evidence for the expansion of the thermolysin-like metalloproteinase in *M. anisopliae*. The isoforms found in hydrated conidia differed in their ability to digest gelatin which can be interpreted as adaptation to target proteins of the host.⁷⁶

In turn, diversifying pathogen-associated proteinases can be expected to cause selection for mechanisms allowing reciprocal

diversification of corresponding host proteinase inhibitors. This is in agreement with the discovery of a number of serine proteinase inhibitors in *G. mellonella* capable of inhibiting serine proteinases produced and secreted by *M. anisopliae*.⁵⁸

Interestingly, pyrosequencing of the immunity-related transcriptome from *G. mellonella* led to the discovery of a number of IMPI homologues (work in progress). Whether the recently identified isoforms of thermolysin and the homologues IMPI peptides have emerged during reciprocal diversification is presently examined. Many, if not most, proteinase inhibitors occur as small gene families with altered specificities among the paralogues. The evolutionary mechanisms acting on proteinase inhibitor variability of the host are apparently the same providing diversification of its antimicrobial peptides. Recruitment of other protein-folding scaffolds to proteinase inhibitor function, gene or domain duplication and shuffling are probably the most important forces driving proteinase inhibitor evolution.^{65,79,80}

Beyond diversification of pathogen or host derived effectors there are also other features which argue for coevolution between pathogen-associated proteinases with host proteinase inhibitors in insects. Because non-specific degradation of host molecules by microbial proteinases results in life-threatening loss of functional proteins and accumulation of non-functional peptidic fragments I predicted an efficient mechanistic countermeasure in insects. Experimental analysis led to the discovery of a mechanism in our model host *G. mellonella* that enables sensing and feedback loop regulation of microbial metalloproteinases, and which can be, therefore, considered as a counter-adaptation to the widespread utilization of thermolysin-like enzymes as virulence factors among different groups of pathogens.⁸¹ The presence of thermolysin in the hemolymph alone is sufficient to elicit humoral immune responses in *G. mellonella* that are qualitatively (spectrum of induced and secreted immunity-related peptides and proteins) and quantitatively (expression levels of antimicrobial peptides) comparable with those observed upon challenge with bacterial or fungal cells, or cell wall components, respectively.⁸² As mentioned above, the IMPI capable of inhibiting thermolysin is induced and secreted into the hemolymph during humoral immune responses along with other proteinase inhibitors and antimicrobial peptides. The immune system senses the presence of thermolysin-like metalloproteinases by particular products of their activity. Pathogens employ thermolysin to breakdown the extracellular matrix of host cells and tissues during pathogenesis. Particular peptidic fragments resulting from hydrolysis of collagen IV turned out to represent danger signals which, in turn, can set the immune system into alarm.^{66,69}

Thermolysin is also a potent activator of the enzyme cascade that controls phenoloxidase activity which catalyzes the formation of melanin. Hemolymph coagulation resulting in

hemolymph clots is a first response to wounding in insects and phenoloxidase has been shown to shape the clot's physical properties by crosslinking of proteins and melanization. Entrapping of bacteria within the clots alone is not sufficient to kill them and requires bactericidal compounds among which some occur as intermediates during synthesis of melanin.⁸³ Because melanization of entrapped bacterial or fungal cells represents an efficient defense mechanism in insects, adapted pathogens should avoid excessive melanization caused by their secreted microbial metalloproteinases. Indeed, as a counter-adaptation to efficient mechanisms mediating both sensing of microbial metalloproteinases and activation of immune responses against their producers, entomopathogenic bacteria and fungi tightly regulate the activity of thermolysin. The mature enzyme can be inhibited by its propeptide.⁸⁴ A fine-tuned timing of enzyme production has been documented by analysis of gene expression during germination, pathogenesis and conidiogenesis and of the parasitic fungus *M. anisopliae*.⁸⁵ Secretion of thermolysin in order to degrade extracellular matrix proteins and to colonize host tissues occurs in a later stage of mycosis when host hemocytes undergo apoptosis initiated by released destruxins. At this phase of infection, the insect hosts occur moribund and are not able to mount an immune response sufficient to protect them from death.^{4,12,86} These findings add to our knowledge about molecular mechanisms that pathogens and parasites use in evasion of host immunity.

Pathogenic mechanisms that manipulate host immunity or escape from host defense are sensitive to parasite fitness and thus dominate as causes of parasite virulence.⁸⁷ Theory predicts that adaptation of *M. anisopliae* and other pathogens to a broad host range should be accompanied by rapid diversification of genes involved in an arms race with multiple hosts, while adaptation to particular host species should promote loss of genes lacking selection pressure because they are not required for infection of a limited host range. Comparative genomic analyses of *M. anisopliae* strains with either broad or narrow host ranges have recently confirmed this hypothesis.⁸⁸ However, identifying coevolving partners from paralogous gene families remains to be elucidated and recent bioinformatic tools will help in the near future to more precisely reconstruct coevolution between pathogen-associated proteinases and host proteinase inhibitors.^{89,90}

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