

**The role of a serralyisin PrtA system in the
infection mechanism of an entomopathogen,
*Photorhabdus***

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3. Abbreviations

BA	Benzamidin
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
Bt	<i>Bacillus</i> toxin
CAPS	3-cyclohexylamino-1-propanesulfonic acid;
Dabcyl	4-(4-dimethylaminophenylazo)benzoic acid;
DTT	1,4-dithiothreitol;
Edans	5-[(2-aminoethyl) amino] naphtalene-1 sulfonic acid;
EDTA	ethylenediaminetetraacetic acid;
FuaALGPA	3-(2-furil)-akrilolil-Leu-Gly-Pro-Ala-OH
IJ	infective juvenile;
mPBS	modified phosphate-buffered saline;
NCBI	National Center for Biotechnology Information
PAT	PrtA substrate
PBS	phosphate-buffered saline;
PAGE	polyacrylamide gel electrophoresis;
PBS	phosphate-buffered saline;
Php	<i>Photorhabdus</i> protease
Pht	Photorhabdus toxin
PMSF	phenylmethylsulfonyl fluoride;
PrtA	protease A;
Ptc	phenylthiocarbamide;
PVDF	polyvinylidene fluoride;
Rtx	Repeats in ToXin;
SDS	sodium dodecyl sulfate;
SPH-3	serine protease homologue 3
Succ.	succinyl
Tc	toxin complex
TRI reagent	guanidine-isothiocyante reagent
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol

4. Introduction

Pathogenic microorganisms are causative agents in many human diseases and death and they also cause losses in agriculture by infecting plants and animals. Each species of pathogens has a characteristic spectrum of hosts. The exploration of the molecular interactions which determine virulence is important to understand host specificity and might be instrumental in the prevention and treatments of microbial infections.

Virulence factors of microorganisms belong to either microbial toxins or microbial enzymes. Secreted enzymes may function as virulence factors which are essential for survival and spread in the host. Among these enzymes, proteases can neutralize the host's defense systems by different ways. However, the roles of only few of these enzymes in the pathomechanism have been documented. For example, inhibitor A of *Bacillus thuringiensis* and the proteases of *Serratia marcescens* specifically cleave insect immune proteins such as cecropins and attacins, while the zinc metalloproteases of *Bacteroides fragilis* and *Clostridium* spp. have direct toxin activity [1, 2]. ZapA, which is an extracellular metalloprotease of the uropathogenic *Proteus mirabilis*, cleaves human immunoglobulins and antimicrobial peptides associated with the innate immune response [3]. Other function of secreted enzymes during infection includes invasion of the host and bioconversion of its tissues for nutrient supply of the growing microorganism. Production of inhibitors as a component of the immune response against the bacterial enzymes can provide protection for the host.

Proteases function in proteolytic systems. A proteolytic system consists of a protease, as well as its natural substrate(s) and its natural inhibitor(s). The full understanding of the physiological role of proteases can be reached only via the knowledge of the whole proteolytic system in which they participate. Since this is very difficult it is not surprising that such systems of neither protease of the pathogens is known with the exception of the mentioned substrate proteins and several inhibitors of pathogen origin. My PhD work is part of research project which is aimed to explore the role of the proteolytic system of a serralysin in *Photobacterium luminescens* infection mechanism.

4.1 Why an insect-pathogen infection model

One of the main factors in the successful examination of biological processes is the selection of a relevant model system. The mammalian infection models of mouse and rat are such since they share high similarity with the human body although their maintenance is expensive and their experimental use is limited by ethical reasons. Therefore, insect models are being developed for e.g., the studies of pathomechanism, because these have the advantages of low cost, genetic and physiological malleability and the lack of ethical problems involved in studying mammals [4]. On the other hand the observations in insect systems can be instructive for studies in vertebrate-pathogen bacteria systems, because many innate immune mechanisms are conserved throughout the animal kingdom [5]. For example, in adult *Drosophila melanogaster*, homologues of the transcription factor NF- κ B are activated upon bacterial invasion in a process mediated by the TOLL family of receptors similarly to immune cells in mammals which induces the expression of defense genes [6, 7]. Studying bacterial – insect interactions can also have agricultural significance through the development of environment-friendly crop-protection technologies based on insect specific microbial toxins and virulence strategies [8].

4.2 *Manduca sexta*: as the host in an infection model system

Manduca sexta (Tobacco hornworm, Lepidoptera) has been widely used as a model for insect biochemical research due to its size and hemolymph volume: the last instar larva reaches 10–12 g, and 1–2 mL of hemolymph (with approximately 10^6 hemocytes) can be collected from it. This species is also easy to rear in the laboratory. Thus it is well suited for studies of hemocytes, hemolymph proteins and their interactions [4, 5]. At the same time *M. sexta* larvae are sensitive to *Photorhabdus luminescens* infection, so they are perfect model to study the pathogenic mechanism and the interaction of the *Photorhabdus* virulence factor(s) and the insect immune system, despite the fact that *M. sexta* lacks the genetics of *Drosophila*.

4.3 Defense system of insects

Metazoans have developed efficient mechanisms to eliminate microbial invaders. Innate immunity is common to all metazoans and serves as the first-line of defense. It consists of recognition of microorganisms by receptors, and rapid effector

mechanisms that involve phagocytosis, nodule formation, and encapsulation, activation of proteolytic cascades and synthesis of potent antimicrobial peptides. The adaptive immune system, as the second line of defense, is restricted to some 45,000 vertebrate species, and involves the complex repertoire of immune receptors in lymphocytes through somatic gene rearrangement and clonal expansion of activated lymphocytes which endow memory. Since insects lack adaptive immunity, they are excellent model organisms for studies of the innate immune system [5, 9, 10]. This system comprises both cellular and humoral reactions (**Fig. 1**).

4.3.1 Immune recognition

Recognition of pathogens is the first step of the immune response which is mediated by pattern recognition proteins (PRP) such as Hemolin (HEM), Peptidoglycan recognition protein (PGRP), Immulectin-2 (IML-2), Pattern recognition serine proteinase (PRSP) and β -1,3-glucan recognition proteins (β GRP-1 and β GRP-2) [5, 11]. These proteins bind to conserved pathogen-associated molecular pattern (PAMP) molecules such as peptidoglycan, lipopolysaccharide, lipoteic acid and β -1,3-glucan present on the surface of bacteria and fungi but not on the host cells, and trigger – after a shorter or longer process of signal transduction - a protective response directly or indirectly through the induction of antimicrobial genes [11, 12].

4.3.2 Cellular immune response

The initial cellular response to wound or invaders such as bacteria, fungi or nematodes is mediated by circulating hemocytes, which are efficient in eliminating particles by either phagocytosis, nodule formation or encapsulation. Phagocytosis is the primary defense mechanism against number of bacteria below a certain threshold level. When this threshold is surpassed, phagocytosis is augmented by nodule formation, whereby both hemocytes and bacteria become entrapped in an extracellular matrix [13]. The resulting large cellular aggregates adhere to tissues, leave circulation and become melanized. Around foreign objects which are too large to be phagocytosed (e.g., parasites, nematodes and mature nodules) the hemocytes initiate a multilayered cellular capsule, a process called encapsulation [14]. Melanin deposits (see in humoral immune response) form in the inner layers of the capsule that restricts growth and movement of the offending organism and may result in its death (cellular melanotic encapsulation). Two classes of hemocytes, the plasmatocytes and granulocytes (the primary

phagocytes) participate in these processes. The cellular encapsulation around foreign organisms starts with granulocytes, followed by multiple layers of plasmatocytes, and ends with a single layer of granulocytes [12].

4.3.3 Humoral immune response

The humoral responses of the insect immune system include melanization, clotting of hemolymph and expression of genes encoding recognition proteins and antimicrobial peptides.

An important component of both the cellular and humoral arms of the immune response is the immune initiated synthesis of melanin by phenoloxidase. Recognition of parasites or pathogens by pattern recognition receptors triggers a serial activation of mostly unknown serine proteinase pathways, which are probably components of a network. One such, not fully known pathway in this system leads to the activation of prophenoloxidase- activating proteinase (PAP). PAP, also known as prophenoloxidase activating enzyme (PPAE), is the terminal proteinase that converts inactive prophenoloxidase (proPO) to active phenoloxidase (PO) [15]. PO catalyzes the oxidation of phenols or diphenols to quinones, which then will polymerize non-enzymatically to melanin, a toxic compound to microorganisms [16]. The quinone intermediates may also participate in cuticle sclerotization, wound healing and killing of parasites or pathogens entrapped in capsules during the cellular immune response.

Due to these important physiological functions, PO and proteolytic activation of proPO have been investigated in many insects for more than three decades [17]. Our understanding of this serine proteinase cascade is still incomplete, because the number of proteolytic steps in the pathway is uncertain. The serine proteinases that directly activate proPO (PAPs) have been isolated and cloned from several arthropod species including *Manduca sexta*. Three PAPs from *M. sexta* are termed as PAP-1, PAP-2, PAP-3 [18, 19, 20]. These serine proteases contain one or more clip domains. Clip domains are 37–55 amino acid residue sequences consisting six highly conserved cysteine residues forming three disulfide bonds. This structural unit is widely found in arthropod serine proteinases and serine proteinase homologues. The clip-domain could be a site for interactions of a proteinase with its upstream activator, its downstream protein substrate, or a co-factor that regulates the enzyme's activity [21]. PAPs are synthesized as inactive zymogens and activated by other clip-domain serine proteinase(s) upstream in the pro-PO cascade pathway (**Fig. 1**). The active PAPs are

regulated by specific inhibitors, because the quinons and reactive oxygen species generated by uncontrolled spread of PPO activation would be harmful to the insect (see later) [22].

Although some of the molecules of the immune protease network have been found and characterized at molecular level, further constituents are supposed, and the order and regulation of activity of the known proteinases are still poorly understood. Our current knowledge is mainly limited to pathogen recognition, the activation of the initial protease(s) and PPO activation. Two serine protease inhibitors, serpin-4 and serpin-5, were used as probes to detect proteases that are activated upon exposure of plasma to bacteria. It was found that they form complex with Hemolymph Proteinase -1 and -6 (HP-1 and HP-6), which are proteases upstream of PAP-s. The formation of complex between the serpins and HP-1 and HP-6 blocked PPO activation [23]. In another study seven known (HP1-HP4, PAP-1, PAP-2 and PAP-3) and 18 unknown serine proteinases (HP-5-HP-22) were found in *Manduca sexta* fat body or hemocytes cDNA library from larvae using degenerate primers, encoding two conserved regions in S1 family of serine proteinases [24]. 15 proteinases have a pro-region longer than 90 residues, and nine of these contain 1 or 2 (regulatory) clip domains. HP-14 is the only one with many modular structures such as five low density lipoprotein receptor class A repeats, a Sushi domain, a unique Cys-rich region, and a serine proteinase-catalytic domain making it similar to complement/MASP of mammals [25]. HP-14 exists in the hemolymph as an inactive zymogen, pro-HP14, as a pattern recognition protein. It was observed *in vitro* that pro-HP14 binds to β -1,3-glucan and β -GRP-2 and autoactivates [26]. It was shown also *in vitro* that HP-14, generated by incubating proHP-14 with beta-1,3-glucan and beta-1,3-glucan recognition protein-2, activated proHP-21 by limited proteolysis. ProHP-21, a 51.1 kDa glycoprotein, contains an amino-terminal clip domain, a linker region, and a carboxyl-terminal serine proteinase domain. Active HP-21 cleaved PPO activating proteinase-2 precursor (proPAP-2), which activated PPO in the presence of serine proteinase homolog-1 and 2 [27]. Thus, HP-14 activation is the initial step of a PPO activation cascade, which represents the first fully elucidated pathway in the immune proteinase system [26]. This mechanism is similar to the lectin-mediated pathway for complement activation in mammals where mannose-binding protein and mannose-binding protein-associated serine proteinase (MASP, which has similar structure to pro-HP14) trigger serine proteinase cascade [26, 28]. These similarities in the PPO activation pathway in arthropods and the complement system in

vertebrates also shows that proteinase autoactivation to trigger innate immune response is an ancient evolutionary adaptation for defense against infection [28].

It was found in *Manduca sexta* that PAP-2 needs cofactor(s) to activate PPO, which are two serine protease homologues (SPH-1 and SPH-2) [15, 17, 29]. These associate with PAP and immulectin-2, a calcium dependent lectin that binds to microbial lipopolysaccharide, forming a PPO activation complex, which – in this case – contains IML-2, SPH, PAP, and PPO. These protein interactions may function to localize proPO activation to the surface of pathogens or the site of entry for microbial infections, and ensure a high local concentration of the quinone products of PO reaction [15]. Serine protease homologues (SPHs) may function in this complex as recruiters of PAP and PPO to the surface of pathogens or parasites.

SPHs contain an amino acid sequence that is clearly related to the chymotrypsin family of serine proteinases, but the active site serine is replaced by a glycine residue resulting in the loss of proteolytic activity. Numerous SPHs have been reported in both invertebrates and vertebrates, but the physiological function of most of them remained unknown. Many SPH-s contain an amino-terminal “clip” domain, like many active serine proteinases in the hemolymph (see above) [15, 30]. Genome projects have revealed that insects have large numbers of SPH genes. For example, *Drosophila melanogaster* has no fewer than 63 genes encoding predicted SPHs [30] including the well studied example, *masquerade* [31]. Masquerade-like SPHs, which include *Manduca sexta* SPH-1 and SPH-2, have an N-terminal clip domain, and appear to play a variety of roles in development and immunity [32, 33, 34]. Most of the SPHs lack the clip domain. In *Drosophila* there are 47 such SPHs compared to only 16 SPHs with clip domains [30]. In addition to the role of SPH-1 and 2 in *M. sexta* (above), clip domain containing SPHs are important regulators of immune responses in other insects and in invertebrates. They are involved in antimicrobial response in horseshoe crabs (*Limulus polyphemus*), pattern recognition in a crayfish, somatic muscle attachment in *Drosophila* embryos, and immune responses in the mosquito *Anopheles gambiae* [33, 35-37]. Clip domain SPHs in a coleopteran insect, *Holotrichia diomphalia*, the beetle *Tenebrio molitor*, and the crab, *Callinectes sapidus* are proposed to have similar role in PPO activation to that of SPH1 and 2 of *M. sexta*, while a clip-domain containing SPH in the venom of a hymenopteran parasitoid, *Cotesia rubecula* inhibits PPO activation in the hemolymph of its lepidopteran host [38-42]. Other known immune-related functions of invertebrate clip domain-containing SPH-s include, cell adhesion (*Penaeus*

inonodon, *Pacifastacus leniusculus*) and opsonisation (*Pacifastacus leniusculus*) [36, 43-45]. In contrast to clip-domain SPHs, the role of the invertebrate non clip-domain SPHs is as yet unknown. An immune related function of three such proteins is only inferred from their enhanced expression during microbial infections. These are SPH-3 in *Manduca sexta*, an azurocidin-like protein in *Trichoplusia ni* (another lepidopteran), and ISP15 in the mosquito *Anopheles gambiae* but the functions of these proteins remain unknown [37, 46]. A range of as yet ill-defined roles played by mammalian SPHs without clip domains involves hemoglobin scavenging, regulation of Langerhans cell function and toxicity to *Plasmodium falciparum* trophozoites by haptoglobin, suppression of dendritic cell function by hepatocyte growth factor, as well as both signaling and antimicrobial activity by the human neutrophil granule protein, azurocidin [47- 52].

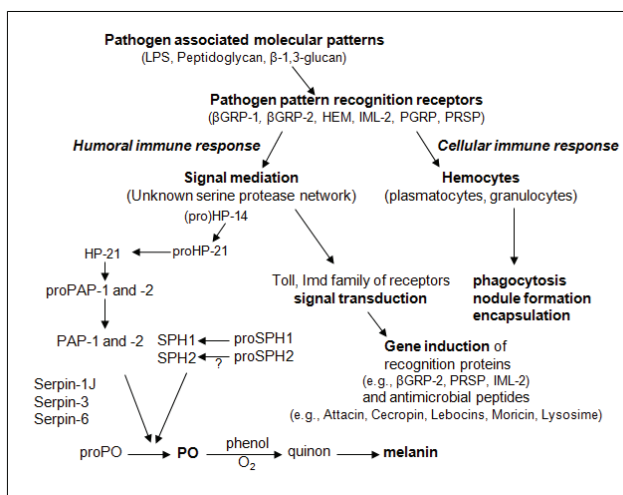
Because quinones and melanin are toxic to both host tissues and pathogens, the conversions of PPO to PO and PO activity have to be kept local to the place of infection. The mechanism which is responsible for this includes a system of interacting proteins (e.g., pattern recognition receptors, serine proteinases, serine proteinase homologs/SPHs, serine proteinase inhibitors/serpins, and pro-PO) [17, 53]. Candidates for regulators of PAPs are members of the serpin superfamily, the mammalian counterparts of which are known to regulate proteinases involved in inflammation, blood coagulation, and complement activation [5, 22, 54]. Serpins have been purified from arthropods too, (*Bombix mori*, *Manduca sexta*, *Aedes aegypti*, *Mythimna unipuncta*, and *Tachypleus tridentatus*). They regulate a number of biological processes including hemolymph coagulation, proPO activation, and induced synthesis of antimicrobial peptides. There are nearly 30 serpin genes in the *Drosophila melanogaster* and 15 ones in *Anopheles gambiae* [55]. Serpins are 370-390 amino acid residue proteins with a reactive site loop 30-40 residues from the carboxyl terminus [56]. Serpins are irreversible covalent 'suicide' protease inhibitors [57]. When a susceptible proteinase begins to cleave a specific bond (designated the P1-P1' bond) in the reactive site loop, the serpin undergoes massive and irreversible conformational changes, in which the exposed reactive site loop sequence inserts into a β -sheet of the protease [5, 57]. In *M. sexta*, six serpins have been identified so far [56, 58-60]. The major serpin in the hemolymph of naive *Manduca sexta* larvae is the serpin-I, which is actually a mixture of 12 variants (serpin-1A through -1K and -1Z), produced from a single gene with alternative splicing [61]. These proteins are identical in sequence,

except for a region encoding the carboxyl-terminal, reactive site loop containing 40-46 residues [59]. The serpin-1 gene is composed of ten exons, with 12 alternate version in the ninth [56]. It was shown by biochemical analysis of the recombinant serpins produced by *E. coli* that the variation in reactive site loop sequences results in differences in protease selectivity [61]. One of the serpin variants, serpin-1J can efficiently block activation of *M. sexta* prophenoloxidase by the inhibition of PAP-3 [20]. To date, the physiological targets of the other 11 variants from serpin gene-1 are unknown. Another serpin of *M. sexta*, serpin-3, also blocks PPO activation by inhibiting PAP-1 and PAP-3 [22]. The biochemical analysis of serpin-6, which was recently cloned from *M. sexta* showed that this recombinant serpin also inhibits PAP-3 efficiently [54]. Two new immune-responsive serpins, serpin-4 and serpin-5 have been identified. They are able to inhibit PPO activation to different degrees but are not efficient inhibitors of PAPs, suggesting that they inhibit serine proteases upstream of PAPs in the activation cascade [2].

Serine proteinase cascade(s) also appears to lead to the activation of genes encoding antimicrobial proteins and small, cationic peptides. The major site of the synthesis of immune-induced antimicrobial peptides is the fat body (equivalent of the mammalian liver) and they are secreted into the hemolymph where they act synergistically to kill invading microorganisms [62]. Several distinct inducible antimicrobial peptides (or peptide families) have been identified in insects. Their activity spectra are directed either against fungi such as Drosomycins, Metchnikowin or against Gram positive bacteria such as Defensins or against Gram negative bacteria such as Attacins, Cecropins, Lebocins, Drosocins, Diptericins, Moricins, Lysozyme. It is assumed that their combined activities largely contribute to the blocking of bacterial growth in the hemolymph [62, 63]. The intracellular regulation and activation pathways of these antimicrobial genes are intensively studied mainly in *Drosophila melanogaster* due to its known genome sequence. These studies suggest that intracellular immune signaling uses components which are similar, sometimes identical to those involved in *Drosophila* embryogenesis, such as the Toll and Imd receptor families [9]. In contrast, these studies have not revealed yet any of the extracellular components of the immune signaling pathways. It can only be inferred that proteases, which are similar to Easter, Snake (clip-domain serine proteinases), Gastrulation defective (a non-clip-domain serine proteinase) and Spätzle (Toll ligand) might participate in them on the grounds that these proteins form the signaling pathway to Toll receptor. Experiments also

revealed the fact that signalization in the innate immunity contains evolutionary conserved elements throughout the animal kingdom: the activation of Toll-like receptors – IL-1 receptor is a pathway leading to the induction of the antimicrobial peptide genes in mammals [9].

Figure 1. Insect innate immunity based on [5, 64]. For details see text. Abbreviations: LPS, lipopolysaccharide; HEM, hemolin; IML-2, immunlectin-2; PGRP, peptidoglycan recognition protein; PRSP, pattern recognition serine proteainase; β GRP-1, β GRP-2, β -1,3-glucan recognition protein-1 and -2; proPAPs, zymogen of prophenoloxidase- activating proteinase; PAP, prophenoloxidase- activating proteinase; proSPHs, precursor of serine proteinase homologues; SPHs, serine proteinase homologues (SPH-1 and SPH-2); ProPO, prophenoloxidase; PO, phenoloxidase;



4.4 *Photorhabdus* as the ideal pathogen in an infection model system

Photorhabdus is Gram negative bacterium species in the family of *Enterobacteriaceae*. It is highly virulent and toxic against insects. Such properties are very important in studies of infection mechanisms because they offer a stable way of generating infection. Also the infection can be relatively easily investigated in the laboratory on *Lepidopteran* host models [4]. This bacterium is a close relative of the human pathogen *Yersinia pestis* the cause of plague. As revealed by genomic analysis,

they share not only the chromosomal backbone of *Enterobacteriaceae*, but also many putative mobile regions encoding virulence factors and proteins of unknown function [65]. Thus, investigation of the *Photorhabdus*-insect interaction might be informative for other pathogens, like *Yersinia* and *Serratia entomophila*, the causative agent of “amber” disease in larvae of New Zealand grass grub [66, 67]. Moreover, some strains of the genus *Photorhabdus* are capable of colonizing humans: in a few cases, as causative agents, *Photorhabdus asymbiotica* were found in human infections [68]. *Photorhabdus* strains are intensively studied for their strong virulence and interesting symbiotic life, as well as for their exploitation in the control of agricultural pest. *Photorhabdus* can be a source of useful genes for transformation of crops. For example, the gene encoding toxin A which is highly toxic to a variety of insects, including some agriculturally important pests was successfully introduced in *Arabidopsis thaliana* to generate the first transgenic plant containing *Photorhabdus* genes [69-71].

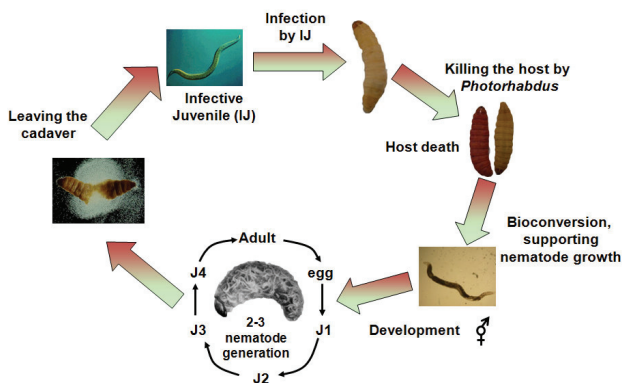
4.4.1 *Photorhabdus* lives in symbiotic complexes with nematodes in the nature

Photorhabdus forms a mutualistic symbiosis with entomopathogenic nematodes of the family *Heterorhabditidae*. The *Photorhabdus*-*Heterorhabditis* complex has a three-stage life cycle (Fig. 2). In the first symbiotic stage, the free-living infective form of the nematode, called the infective juvenile (IJ) carries *Photorhabdus* bacteria in its gut and actively seeks out for insect hosts. In the second, pathogenic stage, in which susceptible insect host is killed by the combined action of the nematode and the bacteria [66, 72]: the nematode gains access to the hemocoel of the insect via the respiratory spiracles or digestive tract [73]. Upon reaching the insect blood system nematode regurgitates the bacteria into the hemolymph. This is the service of the nematode for the bacterium, which - in itself - is unable to invade the hemocoel and infect the insect. Within the hemocoel, the bacteria first defeat the immune system then start multiply and kill the host in most of the cases. In the third, replicative/multiplicative stage the bacterium cells replicate rapidly and start converting tissues of the cadaver into bacterial biomass using mixture of hydrolytic exoenzymes (bioconversion). The cell density approaches the stationary-phase of culture. Uncharacterized bacterial signals then stimulate nematode development and the IJ converts into a self-fertile hermaphrodite. The hermaphrodites lay eggs that develop, through juvenile larval stages J1–J4, into male and female nematodes which feed on the bacterium. Thus, only a single infective juvenile is enough to establish a nematode

culture in the cadaver of the insect host. Nematode growth and development continues for two to three generations until the cadaver as a nutrient source has been exhausted. Then, the J2 juvenile nematodes, probably stimulated by environmental factors, develop into the specialized IJ stage meanwhile reassociate with their symbiotic bacterium partner [74]. When IJ carries the bacteria in its intestinal tract, then emerges from the insect carcass to search and colonize a new insect host.

Photorhabdus maintains suitable conditions for nematode grow and reproduction. These are optimal, when the natural symbiont bacterium partner dominates the microbial flora. Therefore, *Photorhabdus* not only has to produce different insecticidal toxins and virulence factors for overcoming the insect defense but also several antibiotics, such as stilbene derivatives, anthraquinone derivatives, genistine, a furan derivative and a phenol derivative to repel, overcome, or out-compete either other microorganisms, as opportunistic colonists of the same insect, including those that are already present, such as the native gut flora of the insect, as well as scavengers [64, 72, 75].

Figure 2. Life cycle of *Photorhabdus luminescens* bacteria and *Heterorhabditis bacteriophora* nematode symbiotic complex from [66]. For details see text.



4.4.2 An impressive repertoire of virulence factors–strategy of “over-killing”

4.4.2.1 Toxins

8-12 hours after its release into the open blood system (hemocoel) by its symbiotic nematode partner, *Photorhabdus* starts multiply rapidly in spite of the activation of the insect defense system. It has been suggested that the events that lead to bacterial proliferation and insect death are due to numerous factors: the secretion of proteases, lipases, lipopolysaccharide molecules and the anti-hemocyte properties of the bacterial cell-surface as well as the production of a range of toxins of both oral and injectable insecticidal activity such as the high molecular weight Toxin complexes (Tc's), the “makes caterpillars floppy” (Mcf) toxins, the “*Photorhabdus*-insect-related” (Pir) toxins and other proteins encoded by the “*Photorhabdus* virulence cassettes” (PVCs) [4, 76, 77]. Although the nematode may play a role in insect death, it is not surprising that the bacteria alone are sufficient to cause the death of insect as it is seen following the injection of 5-100 cells into the hemocoel.

We have little knowledge of the interaction between *Photorhabdus* and the insect immune system. To escape from the first (mainly cellular) line of immune defense, *Photorhabdus* has two possibilities: it can either hide from the cell-based immune system by entering tissues where the phagocytosis less efficient, and starts multiplying there, or it can suppress the hemocyte activity. It was shown with artificial *Photorhabdus* infection of larval *M. sexta* that the midgut is colonized earlier than other tissues. The colonization and the destruction of the midgut is important to stop the insect feeding, but it might also provide shelter to hide from the majority of hemocytes under the extracellular matrix, next to the midgut epithelium where the humoral immune response is less efficient [4, 66]. Within this niche the bacteria express both the gut active Toxin complex A (Tca, see later) and also an RTX-like metalloprotease PrtA and induce the death of epithelium cells [4, 78]. It was also shown *in vitro*, that *Photorhabdus* bacteria secrete an unidentified, heat-stable protein into the supernatant which is capable of inhibiting its own phagocytosis by *M. sexta* hemocytes. This antiphagocytic factor was also detectable during infection, but the molecular base and the role of this factor in modulating hemocytes behavior remains unclear [4, 66, 78]. The fact that the number of bacteria in the hemolymph increases exponentially after a brief delay, shows that the hemocytes have a little overall effect in suppressing bacterial growth [4, 66]. The immune system recognizes the presence of *Photorhabdus* and mounts antimicrobial defenses that include cellular components (opsonisation,

encapsulation) as well as humoral ones (the transcription of several immune-related genes), however, these are weak and eventually unsuccessful [79]. They can only slow the progress of the infection temporarily. Besides the virulence factors that interfere with the cellular immune response, others can be supposed that are against the molecules in the humoral immune response [77, 79, 80]. Such a virulence factor is an organic solvent-extractable inhibitor which targets two important host defense functions, the enzyme phenoloxidase (PO) and nodule formation. It is a stilbene ((*E*)-1,3-dihydroxy-2-(isopropyl)-5-(2-phenylethenyl)benzene) which also acts as an inhibitor of the growth of microbial competitors both *in vitro* and *in vivo* [80].

Genomic sequence comparisons predicted more toxin and other virulence factor genes in the genome of *Photorhabdus luminescens* subspecies *laumondii* strain TT01 than in any other bacterial genome sequenced to date. Many of these have not been verified experimentally yet. For example, the deduced protein sequence of eight genes is homologous to the *Vibrio cholerae* RtxA toxin which belongs to the RTX (repeat in toxin) family of toxins, a group of related exotoxins produced by a variety of pathogenic Gram-negative bacteria with hemolytic, leukotoxic, and leukocyte-stimulating activities [65, 81, 82]. The products of two other loci, *plu* 4093-*plu* 4092 and *plu* 4437-*plu* 4436, were shown to encode insecticidal toxins when expressed in recombinant *Escherichia coli* which were orally toxic against the caterpillar pest *Plutella xylostella*, three mosquito and a lepidopteran species [65]. Similar gene pairs are found in the genome of *Photorhabdus asymbiotica* and *Yersinia intermedia* too [77]. The proteins encoded by these loci, are termed as ‘*Photorhabdus* insect related’ (Pir) proteins A and B binary toxins [83]. PirB shows similarity to both leptinotarsin from *Leptinotarsa decemlineata* and the Cry pore-forming toxin produced by *Bacillus thuringiensis*, which is widely used as insecticide. This indicates that the Pir proteins can act as important virulence factors but the mode of their action and their role in the infection process of *Photorhabdus* haven’t investigated yet [65, 83-85].

Another toxin gene is the ‘Makes caterpillars floppy’ or *mcf1*. *Mcf1* acts on both the gut and insect immune system. Injection of either *E. coli* carrying the *mcf1* gene or by *Mcf1* toxin itself into caterpillars results in a rapid (within 12 h) loss of caterpillar body turgor and death of the larvae within 24 hours. *Mcf1* may trigger apoptosis [86, 87]. A homologue of *mcf1*, termed *mcf2*, is also identified in *P. luminescens* strain W14 genome. *E. coli* expressing *Mcf2* also kills insects after injection [88].

Photorhabdus high molecular weight toxin complexes (Tc's) are intensively studied because these toxins have been suggested as useful alternatives to those derived from *Bacillus thuringiensis* to generate insect resistant transgenic plants [67, 77, 89-93]. The Toxin complexes are large, multi-subunit orally active insecticidal toxins produced by both gram-negative and gram-positive bacteria [94]. They have been documented in a range of bacteria, some of which are insect-associated (*Serratia entomophilla*, which colonizes insects during its life cycle; *Yersinia pestis*, the causative agent of bubonic plague, which has a flea vector) and others that have no obvious link with insects (*Pseudomonas syringae* a plant pathogen; *Fibrobacter succinogenes*, a commensal of ruminants) [67]. These toxins are effective by both injection and oral ingestion, causing larval death via disruption of the midgut epithelium. The presence of the tc-like genes in both gram-negative and gram-positive and also in bacteria not associated with insects implies that Tc-like proteins play fundamental role in bacteria lifestyle.

4.4.2.2 Proteases

Secreted microbial enzymes can play diverse roles in the interaction between pathogens and their hosts. Among putative virulence factors, secreted proteases are also candidates for the role of pathogenicity determinants because they can help the survival of the bacterium by neutralizing the immune system of the host. In previous studies there were sporadic observations that unidentified proteases of bacteria can selectively destroy insect antimicrobial proteins such as cecropins and attacins, which are known to play an important role in host defense during the early phase of microbial infection of *Lepidopteran* larvae, through their bacteriolytic effect [95-97].

The genome of the TT01 and W14 strains contain numerous sequences that could be identified as protease- or peptidase-encoding genes [65, 98]. However, direct experimental proof of their production is limited merely to several proteases. PrtA is the best known of them. This enzyme is a 55 kDa, RTX (repeats-in-toxin)-like metalloprotease belonging to the serralyisin subfamily of metalloproteases. RTX-proteases are secreted to the external medium through a Type I pathway via an ABC transporter. These proteases are often secreted together with and linked to their cognate inhibitors, such as Inh, also known as PrtI in the case of PrtA. The inhibitors are sometimes encoded in the same operon [99]. Thus, the complete *prtAIBCD* operon includes the *prtA* gene, which is found immediately upstream of a gene encoding its

own inhibitor, *inh*, and also three open reading frames, *prtBCD*, encoding the type I secretion system for PrtA [99, 100]. Internal deletions in recombinant *E. coli* carrying the whole *prtAIBCD* operon result in defective clones which indicates that all of the genes in the *prt* operon is required for synthesis and secretion of PrtA into culture medium [99]. The *inh* gene predicts a 14.8 kDa pre-protein, while the mature inhibitor is a 11.9 kDa protein which accumulates in the periplasm. Inh has high specificity and affinity for PrtA, and inhibits the protease in a one to one molar ratio [100].

Photorhabdus PrtA expression and presence was detected during culture growth and infection by zymogram analysis [78]. Fluorescence, anti-PrtA immunoreactivity labels showed that during *Photorhabdus* infection of *M. sexta* PrtA is localized first in the midgut, then in the fat body, muscle and their associated tracheae [4]. With a development of highly sensitive and specific artificial substrate Dabcyl-EVYAVES-Edans, it was possible to selectively measure activity in biological samples and investigate the dynamics of PrtA production during *Photorhabdus* infection of *Galleria mellonella*. PrtA was first detected 14 h post infection, and the activity was mainly in the tissues and only ten hours later it appeared in the hemolymph [101].

These available data are insufficient to decide whether PrtA might function as a virulence factor. To date, no natural substrate was identified for PrtA that would help us to understand and prove its possible physiological role.

4.5 Virulence factors as tools of investigation

The long list of toxins makes understandable why *Photorhabdus* can kill so effectively an insect. However, these can serve only one of the bacterial activities in the process of taking over the body of the host with the purpose to convert it into a “culture medium”. An equally important function is to survive the complex, well orchestrated and usually very efficient defense mechanisms of insects. Such immune suppressive, hiding, disguising functions of *Photorhabdus* are much less known than toxins which are not against the immune response of host. Therefore, proteases are exceptionally useful for a pathogen, because their peptide hydrolyzing capability provides a very efficient way of inhibiting the function of the mostly proteinaceous carriers in the immune response. Obviously, the simplest way of protection against such a molecular weapon is the production of inhibitor by the host. Thus a tripartite interaction system (proteolytic system) forms between the protease, its native substrate(s) and inhibitor(s).

If, indeed, immune suppression is the function of a proteolytic virulence factor, then exploring its proteolytic system can provide an insight into the immune system of a host. As for instance, among the target proteins new components of the immune system can be discovered and through the effects of a selective (proteolytic) inactivation of these targets (new or the known components of the immune system) their interactions can be studied enhancing our understanding of the system, in addition to understanding the role of a virulence factor.

4.6 Serralysins as putative virulence factors

The investigation of the function of PrtA through exploring its proteolytic system is interesting from that respect too, that PrtA is a serralysin, because serralysins are widely supposed as important virulence factors but without much hard evidence about it.

Serralysins (named after a protease from *Serratia marcescens*) belong to clan MA of metallopeptidases, the metzincins. They are in the M10B subfamily of matrix metalloproteases (M10 family; MEROPS ID: M1.051), the group of enzymes of prokaryotic origin. The catalytic Zn is bound in the N-terminal, metzincin type domain which contains the HEXXHXXGXXH motif with three histidines that ligand Zn²⁺. Beneath this structure is a conserved methionine containing turn, characteristic to metzincins. The C-terminus of the protein contains an RTX (repeats-in-toxin) domain, which includes a glycine-rich tandem repeats of RTX-like consensus sequence GGXGXDX(L/I/F/V)X, essential for secretion [101]. Serralysins are secreted by a wide range of microorganisms, including plant, insect and human pathogens [66, 103]. Serralysins have been identified from *Serratia marcescens* (serralysin), aeruginolysin (an alkaline proteinase) from *Pseudomonas aeruginosa*, ZapA metalloprotease of *Proteus mirabilis* and proteases A, B, C, G and W of various *Erwinia* strains and PrtA from *Photobacterium* [101].

Photobacterium PrtA enzyme shows 66% similarity to the *Pseudomonas aeruginosa* AprA protease (epralysin), however it was designated after a similar protease in *Erwinia chrysanthemi* [99]. These enzymes are supposed to be virulence factors but, except for some inhibitors that are secreted by the bacteria along with their enzymes, the proteolytic system of neither of serralysin enzymes was explored. Only few potential natural substrates have been found for ZapA of *Proteus mirabilis* and the alkaline metalloprotease of *Pseudomonas aeruginosa*, which cleave human IgA and

IgG proteins and a proinflammatory cytokine, interleukin-6 (IL-6) *in vitro* [3, 104]. However, the relevance of these *in vivo*, in the pathogenicity is question because the applied conditions, a large enzyme to substrate molar ratio and the very long incubation time (1:10-1:6000 and 3-8 hours, respectively) do not specify a sensitive cleavage which might be supposed for specific target proteins in a proteolytic system.

5. Goals of my thesis work

The research conducted in István Venekei's laboratory investigates the proteolytic system of *Photorhabdus* protease PrtA, as a tool to investigate the innate immune system of insects. Joining this project, my task was to prove that PrtA might be a virulence factor via suppressing the immune response. The main goal of my work was to find target proteins to PrtA and to prove that they are immune proteins through their identification and/or investigation of their function. Thus, I tried to investigate one small segment of the role of PrtA in *Photorhabdus luminescens* infection mechanism using the following biochemical and molecular biological approaches:

- Detection and comparison of the time course of proteolytic activities in different *Photorhabdus* strains in culture growth using various biochemical methods.
- Establishing the earliest produced protease by different *Photorhabdus* strains is during the bacterial growth in culture and after *Galleria mellonella* infection, *in vivo*.
- Finding such proteins which are natural substrates of this enzyme in *Manduca sexta* hemolymph.
- Identifying these natural substrates after partial purification which is sufficient for N-terminal determination.
- Investigating the role of at least one target protein in *Manduca sexta* immunity by inactivation with RNAi method.

6. Materials and Methods

6.1 Material

6.1.1 Bacterium strains and culturing; preparation of bacteria for insect injection

The identities, taxonomic positions, and origins of *Photorhabdus* strains used in the SDS-PAGE- and native PAGE-coupled zymography experiments and in *Galleria mellonella* injection are summarized in **Table1**. These strains were obtained from the entomopathogenic nematode/bacterium strain collection maintained at the Department of Genetics, Eötvös Loránd University, Budapest. *Escherichia coli* strain *DH5 α* and *Photorhabdus luminescens subsp. laumondii* strain TT01 for *Manduca sexta* injections were cultured at the Department of Biochemistry, University of Bath, Bath, United Kingdom.

Single colonies were used as a starting material, from Luria-Bertani (LB) plates that were grown on for 48 hours, at 30 °C (*Photorhabdus* strains) or 37 °C (*E. coli*). Liquid cultures were grown in LB medium at the required temperature in a rotary shaker at 300 rpm.

For injections, serial dilutions were prepared of overnight *Photorhabdus* and *E. coli* cultures (Brecon/1, Hm/1, Hm/2, NC19/1, NC19/2, TT01 and *DH5 α*) with sterile phosphate-buffered saline (PBS solution; 137.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄ pH 7.4) that contained 1 mM (final concentration) phenylthiocarbamide (PBS-Ptc) to obtain 100 cells in 5.0 μ l (*G. mellonella* injection) or 50 μ l (*M. sexta* injection). The actual numbers of cells were estimated from the number of colonies after plating 50 μ l portions of the final dilutions onto LB agar plates. 5 μ l of each final cell dilution was injected into 10 fifth-instar larvae of *Galleria mellonella* or 50 μ l into 10 fifth-instar *Manduca* larvae using a 100 μ l disposable syringe with a 30-gauge needle. PBS-injected insects served as controls.

Table 1. Examined *Photorhabdus* strains

Entomopathogenic symbiosis: natural partners							
Bacterium:			Nematode:		Origin of nematode or the bacteria		
<i>Photorhabdus</i>			<i>Heterorhabditis</i>				
species	subspecies	strain	species	strain	Geographic	Laboratory	
<i>luminescens</i>	<i>laumondii</i>	TT01/1	<i>bacteriophora</i>	TT01	Trinidad	Boemare	
		Brecon/1	<i>bacteriophora</i>	Brecon	Australia	Gerritsen	
		RH/1	<i>bacteriophora</i>	RH	USA	Hurlbert	
			RH/2			Hurlbert	
	<i>akhurstii</i>		Indica/1	<i>indicus</i>	LN2	India	Burnell
		<i>luminescens</i>	Hm/1	Unknown, lost		USA	Boemare
			Hm/2				Boemare
			Hb/1		Hb	USA	Boemare
			Hb/2			Boemare	
	<i>temperata</i>	-	NC19/1	<i>bacteriophora</i>	NC1	USA, NC	Ensign
NC19/2						Ensign	
-		WX6/1	Unknown, lost		USA, WI	Nealson	
		WX6/2				Nealson	
-		WX8/1	Unknown, lost		USA, WI	Nealson	
	WX8/int				Fodor		
		WX8/2			Fodor		
<i>temperata</i>	<i>temperata</i>	HSH2/1	<i>megidis</i> NWE	HSH-2	Germany	Ehlers	
		HSH2/2				Ehlers	
		K122/1	<i>downesii</i>	K122	Ireland	Clarke	
		K122/2				Clarke	

6.1.2 Insects; preparation of hemolymph plasma and fat body samples

Fourth instar *Galleria mellonella* (greater wax moth, *Lepidoptera*) larvae were obtained from a colony that was reared on bee-wax – pollen diet in our laboratory. For detection of proteolytic activity in the hemolymph, 100 *Photorhabdus* cells were injected into larvae, which were kept at 25°C. 5 µl of hemolymph samples were taken through a pro-leg and were diluted immediately 5-fold with ice cold PBS-Ptc at 12, 22, 28, 42 h after injection.

Manduca sexta (tobacco hornworm, *Lepidoptera*) eggs were kindly provided by the Department of Biochemistry, University of Bath, Bath, United Kingdom. Naïve larvae were maintained individually on a wheat germ-based artificial diet at 25 °C [105]. Injected larvae were kept at 25°C to determine survival. After surface sterilization of the newly moulted, day 0, fifth-stage larvae with 70% ethanol, the insects were bled and dissected to collect hemocytes and fat body, respectively. Hemolymph samples were immediately taken into chilled sterile PBS-Ptc in 1:4 hemolymph to PBS ratio. The cellular fractions were sedimented at 5000 rpm for 15 minutes to obtain cell-free hemolymph plasma. The hemolymph plasma was used for

SDS and native PAGE coupled zymography, protein purification, phenoloxidase activation assays, western analysis, while hemocytes were used for RNA extraction. Fat body samples were collected into a pre-chilled sterile Eppendorf tube and used for RNA isolation.

6.2 Methods

6.2.1 Protease detection during culture growth and insect infection

6.2.1.1 Polyacrylamide gel electrophoresis and zymography

To monitor purification steps, to detect the PrtA cleavage of hemolymph proteins and to determine the mass of the PAT proteins, SDS-polyacrylamide gel electrophoresis was performed using a 10% acrylamide, 0.26% bis-acrylamide separation gel. The samples were run under reducing conditions, using 2×sample buffer (3% SDS, 0.16 M TRIS-HCl pH 6.8, 20% glycerol, 0.01% bromphenol blue and 8.8 mg/mL DTT) at a 2:1 sample:sample buffer ratio and heated for 5 min before loading.

To detect the protease activity, 0.025% casein (Sigma) or gelatin (Bloom 300, Sigma) was copolymerized in the SDS or native gels (SDS or native PAGE coupled zymography). Native gel electrophoresis was made with 10% acrylamide, 0.26% bis-acrylamide in 0.38 M TRIS-HCl pH 8.8 buffer. The samples were mixed with sample buffer (1.14 M TRIS-HCl pH 8.8, 0.01% bromphenol blue and 20% glycerol) at 2:1 sample: sample buffer ratio. After running, the zymograms were soaked in 2-4 changes of 100 mL buffer solution containing 50 mM TRIS-HCl pH 8.0, 10 mM CaCl₂ and 0.1 M NaCl in order to develop the proteolytic bands. Native zymograms were incubated in two changes, the first for 20 minutes and the second for 60 minutes. SDS zymograms were incubated in four changes, the first three for 20 minutes each, and the last one for 120 minutes. The gels were stained with Coomassie Brilliant Blue R250 staining.

6.2.1.2 Enzyme activity measurement

The enzyme activities were measured in the culture supernatant at 30°C in 1.0 mL (final volume) of mixtures containing 50 mM TRIS-HCl pH 7.0, 10 mM CaCl₂, and 0.1 M NaCl (assay buffer). The final substrate concentrations were 50 μM for the 2-furylacryloyl (Fua)-blocked, photometric substrate Fua-Leu-Gly-Pro-Ala (Fua-LGPA; Bachem). The reactions were started by addition of 150 μl of culture supernatant and the decrease in absorbance was monitored at 324 nm until the end of the reaction. The catalytic activity (k_{obs}) was calculated by fitting the final portion of the

curves (where the remaining substrate concentration was less than 1/10 the K_m (40 μM for Php-B) to first-order kinetics by using the Origin 5.0 software (Microcal).

6.2.1.3 PrtA purification

The cloned PrtA was purified from *E. coli* Hb101 strains transformed with pUC19 plasmid containing PrtA from *Photorhabdus ssp. akhurstii* W14 (a generous gift from Richard French-Constant, Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom). 100 mL LB medium was inoculated with several colonies from a fresh LB plate, and incubated for 24 hours at 37°C on a rotary shaker at 200 rpm. The culture supernatant was dialyzed for four hours against 2×5 L of buffer A (20 mM TRIS-HCl pH 8.0, 50 mM NaCl, 10 mM CaCl₂). The dialyzate was centrifuged with 8000 rpm for 15 minutes at 4 °C, then the clear supernatant was loaded onto a 1.6×3 cm polyethylene-imine silica column (Matrex silica PAE 300, Millipore) equilibrated with buffer A. The elution was with a 20 mL 0-1.0 M linear NaCl gradient in buffer A with 0.5 mL/min flow rate. The PrtA containing fractions were clean of other proteins. This procedure was worked out by Judit Marokházi in our laboratory.

6.2.2 Separation and analysis of proteins cleaved by PrtA

6.2.2.1 Preparation of “type” fractions from hemolymph

The pH of 50 mL of hemolymph plasma was adjusted to pH 9.0 using 10 M NaOH. After centrifugation (at 18,000 rpm for 15 min at 4°C), the clear supernatant was loaded onto a 16 cm×1.0 cm DEAE Sephadex anion exchange column. The proteins were eluted with a linear 0-0.9 M NaCl gradient in the equilibrating solution at a flow rate of 0.5 mL/min. 1.2 mL fractions were collected and analyzed with SDS-PAGE.

6.2.2.2 Partial purification of PAT proteins

50 mL of hemolymph sample was precipitated for 2 hours on ice with the addition of saturated (NH₄)₂SO₄ solution (pH 8.0) in two steps to 20% and 40% final (NH₄)₂SO₄ concentration. The precipitates were resuspended in 3.0 mL gel filtration buffer (20 mM TRIS-HCl, pH 8.0, 1.0 mM benzamidine, 1.0 mM phenylthiocarbamide, 0.3 M sodium acetate pH 8.0). Before gel filtration, the samples were dialyzed against 2×3 L gel filtration buffer for 6 hours and then the insoluble material was sedimented

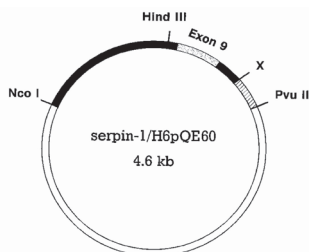
with centrifugation at 14,000 rpm for 20 min at 4 °C. The clear supernatant was applied to 16/60 Sephacryl-S200 HR gel filtration column (Amersham Biosciences) or on Superdex-75 analytical gel filtration column (Amersham Biosciences) and chromatographed at a 0.5 mL/min flow rate. The protein content of the effluent was monitored at 280 nm.

Before ion exchange chromatographies, the protein solutions were dialyzed against 1×5 L ion exchange buffer (20 mM TRIS-HCl, pH 8.0, 1.0 mM EDTA, 1.0 mM phenylthiocarbamide, 1.0 mM benzamidine) for 6 hours. Then, the insoluble material was sedimented with centrifugation (as above). The Matrex Silica PAE 300 column (Millipore; 9.0 x 1.0 cm) was eluted with a 0-05 M NaCl linear gradient in the ion exchange buffer, whereas during chromatography on MonoQTM 5/50 GL FPLC column (Amersham Biosciences) the following NaCl gradient was used in the ion exchange buffer: 0-0.4 M NaCl within 35 minutes, then 0.4-0.5 M NaCl within 5 minutes. The flow rate in ion exchange chromatographies was 0.5 mL/min. The protein content of the effluent was monitored at 280 nm.

6.2.2.3 Purification of expressed Serpin-1 proteins

The purification of the twelve expressed Serpin-1 proteins were performed using His select Nickel affinity columns (Sigma-Aldrich) according to [56] from 30 mL cultures of *E. coli* XL1 blue strain, which was transformed with Bluescript plasmids that contained the Serpin-1 variants (generous gift from Mike Kanost, Department of Biochemistry, Kansas State University, U.S.A.; **Fig. 3**). For transformation 2 µl of plasmid DNA was added to 50 µl of XL1 blue competent *E. coli* cells that were incubated for 40 min on ice. The heat shock was at 42 °C for 1 min. Then, 1 mL of LB was added to the cells, and incubated at 37 °C for 1 hour on a rotary shaker at 200 rpm. The transformants were plated out on LB plates containing 0.1 µg/mL ampicillin and the plates were incubated for overnight at 37 °C.

Figure 3. Recombinant plasmids for expressing *M. sexta* serpin-1 variants. Generous gift from Mike Kanost, Department of Biochemistry, Kansas State University, USA. A cDNA for serpin-1B in expression vector H6pQE-60 was used to reconstruct all 12 of the cDNA variants by substituting an equivalent restriction fragment from each variant cDNA. *Open bar*, plasmid vector; *filled bar*, constant regions of *Manduca* serpin-1 cDNA; *shaded bar*, the region of cDNA corresponding to exon 9; *cross-hatched bar*, vector sequence that differs, depending on how the original variant cDNA was cloned. X represents an *EcoRI* site for clones expressing serpin-1 variants B, F, and J'; X represents an *XhoI* site for clones expressing serpin-1 variants A, C, D, E, G, H, I, J, K, and Z [61].



6.2.2.4 Blotting and N-terminal sequencing

For N-terminal sequencing the partially purified PAT protein containing samples were run in 10 % acrylamide-SDS gels under reducing conditions. Then the gels were soaked for 10 minutes in transfer buffer (10mM CAPS (Sigma) pH 11.0, 10% methanol), and blotted onto Immobilon-P PVDF Transfer Membrane (Millipore) at 200mA for 2 hours. The protein bands on the membrane were visualized by Coomassie Brilliant Blue R-250 staining. The bands of PAT proteins were cut out and subjected to Edman-sequencing in a Microtec-protein sequencer (Applied Biosystems) by András Pathy from the ELTE-MTA Biotechnology Research Group. Identification through database search for similar sequences was made with BLAST using the NCBI database.

6.2.2.5 Digestion of hemolymph and PAT proteins with PrtA

During the initial search for PrtA substrate proteins, hemolymph fractions that contained 0.5-5.0 $\mu\text{g/mL}$ protein, were exposed to digestion with 0.3 ng PrtA (~ 0.3 nM final) or 30 ng chymotrypsin, trypsin and *Clostridium* collagenase (~ 60 nM final) at room temperature in the presence of 50 mM Tris-HCl, (pH 8.0) 10 mM CaCl_2 and 0.1 M NaCl (a reaction buffer in which all of the applied enzymes could exhibit their

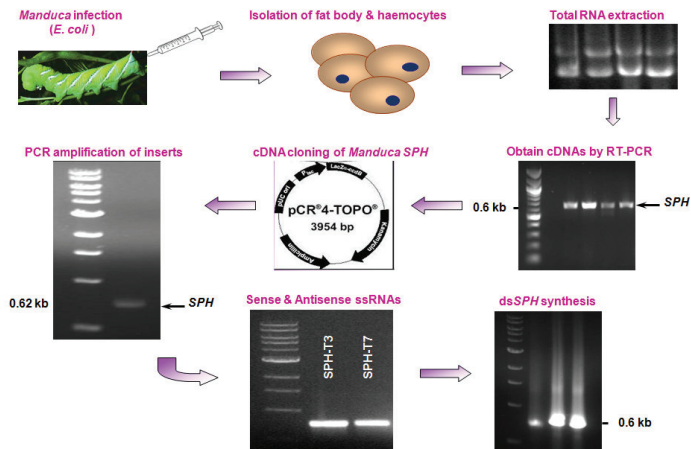
highest activity on synthetic substrates). Samples were withdrawn at 45 and 90 minutes of incubation. In order to find the PAT protein containing fractions after the various isolation steps, samples of appropriate volumes were digested with 0.3 nM PrtA in 20 μ l final volume at room temperature for 90 minutes. The purified serpin-1 variants were subjected to PrtA cleavage at 1-4 μ M Serpin-1 to 30 nM PrtA ratio (1.0-3.7 μ g serpin-1 to 30 ng PrtA) in the reaction buffer at room temperature for 90 minutes. All the samples from the digestions were analyzed with SDS-PAGE.

6.2.3 RNA interference (RNAi)

6.2.3.1 Total RNA isolation

To isolate total RNA, 100 mg of dissected fat body and 30 mg of bled hemocytes was homogenized in 500 μ l of TRI reagent® (Sigma, UK) using a plastic grinder. After homogenization the samples were centrifuged at 12,000 rpm for 10 minutes at 4°C. The removed supernatants were allowed to stand for 5 minutes at room temperature before adding 200 μ l of chloroform. The mixtures were vortexed for 15 seconds and were allowed to stand for 10 minutes at room temperature, then spinned at 12,000 rpm for 15 minutes at 4°C. The top aqueous phases were transferred to new tubes, and 500 mL of isopropanol was added to them and mixed thoroughly. The samples were incubated for 10 minutes at room temperature and centrifuged at 12,000 rpm for 10 minutes at 4°C. After removing the supernatants the RNA pellets were resuspended in 1 mL of 70% ethanol, and sedimented at 7,500 rpm for 5 minutes at 4°C, then air-dried for 10 minutes. The resulted RNA preparation was dissolved in 20 μ l of di-methyl-propyl carbonate (DMPC)-treated water, and treated with RNase free DNaseI (Invitrogen, UK) (1 U/ μ L) to remove DNA contamination.

6.2.3.2 Generation of dsRNA of SPH-3

Figure 4. Generation of dsRNA of SPH-3. For details see text

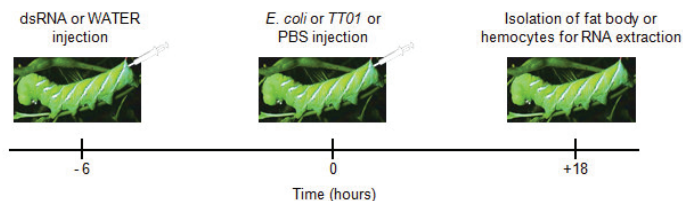
To synthesize dsRNA of *SPH-3*, cDNA was amplified with RT-PCR on total RNA extracted from fat body or hemocytes from insects previously injected with *E. coli* to elicit immune response. RT-PCR was made using OneStep RT-PCR kit (Qiagen, UK). Specific *SPH-3* primers were used, which are shown in **Table 2**. The resulting PCR product was cloned into pCR4-TOPO vector (Invitrogen, UK) and transformed into *E. coli* one shot TOP10 chemically competent cells (Invitrogen, UK). The transformants were plated on LB plates containing 1 µg/mL ampicillin (Sigma, UK). 10 colonies were inoculated into 5 mL LB broth and 1 µg/mL ampicillin and the cultures were incubated for overnight (O/N) at 37 °C and then plasmid DNA was prepared with QIAprep Spin Miniprep Kit (Qiagen, UK) following the manual's protocol. The minipreps were checked by sequencing for the correct nucleotide sequence and used as a template to amplify the insert with T7 (TAATACGACTCACTATAGGG) and T3 (ATTAACCCTCACTAAAGGGA) primers by PCR (GenAmp Kit, UK). PCR conditions: 34 cycles with the following 3 steps in each: 93 °C for 30 sec, 50 °C for 30

sec, 68 °C for 1 min then a final extension at 68 °C for 5 min. The PCR product was purified with Montage PCR centrifugal filter Devices kit (Millipore, UK). These purified PCR products were used to synthesize the sense and antisense RNAs using T3 and T7 ‘Megascript’ kits (Ambion, UK), respectively, according to the manufacturer’s instructions. DNA templates were removed with RNase free DNaseI (Invitrogen) (1 U/ μ L), and the reaction products recovered and purified using lithium chloride precipitation following the kit’s protocol. Single –stranded (ss) RNAs were dissolved in DMPC treated water and complementary strands were annealed by combining equal molar amount of each strand, heating to 70 °C for 15 min and cooling overnight at room temperature. The annealing of ss-RNAs was found to be complete when examined by agarose gel electrophoresis. dsRNAs were diluted to 2 μ g/mL in DMPC treated water and stored at -20 °C until required. The sequence of the dsRNA was from base 19 to 675 (the entire coding region) of the SPH-3 cDNA (Fig.4). The negative dsRNA control (dsCON) was synthesized with the same method as described above.

6.2.3.3 Insect injection protocol

For RNAi, 100 ng (50 μ L, 2 μ g/mL) of dsRNA of *SPH-3* (dsSPH3) in DMPC-treated water was injected into *Manduca sexta* larvae as primary injection, and then, 6 hours later with *E. coli* or *Photorhabdus* (TT01) as secondary injection. Controls used DMPC-treated water without dsRNA in the primary injections and PBS without *E. coli* or TT01 in the secondary injections. After treatment, insects were held at 25°C for 18 hours before isolation of fat body and hemocytes (Fig.5).

Figure 5. Sequence of injections of *Manduca sexta* for RNAi



6.2.3.4 RT-PCRs

To determine the transcription of each gene, semi-quantitative single-step reverse transcription (RT)-PCR was performed with the 'OneStep' RT-PCR kit (Qiagen, UK). Each reaction was carried out in a 50 μ l volume containing 0.6 mM of forward and reverse gene primers and 2 ng of total RNA template. Amplifications were performed on a PTC- 100 thermal controller (MJ Research, USA) under the following cycling conditions: at 50 °C for 30 min (reverse transcription step), 95 °C for 15 min (initial PCR activation step), followed by 35 PCR cycles with the following steps in each 94 °C for 30 sec (denaturation), 50 °C for 30 sec (annealing) and 72 °C for 1 min (extension), then a final extension of 72 °C for 10 min. The different gene specific forward and reverse primers are summarized in **Table 2**.

The RT-PCR product of ribosomal protein S3 (rpS3) mRNA (*Manduca sexta* rpS3 is a constitutively expressed housekeeping gene) was used as a loading control to make sure that the same amount of total RNA as a PCR template was used for each experiment. RT-PCR control reactions for rpS3 were performed as outlined above. PCR reaction without template as negative control (NC) was also carried out in each case. PCR products were separated by 1% agarose gel electrophoresis. Expression of immune-related genes and the extent of dsRNA induced gene silencing were assessed using two insects in each treatment.

Table 2. Nucleotide sequences of primers used in RT-PCR. All sequences read 5' to 3', left to right

Gene	Accession No	Primer	Sequence	Product size (bp)
<i>SPH3</i>	AF413067	Forward	CGTGGCACGATAATGTTGTT	657
		Reverse	AGTCGCTGCGTCAATGTATG	
<i>Hemolin</i>	U11879	Forward	ACAGCAACAACACAGGTGAA	1273
		Reverse	TTAAGCAACAATCACGAGCG	
<i>IML-2</i>	AF242202	Forward	GACTCTTGGGAGTCGTGTGA	953
		Reverse	GACTGTTTGGGTCCTTTTCG	
<i>PGRP</i>	AF413068	Forward	ACGGTATCACTTCCGTCCAC	516
		Reverse	CATTCTGGCCATCTCCTGAT	
<i>PRSP</i>	AY380790	Forward	ACGGATGGCACCCCTGGTGCAGCC	680
		Reverse	TTTGCAACACATCAACGTAAG	
<i>βGRP-1</i>	AF177982	Forward	CCTGACGCGAAGTTAGAAGC	792
		Reverse	AACGCGACCATATTTGAAGG	
<i>βGRP-2</i>	AY135522	Forward	TCTACCCCAAAGGCTTGAGA	802
		Reverse	CACCACCAGAGACCCACTTT	
<i>PPO</i>	L42556	Forward	AAACAACCTCCCAAACGATGC	889
		Reverse	TGTGCATGTTGTTGTGGATG	
<i>Attacin B</i>	BI262658	Forward	GGTCACGGCGCTACTCTTAC	341
		Reverse	TTGGGCATCTCGAACTTCTT	
<i>Cecropin D</i>	BI262670	Forward	TTCTTCGTCCTCGCTTGCTT	161
		Reverse	CCTTTGAAAATGGCGGTTG	
<i>Lebocin</i>	BI262726	Forward	ACGTGCGTAGTGTGAACGAG	170
		Reverse	CGCAGATTATGAGTTACGACGA	
<i>Lysozyme</i>	BI262563	Forward	TACAAACGCCACAAGTTCCA	309
		Reverse	AGGCTTGTAACCAAGGACGA	
<i>Moricin 2</i>	BI262611	Forward	TCGCGTTGTCGCTATTATTTT	327
		Reverse	CAGAAGATTCCGAAGGGAGA	
<i>rpS3</i>	U12708	Forward	CTGGCTGAGGATGGCTACTC	186
		Reverse	TTTCTCAGCGTACAGCTCCA	

6.2.4 Examination of the effect of RNAi of SPH-3 in *Manduca sexta*

6.2.4.1 Western blotting

Manduca sexta hemolymph plasma samples were separated with standard SDS-PAGE. After electrophoresis the gels were transferred electrophoretically (Mini Trans-Blot Transfer Cell, Bio-Rad, UK) to PVDF membranes (Bio-Rad, UK). The samples were transferred in cold Towbin buffer containing 20% methanol, 0.18 M glycine, 25 mM TRIS at 100 volts for 60 min. The blot was blocked in 5% skimmed milk powder (Marvel) in transblotting solution (TBS, 20 mM Tris, 0.5 M NaCl, pH 7.5) for 12 h at 4 °C. The blot was washed 3 times for 10 min in TBS, then incubated for 1 h at room temperature in tween transblotting containing 3% milk powder (TTBS, 20 mM Tris, 0.5M NaCl, 0.1% Tween-20, pH 7.5) containing 1/10,000 dilution of the primary anti-SPH-3 antibody which was raised in rabbit against the synthetic peptide PQFKGRNTNYRNDI (corresponding to SPH-3 amino acid residues 135-148). After subsequent washing (3 times for 10 min in TBS) the blot was incubated in TTBS solution containing 3% milk powder, 1/10,000 dilution of horseradish peroxidase-labelled goat anti-rabbit IgG secondary antibody (Upstate, UK) for 1 h at room temperature. After further washing (3 times for 10 min in TBS) the bound antibodies were detected using chemiluminescence Western blotting kit (Visualizer, Upstate, UK) and the membrane was exposed to X-ray film (Biomax, Kodak, UK) for 1 sec to detect the signals.

6.2.4.2 Mortality bioassay

In survival experiments, after insect injections (**Fig. 5**), the state of the insects were checked at eight time points (12 h, 18 h, 21 h, 24 h, 36 h, 41 h, 48 h, 60 h) after the second injection. The mortality was defined as failure to react to poking with a needle. Ten insects were used for each treatment and the experiment was repeated three times.

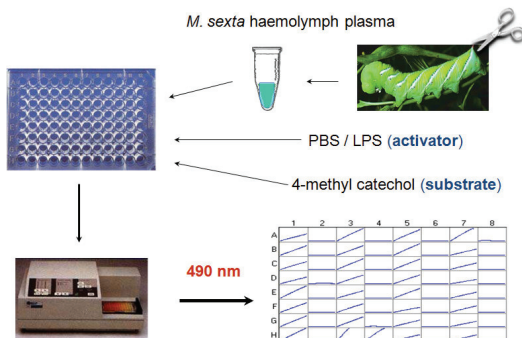
6.2.4.3 Effect of RNAi knockdown of SPH-3 on PO activity by visual examination of hemolymph

18h after the bacterial infection insects were individually bled into pre-chilled sterile polypropylene tubes to collect their total hemolymph fluids (approximately 500 μ l per larva). Hemolymph samples were incubated for 1 h at room temperature. The samples were visually evaluated for the development of their darkening.

6.2.4.4 Phenoloxidase (PO) activity measurement

Total activatable PO activity of *Manduca sexta* hemolymph was quantified with a microplate enzyme assay using Molecular Devices Thermomax microplate reader with flat bottom 96-well plates (Nunc, UK). 18 h after the second injection, cell-free hemolymph plasma was prepared. The reaction mixture containing 115 μ l 50mM PBS buffer (pH 6.5), 10 μ l diluted hemolymph plasma, and 2 μ l *E. coli* LPS (5 mg/mL) (Sigma, UK) was left for 1 h at room temperature on a plate shaker to allow the activation of the enzyme. Then, 25 μ l of 20mM 4-methylcatechol (Sigma, UK) was added to initiate the reaction and then, sterile distilled water make up the mixture to 200 μ l final volume. The change in absorbance was read at 490 nm for 1 h at room temperature with a reading taken every 1 min (**Fig. 6**).

Figure 6. Phenoloxidase activity measurement from *Manduca sexta* plasma samples



6.2.4.5 Nodule formation

Nodule formation was assessed 18 h after immune challenge. Insects were immobilized on ice for 15 min and then dissected under 1% (w/v) NaCl solution saturated with phenylthiocarbamide (Ptc) which prevented general post-dissection darkening. Melanized nodules within the hemocoel were counted using a stereomicroscope and a tally counter.

6.2.4.6 Pathogen growth *in vitro*

To determine the ability of *P. luminescens* to grow in the presence of plasma insects were bled individually 18 h after the bacterial infections, and cell free plasma was prepared following the addition of 20 mM Ptc. All samples were inoculated with $\sim 10^3$ cells (3 μ L) of *P. luminescens* and incubated at 30°C with constant shaking for 24 h. Growth was estimated as optical density at 600 nm.

7. Results

7.1 Comparison of proteolytic activities produced by different *Photorhabdus* strains

7.1.1 Measuring enzymatic activity in culture

Photorhabdus bacteria had been found as protease producers however the number and the type of secreted proteases and the dynamics of production remained unknown. My goal was to find a protease, which might be a pathogenic factor during *Photorhabdus* infection. Such an enzyme must be secreted early and in all strains of *Photorhabdus*. Therefore, I investigated 20 *Photorhabdus* strains and compared their protease production with zymography. I focused on proteases that could be detected in the first 48 h of culture and infection and could, therefore, have a role in virulence. For the detection of protease activity I used both SDS- and native PAGE coupled zymography in the presence of casein or gelatin substrates. (Fig.7, Table 3). *Photorhabdus* bacteria produced more than one protease in the examined period but I couldn't find any other enzyme, which would be secreted earlier than a 55 kDa protease, PrtA. PrtA activity was observed after SDS-PAGE coupled zymography in almost all strains except for several secondary phase variants. There were slight variations in the level of PrtA activity and in the time of occurrence among strains and among growth conditions. In case of four strains – Hm/2, Wx6/2, HSH-2/1 and HSH-2/2 – I couldn't detect any PrtA activity with either SDS or native gel electrophoresis. In case of the most intensive PrtA producers (Brecon/1 and Nc19/2) the activity appeared from 12 - 24 h of culture (mid-logarithmic – late-logarithmic growth phase) and remained detectable for the 44 h of culturing. With the exception of Hm/2 and K122/2 strains, there were at least two activity bands in the molecular mass range from 50 to 55 kDa. The smaller species, which were thought to be molecular variants of PrtA, were not present at the beginning of secretion. In each case, two dominant forms, a larger 55-kDa form (PrtA1) and a 2- to 4-kDa smaller form (PrtA2), could be distinguished. The ratio of these forms and the occurrence of other forms varied slightly with different cultures of the same strain, but the PrtA1 form was always the most abundant (or most active). I also found with SDS-PAGE coupled zymography in the cultures of strains Brecon/1 and K122/1 but not in the cultures of the other strains, another major activity, a 37-kDa protease termed as Php-C. Depending on the strain, it occurred from 21 to 40 hours of culturing (in the late logarithmic and early stationary

phases). In strain Brecon/1, Php-C also showed size variation and was resolved by SDS-PAGE as a larger 37-kDa form (Php-C1) and a fainter, smaller and substantially less active (or abundant) 35-kDa form (Php-C2). Although, Php-C was detected by SDS-PAGE coupled zymography in only two strains, the native PAGE coupled zymography showed that it was actually produced by fifteen strains (**Table 3**).

Figure 7. Comparison of protease secretion in the culture of seven *Photorhabdus* strains (Hm/1; Hm/2; Nc19/1; Nc19/2; K121/1; K121/2 and Brecon) with SDS- and native PAGE coupled zymography. Detection of protease secretion with SDS-PAGE coupled zymography in the presence of gelatin substrate (A) and casein substrate (C); and with native PAGE coupled zymography in the presence of gelatin (B) and casein substrates (D). The numbers on the top of the gels indicate the sampling time point from culture growth. The numbers on the top of the gels indicate the sampling time point from culture growth. A1 and A2 are molecular variants of PrtA; C1 and C2 are molecular variants of PhpC (C).

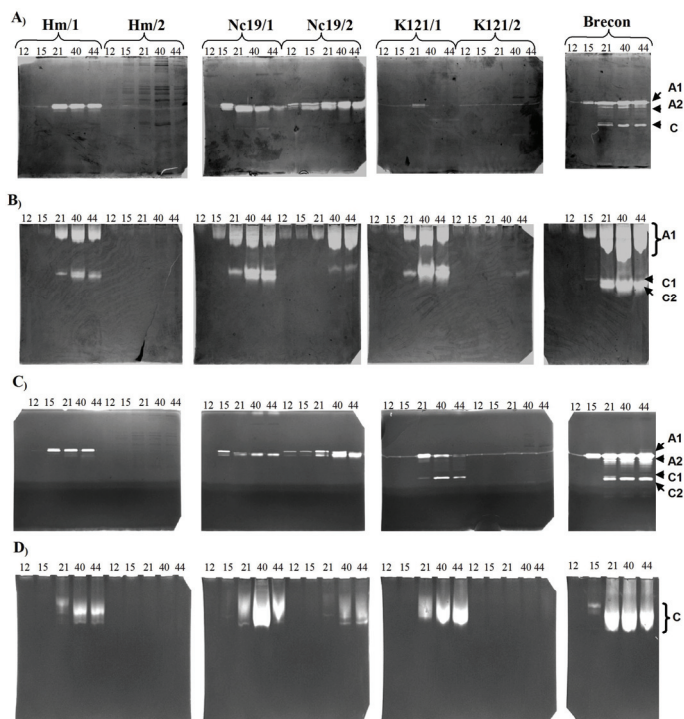


Table 3. Protease secretion by 20 *Photorhabdus* strains and phase variants: summary of activities with three detection methods. Intensity of the highest activity: -, not detectable; (\pm), very weak; (+), weak; +, well easily detectable; ++, strong. The times of first detection are indicated in parentheses, as follows: ml, mid-logarithmic phase (OD₆₀₀ nm between 1.0 and 4.8; h 12 to 21); ll, late logarithmic phase (optical density at 600 nm between 4.8 and 5.3; h 21 to 40); s, early stationary phase (optical density at 600 nm between 5.3 and 6.6; h 40 to 50). On Fua-LGPA substrate the values were determined in supernatants having optical densities at 600 nm between 4.9 and 8.1 (43-h cultures). k_{obs} is the first-order rate constant and is expressed in seconds⁻¹ (see Materials and Methods).

Strain	Zymography following						
	SDS-PAGE			Native-PAGE			Fua-LGPA-ase activity (k_{obs})
	PrtA1	PrtA2	Php-C	PrtA	Php-C1	Php-C2	Php-B
Indicus/1	++ (ll)	(+) (ll)	-	+ (ll)	++ (s)	-	17.0 (ll)
Brecon/1	++ (ll)	+ (ll)	+ (ll)	++ (ll)	++ (s)	++ (s)	11.5 (ll)
TT01/1	(+) (ls)	- (ll)	-	++ (ll)	+ (ll)	-	21.2 (ll)
Rh/1	+ (s)	-	-	+ (s)	++ (ll)	-	8.6 (ll)
Rh/2	(+) (ls)	-	-	(+) (ls)	+ (ll)	-	3.8 (ll)
Hb/1	++ (ll)	(+) (ll)	-	+ (ll)	-	-	1.7 (ls)
Hb/2	(+) (ls)	-	-	(+) (ll)	(+) (ls)	-	3.8 (ls)
Hm/1	+ (ls)	-	-	+ (s)	-	-	4.5 (ll)
Hm/2	-	-	-	-	-	-	9.3 (ll)
Nc19/1	+ (ll)	++ (ll)	-	++ (ll)	++ (s)	++ (s)	9.7 (ll)
Nc19/2	+ (ll)	++ (ll)	-	++ (ll)	(+) (ls)	-	1.5 (ll)
Wx6/1	(+) (ls)	-	-	+ (ll)	+ (ll)	-	4.3 (s)
Wx6/2	-	-	-	-	++ (ll)	-	2.2 (s)
Wx8/1	+ (ll)	(+) (s)	-	+ (ll)	++ (ll)	-	3.4 (s)
Wx8/int.	++ (ll)	(+) (s)	-	++ (ll)	-	-	3.6 (s)
Wx8/2	+ (ll)	(+) (s)	-	+ (ll)	-	-	3.1 (s)
HSH-2/1	-	-	-	-	-	-	2.1 (ls)
HSH-2/2	-	-	-	-	-	-	3.2 (ls)
K122/1	(+) (s)	-	+ (s)	++ (s)	++ (s)	-	9.9 (s)
K122/2	-	-	-	(\pm) (ls)	-	-	0.4 (s)

7.1.2 Enzyme activity measurement with chromogen oligopeptide substrate

I measured and compared the time course of proteolytic activity of 20 strains on Fua-Leu-Gly-Pro-Ala (Fua-LGPA) bacterial collagenase substrate. This artificial chromogen substrate is used to measure the activity of proteases that have primary preferences for amino acids that are C terminal to the scissile bond. Hydrolysis of that substrate at the Leu-Gly bond causes a shift in the absorption spectrum of the Fua chromophore group [106]. I found that all the tested strains produce an enzyme with Fua-ALGPA-ase activity in the late logarithmic and early stationary growth phases (Table 3). Later in the stationary phase (40 to 50 hours, depending on the strain) the activity did not increase further. The Fua-LGPA-ase enzyme, termed as Php-B, was purified and proved to be a 74-kDa intracellular metalloenzyme, known as OpdA,

which could not be detected by either SDS-PAGE or native PAGE coupled zymography [107].

7.2 Measuring protease activity during infection

7.2.1 Infection of *Galleria mellonella* larvae with different *Photorhabdus* strains

In order to investigate the presence of protease activities during infection, I took hemolymph plasma samples from *G. mellonella* larvae infected with five different *Photorhabdus* strains, Brecon/1, Hm/1, Hm/2, Nc19/1 or Nc19/2, for detection of PrtA and Php-C with native and SDS-PAGE-coupled zymography. Choosing these strains I tested how the differences in protease production in culture manifest during infection. In culture growth from Hm/1 and Hm/2 phase variant strains, the Hm/1 secretes both PrtA and Php-C activities, while Hm/2 produces neither of them (no enzyme activity was detectable with SDS- and native PAGE coupled zymography **Fig. 7**).

I injected about 100 cells into each *G. mellonella* larvae and detected the activities of both PrtA and Php-C the earliest at 12 to 42 hours postinfection, respectively (**Fig. 8**). While PrtA was produced again by every strain, I observed Php-C only in the hemolymph plasma of Brecon infected *G. mellonella* 42 h postinfection (**Table 4**). From these experiments I concluded that PrtA was the earliest secreted enzyme that I could detect with my methods both in culture and infection.

Figure 8. Proteolytic activity during *Galleria mellonella* infection with Nc19/1 *Photorhabdus* strain. A representative experiment showing the detection of PrtA with SDS-PAGE coupled zymography. The numbers above the gel indicate the time points in hours of hemolymph sampling from *Galleria* larvae after infection with about 100 *Photorhabdus* cells. PrtA activity appeared first at 28 hours after injection. The same result was obtained in case of Hm/1, Hm/2 or Brecon infected insects (see Table 4). Note: the proteases from *G. mellonella* larvae itself did not interfere with the detection of *Photorhabdus* proteases [1]

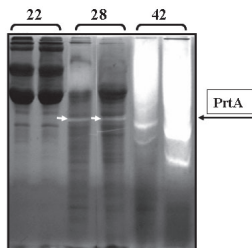


Table 4. The time of first detection of PrtA in the hemolymph plasma samples from infected insects.

Strain	Protease	Time of first detection with zymography following	
		SDS-PAGE	Native-PAGE
Nc19/1	PrtA	28	42
Nc19/2	PrtA	42	42
Hm/1	PrtA	28	42
Hm/2	PrtA	28	42
Brecon/1	PrtA	28	42
	Php-C	42	42

7.3 Investigation of natural substrates of PrtA

As an early secreted enzyme of a pathogen, PrtA might function as a virulence factor. I hypothesized that as a protease it may have an immune suppressive role e.g., through the cleavage of immune proteins. To investigate this possibility I continued my experiments with the exploration of the proteolytic system of *Photorhabdus* PrtA searching for target proteins in the hemolymph of *Manduca sexta*. Thus, I subjected hemolymph plasma from naïve *M. sexta* larvae to *in vitro* digestion with purified PrtA.

Since the number of proteins in the hemolymph is large and some are present in high concentration, it would have been hard to detect the cleavage of those components that are in small amount. Therefore, first I performed protein separation on a DEAE anion-exchanger. By SDS-PAGE analysis of the fractions I could distinguish four groups of the fractions (“type” fractions) that differed in at least one protein band from each other. Fractions I, II, III and IV represents these groups (**Fig 9**).

I treated these four “type” fractions with purified PrtA at a ratio of 0.5 to 5.0 μg hemolymph protein to 0.3 ng PrtA (~ 0.3 nM final). In order to see specificity and sensitivity of PrtA cleavage I also subjected the “type” fractions to digestion with pancreatic trypsin, chymotrypsin and *Clostridium histolyticum* collagenase (60 nM final concentration) for shorter and longer incubation (45 and 120 min). **Figure 10** shows that PrtA hydrolyzed ten proteins in “type” fractions I-III (no cleavage was observed in “type” fraction IV). Since one of these proteins was also cleaved by another protease (collagenase; ~ 110 kDa protein in fraction II on **Fig 10B**), I concluded that nine proteins might be specific substrates to *Photorhabdus* PrtA (**Fig. 10**). I provisionally distinguished these proteins with the name PAT-*x*, where PAT means PrtA Target, and *x* shows the molar mass estimated by the protein’s relative mobility in SDS-PAGE.

Figure 9. DEAE Sephadex anion exchange chromatography of *Manduca sexta* non-immunized hemolymph plasma. A: SDS-PAGE analysis of the fractions shows four fraction types (“type” fractions I-IV), which contain unique band pattern. **B:** SDS-PAGE analysis of the four “type” fractions (I-IV) generated by DEAE Sephadex chromatography. The fraction numbers are shown on the top of the gels corresponding to A. The proteins which were cleaved by PrtA are labeled with arrows. MW, molecular mass standard.

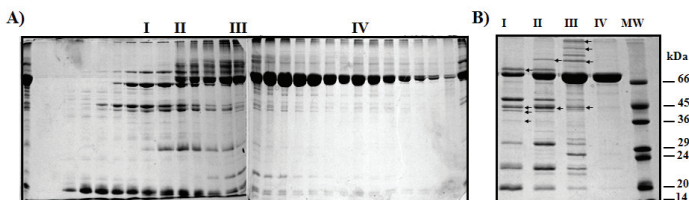
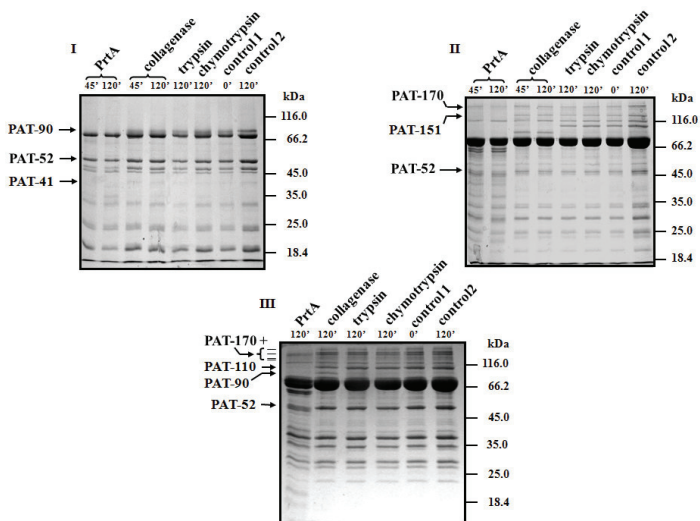


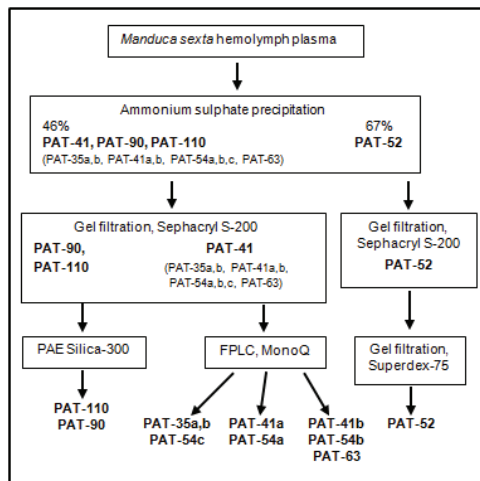
Figure 10. Selective PrtA cleavage of proteins in the three “type” fractions (I-III) from DEAE Sephadex chromatography. SDS-PAGE analysis. The conditions of the reactions shown above the lanes are the type of the protease applied (control is without protease) and incubation time. Arrows point to PAT proteins that are selectively cleaved by PrtA. The numbers are estimated to the molecular masses of PAT proteins. These are not shown for three proteins, which are larger than PAT-170 in type fraction III because of the imprecision of estimation. Molecular weights (kDa) are indicated on the right hand side of each gels.



7.4 Isolation and identification of PAT proteins from *Manduca sexta* hemolymph

To understand the possible role of PrtA during the infection process of *Photorhabdus* I investigated the substrate side in the proteolytic system of PrtA further with the isolation and identification of its target proteins (PAT proteins). To this end I developed a purification procedure for PAT proteins (Fig. 11) to reach such a separation level that is sufficient for their N-terminal sequencing after SDS-PAGE and blotting and thus, for their identification from the protein database.

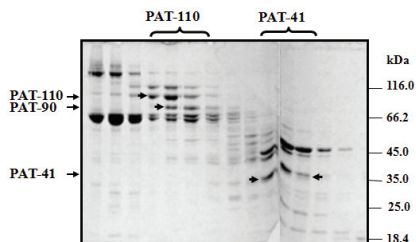
Figure 11. Procedure for the separation of PAT proteins. PAT protein names in boldface letters in a purification step indicate those proteins that were detectable following that step.



7.4.1 Purification of PAT-110

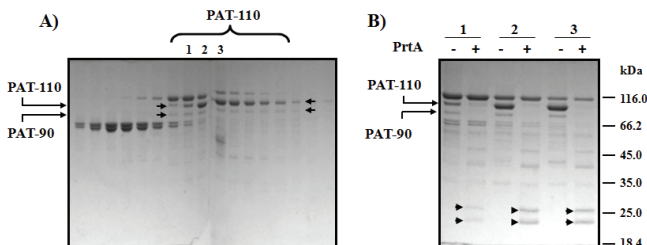
I started the purification of PrtA target proteins with a two-step $(\text{NH}_4)_2\text{SO}_4$ precipitation. The 46 % precipitate contained all of the PAT proteins, including PAT-110, PAT-90 and PAT-41, but not PAT-52, which remained in the supernatant. The second purification step to separate PAT-110 from the other hemolymph proteins was gel filtration of proteins in the 46 % $(\text{NH}_4)_2\text{SO}_4$ precipitate on Sephacryl S-200 column (Fig. 12). This step separated efficiently PAT-110 from PAT-41 and a number of other hemolymph proteins, but PAT-90 remained with PAT-110. From this step I made the further purifications of PAT-110 – PAT-90 and PAT-41 separately (Fig. 11).

Figure 12. Gel filtration of proteins in the 46 % $(\text{NH}_4)_2\text{SO}_4$ precipitate on Sephacryl S-200 column. SDS-PAGE analysis of the fractions. Arrowheads indicate the appearance of PAT-110, PAT-90 and PAT-41 during gel filtration procedure. The fractions containing PAT-110 and PAT-41 are indicated on the top of the gel.



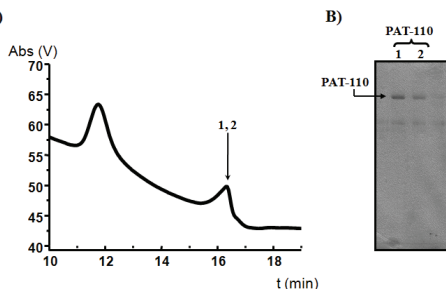
For further purification of PAT-110, I combined the fractions of gel filtration containing PAT-110 (and PAT-90) and subjected to PAE Silica chromatography. The SDS-PAGE analysis of the fractions shows that PAT-110 and PAT-90 remained in the same fractions, but they were pure from many other hemolymph proteins (**Fig.13A**). When I treated the fractions containing PAT-110 and PAT-90 with PrtA (**Fig.13B**), only these two proteins were hydrolyzed. Their hydrolysis was complete under the applied conditions (0.3 ng PrtA to 0.5-5.0 μg hemolymph protein ratio and 90 min incubation time) showing that they were very sensitive to PrtA digestion. The cleavage products of PAT-110, a 27 kDa and a 20 kDa protein, that accumulated temporarily indicated that PrtA can have more than one cleavage site in that protein.

Figure 13. PAE Silica anion exchange chromatography of PAT-110. **A:** SDS-PAGE analysis of the fractions. Arrowheads indicate the appearance of PAT-110 and PAT-90 during the chromatography procedure. The fractions containing PAT-110 is indicated on the top of the gel. **B:** SDS-PAGE analysis of PrtA treatment of three fractions (1, 2, 3). The fraction numbers above the lane correspond to A. Incubation in the presence (+) or absence (-) of PrtA is indicated on the top of the gel. Arrowheads show the PAT-110 cleavage products.



Fractions from PAE chromatography containing PAT-110 and PAT-90 were still contaminated with several other proteins. To eliminate these and obtain pure PAT-110 and PAT-90 fractions I performed Mono Q anion exchanger chromatography. PAT-110 was eluted in a pure form from the column at approximately 0.2 M NaCl concentration, whereas PAT-90 was lost (**Fig. 14**). Although the yield was very low, the amount was sufficient for blotting and N-terminal sequencing.

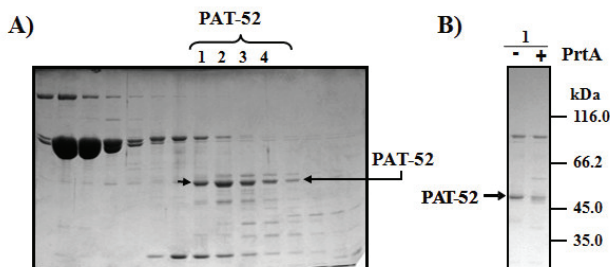
Figure 14. Mono Q anion exchange chromatography of PAT-110. **A:** Profile of protein elution. The fractions (1, 2) containing PAT-110 was eluted from the column at 16 min. **B:** SDS-PAGE analysis of the fractions. The fractions containing PAT-110 are indicated on the top of the gel. The fraction numbers above the lane correspond to A.



7.4.3 Purification of PAT-52 protein

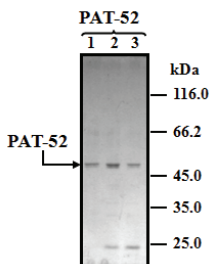
After 46% precipitation with ammonium sulfate PAT-52 was separated from PAT-110 and other PAT proteins (**Fig. 11**) and remained in the supernatant. Then, it was precipitated with additional 20% $(\text{NH}_4)_2\text{SO}_4$. I resuspended the precipitate and purified further first with gel filtration on a Sephacryl S-200 column (**Fig. 16A**). The SDS gel analysis shows that PAT-52 was separated from the proteins with higher molecular weight but not from the smaller ones. The treatment of the fractions with PrtA showed that PAT-52 was the only protein which was cleaved by PrtA, although it did not hydrolyzed completely (see later) (**Fig. 16B**).

Figure 16. Gel filtration of PAT-52 on Sephacryl S-200 column. A: SDS-PAGE analysis of the fractions. The fraction numbers are shown above the lanes (1-4). The fractions containing PAT-52 are indicated on the top of the gel. **B:** SDS-PAE analysis of the PrtA treated (+) and non-treated control (-) fractions. The fraction number (1) above the lane corresponds to A.



I continued the purification with fractions of Sephacryl S200 column which contained PAT-52 (**Fig. 16**) on a Superdex-75 gel filtration column to separate PAT-52 further from the minor hemolymph proteins still present. **Fig. 17** shows that the level of the resulted purity was sufficient for blotting and N-terminal sequencing.

Figure 17. Superdex-75 gel filtration chromatography of PAT-52. SDS-PAGE analysis of the fractions. The fractions containing PAT-52 are indicated on the top of the gel.



7.4.4 Determination of N-terminal sequences of PAT proteins

The isolation and concentration of eight proteins such as PAT-52 and PAT-110 after complete purification, then PAT-35ab, PAT-41a, PAT-41b, PAT-54a, PAT-63 and PAT-90 after partial purification permitted the N-terminal sequence determination from blotted PVDF membrane following SDS-PAGE. Surprisingly, the sequencing resulted in nine determined N-termini because one of the samples (PAT-35ab), however, it showed one single band on the SDS gel, but it gave double signal in each sequencing cycle indicative of two proteins distinguished as PAT-35a and PAT-35b. This increased the total number of PAT proteins to sixteen. Having the N-terminals of the nine PAT proteins determined, I looked for homologues of the obtained amino acid sequences in the NCBI protein databases using the Basic Local Alignment Search Tool (BLAST) program. **Table 5** summarizes those PAT proteins for which I obtained sequence information together with the database hits as well as their known or supposed function. With the exception for PAT-41b, PAT-90 and PAT-110, the interrogation of the Protein Data Bank yielded matches with high confidence. For the double sequence of PAT-35a and b Miklós Képpó generated all the possible sequence variants ($2^{10} = 1024$ sequences) with the help of a C-script then loaded them on the BLAST server. He restricted the search to *M. sexta* sequences with an upper limit of e-value at 0.1. The search resulted in two proteins, scolexin A and scolexin B. Their polypeptide chains are of the same length and exhibit more than 90% sequence identity

explaining why PAT-35 a and b remained together during the isolation steps and in SDS-PAGE.

Regarding the function of each identified PAT proteins I can conclude that all of them seem to have immune-related functions, which either had been known, supposed or was proven later as in the case of PAT-41a (see below). (The sequencings were made by András Patthy).

Table 5. N-terminal sequences of nine PAT proteins in comparison to the closest hits from the protein database. Abbreviations: SPH-3, serine protease homologue-3; HAIP, hemocyte aggregation inhibitor protein; β -GRP-2, β -1,3 glucan recognition protein-2.

PAT protein	N-terminal sequence Database sequence	Database name	BLAST e value	(Supposed) function	Accession number
PAT-35ab	DANDIDIQQN				
	GPGANQLNLK	Scolexin A	4×10^{-2}	Immune	AAD14591
	DPGANDIQLN GANDIQLNQK	Scolexin B	6×10^{-2}	Immune	AAD14592
PAT-41	DQGSPDLPLATEPPT				
	DQGSPDLPLATEPPT	SPH-3	3×10^{-4}	Immune	AAO21508
PAT-41b	GVIKEESI	Unknown		Unknown	
PAT-52	ETDLQKILRESNDQFTA				
	ETDLQKILRESNDQFTA	Serpin-1	1×10^{-5}	Immune	AAC47343
PAT-54	TPASVPRKVLXYYSK				
	TPASVPRKVLXYYSK	HAIP	2×10^{-3}	Immune	AAB32418
PAT-63	LEVPDALKEAIYPKG				
	LEVPDALKEAIYPKG	β -GRP-2	2×10^{-2}	Immune	AAN10151
PAT-90	QXNESLVI	Unknown		Unknown	
PAT-110	QRXESLVISXVEDXGXAAI	Unknown		Unknown	

7.4.5 Analysis of the cleavage products of PAT proteins

As PAT proteins became more concentrated and better isolated from other proteins, some features of their cleavage could be observed. For example PAT-52 could never be hydrolyzed completely (**Fig.16B**), even a longer (120 minute) exposure to PrtA. In contrast, PAT-110 and 90 proved to be very sensitive: they were completely cleaved in the presence of even 0.03 ng PrtA within less than 40 minutes. Importantly, when I exposed pure PAT-110 to trypsin or chymotrypsin digestion under the same

conditions as those with PrtA, it remained intact showing that this protein (like other PAT proteins) is not generally sensitive for proteolysis.

The appearance of cleavage products was mostly temporary indicating a further degradation by either PrtA or contaminating proteinases that were present in the hemolymph and could start acting on PAT proteins only after the initial cleavage by PrtA. However, the cleavage product of PAT-170, 54a, 41a, 35a,b and especially PAT-52 has longer half life suggesting that these might not be degraded further by PrtA. In the case of the latter the cleavage product was just a little smaller than the intact PAT-52 (**Fig.16B**) showing that PrtA clips only 10-15 amino acids from one of the molecular termini (see below).

I investigated the cleavage of PAT-52 (*M. sexta* serpin-1) further to explain why a fraction of this protein always remained uncleaved by PrtA. This protein is present in the hemolymph in twelve C-terminal variants [56], and even the purified fraction might contain several of them. Therefore, the most likely explanation is that only some of the variants are sensitive for PrtA cleavage. To verify this and to establish which ones are cleaved, I expressed and purified all of the serpin-1 variants and exposed them to PrtA digestion. Several variants (B, E, F, J, and Z) were, indeed, not digested; the others in turn were hydrolyzed with an occasional accumulation of 1.0-1.5 kDa smaller, short lived cleavage products. PrtA must discriminate serpin-1 variants through their differential, 40-50 amino acid long C-terminal end, which contains the reactive site loop, confers the protease selectivity [61] and – as it is revealed by their different PrtA sensitivity – also the susceptibility to proteolytic cleavage by a noncognate (i.e. non serine) protease. The N-terminal sequence of the cleaved and the uncleaved forms was the same, which proves that PrtA clipped 10-15 amino acids from the C-terminus of serpin-1. I obtained the same result when the N-terminal sequence of PAT-52, partially purified from *M. sexta* hemolymph and exposed to PrtA hydrolysis, was determined. It was the same as that of the intact protein (ETDLQKILRESNDQFTA).

7.5 Identification of the function of Serine Protease Homologue-3

I identified PAT-41a as Serine Protease Homologue-3 (SPH-3), a *M. sexta* hemolymph protein of unknown function. However, it was reported as “immune related” on the basis that it has proved to be immune inducible [108]. To verify its function in the immune system of *M. sexta* I exploited RNA interference technique.

7.5.1 Induction and RNAi-mediated knockdown of SPH-3 in *M. sexta*

First, to corroborate that *SPH-3* is an immune inducible gene in *Manduca sexta* I carried out RT-PCR using specific *SPH-3* primers and RNA extracts from fat body and hemocytes. The RT-PCR result was consistent with earlier observations that SPH-3 was expressed in naïve, non-immune induced *Manduca larvae* at a very low level, but it was markedly up-regulated in both fat body and hemocytes of insects which had previously been challenged with either non-pathogenic *E. coli* or pathogenic *Photographus* TT01 bacteria (**Fig. 18**). Thus, no SPH-3 transcription was seen in negative controls including insects injected with PBS or untreated insects.

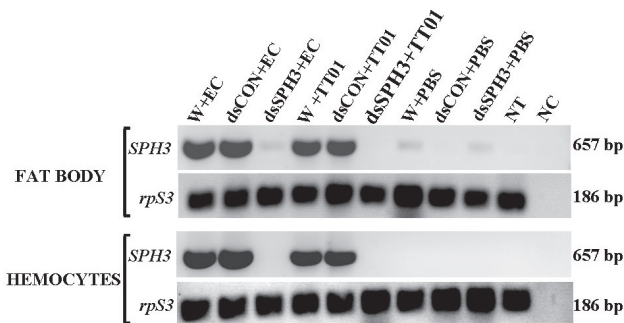
Figure 18. Induction of SPH-3 encoding gene in *Manduca sexta*. RT-PCR on fat body and hemocyte total RNA was extracted 18 h after bacterial challenge with *E. coli* (EC) or *Photographus* (TT01) or injection of PBS. A no-template RT-PCR control (NC) and the non-treated (NT) negative control are included. *Manduca sexta* ribosomal protein gene S3 (rpS3) was used as a loading control. The sizes of PCR products are indicated. (For the specific primers used see **Table 2**)



Second, to investigate if SPH-3 is required for normal immune function I knocked down *SPH-3* in *Manduca sexta* with RNA interference. For the experiments I constructed a dsRNA reagent specific for SPH-3 (dsSPH3). When I checked SPH-3

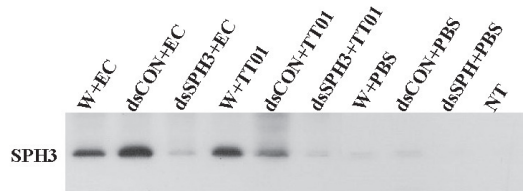
expression (as above) 18 h after dsRNA treatment I found complete knockdown i.e. upon injection with either non-pathogen or pathogen microorganism the gene of SPH-3 remained inactive (**Fig. 19**). This inhibition in the induction of SPH-3 gene was specific to dsSPH3 treatment, and it was not observed on treatment with dsRNA of a plant catalase (dsCON; **Fig. 19**). This also showed that the inhibition of SPH-3 gene induction was not a general effect of dsRNA injection.

Figure 19. RNAi-mediated knockdown of SPH-3 in *Manduca*. 6 h before bacterial challenge insects were pretreated with the injection of dsRNAs of either SPH-3 (dsSPH3) or catalase (dsCON as dsRNA control), or of DMPC-treated water (W as injection control). The immune challenge was the injection of 10^3 cells of either *E. coli* (EC) or *Photorhabdus luminescens* TT01 (TT01). As negative control (no bacterial challenge), insects were pretreated the same way, but 6 hours later they were injected with PBS (PBS). Total RNA was extracted from fat body 18 h after second injection (with bacterial challenge or PBS). Non treated control (NT) and RT-PCR control (NC) are also shown. (For further details see Methods). *Manduca sexta* ribosomal protein gene S3 (rpS3) was used as a loading control. The sizes of PCR products are indicated. RT-PCR was performed in duplicate for each treatment; results for single individuals are shown.



Then, I investigated the effect of dsSPH3 treatment on protein level also, using immunoblot with anti-SPH-3 antibody. The result was in a complete agreement with that of RT-PCR: SPH-3 protein level in hemolymph plasma is also strongly reduced by RNAi knockdown of SPH-3 (dsSPH3), but not by dsCON. At the same time I observed very low levels of SPH-3 protein in the non-induced controls which means that there is some SPH-3 expression even in naïve larvae, which correlates with the fact that I purified SPH-3 protein from naïve *M. sexta* hemolymph plasma (**Fig. 20**).

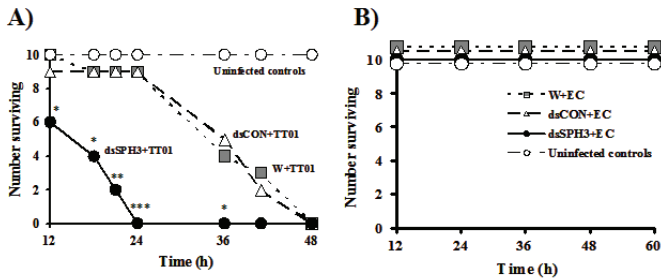
Figure 20. Western blot analysis of hemolymph SPH-3 protein level, using specific anti-SPH-3 antibody. The treatments and injection protocol were the same as given in Figure 19. (For further details see Methods.) The experiment was made in duplicate for each treatment; results for single individuals are shown. Specificity of staining was checked by omitting the primary antibody, which led to complete absence of bands (not shown).



7.5.2 Mortality bioassay

Having knocked-down of SPH-3 transcription successfully the next question was whether it has an effect on *M. sexta* mortality. To test this I carried out mortality bioassay in which *M. sexta* larvae were injected first with either DMPC-treated water (W), dsRNA control (dsCON) or SPH-3 dsRNA (dsSPH3) and 6 h later with 10^3 cells of *P. luminescens* TT01 or PBS. As it was expected from earlier results 10^3 cells of *P. luminescens* TT01 killed *Manducas* within 48 h when they were pretreated with water or dsCON before TT01 injection [80]. By contrast RNAi knockdown of SPH-3 (dsSPH pretreatment) caused a drastic decrease in the ability of the insect to survive experimental *Photorhabdus* infections since all insects died within 24 h. The time for 50% survival decreased from 24-36 h in control insects to only 12-21 h in the dsSPH3-treated insects. The difference in the number of survivors at 21 h was highly significant. Injection of dsRNAs alone without bacterial infection (dsSPH+PBS), or injection of water and then PBS (W+PBS) did not cause death. Similarly when nonpathogenic *E. coli* was used instead of *Photorhabdus* no insect death was observed and the survival rate was the same as that of non-pretreated insects (Fig. 21).

Figure 21. Time course mortality bioassay following RNAi of SPH-3. **A:** RNAi-mediated knockdown of SPH-3 is associated with decreased survival following *Photorhabdus* (TT01) exposure. The injection protocol of insects was the same as given in the legend to Figure 19. Asterisks show significant differences between SPH-3 knock-down insects and the infected control groups. **B:** The survival rate of insects infected with nonpathogenic *E. coli* was 100%. Groups of ten insects were pre-treated with dsRNA 6 h before administering the same dose of *E. coli* as the lethal inoculum of *Photorhabdus*. Uninfected control groups were the same in both (A;B) experiments (W+PBS, dsSPH+PBS, dsCON+PBS, and NT). The experiment was repeated three times with same results.

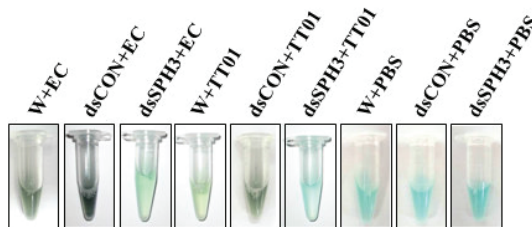


7.5.3 SPH-3 is required for prophenoloxidase (PPO) synthesis

Since phenoloxidase (PO) activation in hemolymph plasma is an important and easily-observed component of immune defense, I examined PO activity in cell-free hemolymph of insects infected with bacteria. Prophenoloxidase (PPO), the proenzyme of phenoloxidase (PO) is constitutively present in hemolymph plasma at a low level and is overtranscribed on immune challenge (see Introduction). To test whether knocking down of SPH-3 by RNAi affects PO level, first I examined hemolymph total PO activity visually, through the observation of hemolymph melanization (**Fig. 22**). RNAi knock-down of SPH-3 followed by infection with *E. coli* caused hemolymph of treated insects to remain unmelanized after bleeding (dsSPH3+EC). By comparison hemolymph from infected insects given control dsRNA or water, turned black within one hour (dsCON+EC; W+EC). I also examined hemolymph from insects infected with *Photorhabdus*. In the case of infected controls (dsCON+TT01; W+TT01), the result is complicated by the fact that *Photorhabdus* produces molecules that inhibit plasma PO [80]. Consequently, the extent of PO-mediated darkening observed in hemolymph from these insects was less than in the case of *E. coli*-infected insects. Nevertheless,

hemolymph from *Photorhabdus*-infected insects pre-treated with SPH-3 dsRNA darkened much less than that of infected controls. This might be caused by the inhibitor of PO produced by TT01, the lack of PPO activation or the suppression of PPO production. Under the conditions of this assay, hemolymph from uninfected controls did not darken at all.

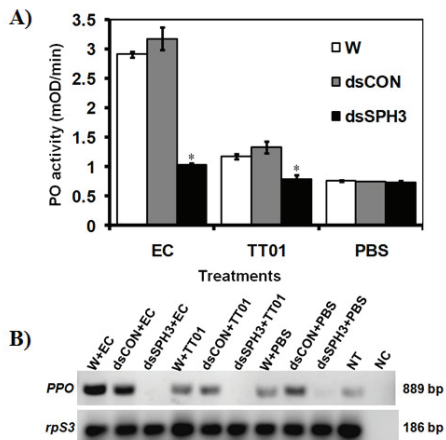
Figure 22. RNAi of SPH-3 prevents melanization. PO activity as shown by visual examination of hemolymph exposed to air for 1 h after bleeding. Treatments and controls were as described in the legend to Fig. 19. Note the reduced melanization in the hemolymph from *Photorhabdus*-infected insects (W+TT01 or dsCON+TT01) compared to *E. coli*-infected individuals (W+EC, dsCON+EC) (for further explanation see text).



Thus, the effect of SPH-3 knock down was similar to that of *Photorhabdus* injection regarding PO activity and melanization. This raised the immediate question whether SPH-3 is required for either the activation or the synthesis of prophenoloxidase (PPO). The hypothesis was that if SPH-3 is primarily required for PPO activation, then the total amount of activatable PO should not be reduced in the SPH-3 knock-down insects, regardless of whether they have been infected or not. On the other hand, if the main role of SPH-3 is to allow the synthesis of additional PPO (i.e. it participates in the induction of the gene upon infection), then the total amount of activatable PO in the SPH-3 knockdown insects should be markedly lower than in control insects (without knock down) when both types of insect have been exposed to infection. At the same time the dsSPH3 treated and untreated insects should not be different in activatable PO in the absence of bacterial infection from the negative controls i.e. to insects without bacterial challenge (W+PBS, dsCON+PBS and dsSPH3+PBS). To test this I carried out PO measurements to determine PO and PPO levels in the hemolymph. For the latter PPO conversion to PO was induced *in vitro* by the PPO-activating agent, bacterial lipopolysaccharide (LPS) (Fig. 23A). When total PO activities from dsSPH3 pretreated

and *E. coli* or *Photorhabdus* TT01 injected insects were compared, they did not differ and were similar to the activity of negative controls (W+PBS; dsCON+PBS; dsSPH3+PBS). However, the difference was substantial relative to positive controls (no dsSPH3 pretreatment+ *E. coli* infection), because these latter exhibited a significant increase in PO activity upon bacterial challenge. When *Photorhabdus* TT01 was used for infection in the positive control, the increase in PO activity was much less probably due to the PO inhibitor of *Photorhabdus* TT01 [80] (and perhaps the suppression of SPH-3). The simplest interpretation of this result is that total PO activity is lower in dsSPH3-treated insects, because there is less proenzyme (PPO) in the hemolymph plasma. This might be because SPH-3 is needed to PPO synthesis rather than to PO activation. To investigate this possibility I used RT-PCR to detect the mRNA level of PPO in fat body cells (Fig. 23B). The results show that the treatment with dsRNA of SPH-3, indeed, down-regulated the mRNA level of PPO, so that not only its induction was suppressed after bacterial infection but also the basal PPO mRNA level was also abrogated.

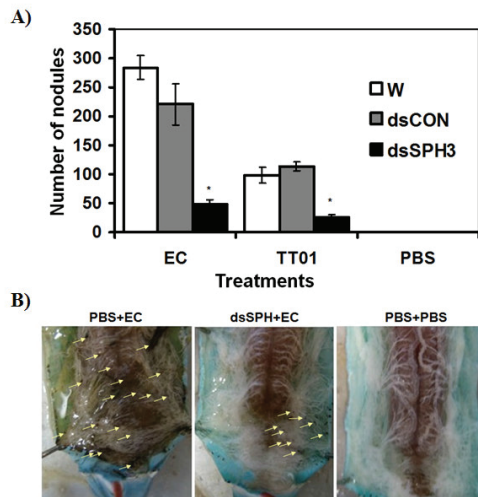
Figure 23. Effect of dsSPH3 on PO activity. The protocol of pretreatment and infection of insects as well as hemolymph sampling were the same as given in the legends to Figure 19. (For further details and mRNA extraction from fat body see Methods). **A:** Total PO activity measurement in hemolymph plasma samples on 4-methylcatechol after 60 minutes activation of PPO with the addition of LPS. **B:** Fat body PPO mRNA levels detected with RT-PCR



7.5.4 SPH-3 is required for nodule formation

Like other insects, *M. sexta* caterpillars form melanotic nodules around sites of bacterial invasion. This is an important defense mechanism against infection in which PPO also takes part [78]. Following the same protocol of treatment and infections as above, I investigated the number of melanotic nodules formed *in vivo* in *M. sexta* larvae. Insects pre-treated with SPH-3 dsRNA (dsSPH3) have fewer nodules than water (W) or dsRNA control (dsCON) pre-treated insects (**Fig. 24**). This result was consistent with the reduction in the amount of PPO in the hemolymph of the SPH-3 knock down insects but it cannot be excluded that the smaller number of nodules may have been due to another way of SPH-3 participation in nodule formation (e.g. via influencing the production of cell adhesion factors).

Figure 24. Number of melanotic nodules formed in *M. sexta* larvae. The experimental conditions (insect pretreatment infection and the time of sampling were the same as described in the legends to Figure 19.) Five insects were used for each treatment and each assay was replicated three times. Asterisks indicate significant differences between the SPH-3 knock-down insects and the two control groups.

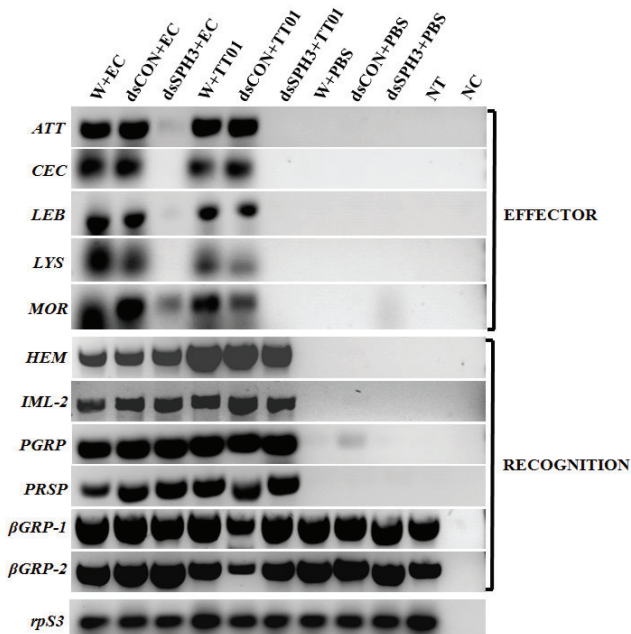


7.5.5 SPH-3 is required for synthesis of antimicrobial effectors but not recognition proteins

The results above showed that SPH-3 plays an important role in *M. sexta* immune responses to Gram-negative bacteria. Thus, the next question was how basic the role is, i.e.; how wide the range of processes is that SPH-3 is involved in? Therefore first I looked by RT-PCR if knocking-down of SPH-3 influenced the mRNA level of the known immune effector antimicrobial proteins and peptides other than PPO/PO, such as Attacin, Cecropin, Lebocin, Lysozyme, and Moricin which are induced by immune challenge in *M. sexta*. I found that the SPH-3 RNAi treatment markedly reduced or abrogated the immune-stimulated transcription of all these genes (**Fig. 25**). Essentially they remained silent even upon bacterial infection. This shows that the induced expression of SPH-3 is required for immune signaling upstream of events that control the expression of a wide range of immune effectors. Next, I tested whether RNAi of SPH-3 influenced the function of immune related recognition proteins also. I carried out a series of RT-PCRs using specific primers to detect mRNA encoding six different recognition proteins. Hemolin, Immulectin-2, Peptidoglycan Recognition Protein (PGRP), Pattern Recognition Serine Protease (PRSP, also known as HP-14, hemolymph proteinase-14) are recognition proteins in *M. sexta*, which are immune inducible after bacterial challenge while other two recognition proteins, β -1-3-glucan recognition protein-1 (β -GRP-1) and β -1-3-glucan recognition protein -2 (β -GRP-2) are constitutively expressed. I found that RNAi of SPH-3 had no effect on the mRNA levels of any of these proteins (**Fig. 25**).

Thus, the simplest explanation of my observations is that SPH-3 participates somehow in the signal mediation from the recognition of pathogen towards the control of genes of the immune effectors.

Figure 25. RT-PCR results indicating levels in fat body of mRNAs encoding six microbial pattern recognition protein genes: *Hemolin (HEM)*, *Immulectin-2 (IML-2)*, *Peptidoglycan Recognition Protein 1A (PGRP-1A)*, *Pattern Recognition Serine Proteinase (PRSP)* and β -1-3-glucan recognition proteins (β -GRP-1 and -2), and six antibacterial effector peptide genes: *Attacin (ATT)*, *Cecropin (CEC)*, *Lebocin (LEB)*, *Lysozyme (LYS)*, *Moricin (MOR)*. The pretreatment, infection and sampling protocol of *M. sexta* larvae was the same as given in the legends to Figure 19 (for further details see Methods). *Manduca sexta* ribosomal protein gene S3 (*rpS3*) was used as a loading control. Size of PCR products is indicated. RT-PCR was assessed in duplicate for each treatment; results for a single individual are shown.

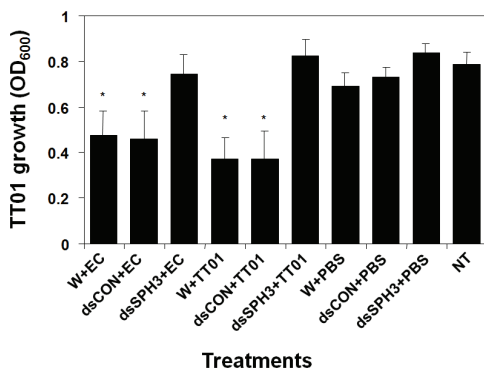


7.5.6 Knock-down of SPH-3 enhances the survival of *Photographus* in hemolymph

Since the role of immune inducible antimicrobial effectors is to restrict the growth of invading microbes in the insect's hemolymph [10], the results above showing the down regulation of all the examined effector genes imply that the knock-down of

SPH-3 expression can lead to enhanced growth of pathogenic bacteria. To test this, I pre-treated insects as above and then induced antimicrobial effector synthesis by infecting them with either *E. coli* or *Phototrhabdus* as previously. 18 h post-infection I prepared bacterium-free plasma from these insects by centrifugation and inoculated them with *Phototrhabdus* TT01 bacteria. I found as expected, that the pre-exposure of insects to *E. coli* or *Phototrhabdus* infection i.e. the immunization significantly reduced the growth of *Phototrhabdus* in the plasma, but this immunizing effect was completely abrogated by prior knockdown of SPH-3 (**Fig. 26**) so that *Phototrhabdus* grew to the same extent in the plasma of dsRNA of SPH-3 pre-treated insects as in the plasma of naïve insects (that had not been exposed to bacteria).

Figure 26. Pathogen growth assay. Final cell number (optical density at 600 nm, OD₆₀₀) of *Phototrhabdus* after 18 h growth in cell-free plasma collected from variously treated *M. sexta* larvae. The protocol of pretreatment, infection and hemolymph sample taking was the same as described in the legends to Figure 19. Asterisks show values that do not differ from each other, but which differ significantly from all other values.



8. Discussion

Proteolytic systems might play an important role in the pathomechanism of infections. The role of proteases in biological processes can only be understood via examining the whole proteolytic system in which they participate. My PhD work is part of a study aimed to explore the possible role of a proteolytic system in bacterial virulence using a natural host-pathogen model system, which is between a highly pathogenic bacterium, *Photobacterium luminescens*, and an insect host, *Manduca sexta*, and to gain insight into the pathogenicity with biochemical and molecular approaches.

To explore the proteolytic systems of *Photobacterium*, I started my work with the investigation of a *Photobacterium* protease, PrtA whether it might function as a virulence factor. Then, in order to obtain some information about its target protein preference and, through this, the potential role in the infection of this enzyme, I looked for and identified its natural substrates in *M. sexta* hemolymph. Finally, I investigated the role of one of the PrtA targets, SPH-3, in *M. sexta* immune response if it reveals new aspects of the insect immune defense.

8.1 Investigation for a possible virulence factor

As a first step in the investigation of the proteolytic system of *Photobacterium*, which may be involved in the pathogenic process, I examined the protease production of 20 *Photobacterium* strains, including eight phase variant pairs. This was the first time when such a high number of *Photobacterium* strains was compared for their protease production biochemically, using a combination of different detection methods. The hypothesis was that a protease which might function as a virulence factor should be the earliest produced enzyme by all strains. I found three major activities during the bacterial growth in culture, termed as PrtA, Php-B and Php-C. Php-B was produced by all strains while PrtA and Php-C production exhibited substantial and seemingly strain specific differences; ranging from undetectable (HSH/2 strain) to a very intensive production in both phase variants of a strain NC19 (**Table 4**). In all cases where I detected enzyme activity, the enzyme responsible for the most prominent zymographic activity, was also the enzyme that was secreted earliest in both culture growth and infection. This was PrtA, an Rtx-like protease belonging to the serralyisin subfamily of metallopeptidases. These results suggested that PrtA can be a protease which might function as a virulence factor although it was not detected in all strains. The lack of

PrtA activity was rather characteristic for the secondary phase variant *Photorhabdus* strains which are, at the same time, very pathogenic to insect hosts, but they are not able to form symbiosis with nematodes. The simplest explanation for larger differences or the absence or presence of PrtA activity might be the formation of an enzyme-inhibitor complex, (like the one between PrtA and the Inh protein of strain W14) as it has already been proposed as a potential cause of the reduced protease activity of some *Photorhabdus* strains or secondary-phase variants [100, 109]. While this might be the interpretation for a reduced (or missing) PrtA activity detected with native PAGE-coupled zymography, it cannot explain the missing zymographic activity observed after SDS-PAGE, because the presence of SDS disrupts the non-covalent enzyme-inhibitor complex and the enzyme can be well separated from the inhibitor by the time of zymogram development. Further possibilities are a non-functional secretion or missing or inactive PrtA gene. I did not test whether there was a non-functioning secretion (resulting in an accumulation of PrtA in the cytosol), and I did not examine either the activity of the gene or the possibility of a differential posttranslational regulation [110], which would have been needed to clarify the cause of variations in PrtA production.

The fact that *Photorhabdus* bacteria are extremely virulent specialist pathogens of insects – in the laboratory only 10-100 bacteria are sufficient to kill the host [111] – must involve a mechanism by which the bacterium can evade the immune defense and PrtA can still be one of the factors participating in it. In support of this assumption are the facts that *Photorhabdus* starts producing PrtA early during infection (see above), and PrtA did not exhibit activity on native proteins (fibrinogen, albumin and collagen types I and IV) [101], which might have been expected if it had function in the bioconversion of host tissues as a non-specific protease [78, 99], or if it were involved in the degradation of extracellular matrix [4]. The contribution of PrtA to pathogenicity does not include a direct toxic effect either [99], as in the case of several other metalloproteases which are lethal toxins.

8.2 PrtA might function as an immune suppressor

To prove that PrtA is a virulence factor with e.g., with immune suppressive function, I looked for potential target proteins in the hemolymph of *Manduca sexta* that are specifically cleaved by PrtA.

I obtained sequence information for nine of the sixteen proteins that were sensitive to PrtA treatment *in vitro*, and by searching databases I could identify six of

them (**Table 5**). PAT-63 is the β -1,3 glucan recognition protein-2 (β -1,3-GRP-2) of *M. sexta* [112]. Similar proteins have been found in different families of invertebrates [112-116]. By binding β -1,3-glucans (e.g., laminarin and crudlan) and/or lipopolysaccharides, such pattern recognition proteins function as immune receptors which trigger proteolytic cascade(s) for the activation of prophenoloxidase [53, 112, 113, 115-117] and an unknown signaling pathway for the production of immune-inducible antibacterial peptides and proteins [115]. PAT-54a, the hemocyte aggregation inhibitor protein (HAIP) had been found through its effect on hemocytes *in vitro* [118]. It might be involved in cellular immune responses like nodulation and encapsulation of pathogens. PAT-35 a and b, *Manduca sexta* scolexin A and B, are chymotrypsin-like proteinases [119, 120] which are thought to induce coagulation reactions during nodulation and encapsulation. PAT-52, *Manduca sexta* serpin-1, was found to inhibit *in vitro* various serine proteinases [58, 61]. Of the twelve C-terminal sequence variants, which are generated via alternative splicing [105], functions were found only for serpin-1I and J, which inhibit hemolymph proteinase-14 [53] and prophenoloxidase-activating proteinase-3 [20], respectively. Thus, they are involved in the regulation of one of the most important instant immune responses of insects, melanization which is caused by phenoloxidase activity. The twelve serpin-1 variants, which differ only in their 40-50 amino acids long C-terminal sequence, were cleaved with different rates by PrtA. This specifically shows a differential sensitivity to PrtA, while generally indicates that the C-terminal segment, which confers the protease selectivity to serpin-1, may also be the determinant of their stability towards a non-cognate protease. PAT-41, serine proteinase homolog-3 (SPH-3), belongs to a large group of proteins that are similar to serine proteinases in their amino acid sequence but are catalytically inactive due to the replacement of the catalytic residue(s) [30]. Members of the group are also found in vertebrates. Most of them contain an N-terminal, disulfide knotted extension called clip-domain. With the exception for several clip-domain SPH-s, their function is unknown. SPH-3, a non-clip-domain SPH, has been found as an immune inducible protein [22]. I suppose that PAT-41, 110 and (most of) the other, unidentified PAT proteins also have immune related function, and may be new, as yet unknown participants in the immune system.

8.3 SPH-3 might play an important role in the immune defenses of *Manduca sexta*

Among the PrtA target proteins SPH-3, a serine proteinase homologue (PAT-41a) had originally been found in a survey of immune-induced mRNAs in *M. sexta* fat body cells by M. Kanost's group but then they did not investigate its function in the immune response [108]. Thus, to find further evidence for the possible role(s) of PrtA in the infection mechanism of *Photorhabdus* through studying the function of its target proteins, SPH-3 seemed an attractive choice. An additional interest in such study was the fact that the function of only several SPH-s is known despite their abundance in the animal kingdom.

I employed SPH-3 specific dsRNA treatment of *M. sexta* larvae to suppress SPH-3 production through systemic RNAi, a method which had previously been used for the investigation of various immune proteins [10, 11, 79]. In those studies it was found that knock-down of individual effectors in *M. sexta* using RNAi generally had only a small effect on the insect's ability to resist *Photorhabdus* infection. In contrast, suppressing expression of individual recognition genes (PRPs – e.g.; Hemolin, IML-2 and PGRP) increased substantially the death rates. This is in accord with the supposition that there are only few pathways from immune detection, each of which probably control a number of effector functions. I have shown that the effect of SPH-3 knock-down on resistance to *Photorhabdus* in itself is large, and thus SPH-3 functionally resembles a pattern recognition protein (PRP) rather than an antimicrobial effector. However, the unusually large impact of SPH-3 inactivation on *M. sexta* survival raised an alternative possibility also, that SPH-3 can play a role in the signal mediation, between the recognition and effector functions. Consistent with such a role, I found that inhibition of SPH-3 expression had no effect on the mRNA levels of any of four examined PRPs, but that SPH-3 knock-down strongly repressed the immune-related expression of all five tested antimicrobial effectors. I confirmed that the decreased ability to resist *Photorhabdus* is due to the hemolymph plasma's lower content of antimicrobial peptides and proteins, by showing that SPH-3 knock-down leads to enhanced ability of *Photorhabdus* (and *E. coli*) to grow in *M. sexta* hemolymph plasma *in vitro*.

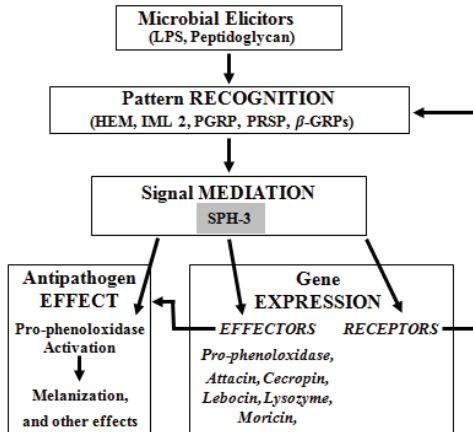
Among the reduced antimicrobial functions were the diminished hemolymph total PO activity in infected (but not uninfected) insects, showing that SPH-3 is required for the up regulation of PPO synthesis.

My findings point to a central role for SPH-3 in immune signaling in *M. sexta* which underlines the importance of ensuring that the RNAi effect of the dsRNA used is specific to the targeted gene. In this case, the observed effects can be considered as specific ones to SPH-3 knock-down because (i) a control dsRNA (corresponding to an irrelevant gene from a plant) did not have the same effect, and (ii) the immune-induced over-transcription of PRSP, which has considerable sequence similarity (51% at the amino acid level) with SPH-3, was completely unaffected by the SPH-3 RNAi pre-treatment.

The result of my experiments that RNAi knock down of SPH-3 mRNA drastically reduced the mRNA levels of all the tested effector molecules, but did not affect those of the pattern recognition proteins, suggests that SPH-3, as a component of the extracellular signaling pathway, participates in the mediation of information from the recognition of microbial invaders specifically to the control of antimicrobial effector synthesis. This is of interest for several reasons: (i) SPH-3 is involved in the regulation of gene expression whereas SPH-s of known function are not known to do this; (ii) the range of affected immune-related genes is wide; (iii) the surprising restriction of the signaling route only towards the antimicrobial effector genes, implies the existence of (at least) two distinct immune signaling pathways in *M. sexta*; (iv) since SPH-3 is catalytically inactive it might function as either a ligand or an adaptor molecule in the immune signaling pathway.

To date extracellular signal mediation is believed to be a single process which controls both receptors and effector genes through a network of intracellular transducer molecules. My observations indicate for the first time that signal mediation towards the effector genes may be distinct from the pathway leading to the recognition gene expression. This observation might reveal new aspects of the insect immune system (**Fig. 27**). This notion requires a rethinking of the structure of immune signaling and prompts the investigation of the possibility a new pathway which is independent from the known ones and may be the main controller of the immune effector genes.

Figure 27. Proposed model of the role of SPH-3 in *Manduca sexta* immune pathways. Proposed model for the role of SPH3 in *Manduca* immune pathways. SPH-3 acts downstream of the pattern recognition proteins and is involved in signal mediation from pathogen recognition towards the gene regulation of immune effectors, but not of recognition proteins.



The existence of intracellular immune signaling pathways is already well established in *Drosophila*, where pathways form the Toll and Imd receptors each regulate the induced expression of partially overlapping sets of effector genes in response to fungal/Gram positive and Gram negative bacterial infections, respectively [121]. However, the extracellular part of these pathways, i.e. the link between the recognition of microorganisms by the PRR and the activation of the intracellular Toll (and also the Imd) pathway is still missing. In the developing *Drosophila*, Toll activation is triggered by a cascade of serine proteases consisting of Gastrulation defective, Snake and Easter, a process which is similar to the coagulation cascade in mammalian blood. This cascade ends with the cleavage of the clip domain of the cytokine-like protein Spätzle [122]. Being catalytically inactive, SPH-3 cannot function as an enzyme in a proteolytic signaling cascade. It is possible; however, that it is a functional counterpart in *Manduca* of Spätzle (or a related molecule) in *Drosophila*, i.e. a ligand in an extracellular signaling pathway towards an unknown receptor. A Spätzle homologue is present in *Bombyx mori* (also a *Lepidopteran*, like *M. sexta*) and the

involvement of Spätzle homologue in the signaling pathway has been shown in both *Bombyx* and *Manduca* [123]. A Spätzle processing enzyme is present in *Bombyx* [124].

The possibility of a functional similarity between SPH-3 and Spätzle is not supported by the pairwise amino acid sequence comparison. It showed no significant similarity between SPH-3 and Spätzle (either *Drosophila* or *Bombyx*) but, surprisingly, does show similarity between SPH-3 and Easter, which is a catalytically active, clip domain containing serine proteinase of *Drosophila* in the signaling pathway towards Toll receptor. The sequences are 21.7% identical even when the full (clip domain containing) sequence of Easter is included in the comparison. (Without the clip domain the identity is 25.3%; **Table 6**)

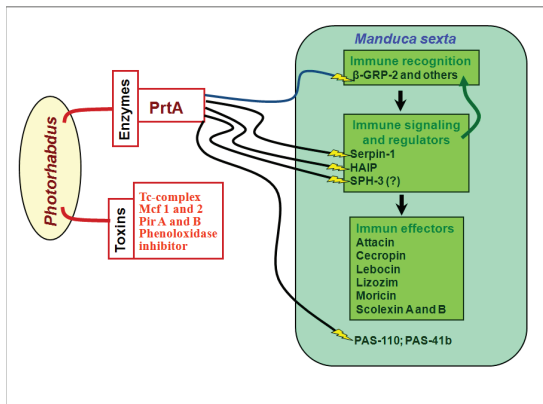
Table 6. Results of the sequence comparison of SPH-3 with Spätzle, Gastrulation defective, Easer and Snake.

	Identity with SPH-3	Identity with SPH-3 without clip-domain
Spatzle	18.6%	-
Gastrulation defective	15.5%	-
Easter	21.7%	25.3%
Snake	15.2%	21.4%

9. Conclusion

The identity of the six PAT proteins and the experimental data on them indicate that they (may) have immune related function involving the three aspects of the immune defense: (i) immune recognition (β -1,3-GRP-2); (ii) immune signaling and regulation (HAIP, SPH-3, and serpin-1), and (iii) antimicrobial effector activity (scolexin A and B, and the “SPH-3 signaling pathway” controlled effectors). In as much as my observations *in vitro* can reflect the activity of PrtA *in vivo*, the functions of the identified PrtA target proteins indicate, for the first time, a role to serralysin, which is a multiple participation in the virulence mechanism of a pathogen. Through the cleavage of a number of immune proteins, this mechanism is a complex suppressive role on the innate immune response via interfering with both the recognition and the elimination of the pathogen during the first, infective stage of the host-pathogen interaction.

Figure 28. The possible roles of PrtA in the infection strategy of *Photorhabdus*. My observations suggest a multiple participation of PrtA in the virulence mechanism of *Photorhabdus*.



My results also suggest that natural target proteins might be found to other serralysins including these enzymes of even human pathogens, also among the components of rather the innate than the adaptive immune system. This supposition

seems reasonable also because some innate immune mechanisms are conserved and many are similar throughout the animal kingdom. The first challenge for the pathogens during an infection is the innate immune response, thus it might be a winning strategy, and effective virulence mechanism to weaken or destroy the components of it.

10. Abstract

In my thesis work I used an easily accessible, low cost insect-entomopathogen bacterium model system for studying the role of a protease in the virulence mechanism. The insect, *Manduca sexta* is widely used as a model for insect biochemical research due to its size and hemolymph volume, while the bacterium, *Photorhabdus*, is an intensively studied pathogen for its nematode-symbiotic lifecycle and very high virulence.

Despite the fact that proteases can play various roles in establishing and propagating infection, their function in these processes is rarely investigated and therefore not known. The assessment of these is possible only through determining at least the target proteins in their proteolytic system.

To find a virulence associated protease, I investigated the protease production of *P. luminescens* with various methods, during both culturing of 20 strains and infection. Three proteolytic activities could be distinguished this way. One of these enzymes was PrtA, a metzincin metalloprotease, a member of the serralyisin family which was the earliest secreted enzyme by *Photorhabdus* in both culture growth and during infection. It was hypothesized that PrtA might function as a virulence factor. To prove this, I investigated its natural substrates in *Manduca sexta* hemolymph. I found sixteen PrtA target (PAT) proteins which were selectively cleaved by PrtA. I purified nine of them partially and one of them fully. With the help of their N-terminal sequence I could identify six PAT proteins from the NCBI protein database. Each of these has immune related function involving the three aspects of immune defense: recognition, signal mediation and effector functions.

I investigated further one of the PrtA target proteins, SPH-3 (serine protease homolog-3) which had been known as an immune-inducible protein, but its function had not been studied. I found that SPH-3 plays a very important role in the immune signaling in *Manduca sexta*: RNAi knock down of SPH-3 mRNA drastically increased the mortality of the insects infected with *Photorhabdus*. This was accompanied by dramatically reduced mRNA levels of all the tested immune effector (but not the pattern recognition, immune receptor) molecules. I concluded from this that SPH-3 act as an extracellular component in a signaling pathway, which is responsible for the control of antimicrobial effector synthesis. Such a group specific immune signaling,

which distinguishes the immune receptor from the immune effector genes is not known yet in the insect immune system.

Based on my results I suppose that PAT-41, 110 and (most of) the other, unidentified PAT proteins also have immune related function, and may be new, as yet unknown components in the immune system. In conclusion, my observations support the assumption that PrtA is a virulence factor of *Photobacterium* which might have an immune-suppressive role during infection.

11. Összefoglalás

Munkám során olyan *Manduca-Photorhabdus* rovar-entomopatogén baktérium modellrendszerrel használtam egy proteáz virulenciában betöltött szerepének tanulmányozására, ami széles körben használt, könnyen hozzáférhető és alacsony költségű. *Manduca sexta* méretének, és a belőle nyerhető hemolimfa mennyiségének köszönhetően a biokémiai kutatásokban széles körben alkalmazott rovar modell. A *Photorhabdus* rovarpatogén baktériumokat sokan vizsgálják erős virulenciájuk, és érdekes fonalféreg-szimbióta életmódjuk következtében.

Annak ellenére, hogy a fertőzések kialakításában a bakteriális fehérjebontó enzimeknek sokféle szerepe lehet, funkciójuk csak kevésbé vizsgált, így szinte nem is ismert. A patomechanizmusban betöltött szerepüket szubsztrátspecifitásuk és proteolitikus rendszereik felderítésével lehetne jobban megismerni.

Első céloom egy olyan proteáz keresése volt, amely részt vehet a *Photorhabdus* által kialakított fertőzésben. 20 *Photorhabdus* baktérium törzs proteáz termelését hasonlítottam össze különböző módszerekkel kultúrában, és fertőzést követően egyaránt. Három proteolitikus enzimet különítettem el. Ezek közül a PrtA, egy serralysin metalloproteáz volt az, ami a legkorábban termelődött mind kultúrában mind a fertőzést követően egyaránt. A hipotézisem szerint az az enzim, amely a legkorábban termelődik, tehát a PrtA, lehet az a proteáz, amely virulencia faktorként fontos szerepet játszhat a fertőzés kialakításában. Ennek bizonyítására olyan természetes szubsztrát fehérjéket kerestem *Manduca sexta* hemolimfában, amelyeket a PrtA szelektíven hasít. Összesen tizenhat ilyen célfehérjét találtam, amelyek közül kilencet teljesen, illetve részlegesen megtisztítottam. N-terminális szekvenciájuk alapján hat fehérjét tudtam azonosítani az NCBI fehérje adatbázisból. Mind a hat azonosított protein immunfehérjének bizonyult.

Továbbiakban az egyik PrtA target fehérjének, az SPH-3-nak (szerin proteáz homológ-3) a rovar immunrendszerben betöltött lehetséges szerepét vizsgáltam. Kísérleteim alapján elmondható, hogy az SPH-3 jelentős szerepet tölt be a *M. sexta* immun-szignalizációs útvonalában; az SPH-3 RNSi-vel történő kiütését követően jelentősen megnőtt a rovarok *Photorhabdus* fertőzést követő mortalitása. Ezzel egyidejűleg az immunválaszt hordozó, összes jelenleg ismert antimikrobiális fehérje és peptid génjének expressziója leállt, míg az immun receptorok expressziója, ill. indukciója változatlan maradt. Ebből arra következtettem, hogy az SPH-3 központi

szerepet tölthet be olyan sejten kívüli immunjelátviteli folyamatban, amely az antimikrobiális effektorok szintéziséért felelős. Ilyen specifikus immun-szignalizációs útvonalra, amely megkülönbözteti az immun receptor és az immun effektor gének indukálását, eddig még nem volt példa a rovarok immunrendszerében.

Eddigi eredményeim alapján feltételezem, hogy az adatbázisban nem szereplő PrtA target fehérjék (PAT-41 és PAT-110), ugyancsak immun-fehérjék, amelyek új, vagy eddig még ismeretlen résztvevői lehetnek az immunrendszernek. Összegzésként elmondható, hogy megfigyeléseim alátámasztják azt a feltételezésemet, hogy a PrtA proteáz rendelkezhet immunszuppresszív szereppel a *Photorhabdus* fertőzési mechanizmusában.

12. References

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