

Doctoral Thesis

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

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Introduction

Pathogenic microorganisms have a characteristic spectrum of hosts. The understanding of the host specificity, which might be instrumental in the prevention and alternative treatments of microbial infections, requires the exploration of the molecular interactions which determine virulence.

Virulence factors of microorganisms belong to either microbial toxins or enzymes. Secreted enzymes can 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 e.g., they can efficiently interfere with the function of the proteinaceous immune molecules. Despite the large number of known secreted proteases, there are only sporadic observations for the function of these enzymes. These include inhibitor A of *Bacillus thuringiensis* and the proteases of *Serratia marcescens*, which specifically cleave insect immune proteins such as cecropins and attacins, while the zinc metalloproteases of *Bacteroides fragilis* and *Clostridium* spp. have direct toxin activity.

Among the enzymes secreted by a wide range of pathogens are the Serralysin-type Zn-metallo-proteases (in the M10 family of metalloproteases). These are supposed to be nonspecific enzymes, involved in e.g., bioconversion of the host tissues, because they exhibited a relaxed residue preference on a variety of synthetic peptide substrates and denatured oligopeptides of biological origin. However, serralysins like most of the proteases encounter substrate proteins under physiological conditions, where they are in native conformation. The cleavage of such proteins is substantially restricted by the difficulty of disrupting the native structure around the cleavage site in a 6-8 amino acid long segment, which is needed to achieve proper substrate binding to the active site of the protease.

Therefore, the observations on artificial substrates are poorly informative about the function of Serralysins (and other proteases) and do not exclude the possibility that they can have specific target proteins of special function(s). Excepting some inhibitors that are secreted by the bacteria together with the proteases other interacting proteins of Serralysins, including their natural substrate proteins are essentially not known. Due to this, the generally supposed virulence factor role of serralysins is still not confirmed experimentally. The only observation in this regard is the *in vitro* cleavage of IgA and IgG proteins and several human defensins as well as some cell matrix and interconnecting filament proteins, by ZapA of *Proteus mirabilis* and serralysin of *Serratia marcescens*. However, the *in vivo* significance of these reactions remains to be established because the applied conditions, a large enzyme to substrate molar

ratio, and the very long incubation time (1:10-1:6000 and 3-24 hours, respectively) do not indicate a sensitive cleavage which might be expected in the case of specific target proteins.

PrtA is the best known and studied of the enzymes produced by *Photorhabdus* entomopathogenic bacteria. As a 55 kDa, RTX (repeats-in-toxin)-like metalloprotease, PrtA belongs to the serralsin subfamily of metalloproteases. It was described first from a plant pathogen, *Erwinia chrysanthemi*. Later it was found in other pathogens including *Photorhabdus* and *Xenorhabdus* strains which are insect pathogens. The secretion of PrtA by these organisms has been investigated in both culture growth and infection. During infection anti-PrtA immunoreactivity labels showed the localization of PrtA first in the midgut, then in the fat body, muscle and their associated tracheae. With a development of a highly sensitive and specific artificial substrate the investigation of the dynamics of PrtA production became possible. PrtA was first detected 14 h post infection. The activity also was found mainly in the tissues and only ten hours later it appeared in the hemolymph.

These data are insufficient to decide whether PrtA might function as a virulence factor and they also cannot tell the role(s) that PrtA might play during infection. For a (full) understanding of the function of a proteinase (or any other enzyme) the exploration of its interacting system is needed. In the case of proteases, this is their “proteolytic system”, which is constituted by – besides the protease – all the possible substrate proteins and inhibitors. Due to the nature of the proteolytic system of proteases secreted by pathogens, mapping such systems of pathogen also can reveal new components and/or functions of and shed a new light on the host’s defense mechanisms.

For such studies an infection model is needed. Of these, the insect host and entomopathogenic bacterium is a possibility, which has the advantage of easy accessibility and low cost. At the same time for the very similar molecular components, primarily on the side of the pathogen, but to some degree on the side of the host too, the observations can be instructive for studies in vertebrate host using pathogenicity models. The model host in my experiments was *Manduca sexta* (Tobacco hornworm, *Lepidoptera*), while the pathogen was *Photorhabdus luminescens* (*Enterobacteriaceae*). *Manduca sexta* is widely used as a model for insect biochemical research due to its size and hemolymph volume. *Photorhabdus* is also intensively studied for its nematode-symbiotic lifecycle and very high virulence.

Goals of my thesis work

The main goal of my work was to find and identify target proteins to a serralysin protease, PrtA, and through this to investigate the question whether PrtA might function as a virulence factor. I supposed that the knowledge of the target proteins and their function would give an insight into the possible role of a serralysin-type enzyme and prove that it might take part in the pathomechanism of a microorganism.

Specific aims

- Detection and comparison of the time course of PrtA and other proteolytic activities in different *Photorhabdus* strains in culture growth and during insect infection to find protein(s) that can have a function in the early phase of infection.
- Searching for proteins, which are natural target proteins for PrtA in *Manduca sexta* hemolymph and investigation of their cleavage *in vitro*.
- Purification or isolation of the found proteins sufficiently for N-terminal determination.
- Identification of the proteins and their functions with database search and experiments.

Materials and methods

1. The bacteria and insects

- *Photorhabdus* strains from the entomopathogenic nematode/bacterium strain collection maintained at the Department of Genetics, Eötvös Loránd University, Budapest, Hungary.
- *Manduca sexta* (Tobacco Hornworm, *Lepidoptera*, *Sphingidae*) eggs were hatched and larvae reared in our laboratory. The eggs were kindly provided by the Department of Biochemistry, University of Bath, Bath, United Kingdom.
- *Galleria mellonella* culture was maintained in our laboratory.

2. Investigation of protease production of *Photorhabdus*

- Native-PAGE and SDS-PAGE coupled zymography as well as a range of chromogenic substrates were used.

3. Investigation of natural target proteins of PrtA

- PAT proteins were purified with different chromatographic steps for N-terminal sequencing.
- Hemolymph samples and (partially) purified proteins were exposed to mainly heterologously expressed PrtA purified from culture of *E. coli* Hb101 strains transformed with pUC19 plasmid which contained PrtA cloned from *Photorhabdus* ssp. *akhurstii* W14 (a generous gift from Richard French-Constant, Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom). The cleavage of proteins was analyzed with SDS-PAGE and N-terminal sequencing.
- The (C-His(6) tagged) twelve of *M. sexta* Serpin-1 was expressed in *E. coli* XL1 blue strain, which was transformed with Bluescript plasmids that contained the Serpin-1 variant-encoding sequences (generous gift from Mike Kanost, Department of Biochemistry, Kansas State University, U.S.A.).
- The N-terminal sequencing was made by Dr András Patthy (Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary).

4. Investigation of the role of serine proteinases-3 (SPH-3) in *Manduca sexta* immunity with RNA interference (RNAi).

- RNA interference (RNAi) of SPH-3

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- The dsRNA of SPH-3 was generated with standard procedure.
 - Total RNA isolates from fatbody or hemocytes were used for the RT-PCR analysis of the effects of SPH-3 RNAi on the activity of twelve genes.
 - For the investigation of immune physiological effects of SPH-3 knock-down, *in vitro* pathogen growth, mortality and nodule formation bioassay and phenoloxidase activity measurements were performed.

Results

1. Investigation of protease production of *Photorhabdus*

- For the first time, the protease activities of 20 different *Photorhabdus* strains were compared during culture growth and insect infection.
- With SDS and native PAGE-coupled zymography two activities, termed PrtA (a Serralysin) and Php-C (Oligopeptidase A), were detected and with chromogen oligopeptide substrate one other activity, termed Php-B.
- PrtA was the earliest secreted enzyme both in culture and infection meeting the best the expectations for a virulence factor.

2. Searching for and identification of natural target proteins to PrtA

- I found sixteen target proteins in *Manduca sexta* hemolymph which are selectively cleaved by PrtA. Provisionally I gave name “PAT-X” to these proteins, where PAT is the acronym of PrtA target, and X refers to their molecular mass.
- I purified nine of them partially or fully, and I could identify six of them from the NCBI protein database based on their N-terminal sequence.
 - PAT-63, the β -1,3 glucan recognition protein-2 (β -1,3-GRP-2) of *M. sexta*.
 - PAT-54a, the hemocyte aggregation inhibitor protein (HAIP) of *M. sexta*.
 - PAT-35a and b, scolexin A and B of *M. sexta*, chymotrypsin-like proteinases.
 - PAT-52, *Manduca* serpin-1, which has twelve C-terminal sequence variants.
 - PAT-41, serine proteinase homolog-3 (SPH-3) of *M. sexta*,
 - The interrogation of the database with the sequence of PAT-110 (and PAT-90) did not yielded hits, these hemolymph proteins of *M. sexta* are not

known yet. The other, unidentified PAT proteins also might have immune related function, and may be new, as yet unknown participants in the immune system.

3. Investigating the function of one of the PAT proteins, a Serine Protease Homologue, SPH-3

- Serine protease homolog-3 had been known as an immune inducible protein. I confirmed this with showing a markedly up-regulation of SPH-3 in both fat body and hemocytes of insects which had previously been challenged with either non-pathogenic *E. coli* or pathogenic *Photorhabdus* TT01 bacteria.
- I knocked-down *SPH-3* in *Manduca sexta* with dsRNA injection, which produced the following pathophysiological effects indicating a central role for SPH-3 in the immune response:
 - The ability of the insect to survive experimental *Photorhabdus* infections drastically decreased. The time for 50% survival decreased from 24-36 h in control insects to only 12-21 h in the dsSPH3-treated insects;
 - Whereas the mRNA levels of any of the six tested pattern recognition proteins such as Hemolin, Immulectin-2, Peptidoglycan Recognition Protein (PGRP), Pattern Recognition Serine Protease (PRSP, also known as hemolymph proteinase-14), β -1-3-glucan recognition protein-1 (β -GRP-1) and β -1-3-glucan recognition protein -2 (β -GRP-2) did not change, the mRNA levels of all the six tested effector molecules such as attacain, cecropin, lebocin, lysosyme, moricin, prophenoloxidase dropped to zero.
 - The melanization of hemolymph and the number of nodules 18 hours after infection decreased along with the *in vitro* the bacterium growth inhibiting effect of hemolymph.

Discussion

The fact, that PrtA was the earliest secreted enzyme by all the 20 tested *Photorhabdus* strains during culture growth and insect infection, raise the question whether it might play role in the infection process of *Photorhabdus*. To an answer of this question I looked for and found sixteen proteins (the PAT proteins) and I could identify six ones that proved specific targets to PrtA in *Manduca sexta* hemolymph. All of the identified proteins have immune related function.

I investigated further one of the target proteins, serine proteinase homolog-3 (SPH-3, found as PAT-41). This protein 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) [64].

The drastic reduction in the immune effector (but not the immune recognition) function and the concomitant increase in susceptibility to infection, upon systemic RNAi of SPH-3 indicate a central role to SPH-3 in the immune defense of *M. sexta*: SPH-3 may participate specifically in the extracellular signaling between the recognition of microbial patterns and the initiation of antimicrobial effector synthesis. Thus SPH-3, though an extracellular protein, is involved somehow in the control of gene expression, which has not been known for SPH proteins. The fact that only the expression of immune effector genes was affected by SPH-3 knock-down is of interest because to date signal mediation thought to be a single process which controls both receptors and effector genes. However, my findings suggest that the signal mediation toward the effector genes is distinct from the pathway leading to the recognition gene expression, which might reveal new aspects of the insect immune system.

The cleavage of PAT proteins by PrtA can collectively have a direct compromising effect on the three aspects of the immune defense: (i) immune recognition (β -1,3-GRP-1 and 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). As much as my *in vitro* observations reflect the processes *in vivo* they may indicate, for the first time, a role to a serralyisin. This might be 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. My results also suggest that the natural target proteins might be found to other serralyisins, including these enzymes of

human pathogens too, also among the components of rather the innate than the adaptive immune system. Employing such an enzyme as virulence factor is clearly advantageous since the first challenge for the pathogens during an infection is to overcome the elements of the innate immune system.

Scientific publications relating to my thesis work:

- **Gabriella Felföldi**, Judit Marokházi, Miklós Képiró, and István Venekei Identification of natural target proteins indicates functions to a serralsin-type metalloprotease, PrtA, in anti immune mechanisms Applied and Environmental Microbiology 2009 May;75(10):3120-6. (IF, 4.004)
- Marokházi J., Lengyel K., Pekár S., **Felföldi G.**, Patthy A., Gráf L., Fodor A. and Venekei I. (2004) Comparison of proteolytic activities produced by the entomopathogenic *Photorhabdus* bacteria: strain and phase dependent heterogeneity in the composition and activity of four enzymes. Applied and Environmental Microbiology, 70(12):7311-20. (IF, 4.004)
- Eleftherianos I, Gokcen F, **Felföldi G**, Millichap PJ, Trenczek TE, French-Constant RH, Reynolds SE. The immunoglobulin family protein Hemolin mediates cellular immune responses to bacteria in the insect *Manduca sexta*. Cell. Microbiol. 2007 May;9(5):1137-47 (IF, 2007: 5.293)
- Eleftherianos I, **Felföldi G**, French-Constant RH, Reynolds SE. Induced nitric oxide synthesis in the gut of *Manduca sexta* protects against oral infection by the bacterial pathogen *Photorhabdus luminescens*. Insect Mol Biol. 2009 18(4):507-16 (IF, 2.784)

Scientific presentations relating to my thesis work:

- Felföldi Gabriella, Marokházi Judit, Lengyel Katalin, Patthy András, Gráf László, Fodor András, Venekei István: Proteolitikus enzimek szerepe a *Photorhabdus* rovarpatogén baktérium fertőzési folyamatában Magyar Biokémiai Társaság Molekuláris Biológiai Szakosztályának éves munkaértekezlete Sopron, 2004. 05. 10-13, poster
- Judit Marokházi, **Gabriella Felföldi**, Nikolett Mihala, Ferenc Hudecz, András Patthy, András Fodor, László Gráf, István Venekei: Identification of cleavage site and natural substrate specificity of PrtA, a serralsin-type metalloprotease from the

entomopathogenic microorganism *Photorhabdus* 30th FEBS Congress - 9th IUBMB Conference Budapest, Hungary 2005. Július 2-7, poster

- **Gabriella Felföldi**, Ioannis Eleftherianos, Richard French-Constant, István Venekei, Stuart E. Reynolds: The role of Serine Protease -3 (SPH-3) in *Manduca sexta* shown by RNAi. 7th International Workshop on the Molecular Biology and Genetics of the Lepidoptera Orthodox Academy of Crete, Crete, Greece, Aug. 20-26, 2006, presentation
- **Gabriella Felföldi**, Ioannis Eleftherianos, Richard French-constant, Stuart E. Reynolds: The Serine Protease Homologue -3 (SPH-3) mediates immune response in *Manduca sexta*. '06 Royal Entomological Society National Meeting University of Bath Bath, United Kingdom, Sept. 20-22, 2006, poster