Synopsis of Doctoral Thesis

Secreted proteolytic enzymes of the entomopathogenic bacterium *Xenorhabdus*

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Background and Rationale

Detailed molecular level exploration of interactions between microorganisms and their hosts is indispensable to the understanding and distinction of various infection processes. These range from hostile to cooperative type, such as pathogenic or symbiotic relationship, through mutual tolerance. The knowledge of participating molecular factors and the underlying molecular mechanisms can be exploited in pathogen control for either suppression or support of infection. (Examples for the two cases are, respectively, the prevention and treatment of diseases caused by microorganisms and the environmental friendly biological pest control using e.g. insect pathogen organisms.)

The important, exciting and yet unanswered questions regarding the host-pathogen interactions – among others – are: i.) Why a host tolerates certain pathogens in some situation and not in others. Alternatively, viewed the interaction from the other side, why a pathogen, persistent in the host, can initiate disease only occasionally? That is, what are the molecular determinants of the fate of infections? ii.) What are the determinants of host specificity? How the virulence factors influence the pathogenicity of a microorganism and with this the range of hosts?

Compared to the significance of these questions from the aspects of both basic and applied science, it is surprising how little is known about the molecular details of infections. This is even more striking relative to the expectations set by the era of systems biology: the desired level of knowledge is not less than what is generated by exploring the full range of interacting partners of all the virulence factors of a pathogen and all the defense mechanisms of a host, which have role in the host-pathogen interaction. However, it is extremely difficult to meet this expectation, even with using high throughput methods, because of the large number of participating molecules and their interactions. This is not surprising because - in order to maximize their survival - both the pathogen and the host employ a molecular arsenal during the course of their interaction: the pathogen

secretes a range of various virulence factors while the host uses multiple ways of defense, resulting in an immune response avoidance and “over-killing” on the pathogen side and a complex network of mechanisms, the immune system on the host side.

From enzymological point of view, proteins with catalytic capabilities can play many roles that are useful for a pathogen in every phase of infection. Protease, lipase, glycosidase activities can be instrumental in the penetration of the tissues of host and the host itself, in avoiding and suppressing the immune response of host and in the bioconversion of the molecules of host for the nutrient supply of the pathogen. Numerous enzymes of pathogens are known or supposed as virulence factors but only several targets (substrates) and inhibitors of only few of these enzymes is known. In order to understand the role of an enzyme during infection the complete exploration of its interaction system would be needed (called “proteolytic system” in the case of proteases), which consists of all the substrates and inhibitors of the enzyme.

In the laboratory where I made my Ph.D. work the role of pathogen secreted proteolytic enzymes is studied in such an infection model which features both the hostile (pathogenic) and the cooperative (symbiotic) type of host-microorganism interaction.

Introduction

A good infection model is safe in two respects: i.) if the pathogen is very efficient, i.e. it is highly virulent and, at the same time, ii.) if it is not dangerous to work with. Further requirements are the easy accessibility (e.g. reproduction of the model host) and the low cost. All these are satisfied by the insect pathogen – insect system which is used in the Biochemistry Department of Eötvös Loránd University. Although the defense system of insect hosts - lacking adaptive immunity - is relatively simple but their innate immunity has the same basic design containing very similar or same parts and molecular components as the more complex defense systems which makes observations in the insect model useful in others. Also, the pathogen side of this system seems distant from those that infect humans or agriculturally important animals and plants. However, it is known that most of the enzymic virulence factors of microorganisms are similar or the same.

The pathogen component of the model I used was *Xenorhabdus* which - together with the very close relative genus, *Photorhabdus* - is so highly pathogenic to insects that no lethal cell dose of the bacterium can be determined. Once the bacterium is in the hemocoel many times one single cell is enough to kill an insect. In nature neither *Xenorhabdus* nor *Photorhabdus* are capable of invading insects. They are absolutely dependent in this on their entomopathogenic nematode partner *Steinernema* and *Heterorhabditis*, respectively. In the first part of infection the bacterium cells kill the insect through mostly unknown mechanisms. Then they multiply on the nutrients that they produced via the bioconversion of the insect tissues, and serve as food for the symbiotic nematode partner.

In an earlier work the secreted proteases of *Photorhabdus* had been screened with a combination of detection methods to start an investigation of the role of proteases in infection, and to find such enzymes that are important during the early phase of infection. Only two enzymes had been found with the applied methods, a thermolysin and a PrtA peptidase [1]. The latter belongs to the M10B subfamily of Zn-metallo-peptidases (the serralysins). These enzymes are secreted by many microorganisms including plant and human pathogens but their precise role

remains unknown. Photorhabdus PrtA was secreted early enough to participate in the establishment of infection [2]. Indeed, when tested on hemolymph proteins in vivo, Photorhabdus PrtA cleaved specifically sixteen minor protein components [3]. Eight proteins were sequenced on their N-terminus. Six of the sequenced proteins are known to have immune or immune related function suggesting an immune suppressive role to PrtA.

Xenorhabdus and *Photorhabdus* are sister genera in the family of Enterobacteriaceae that have the same type of host organism and also follow an essentially identical life strategy. Therefore they offer a rare opportunity to investigate how far the similarity between two so closely related pathogens can go in their molecular level pathomechanism. Since no such comparison has been made so far one can only suppose that they are very similar, almost identical in this respect too. The purpose of my thesis work was to collect data about the secreted proteases of *Xenorhabdus* bacteria and to compare *Xenorhabdus* and *Photorhabdus* in the function of these proteases which is only a small part of the complex system of interactions between these pathogens and their hosts but might have significant influence on the infection process.

SPECIFIC AIMS

1) Investigation of protease activities secreted by *Xenorhabdus* strains with the same detection methods that had earlier been used in the case of *Photorhabdus* strains [1] to establish the type and dynamics of activities.

2) Investigation of production of the early secreted activities during insect infection.

3) Looking for target proteins to those activities that appear early during infection, i.e. those that might have role in establishing the infection.

4) Development of efficient purification procedure for those activities that might have function in the early pathogenicity phase of infection (needed to goals under point 3 and 5 and to the identification through N-sequencing).

5) Enzymatic characterization of the protease(s) under points 3 and 4 through investigation of cleavage site specificity (also to find sensitive and specific substrate) and through investigation of inhibitor effects.

METHODS USED

1) I screened Xenorhabdus strains with the following detection methods:

- Gelatinse assay. This is a fast qualitative and non-selective microbiological detection method of protease production of bacterium colonies. It made possible a limited comparison of my results with those of others, because this or very similar methods had often been used in earlier works.

- Enzymography following SDS-PAGE. This is a semi-quantitative method, which is able to separate activities, and makes possible to investigate the dynamics of protease production. I used two denatured proteins, gelatin and casein, as substrates copolymerized in the gels.

- Enzymography following native PAGE. I used this semi-quantitative method to find those activities that might be sensitive to the conditions of SDS-PAGE. Here I also used gelatin and casein as substrates.

- Activity assays on several synthetic substrates to detect collagenolytic and other peptidases with tryptic or chymotryptic type activity.

2) For insect infection I used *Galleria mellonella* larvae that I injected with 50-100 bacterium cells. I collected hemolymph at various times post-infection with cutting the pro-leg of larvae.

3) For finding target proteins to protease B I used some commercially available proteins (two collagen types, albumin and fibrinogen) and hemolymph

from *Manduca sexta*. Before testing them for cleavage I fractioned the hemolymph proteins on a DEAE anion exchange column.

4) For enzymatic characterization of protease B I used standard saturation kinetics on a range of commercially available synthetic substrates. For the identification of cleavage site in the best substrate HPLC separation and mass spectrometric analysis of the cleavage products were used.

5) Other methods: Bacterium cultures were grown in LB medium at 30°C. Protease B was purified with standard liquid chromatographic methods. The N-terminal of protease B was sequenced by András Patthy. We have the mass spectrometric analysis made in the Proteomic Laboratory of the Biology Institute of ELTE.

RESULTS AND DISCUSSION

1) *Protease secretion in the culture of 17* Xenorhabdus *strains*. This was the first systematic biochemical investigation of protease secretion of *Xenorhabdus* strains. With enzymographies I could distinguish four secreted protease activities (activities A-C, after SDS-PAGE and activity 1, on casein substrate after native PAGE) and two more with synthetic substrates (succ.-Ala-Ala-Pro-Phe-Sbzl-ase and Fua-Ala-Leu-Val-Tyr-ase activities) that proved different according to their inhibitor sensitivity and dynamics of production. With the exception of activities A and C the other ones were secreted by every strain. Thus according to the methods I used *Xenorhabdus* secretes a higher number of proteases than *Photorhabdus* which secretes only two enzymes, a PrtA peptidase and a thermolysisn. As for the correspondence of activities between these two bacteria, while I did not find thermolysin activity in *Xenorhabdus* the inhibitor sensitivity and molar mass shoved activity B of *Xenorhabdus* (which I called Xenorhabdus protease B) and PrtA peptidase of *Photorhabdus* (Photorhabdus PrtA peptidase) the same type of enzyme.

2) *Protease secretion in* G. mellonella *during* Xenorhabdus *infection*. Since the secretion of protease B, and the succ.-Ala-Ala-Pro-Phe-Sbzl-ase and Fua-Ala-Leu-Val-Tyr-ase activities were started from the early logarithmic phase (and the activity of protease B even declined from the 24^{th}-28^{th} hour of culturing) I thought these activities might have role in the early stage of infection. So I investigated their production in *Xenorhabdus* infected insect larvae. While the data were not informative for succ.-Ala-Ala-Pro-Phe-Sbzl-ase and Fua-Ala-Leu-Val-Tyr-ase activities, due to their high background value, I observed the appearance of protease B activity from ~15 hours post-infection, i.e. ~10 hours before insect death.

3) *Target proteins of Xenorhabdus protease B*. I found that purified protease B did not cleave collagen types I and IV as well as albumin and fibrinogen (which one might expect in the case of a general peptidase) but it specifically cleaved eight proteins in the hemolymph of *M. sexta*. This target protein "profile" of protease B was exactly the same as that of Photorhabdus PrtA peptidase and was a further indication that these enzymes are closely related. Since many of the PrtA cleaved hemolymph proteins were identified, a comparative SDS-PAGE analysis of protease B digested proteins allowed the identification of three protease B cleaved hemolymph proteins. These have immune (related) functions. Thus I concluded that a role of protease B might be (similarly to Photorhabdus PrtA) the suppression of immune response of infected host.

4) *Identification of Xenorhabdus protease B*. The 15 amino acid long N-terminal sequence of protease B, purified form *X. kozodoii* v. Morocco strain, showed the best match (73% sequence identity) with PrtA peptidases from two *Xenorhabdus* strains (*X. nematophila* and *X. bovienii*). Thus I concluded that protease B of *X. kozodoii* v. Morocco, similarly to the peptidases of the other two *Xenorhabdus* strains, is a Xenorhabdus orthologue of Photorhabdus PrtA peptidase. PrtA peptidases belong to the bacterial subfamily (M10B subfamily, the

serralysins) in the M10 family of Zn-metallo-peptidases (the interstitial collagenases).

5) *Enzymatic characterization of Xenorhabdus protease B (Xenorhabdus PrtA peptidase)*. I tested the activity of protease B on 23 synthetic substrates, and although I could not find specific and highly sensitive substrate, good enough to measure activity in biological samples, I observed several interesting features of protease B, which distinguish protease B from its Photorhabdus orthologue:

i) Protease B prefers a more polar environment of the cleaved bond than Photorhabdus PrtA which might partly explain why protease B could cleave the best substrate of PrtA while PrtA was unable to cleave the best substrate of protease B.

ii) The inhibitor sensitivity profile of protease B is the same as that of Photorhabdus PrtA except for two cases: protease B is inhibited by Zn^{2+} (unlike PrtA) and cannot be completely inhibited by EDTA (PrtA can be). Since the structure of the active site in these enzymes are evolutionary highly conserved there is only one difference between the two enzymes in the vicinity of the catalytic Zn, at position 191: while protease B contains Asp here, PrtA contains Asn. We suppose this difference alone can explain the dissimilar effect of EDTA and Zn^{2+} on these two enzymes.

iii) The addition Co^{2+} and Cu^{2+} not only rescues the 1,10-phenantroline inhibited activity of protease B (as in the case of PrtA) but results in a super-normal activity, similar to that on the addition of these ions to not inhibited enzyme (these activations are not observed in the case of Photorhabdus PrtA).

These enzymatic properties, however, are not unique among Zn-metallo-peptidases, but while a resistance to EDTA inhibition and activation by Co and Cu ions have been described in the case of some serralysins, the inhibitory effect of Zn-ion was not.

CONCLUSIONS

Although they cover only a tiny part of the complex interaction between entomopathogenic bacteria and insect host and, therefore, permit only a limited comparison of the two genera, *Xenorhabdus* and *Photorhabdus*, the available data (my work included) indicate – I think – such degree of difference which is unexpected compared to the very close relationship and identical life strategy of these bacteria. It is seen not only at the level of the (virulence factor) system, in the number and type of the secreted proteases (contrary to the similarities in the infection process), but also at the level of one of the system components, in the properties of a serralysin type protease (contrary to the number of same target proteins). Such variability, albeit common in nature, is interesting and has (even exploitable) consequences as it supposes further differences in e.g. toxin or antibiotics production.

PUBLICATIONS

Massaoud, M. K., Marokházi, J., Fodor, A. and Venekei, I. (2010) *Proteolytic enzyme production by strains of insect pathogen* Xenorhabdus *and characterization of an early-log-phase-secreted protease as a potential virulence factor.* Applied and Environmental Microbiology **76**, 6901-6909

Massaoud, M. K., Marokházi, J. and Venekei, I. (2011) *Enzymatic characterization of a serralysin-like metallo-protease from the entomopathogen bacterium,* Xenorhabdus. Biochem. Biophys. Acta **1814**, 1333-1339.