Přírodovědecká fakulta
Faculty of Science

Jihočeská univerzita v Českých Budějovicích
University of South Bohemia in České Budějovice

Review of the Ph.D. thesis submitted by Mgr. David Hartmann

Selected proteolytic aspects as targets to combat ticks and tick-borne pathogens.

Supervisor: RNDr. Daniel Sojka Ph.D.

Reviewer: RNDr. Zdeněk Franta Ph.D. Faculty of Sciences, University of South Bohemia, České Budějovice

Ph.D. thesis submitted by Mgr. David Hartmann consists of ten parts (introduction, papers I to V, concluding remarks, abbreviations, thesis references and curriculum vitae). It has 154 pages in total, and is built on a foundation of five papers. Four of them have been published in respectable peer-review international journals. The last paper dealing with the "late" isoform of cathepsin L is present in the form of prepared manuscript. Presented papers show collaborative effort between applicant´s laboratory and renowned experts in the field of parasite proteolytic enzymes and are of high quality. Mgr. David Hartmann is the first author on one published paper as well as on prepared manuscript and second author on remaining three papers. Applicant contribution to individual papers is highlighted specifically and proves that Mgr. David Hartmann mastered a broad portfolio of both in-silico and experimental techniques of molecular biology and biochemistry (e.g. qPCR analysis, recombinant protein purification or 3D modelling)

The attractive topic of the thesis focuses on utilization of proteolytic apparatus as a therapeutic target against tick and tick transmitted pathogens. Applicant´s laboratory has broad experience in the field of tick physiology (specifically tick proteolytic machinery) and presented work shows rational development towards the detailed characterization of individual enzyme isoforms and evaluation of their therapeutic potential.

Thesis overview is written on 35 pages. It starts with basic information about individual protease groups and cell proteolytic system as well as a short review of protease-based therapy, which is gaining more attention nowadays. These parts are followed by selected proteolytic factors of ticks and tick borne pathogens, focusing on introduction of individual tick proteolytic enzymes having role in either digestion or immunity. The last part of introduction deals with proteases of selected tick-transmitted pathogens; however, the data about tick borne pathogens are limited to *Babesia spp.* This is most likely because Babesia proteasome is intensively studied by aplicant´s laboratory, but the name of this part could be changed accordingly.

The thesis reads well and does not contain many typos or other formal problems. My only concern towards the thesis is the quality of pictures in prepared manuscript, which is rather low. The level of English text seems good to me (as far as I can judge).

Přírodovědecká fakulta
Faculty of Science

Jihočeská univerzita v Českých Budějovicích
University of South Bohemia in České Budějovice

I have a few questions / comments to the applicant:

1 – I am missing any information about tick metalloproteases in the introduction. Could you comment on role of these enzymes in tick blood meal digestion and/or immunity?

2 – IrAE has strict pH specificity and its activity is irreversibly inhibited when exposed to pH>6. Interestingly this enzyme has been found in gut lumen of *I. ricinus*. The pH of tick gut lumen shall be 6,5 meaning that IrAE is inactive here. Can you speculate about the role of inactive enzyme in this environment?

3 – What is known about the blood cells lysis? How this process occurs inside tick gut? Are there any enzymes involved?

4 – While feeding, tick produce many anticoagulant and anti-platellet aggregation molecules. Is there anything know, whether these molecules are ingested with the blood and could these have anticlotting activity inside tick gut?

5 – There are several isoform of individual enzymes. Is there any evidence how does tick control their temporal and spatial distribution?

6 – As you state in your thesis many proteases can substitute for each other. What will be in your eyes the best strategy to develop effective vaccine?

Despite all my comments, I believe that Mgr. David Hartmann achieved a high level of scientific competence as evidenced by his four publications (one as a first author) and that the results gained during his Ph.D. studies are a valuable contribution to the field of tick-pathogen interaction.

Finally, I am glad to conclude that presented work meets all the criteria and I have no hesitation in recommending **to accept** the thesis of Mgr. David Hartmann for Ph.D. defense.

In České Budějovice, 23.09.2021

Zdeněk Franta

Zdeněk Franta

Branišovská 1760 370 05 České Budějovice, Česká republika T/ +420 387 776 230; zfranta@prf.jcu.cz www.prf.jcu.cz

External reader's review

Ph.D. Thesis "**Selected proteolytic aspects as targets to combat ticks and tick-borne pathogens**" (2021) **by David Hartmann**, School of Doctoral Studies in Biological Sciences, University of South Bohemia in České Budějovice, Faculty of Science

Reviewer:

RNDr. Libor Mikeš, Ph.D., Department of Parasitology, Faculty of Science, Charles University, Prague

The presented Ph.D. thesis addresses proteases and proteolysis-based systems in parasites, with particular focus on those in the hard ticks, vectors of several pathogens of both medical and veterinary importance. The thesis includes an introductory review of the literature that acquaints the reader with general aspects of proteolysis including terminology, sorting and nomenclature of proteolytic enzymes, mechanisms of catalysis and examples of protease use by parasitic organisms with a particular regard to ticks and their peptidases which were in focus of the student's research activities (cathepsins L and D, and legumain). Proteolytic systems of the cell are introduced to prepare the reader theoretically for reading the included papers dealing with the proteolytic systems tied with proteasome and heterophagy. A deeper insight is devoted to processing of host blood by ticks, which is a complex machinery based on cooperation of several endo- and exo-peptidases. A chapter also concerns tick innate immunity, in which the proteases play important roles, and other chapters deal with modern protease inhibitor-based approaches to the control of pathogens, including targeting of eukaryotic parasite proteasomes. I find this introductory part informative, well written in a good-standard English, being comprehensible, explanatory, and readable. Minor objections include an unusual format of citations used in this part (also in the publication no. 5, the "manuscript in preparation"), also logical interconnection of some chapters could be improved to increase the fluency of the text. The number of typographical errors is relatively low, inaccuracies in claims/statements/interpretations and inappropriate generalizations are relatively rare. It is not worth mentioning individual errors; anyway, I have marked them in the hard copy of the thesis which I had at my disposal, and which is now available for inspection by the defence committee. The graphical

accompaniment of the introductory part is of good quality, the sources are properly cited. Probably, a schematic picture of tick body organization could accompany the word description in the end of chapter 1.6.

There are five papers attached to the thesis, in which the student participated either as the first author - papers no. 1 and 5 (manuscript ahead of submission), or as a co-author - papers no. 2, 3, and 4. According to the included statements of corresponding authors, the student's contribution to all the publications was significant. The already published papers appeared in recognized high-quality impacted journals in the field of parasitology and comparative immunology. Except for paper no. 3, which is a comprehensive review of parasite cathepsins D and their relevance as therapeutic targets, the publications present original results of a world-class research focused on somewhat enigmatic isoforms of tick asparaginyl endopeptidases (legumains), an isoform of tick cathepsin L (IrCL3) involved in the late phase of host blood protein digestion and proteolysis-independent modulation of blood clotting, finding of promising therapeutic compounds capable of interfering with the proper functioning of the proteasome of *Babesia* spp. pathogenic apicomplexans, and, last but not least, characterization of a homolog of horseshoe crab factor C in ticks, which is involved in invertebrate innate immunity.

I will not repeat the details of the interesting and important research, which will be a part of the student's defence presentation. Instead, I would like to point to the wide range of methods performed by the student, which include maintenance of ticks and cultivation of *Babesia*, collection of parasite material for molecular/biochemical experiments, molecular methods including qPCR, gene cloning and production of recombinant proteins, methods of enzymology, protein purification and separation, preparation of antibodies and immunohistochemistry etc. Together with David's ability to interpret the results, to work individually and as a part of the team, and to write a high-quality scientific text, I assume him to be a good candidate for acquiring a Ph.D. degree. After reading his thesis and considering its quality, it is my pleasure to fully recommend the Thesis for the defence.

In the end, I would like to put some comments and questions, most of which concern the manuscript in preparation (publication no. 5) dealing with IrCL3:

1) The manuscript still contains numerous typographical or grammatical errors, sentences missing verbs or having useless conjunctions,

references to some figures are missing, generic and species names are sometimes not written in italic, some of the results do not have their corresponding methodology described in the Methods, etc. I marked these in the text of the manuscript. Page 101 - biological replicates are not described.

2) It has been revealed that three CL isoenzymes occur in *I. ricinus*. The existence of IrCL2 is only mentioned and there is even no hypothesis given concerning its possible function and no comment on its primary structure and occurrence of its orthologs in other ticks. So, what do you actually know about it?
3) Page 109, chapter 2.15 – could you explain why there are different time periods and amounts of IrCL3 used for the incubation with hemoglobin and serum albumin?
4) Since S2 pockets are highly involved in the specificity of papain-like peptidases, the crucial amino acids of the S2 binding pocket could be marked in the alignment of CL peptidases on p. 112. How do the three Ir CLs differ in the composition of the S2 pocket?
5) In Fig. 7B - have you identified the bands of ca. 17 and 20 kDa? Their intensity also changes in time... Since the intensity of the band of mature IrCL3 hardly increases in time, in contrast to the pro-sequence band, does it mean a substantial autodegradation of the mature enzyme?

Prague, 28th September 2021 Libor Mikeš

Masaryk University
Faculty of Science, Department of Botany and Zoology
Kotlářská 2, Brno, 611 37
Tel: +420 549498841
Email: martin.kasny01@gmail.com

MUNI

REVIEWER´S DISSERTATION THESIS REPORT

Author: **Mgr. David Hartmann**

Title: **Selected proteolytic aspects as a targets to combat ticks and tick-borne pathogens**

Area of thesis focus:

- **processing of host blood as a source of nutrients and energy (hematophagy) as a continuum of the long-term goal of the Laboratory of Vector Immunology, that established the currently accepted model of multienzyme degradation of host blood proteins by ticks**
- **proteases in innate immunity**
- **validation of *Babesia* proteasome as a potential therapeutic target against the tick-transmitted apicomplexan parasites.**

Basic parameters:
Language: **English**
Number of pages: **154** (part without publications 35)
Number of publications: **2 first author** (1 in preparation), **2 co-author**

The submitted thesis follows the long-term research of the group around Dr. Petr Kopáček (Biology Centre CAS, Institute of Parasitology, Biology of Disease Vectors, Laboratory of Vector Immunology, Institute of Organic Chemistry and Biochemistry ASCR) focused on function of molecules involved in the tick innate immunity, pathogen transmission and blood digestion.

The topic of the thesis is actual and interesting for scientific community. It overlaps to the human medicine and it is related to the relevant area of parasitology, underlying the importance of proteolysis, proteolytic enzymes and other molecules involved into essential proteolytic processes.

INTRODUCTION (page 1-35):
The author briefly introduces the proteolysis in the context of parasitology and follows in six subchapters with description of proteolysis from various points of view.

- **Parasitic diseases (page 1)**

- **Proteolysis, proteases and their substrate specificity (page 1-8)**
  - Main principles of proteolysis
  - The relevancy of MEROPS database
  - Classification of proteases (according to mechanisms of catalysis, position of cleavage, molecular structure)
  - Substrate specificity

comments and questions:

- In the term of description of particular catalytic groups of proteases, such as Serine proteases, Cysteine etc. I miss the presence of characteristics related to proteases of thick (esp. *I. ricinus*) – numbers, (special) functions, importance etc.!

- **Proteolytic systems of the cell (page 9-13)**
  - Two major systems for intracellular protein hydrolysis - the ubiquitin-proteasome system and the autophagy-lysosome pathway
  - Endocytosis

  comments and questions:
  - No information related to ticks!

- **Protease based therapy (page 13-16)**
  - Inhibitors of proteases as promising and successful drugs for various diseases
  - Potential of protease based inhibitors to interfere with HIV virus reproduction
  - Potential of protease based inhibitors to effective therapeutic solutions for COVID-19
  - Potential strategy against parasite infections – *Trypanosoma cruzi* (K11777)

  comments and questions:
  - No information related to ticks!
  - Please could you comment this information (page 13) in the connection with the citation. *"It is estimated that 5–10% of currently used drug targets are proteases (Sojka et al., 2021)."*

- **Proteases of parasitic organisms (page 16-17)**
  - General information of host-parasite interactions - proteases as one of the key factors of parasite virulence

  comments and questions:
  - No information related to ticks!
  - Please could you comment the sentence (page 16). *"Proteases could also be useful as a diagnostic tool for the confirmation of disease or as a marker for disease progression."* Diagnostics?

- **Selected proteolytic aspects of ticks and tick borne diseases (page 17-35)**

- **Processing of host blood**
  - Introduction of model species, hard thick, *Ixodes ricinus*
  - Heterophagy uptake of blood protein components with epithelial cells followed by intracellular digestion with patricipation o multienzyme network of cysteine proteases (cathepsins B, L, C), asparagynyl endopeptidase and aspartic peptidases (cathepsin D)
  - Characteristics of particular proteolytic enzymes

  comments and questions:
  - Please could you comment the sentence (page 19). *"Many invertebrate parasitic organisms are hematophagous species, most notably protozoa, the polyopisthocotylean monogeneans, many digenean flukes, nematodes, hirudinean annelids, insects, acarines, and crustaceans."* Really many except the arthropods?
  - Are there available the transcriptomic/proteomic data for developmental stages of *I. ricinus*?
  - Directly in the text of the thesis I miss the concluding remarks related to the particular proteolytic enzymes of *I. ricinus*
  - Please could you comment the sentence (page 27). *IrCL1 thus remains a promising target against ticks and tick-borne pathogens (Franta et al., 2011), and its inhibition by papain-like inhibitor E-64 using membrane (in vitro) tick feeding leads to significant phenotypic changes (Jan Perner, personal communication).* Is there something new in this topic; e.g. further tests with inhibitor of IrCL1/IrCL3 mialostatin or the other?

- **Proteases and tick immunity**
- Introduction of model species, hard thick, *Ixodes ricinus*

comments and questions:
- Please could you comment the sentence (page 29). *"The most difficult challenge for the tick immunity represents a great variety of pathogens to which ticks are exposed during on-host feeding."* Please could you specify several such pathogens, pathogenic to tick?

- **Proteases of selected tick-transmitted pathogens – selective inhibition of *Babesia* proteasome as a novel therapeutic**
- Proteasome

comments and questions:
- What is the molecular structure similarity of human, tick, amicomplexan proteasome?

GENERAL COMMENTS

The submitted thesis is primarily focused on selected areas of current research related to ticks and proteolysis.

According to my opinion the thesis comprises several points which should be addressed.

- I have serious doubt that the title of the thesis is logically correct: "...proteolytic aspects as a targets ..."
- The aims of the thesis are not defined.
- The complex information related to the "aspects of proteolysis of ticks" is low and at least half of the introduction does not include any contextual comparison to the known findings related to ticks.
- I would appreciate the point summary behind each included publication – the most important findings. (I appreciate Concluding remarks, page 136-137)
- I would like to ask you for the status of the third first author publication – in preparation
- I think that the chapter related to the evasion of the host´s immunity should be covered

The submitted work is based on the results summarized in 5 publications (3 first-author and 2 co-author), from which four were published in the impacted journals.

I am convinced that David Hartmann demonstrated considerable erudition in the field of proteolysis enzymes and also the ability to answers the various scientific questions by the results of well performed experimental work.

The submitted thesis documents that MSc. David Hartmann has fulfilled all the criteria to be awarded by the Ph.D. degree.

Praha 22.9.2021

Martin Kašný, Ph.D.