Opponent's review of the doctoral dissertation/PhD thesis
Author: Mgr. Lenka Ulrychová
Title: Characterization of serine proteases of *Schistosoma mansoni* involved in interaction with host

Supervisor: Mgr. Martin Horn, CSc., Institute of Organic Chemistry and Biochemistry, CAS, Prague
Advisor: RNDr. Jan Dvořák, Ph.D., Faculty of Environmental Sciences Czech University of Life Sciences Prague

Oponent: RNDr.Daniel Sojka, PhD., Institute of Parasitology, Institute of Parasitology, Czech Academy of Sciences, České Budějovice

The dissertation of Mgr. Lenka Ulrychová is on a topical subject, it is based on the long-term thematic concept of the laboratory of Dr. Mareš and her supervisor Dr. Horn at IoCB, CAS, Prague, and because proteolytic aspects of parasites are also the topic of my long-term scientific interest, I was pleased to accept the thesis for revision.

The evaluated dissertation has 151 pages of text including a total of five inserted publications in international scientific journals and two chapters in scientific books (monographs), of which Mgr. Lenka Ulrychová has authored or co-authored. The language level of the provided text is excellent, with minimal spelling errors and typos. A short introductory chapter (INTRODUCTION) is followed by an extensive literature review, the aims of the thesis (AIMS OF THE THESIS), the results and embedded publications, a final discussion (DISCUSSION) and a final summary (CONCLUSION).

After a short chapter **INTRODUCTION**, which briefly describes the dissertation outline and topics, there follows a **LITERATURE REVIEW**, which describes in detail the issues of *Schistosoma* blood flukes, followed in the second part by a description of the surface-tegument of the parasites and a discussion of the knowledge of the interactions of blood flukes with the host during infection and the issue of transcribed mRNAs and their detection by in situ hybridization. Following this, the author discusses the issue of transcriptomes and extracellular vesicles containing RNA molecules. In the last part of the review, the serine proteases of schistosomes are then discussed, and a section discusses the related topic of the previous subchapters, namely the role of schistosome serine proteases in the tegument and parasite-host interactions. The text is well structured, clear, and readable. I have no major comments on this section. In my opinion, the review provides a solid basis for a possible separate publication (review).

The following **AIMS OF THE THESIS** host-parasite would, in my opinion, suffice to be expressed in 6 points in the second part, the introductory paragraph just repeats some things from the previous chapters. The six general points are clearly defined and correspond to the structure of the thesis and the results achieved.

RESULTS, the main body of the doctoral thesis, are presented on exactly 100 pages. The chapter summarizes the provided reprints of five authored and co-authored papers in impacted journals and two monograph chapters. The first publication in Parasites&Vectors, where the PhD candidate L. Ulrychová is the first author, identifies and maps the expression of serine proteases (SmSPs) in *Schistosoma mansoni* adult flukes, revealing their distribution in various tissues through a highly sensitive fluorescence in situ mRNA hybridization technique. This is

very helpful approach because the general expression levels are quite low and the dissection possibilities of flukes for tissue analyses via e.g. via qPCR and Western blot profiling are rather limited if not impossible. The second provided publication in Biochimie, where the Phd candidate is a co-author, explores the intricate peptidase activity in *Schistosoma mansoni* at various developmental stages, uncovering unique endo- and exo-peptidase actions in their excretory-secretory products, which could inform new therapeutic or vaccine strategies against schistosomiasis. Third study published in Plos NTD, the PhD candidate is one of the co-authors, provides insight into the diverse family of S1 serine proteases (SmSPs) in *Schistosoma mansoni*, revealing their complex expression patterns and roles in the parasite's life cycle through genomic, transcriptomic, and proteomic analyses. It highlights the significant expression of SmSP2 in larval and adult stages and identifies a unique protease, SmSP5, as a chymotrypsin-like enzyme, bridging a gap in the phylogenetic understanding of schistosome proteases. The fourth presented paper, also published in Plos NTD, where L. Ulrychová appears among the co-authors, further focuses on SmSP2, *and* reveals its structure, proteolytic activity, and roles in modulating host vasodilation and fibrinolysis. SmSP2 is detected in the surface tegument, esophageal glands, and reproductive organs of the adult parasite by immunofluorescence microscopy, and in the excretory/secretory products by immunoblotting, highlighting its potential as a target for schistosomiasis treatment and vaccine development. The last presented paper published in Plos NTD, where the Phd candidate is listed as a co-author, is dedicated to the interesting prolyl oligopeptidase (SmPOP) from the serine peptidase family S9, this family are known virulence factors for protozoan trypanosomatids but have not been studied in parasitic flukes. This study presents the first localization and functional analysis of SmPOP, alongside chemical tools for measuring its activity, highlighting its role at the host-parasite interface when SmPOP was localized in the parenchyma and tegument (including tubercles) of adult worms and schistosomula and thus might have a role in cleaving host bioactive peptides, which may contribute to the parasite's long-term survival. The development of nanomolar inhibitors against SmPOP suggests its potential as a novel target for schistosomiasis treatment. I positively evaluate indicated PhD applicant contribution to the preface of all four co-authored papers, that clearly indicated a significant involvement of the applicant in all four listed studies. The two listed book chapters (publications 6 and 7) authored and co-authored by the applicant, respectively. Publication 6 details an enhanced RNA in situ hybridization protocol for sensitive fluorescence detection of mRNA in adult *S. mansoni* tissues, utilizing digoxigenin-labeled RNA probes and fluorescent tyramide signal amplification for visualizing low abundance gene transcripts, with potential applicability to other trematode species. The co-authored Publication 7 outlines optimized protocols for collecting excretory/secretory (ES) products from various developmental stages of *S. mansoni*, which play crucial roles in facilitating host invasion and modulating host responses to ensure parasite survival. Contribution of the PhD candidate is again well declared in the preface.

DISCUSSION succinctly synthesizes the findings, placing them within the broader context of ongoing protease research. Although some parts of the introduction are reiterated, the discussion effectively highlights the significance of Ulrychová's work.

CONCLUSION than summarizes the achieved goals and I agree with the point-to-point addressing of each raised aim as they have been fulfilled and main results achieved.

To summarize, Mgr. Lenka Ulrychová's PhD thesis thus represents a significant and high-quality contribution to the study of *Schistosoma mansoni* proteases. Despite being the first author on a limited number of the included publications, her contributions are substantial and

well-documented. The thesis meets the rigorous standards required for the award of a PhD degree from the Faculty of Science, Charles University Prague, and I fully support its acceptance.

My questions and comments about the thesis are as follows:

1. Although the overall quality of the written text is close to perfection, some small formal errors are still present, e.g. the title page Charles university should be written as Charles University in English, I guess.

2. I understand that the term 'Excretory/secretory (ES) products' holds specific significance within the helminthology community. However, coming from a cell biology background, I believe there's room to more clearly define what this term encompasses, especially since it is central to the thesis's first aim and is referenced multiple times throughout the text. The description provided, 'a variety of biomolecules from fluke bodies that are released into the surrounding host environment,' remains quite non-specific. Could you perhaps elaborate on this term? A more detailed discussion on the nature and scope of ES products, that is present in the Introduction of the book chapter in publication 7, but the text is shortened right in the point of explanation, would greatly enhance the clarity and impact of your thesis.

3. Figure 3 of the thesis depicts the mechanisms of host homeostasis, illustrating the individual steps of coagulation and fibrinolysis, and how these processes interact directly with complement activation. Given that all these processes are serine protease-dependent, it would be valuable to clarify how other than SmSP2 (Figure 8, publication 4) *S. mansoni* serine proteases could potentially interact with these host systems to evade immune responses. Furthermore, are inhibitors, potentially released by the fluke, also playing a role in this interaction? Expanding on these points could provide a more comprehensive understanding of the fluke's evasion strategies and add depth to the discussion on host-parasite interactions.

4. The thesis mentions the role of tegument-produced cysteine proteases, specifically cathepsin B2 and calpain, suggesting their involvement in either metabolism or host-parasite interactions. Could you elaborate on how these proteases contribute to the dynamics of host-parasite interactions? Additionally, it would be interesting to explore whether there is any synergy between these proteases and the SmSPs (*Schistosoma mansoni* Serine Proteases) discussed in your study, particularly in relation to the immune evasion strategies employed by the blood fluke. A more detailed discussion on these points could significantly enhance our understanding of the complex mechanisms underpinning immune evasion.

5. In the introduction, where you detail the individual serine proteases from blood flukes, it might be beneficial to include a table or, even better, a phylogenetic tree analysis that mirrors a simplified version of Figure 5 from Publication 3. This addition would provide a more comprehensive and clearer overview of the diverse landscape and distinct characteristics of the serine protease isoenzymes being discussed. From my assessment, the serine proteases SmSP1-5 appear to fall into three unique groups: (i) the 'Plasma-Kallikrein'-like isoenzymes SmSP1 and SmSP3; (ii) the insect plasminogen-like transmembrane isoenzymes SmSP2 and 4; and (iii) the newly identified group of SmSP5, which is akin to arthropod chymotrypsin-like homologues and demonstrates a preference for bulky hydrophobic residues. This preference sets it apart from the trypsin-like proteases of SmSP1 to SmSP4, which favor basic residues. Moreover, the description of an incomplete N-terminal Cub domain in SmSP3, which is reportedly not co-expressed with the protease domain except in egg stages, prompts

further inquiry. Drawing on my experience with multidomain serine proteases in ticks, I wonder if this could indicate a potential assembly issue or oversight, possibly obscuring the true N-terminus. What approaches have been taken to confirm the actual starting codon and 5' UTR of SmSP3 -is the prediction same for other fluke species? Furthermore, has the possibility of regulated alternative splicing creating splice variants in different life stages been explored?

6. The *S. mansoni* serine protease 2 (SmSP2, GeneDB Smp_002150) is comprehensively described in Publication No. 4 as a secreted enzyme that plays a critical role at the host-parasite interface. From its localization in the tegument and the excretory/secretory products has been well documented. From the qPCR profiling (Figure 3, publication 3) it seems like SmSP2 is the only studied protease significantly expressed in all life stages including Miracidia - could this be explained? Additionally, SmSP2, successfully purified from worm extracts and expressed in both bacterial and yeast systems, has been confirmed in vitro for its role in modulating host vasodilation and fibrinolysis. suggests that SmSP2. However, to advance this potential, as a promising candidate for novel chemotherapeutic interventions, in vivo validation of SmSP2 as a therapeutic target might be necessary. Could further confirmation be pursued through in vivo studies?

7. The characterization of SmPOP (publication no.5) stands out as the most comprehensive and compelling study among the presented works. Results and used methods are well explained. I would like to know, what criteria were used to select the specific set of hormones and peptides for testing in worm cultures? Additionally, is there potential for a more intricate degradomic study to be conducted on SmPOP's impact on the host's immune and homeostatic systems?

8. The qPCR profiling in Figure 3 of Publication 3 indicates that the studied SmSPs are significantly expressed in both eggs and adult stages, yet notably, most are more abundantly expressed in eggs. This observation raises up questions about the biological implications of such expression patterns. Could the elevated expression levels in eggs, compared to adults, be linked to specific interactions between the eggs and host tissues? Additionally, are there any recent findings that further elucidate the role of these SmSPs in egg-host tissue interactions?"

9. In your only first-authored research publication (No. 1), the application of in-situ hybridization has unveiled novel findings regarding the localization of all studied SmSPs within the gonads of both male and female flukes. The discussion suggests the possibility of mRNA being transported via sperm from males to fertilized oocytes, highlighting a fascinating area of study. Given the potential significance of SmSPs in early embryogenesis, as evidenced by their later high expression levels in eggs as demonstrated in Publication 3 (figure 3), this aspect warrants further in-depth investigation in vivo. Could additional studies be conducted to explore this phenomenon more thoroughly?

10. Is the author -PhD candidate going to continue in this great topic and if so, what are her immediate scientific plans and goals?

In České Budějovice on 24.2. 2024 by

RNDr. Daniel Sojka, PhD.