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The master thesis of Hana Slabá describes TILIr, novel serpin and its relatives from the superfamily of serine protease inhibitors from *Ixodes ricinus* and it is written on 48 pages. The thesis consists of 6 chapters (introduction, materials and methods, results, discussion, conclusion and references) and has a good quality of English without typing and spelling errors.

The first chapter, named Introduction has 11 pages and the author characterizes tick immune system, gradually focuses on serpins, trypsin inhibitors and tick serpins. This chapter clearly describes the issue of the thesis.

Chapter Materials and methods: Hana describes many molecular and biochemical approaches, such as RNA isolation, cDNA synthesis, expression of recombinant protein and others. All methods are well described, the chapter does not lack well arrange tables of kits, primers, chemicals and bacterial cultures.

Chapter Results: Author present her results on 11pages, each result is accompanied by clear picture. Author analyzes all obtained data in *in silico* subchapter. I have some reminders to this chapter: How author explains differences between the levels of expression of TILIr and its isoforms in developmental stages (pictures 5,6,7) and secondly different sizes of PCR products of specific primers of isoforms on the pictures 6,7?

Chapter Discussion: This Chapter offers an extensive view into biological functions of serpins and author pastes her results in this mosaic.

Finally I have some questions: Why author did not use quantitative PCR for the expression analysis? Can author explain the role of TILIr and its isoforms, like antitrypsin and anticoagulation protein, in the gut?

Through the above comments and questions, it is obvious, that Hana managed many methods and got relatively large amount of results. According my opinion, the Master thesis of Hana Slabá meets all requirements of Faculty of Science at University of South Bohemia and therefore I recommend it for the defense.

In České Budějovice 9.1.2017

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Opponent's review of the master's thesis of Bc. Hana Slabá named

Characterisation of novel serpin TILIr and its relatives from superfamily of serine protease inhibitors from *Ixodes ricinus* tick

The thesis of Bc. Hana Slabá deals with topic, which I find very interesting – inhibitors of serine proteases from ticks, namely the tick *Ixodes ricinus*. I will divide my review to formal and content parts.

Formal aspects:

Thesis has 53 pages (including annotation page and table of contents) and is divided into usual sections – Introduction, Aims, Methods, Results, Discussion, Conclusion and References – with appropriate length. Table of contents is incomplete, the Discussion is not included.

While Methods and Results sections are divided to detailed and numbered subdivisions, similar division in the Introduction is missing, despite using subheadings. The Discussion is not divided at all. This inconsistency looks like the author had problems with formatting in text editor or that it was finished in a hurry, without paying attention to the layout and the look of the thesis.

The thesis is written in English, which is definitely plus. English is OK, however sometimes a bit "clumsy" and difficult to read. Some grammar mistakes/typos are present, such as for example:

Page 1, second paragraph: responce – should be response

Page 9, paragrapgh 2: "Although not so advanced as...." Should be "as advanced as"

Page 36, paragraph 3: "TILIr and neither one of its isoforms contain no asparagines..." – should be contain any asparagins.

Content aspect:

Overall impression from the work is not very positive. There is big difference between the title and work content itself. Major flaw is that it seems like the author doesn't know that serpins and TIL domain inhibitors are two completely different families of serine protease inhibitors. Moreover, serpins are considered as the synonymum for all serine protease inhibitors, but this is incorrect assumption. **Author should explain during the defense, what families of serine protease inhibitors can be found in ticks and tell the difference among them.** This confusion unfortunately flows through whole thesis and I will have to return back to this major mistake, because it unfortunately ruins the results of experimental work, which are actually not bad.

Introduction:

The introduction is inconsistent and unbalanced, individual sections do not make sense together as a whole. At first, the author describes the phenomenon of differential expression of genes. Very short description of tick immune system is followed by quite detailed description of the group of antimicrobial peptides and its role in tick immunity (the immune interaction between tick and host is not described at all). Surprisingly, cystatins are included also among antimicrobial peptides, which is more than questionable idea. **I want author to explain, what is the main described function of tick cystatins, as shown in many experimental works.** Section 1.4 deals with serine protease inhibitors, but immediately they are limited to serpins (see my first comment highlighted in bold). Seven pages are dedicated to description of serpins, their structure, function and mechanism of action, while only

half page is dedicated to TIL domain inhibitors that are however, in the center of the experimental work. Obviously, this ratio should be the other way round. Moreover, the author sometimes mistakes proteinases and proteinase inhibitors (section 1.5 second paragraph). I consider this as a typo or inadvertent error, but author should be more careful. Project objectives mix again serpins and TIL domain inhibitors together. From strict point of view, I would have to state that author did not fulfill objectives 1, 2 and 3 in her thesis.

Materials and methods:

The table of used chemicals (page 14) is chaotic, right side is sometimes used for explanation of abbreviations, sometimes for the name of company, sometimes for precise composition of the medium or buffer, sometimes just for further specification (e.g. antibiotics/ampicillin) and many other variants are present.

Table IV is completely inappropriate, considering that the stated information is mentioned anyway again in one simple sentence in the Results – 4.10.

Methods are written nicely and it is the best section of the work. It is obvious that the author performed experiments independently. I have, however, several questions to this part:

3.2.1. – Author states she dissected organs from ticks and also that she used all developmental stages. Please specify, which stages were really dissected for organs and which were homogenated as whole bodies.

3.2.13. – Protein was collected from the soluble intracellular fraction. How did you know that the protein is not expressed into the inclusion bodies? There should be SDS-PAGE gel to show the presence of recombinant in the intracellular soluble fraction and also the inclusion bodies fraction.

3.2.15. – Affinity chromatography was used to purify the protein, but I found no mention about the introduction of His-Tag. Is it the part of used vector?

Results:

Again, there is missing information about which stages were dissected and which were used as a whole.

4.3. The sequences in figures should be somehow annotated, there should be at least translation included with some description of secondary structures or important parts of the genes. Nowhere is written, why the fragment AAATAAAA is highlighted in the sequences. The alignments figures are of poor quality and also the description is missing. What "+" means in protein alignment? Why the first sequence of each isoform is full and the second has missing amino acids?

The figures from PCR are very hard to comprehend and understand, images are of variable quality and insufficient description. In many of them, I miss the marker to see the size of the PCR product

4.4. Insufficient description of the figure 3. What "N" means?

4.5. From figure 5 legend, it seems that "gene" expression is downregulated after attachment and feeding. Please explain.

4.7. After long search I found the information about molecular weight of the TILIr in this section (it should be together with other descriptive information, such as PI, signal peptide prediction, glycosylation etc.). Is it the size with or without His Tag? Figure 12 has insufficient description, all figures should be self explanatory.

Functional assays: It is unfortunate that antitrypsin assay was not statistically evaluated. How the author would analyze and evaluate antitrypsin experiment?

Figure 18: How is it possible that different calculations of PI give so huge differences

Discussion:

The discussion deals from a large part, similarly to the introduction, with serpins, while experimental part has nothing to do with serpins. On the other hand, it does not discuss obtained results at all. I would like to know, why so much space is dedicated to group that is not even barely related to TIL-domain inhibitors. And if it is because of the comparison of different serine protease inhibitors, why there is no mention of Kunitz domain inhibitors that are very well described from ticks as antihemostatic proteins and form the largest group of secreted proteins in ticks.

There are several factual errors in the discussion: Page 35, 3. Paragraph: "Many serpins appear to have introns in their structure."

Page 37, paragraph 1: Thrombin and **trypsin** play important roles in mediating host defense.

Page 37, paragraph 4: "This study adds another trypsin inhibitor to serpin family in ticks." I am afraid that this statement clearly supports my concern that the author thinks TILIr belongs to serpin superfamily, which is a major factual mistake. Actually, I am surprised, how this could remain in the thesis (which is unfortunately based almost solely on this mistake, at least in the introduction and discussion).

Conclusion:

In addition to "serpin issue", author concludes that TILIr was shown as thrombin inhibitor, based on an anticoagulation test using whole blood sample. Is it really possible to make such a statement based on that specific test?

The information given in the last sentence of the conclusion belongs to Results section, not to Conclusion.

Final evaluation:

In my opinion, the thesis documents author's strengths and weaknesses. The experimental part, if properly presented, would be definitely sufficient for a good thesis. The author did semi-quantitative PCR, cloning, protein expression, functional assays and *in silico* analysis. I believe that author learned a lot and that the amount of results is sufficient for a master thesis. On the other hand, the experimental work is ruined by weak data presentation and overall presentation of the topic. Inconsistent introduction, sloppy table of chemicals, insufficiently described results and the discussion that was led in wrong direction, bring final disappointment from unexploited potential.

It is hard to recommend this thesis for a defense, but I will do so, if Bc. Hana Slabá will be able to convince me during her presentation, which definitely should not follow the structure and content of her thesis.

In České Budějovice, 10.1.2017

RNDr. Jindřich Chmelař, Ph.D.