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Production and functional characterization of tick salivary protease inhibitors

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Serine and cysteine peptidase inhibitors are important components of tick saliva playing specific roles in modulating physiological and immune responses of the vertebrate host during tick feeding. As I mentioned in the revision statement of Jan Kotal's Bachelors thesis some two years ago, it is a great pleasure to see the progress in topics initially studied here at the institute of Parasitology by prof. Kopecký and now strongly pushed forward by Jan's supervisor Dr. Kotsyfakis. My comments and questions to the presented master thesis are following:

Formal issues:

A. **Less could be more…**I do understand, that there are no general instructions to the format of master diploma works at the Faculty of Science. However, I guess that the committee would agree with my strong feeling that the thesis is way too long. All together 102 pages, including 77 figures, and 6 tables doe's not reflect the output of the work. We all also understand how much work and effort was given to the part describing expressed protein refolding optimization. However, the knowledge learned from these experiments should be ideally compressed to a single table and described briefly in one or two chapter paragraphs. Describing optimization separately for each expressed protein covers almost a half of the thesis (pages 30-64). Another negative effect of the length of the thesis is that the general idea of the project appears unclear and rather hidden by the enormous number of SDS page gels, graphs and tables.

B. **Due to the length of the work, it became an exacting task for the author to have the text and figures in correct form**. Although the overall impression is very good, one can still find sporadic mistakes in formatting, E.G.:

- differences in graph titles page 68, figures 61 and 62
- formatting of chapter headlines (2.12.1.-2. – not underlined)
- 2.12.1. RNA isolation: *"approximately3min.The"*
- or the truly disunited formatting of citations (!)
- unusual feature - figure legends above figures (?)

Content: The work is divided into 4 chapters (excluding References).

A. **Chapter of Introduction is very intelligibly written with appropriate length and indicates author's overall understanding to the given topic**. Tick protein name shortcuts from overtaken image in Fig.1 should be either removed or further explained in the text or in an extra table.

B. **Materials and methods: Very comprehensive and well written**, nice is also the useful table with used consumables. BTW. I would highly recommend to use Thermo Scientific PageRuler pre-stained ladder instead any Lifetechnology/Invitrogen protein markers so as See Bee 2 plus. PageRuler is more concentrated, stable and a lot cheaper in comparison to all Lifetechnology products. I also really like the use of the quite

novel assay kit ProteoStat® Fluorescent Reagent (Enzo Lifesciences) in order to estimate the efficiency of protein refolding.

C. **Results – An enormous amount of work for a Ms. thesis, however it should be shortened to keep the intelligibility of previous chapters**. The initial statement about statistical evaluation belongs to methods or figure legends. My major comments about enormously extended Result chapters describing refolding optimization are mentioned in **Formal issues: A**., already. I wonder where the strange SDS PAGE band shading (e.g. figure 52) comes from, could it be an artefact from the scanner? I really do think that the chapter (3.5.) is not very well experimentally designed and it would have been better not to include these results in the work at all. In previous chapters the author describes expression and refolding of CystA, CysB, SerA and SerC, while in assays (Fig. 70-73) we see Sialostatin L, Ser B and some IrS2 which I have not found explanation for neither in legends or the rest of the thesis (probably Iris 2, I guess). Besides the lack of negative controls I really miss verifying dose-dependency of each tested protein. Thus I would prefer to see single protein assays performed with proper controls and optimization. My other questions to obtained results are given down below in **Questions to author**.

D. **I wonder why there is no chapter of Discussion after so many pages of pure Results (Chapter 3).** Several pages of Conclusions (chapter 4) are only a partial replacement since we see mostly discussing own results. I miss putting the aims and results of this work in a more general content. **Conclusions** should just shortly conclude and could follow Discussion.

Evaluation: Although I have all these comments I highly recommend this work to be defended with the final mark demanding on the oral presentation of this work.

Questions to author:

1. Refolding: for some of your proteins (CystA) you choose quite high pH (10) and the presence of 300mM salt in the refolding buffer. Are you not afraid that these quite strict conditions especially in the presence of L-arginine actually prevent any folding procedures? Would you also consider highly acidic refolding conditions without the presence of salts, is there any evidence for such refolding in the literature?
2. Vaccination: Chapter 3.2. – Why for the vaccination experiments are used CysA and CysB, although they heave no effect, while in the following chapter - the *in vitro* proliferation of Borrelia - only CysB is tested? Have you tested also other prepared proteins against Borrelia *in vitro*, was there any effect?
3. Borrelia *in vivo*: How do you inject Borrelias, could this have an impact? Is the used line B31 really infectious for mice, have you performed some tests –do you know how to test this? How do you explain the difference in the two normalization approaches- significant change in mice injected with ovoalbumine normalized to the amount of used tissue?
4. Effect on Cell activation – although we do not see significant effects with CystB, sialostatin L and SerB, why are Cyst A and SerC missing in the experiments? Have you tested them, too? Have you tested dose-dependency of the added recombinant proteins and were the experiments performed in biological triplicates?

In České Budějovice, 13.1. , 2014 worked out by

RNDr. Daniel Sojka, PhD.

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STATEMENT OF THE DIPLOMA THESIS REVIEWER

Name of the student:	**Bc. Jan Kotál**
Thesis title:	**Production and functional characterization of tick salivary protease inhibitors**
Supervisor:	**Michail Kotsyfakis, Ph.D.**
Reviewer:	**RNDr. Ján Štěrba, Ph.D.**
Reviewer` affiliation:	**Faculty of Science, University of South Bohemia**

The diploma thesis of Jan Kotal deals with production of recombinant protease inhibitors from the saliva of the tick *Ixodes ricinus*, their refolding, and subsequent characterization of their potential biological activity (inhibition of the host immune system) but also the so-called SAT effect using the murine model. Other proteins produced by Honza's supervisor were also tested.

The thesis has a standard structure, but not a standard length of different sections. The amount of the results (of which only a small part is included) is very big and it is obvious, that Honza worked very well and very hard. The Introduction is rather short in comparison with the Results, but it contains most of the needed information. Also, it is not usual to combine the Introduction with Results and Goals (for example in 1.6.2). Length and depth of the Discussion corresponds to results.

It is a usual defect of many of the experimental theses at our Faculty, that students work a lot wanting and needing a lot of results and at the end there is not enough time to write down everything perfectly and correct all the mistakes. Honza's thesis is not an exception and it contains a lot of mistakes, inconsistencies, also references contain some differences in format. Among others, here are some examples:

- shortcuts/abbreviations should be used in the whole text, and they should be introduced for the first time, when the abbreviated word/phrase is used (not like the abbreviation for guinea pigs introduced in 1.6.2),
- figure captions should be below figures and they should be more descriptive,
- be aware of differences of words which are similar in Czech but mean different things in English - such as number-amount for example,
- there is a lot of missing spaces throughotu the text - maybe it is the result of export into pdf format, anyway, these things should be checked and corrected,
- once the genus name is used in the text (in this case thesis), it should be abbreviated the next times (*Borrelia burgdorferi* for the first time, *B. burgdorferi* afterwards)

The thesis presents high quality research with many interesting results, but it contains also a lot of more important errors. Here are my major comments and also questions, which should be addressed by the student:

- 1.1.3 - identification of *B. burgdorferi* sensu lato (which is a complex of 18 genospecies) to be the causative agent of Lyme disease in Europe and *B. burgdorferi* sensu stricto (one of those genospecies) in USA is very simplified and not correct,
- 2.2, 2.3 - NuPAGE is not a method, but a commercial name for several products used in SDS-PAGE,

- information on plasmids used for recombinant proteins production, on proteins sequence etc., should be presented - without it, it is complicated to evaluate some of the results. In the case, when you do not want it to be widely accessible, it can be omitted from the pdf of the thesis on the web, but still it should be accessible for the reviewers,

- Tab. 1 - why are the chemical formulas given only for some chemicals?

- Tab. 1 - what exactly are the primers "FLAGELLIN primers and probe 69 bp" What is their sequence, how were they designed or which reference was used?

- 2.4 - Triton X-100, not Triton-X 100,

- 2.5 - the function of DTT as reduction agent was definitely described earlier than in 1996,

- 2.6 - based on the pIs of your recombinant proteins maybe more pH values of the refolding buffers could be tried at the beginning. Why did you chose the buffers you used?

- 2.6 - how did you determine the "size" of the pellets (after refolding) - from the text I assume it was by eye? Wouldn´t weighing of the pellet be a better and more exact way to determine the "size"?

- 2.7 - this section describes the methods and therefore for chromatography information on columns used, flows etc. should be also included. Without these data it is complicated to evaluate (does not matter, that it was done by the supervisor, at least some basic description should be included),

- 2.8 - what do you mean by "flagellin gene"? Gene in terms of DNA and so really the gene or mRNA? Nucleic acid isolation is not described, but from the context and description of qPCR I assume it was the DNA. On the other hand, for pro-inflammatory genes it was most probably mRNA - is it right? And the mRNA was isolated using TRIZOL, right?

- 2.8 qPCR for pro-inflammatory genes was done also using RotorGene? Most probably not as you are writing about 384 wells plates. Why only duplicates? For qPCR it quite unusual,

- 2.9 - Institute of Parasitology, not institute of Parasitology,

- 2.9 - "ticks that died during the experiment were excluded from further analysis" - Why? And what do you mean exactly? Ticks that died during feeding on vaccinated guinea pigs? If yes, doesn´t it affect the results if you do not include the dead ticks in your results and you will not draw any conclusion from it?,

- 2.9 - Which were done in duplicates and which in triplicates?

- 2.10 - what does it mean, that the experiment was done in three cycles? That 3x 50 mice were used, or that 50 mice were used in three experiments together (but then it would mean that some of the groups in some experiment contained only 3 mice)?

- 2.10 - what was the passage of *Borrelia* used in the experiments? The infectivity of *Borrelia* decreases with increasing passage number and decreases greatly in passages higher than 10. Without this knowledge it is impossible also to evaluate results in 4.4,

- SAT means saliva activated transmission, not saliva assisted transport (2.8) or saliva assisted transmission (2.10),

- 2.12.3, 2.12.4, 2.12.5 - better description of methods is really missing - I just hope, that the mouse was already dead when you removed the legs etc. :-),

- 2.13 - FACS buffer is not described in Table 1. PBS usually does not contain Ca^{2+} and Mg^{2+}, therefore the description "FACS buffer (PBS (- Ca^{2+}, Mg^{2+}, Gibco) is rather confusing,

- 2.13 - most probably you mean anti-Ly-6G antibodies (if written without "anti-" the code means the code of the clone producing monoclonal antibody, not the antigen); also in Table 1,

- 3.1.1.1 - "This is a gel of pellets of the bacterial cells ..." Much better and simpler would be just write about the inclusion bodies without the description of their preparation. This mistake is also repeated in the next sections, as you used Copy-Paste,

- You should avoid statements such as "The best overexpression" (3.1.1.1), "the better outcome of refolded protein" (3.1.1.5), "The best was obviously pH 10.0" (3.1.1.6), "... still looks good ..." (3.1.1.9),

- 3.1 - much better description of ultrafiltration and the fractions which you used should be given. It is possible to logically conclude from the text, but you have to write in an understandable

way,

- 3.1.1.4 - supernatant and pellet are the fractions you will get from centrifugation, not ultrafiltration,

- 3.1.1.7 - how did you find out, that the fraction captured by the 30 kDa cutoff filter contained also CystA monomer? Why do you think it happened?,

- In the Methods (2.7) you write about three chromatography steps used for purification of refolded proteins - however, in 3.1.1.7 you show only one chromatogram. Also, this chromatogram is only partial and if you do not show the whole chromatogram, then it is impossible to conclude on the purification. It also shows much more CystA aggregates than monomers (Fig. 9b) - was such a result acceptable for your subsequent experiments?,

- 3.1.1.7 - Figs 9a and 9b are clearly separated - why numbering both of them as 9?

- 3.1.1.7 - Fig. 9b - "The amount of protein is shown as the absorbance at 280 nm" - incorrect description,

- 3.1.1.7 - Fig. 9b - what does Sg-1 stand for. Most probably it is not Stargate 1 :-),

- 3.1.1.8 - Statements about the 50 kDa and 65 kDa proteins are illogical. Could you explain it a little better? ("The bands at 65 and 50kDa ...") In 3.1.1.9 you suggest that these proteins are multimers of CystA - do you have any mass spectrometry or Western blot confirmation?

- 3.1.1.9 - "The increase in the refolded protein concentration is slower which suggests forming more aggregates ..." - makes no sense, when measuring protein concentration you measure both monomers and aggregates,

- 3.1.1.11 - figures should be mentioned in the text in increasing order - so for example first Fig. 20, then Fig. 21, then Fig 22,

- 3.2 - maybe weighing of eggs would be more exact,

- 3.3 - were the skin parts excised the same/comparable? How big part of the skin was excised and what was its shape (a circle for example)? Without this information it is impossible to evaluate the results. Comparison of Bb/actin and Bb/mg skin shows that the excised skin parts were different in mass and therefore these results are not informative at all. Values used for preparation of Figs. 67 and 68 should be included in the thesis, then it would be possible to evaluate it,

- 3.3 - stating that there was "almost no difference between TBS, OVA and CystA" is a too strong statement for these results anyway,

- 3.5 - I am not sure about the statistics (I am not a statistician), but maybe your results should be consulted with somebody more experienced - I don´t think ANOVA is the right for evaluation of your results. With other method the results would maybe show statistically significant differences (for example in Fig. 70). Example of qPCR results should be shown as well,

- 3.5.2, Fig 72 - it is surprising, that IL-1β was not activated by LPS in neutrophils - literature shows its activation in humans as well as mice (for example Malcolm et al. 2003 or Lu et al. 2012 after a quick web search). It is not discussed in Discussion,

- 3.6 - an example of FACS experiments results should be shown. Also information on how the different cell types were identified in FACS profiles,

- 4.2.1 - the difference in the pH values of 1 of the best refolding buffers for CystA and CystB is not surprising at all as the pI of the proteins also differs by approx. 1, and the difference of pH of 1 means of course 10x lower conc of OH^- and also 10x higher H^+ concentration,

- 4.2.1 - you mean intramolecular or intermolecular interactions when reasoning about the aggregation of CystA?

- 4.2.1 - As you write, your best buffers for CystA and CystB refolding share the alkaline pH. However, did you not try also acidic pH of buffers (as suggested also by Coutard et al. 2012, one of the references in your thesis)? Why?

- 4.2.2 - are there any indices in your results, which suggest the presence of SerA dimers at IB concentration of 1.3 mg/ml? Could the inclusion of GSH/GSSG in the refolding buffer help with the problem? Is there any indication of it in your results?

- 4.3 - sampling of blood in more time points would be helpful in evaluation the vaccination efficiency,

- 4.3, vaccination overall - did you try to find out if your recombinant proteins contain good antigenic epitopes?

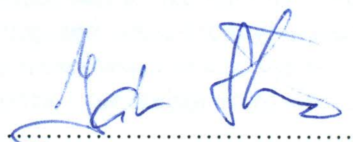
- 4.6, 4.7 - I would expect discussion of these results with literature. Could you provide it?

- 4.9 - collection of lung biopsies in the future would be also helpful

- Final conclusion - on the contrary, there is scientifically significant discovery in your thesis, negative results are also good results and you have also positive results.

Overall, Jan Kotál submitted a thesis of high quality and showed knowledge of a wide variety of molecular-biological and biochemical techniques which helped him to obtain a huge amount of interesting results. Even though the thesis contains quite big number of mistakes I recommend the thesis for the defense. I will decide on the grade based on the answers to my questions.

In České Budějovice, 16. 1. 2014



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