OPONENTSKÝ POSUDOK
doktorskej dizertačnej práce

(ďalej v anglickom jazyku)

Title:	**Crystallographic study of biotechnologically attractive haloalkane dehalogeneses DpcA and DmxA**
Author (PhD defender):	Katsiaryna Doleželová (Tratsiak), MSc.
Supervisor:	Assoc. prof. Ivana Kutá Smatanová, Ph.D.
Place:	University of South Bohemia, Faculty of Science, České Budějovice
Date of defense:	June 6, 2019
Opponent:	Ivana Nemčovičová, Ph.D.

Within this thesis, two new biologically attractive haloalkane dehalogenases DpcA from *Psychrobacter cryohalolentis* K5 and DmxA from *Marinobacer* sp. ELB17 are characterized crystallographically. The scientific project itself is an important part of the big collaboration efforts to study HLD globally. This work simply combines standard techniques used to study the structures of enzymes. The first part of the thesis is focused on crystallization, ligand co-crystallization, diffraction data collection and processing of both DpcA and DmxA enzymes. The second part described an important contribution to the understanding of the molecular basis of cold adaptation in proteins.
The thesis is based on 3 scientific papers, currently 2 records in PubMed and 1 manuscript under review. The successful crystallization of both enzymes was published by Katsiaryna Tratsiak et al. already in 2013 in structural biology communication journal 'Acta Crystallographica F' (IF = 0.989). While the crystal structure of DpcA was solved 6 years later also by Katsiaryna Tratsiak et al. (in 2019) and published in the same journal. It just raising the question, what caused the delay in solving such great-resolution structure (even when resolution was so perfect, nearly 1Å).

Katsiaryna's efforts were mostly focused on preparation of DpcA and DmxA protein crystals for X-ray diffraction experiments and these were successfully prepared within the study. While the production of recombinant or native proteins seems to be out of her skills, however she actively participated in the most critical step, which is in this case, the preparation of the crystals. Therefore, I really appreciate Katsiaryna's work on developing the strategy to crystallize and further optimize the ligand co-crystallization and the soaking methods in order to obtain the protein structures in such a high 'atomic' resolution (DpcA crystals diffracted to the resolution of 1.05 Å and DmxA of 1.45 Å). Moreover, Katsiaryna's work on the particular X-ray diffraction data collection and data analysis and solving the structures by molecular replacement also dominates the outcomes of this thesis.
This study efforts of Katsiaryna have revealed that DpcA solved structure possesses the shortest access tunnel and one of the most widely open main tunnels among structural homologs of the HLD-I subfamily. In addition, the comparative analysis revealed major differences in the region of the alfa-4 helix of the cap domain, which is one of the key determinants of the anatomy of these tunnels. Katsiaryna also participated on analyzing the second macromolecular structure. They found DmxA possesses a unique composition of catalytic machinery presented by halide-stabilizing

residue Q40 instead of the conventional N that is typical for the other members of the subfamily. Therefore, I can say, the solved crystal structures can definitely contribute to better understanding of the structure–function relationships of cold-adapted enzymes as well as the enzymes of extraordinary stability and all these may guide the modification of these enzymes for various biotechnological applications, which just confirms the attractivity of studied topic.

Overall, Katsiaryna obviously contributed extensively to the experimental as well as computational-structural part. She got exposed to various experimental techniques by her own hands and during her PhD study she also conducted a couple of short stays outside of her home lab. I would like to also appreciate Katsiaryna's contribution in writing the manuscripts. All those are good attributes to become one day an independent scientist.

Comments:
The thesis has all together 165 pages divided into 6 main chapters and the supplementary material. The individual chapters are more-less distributed well, however, the main part, e.g. chapters 3–5 are quite confusing while reading. For example, it was a bit confusing to read standard text, then text based on paper, it just gets reader out of flow. It would be better to attach whole papers as they are, not fraction those in chapters, as well as the 'Materials and methods' chapter then losing its point, as it contains the same information. Also, some parts of the methods are mixed with results (for example Fig. 7 and 8 should be placed in results section) and the 'Abstract' and 'Conclusions' could be written better (with logical flow).

Questions:

1. As stated, the crystals of DpcA and DmxA suitable for the X-ray diffraction experiment were obtained in a newly discovered precipitant composition. What are the differences in crystallization conditions obtained for other HLD reported earlier (choose 5)? Compare pH, temperature and precipitant composition. To what extend can this comparison guide the crystallization of other HLD?
2. How and what information obtained from these molecular structures will be used to engineer improved catalytical characteristics and broader environmental activity range of these enzymes? Tell us the perspectives.
3. Why are these enzymes so promising candidates for environmental applications? Please comment on at least 3 properties/characteristics of these enzymes.

Finally, I can state that the candidate fulfills necessary criteria for being awarded a PhD degree. Therefore, **I can recommend Katsiaryna Doleželová (Tratsiak)'s thesis for the PhD defense**.

(Záver: Katsiaryna Doleželová (Tratsiak) vo svojej práci preukázala svoje tvorivé schopnosti i vedomosti a preto táto práca spĺňa základné požiadavky kladené na dizertačné práce v danom odbore. **Odporúčam prácu k obhajobe**.)

Bratislava, 16.05.2019

Ivana Nemčovičová

Ivana Nemčovičová, Ph.D.
Head of Department of Viral Immunology
Biomedical Research Center
Slovak Academy of Sciences

ČESKÉ
VYSOKÉ UČENÍ
TECHNICKÉ V PRAZE

Fakulta jaderná
a fyzikálně
inženýrská

Břehová 7 115 19 Praha 1

Oponentský posudek

Disertační práce "*Crystallographic study of biotechnologically attractive haloalkane dehalogenases DpcA and DmxA*" Katsiariny Doleželové (Tratiak) má celkový rozsah 165 stran, je napsána v jazyce anglickém. Zabývá se krystalografickou studií dvou enzymů s podobným potenciálním využitím, avšak různými biofyzikálními vlastnostmi. Pochopení jejich odlišností od již známých enzymů může významně pomoci v boji proti znečištění životního prostředí. Z tohoto pohledu je cíl a význam práce aktuální a velmi důležitý.

Disertační práce se skládá z následujících částí: prologu (4 strany), teoretického úvodu (47 stran), materiál a metody (6 stran), výsledky a diskuze (82 stran), shrnutí (6 stran). Zbytek textu tvoří odkazy na literaturu a doplňkový materiál.

V části teoretického úvodu autorka používá některá příliš ostrá vyjádření. Například použití barviva Izit (strana 36) není zdaleka vždy prokazatelné. Dále limity pro obsah solventu v proteinových krystalech (strana 36) jsou nastaveny příliš pevně a odkazují se sice na původní, avšak již zastaralou literaturu. Obdobně informace horního limitu použitelnosti techniky NMR (strana 44).

Většina teoretického úvodu je psána dostatečně do hloubky a snaží se v přiměřené míře pokrývat veškerou oblast strukturní biologie. Autorce bych však vytkl a zároveň vznesl první drobnou připomínku, jestli si uvědomuje, která oblast vztahující se k proteinové krystalografii je uvedena pro kryo-elektronovou mikroskopii na obrázku 13, strana 47, a současně není rozepsána pro struktury proteinů?

Sekce materiál a metody obsahuje pouze 6 stran, což nejspíše mylně naznačuje, že rozsah odvedené práce je velmi omezený. Lze odhadnout celkové množství krystalizačních pokusů a zpracovaných dat, které vedly k úspěšnému dokončení této práce?

V této sekci vytýkám autorce dvě různé vlnové délky 0.978 a 100 K (strana 54) a přes pochopitelné pravopisné chyby v anglickém jazyce ještě opakování členu "the" na straně 55.

K práci mám dále následující připomínky:

1) Na straně 63 je uvedeno, že byly použity různé vlnové délky pro obdobná měření. Z jakého důvodu?
2) Na straně 63 je rovněž neuspořádáno pořadí experimentů – difrakce, krystalizace, opět difrakce.
3) Na straně 67 je velmi nepřesně vyjádřena míra shody a podobnosti mezi proteinovými sekvencemi.
4) Na straně 68 není uvedeno jedno z Rmerge/Rmeas. Strana také obsahuje typografický omyl „ó".
5) Na straně 69 chybí závorky.

ČESKÉ
VYSOKÉ UČENÍ
TECHNICKÉ V PRAZE

Fakulta jaderná
a fyzikálně
inženýrská

Břehová 7 115 19 Praha 1

6) Na straně 77 je uvedeno „final Rcryst, Rfree", znamená to tedy, že struktury nebyly upřesňovány v posledním cyklu pomocí úplně všech reflexí? Text tuto skutečnost nevyjasňuje.
7) Na straně 79 je uveden threshold 3.5. O co se jedná? Nechybí fyzikální rozměr?
8) Lze porovnat optimální podmínky pro enzymovou katalýzu s krystalizačními podmínkami?
9) Existují jiné struktury odlišných dehalogenas, u kterých by byla pozorována vazba ligandu?
10) Na straně 110 se vyskytuje tvrzení, že residuum Q40 se nemůže účastnit vazby ligandu. Přesto mutace tohoto rezidua mění aktivitu – substrátovou specificitu enzymu. Jak si autorka vysvětluje tuto skutečnost? Nemůže přece jen docházet ke strukturní reorganizaci tak, aby v případě vazby ligandu k aktivní účasti Q40 došlo?

Než uvedu závěr, chtěl bych ještě upozornit na osobní dojem, že části diskuze připomínají spíše opakování výsledků. Samotné vyřešení struktury, ačkoliv je pochopitelně pro další analýzu naprosto nezbytné, ještě samo o sobě nevysvětluje příčiny extrémního chování obou enzymů. K tomu je zapotřebí provést ještě důkladnější srovnávací analýzu, případně provést mnoho dalších měření, nebo zahrnout analýzu dalších mutantů enzymů.

Přes několik výtek, které byly výše uvedeny, považuji předloženou práci za kvalitní, dobře zpracovanou, zajímavou a především důležitou pro další rozvoj oboru biotechnologií. Získané výsledky jsou cenné a poskytují náhled na příčiny v odlišnostech obou enzymů. Na práci lze dále stavět v dalším výzkumu. V případě, že budou všechny mé dotazy dostatečně zodpovězeny, splňuje předložená práce požadavky kladené na disertační práce doktorského studia, proto doporučuji práci přijmout jako podklad k obhajobě.

V Praze, 17.5.2019

Petr Kolenko

.....

doc. Ing. Petr Kolenko, Ph.D.

Review of the thesis

Crystallographic study of biotechnologically attractive haloalkane dehalogenases DpcA and DmxA

Author: Katsiaryna Doleželová (Tratsiak)

The thesis begins with a prologue containing a few quotes and formulation of main aims of the thesis – crystallization of DpcA and DmxA crystals, obtaining high-resolution X-ray diffraction data and studies of structural features and also thermal stability of these extremozymes. The aims may be better included after the introduction showing the importance of these enzymes. They are applied to catalyse the conversion of highly toxic halogenated compounds and therefore can have high practical applications.

The thesis consists of several parts. Introduction (about 45 pages). Materials and methods (3 pages), three papers two of which has been published in Acta Crystallographica F and the third one is prepared for FEBS journal. Everything is summarized in Conclusions.

The Introduction is well-structured starting with description of enzymes, their function and activity. Then the significance of haloalkane dehalogenases is characterized. The topics are studied in the group already for some time. Table 1 listing different haloalkane dehalogenases clearly shows that there is no unique crystallization method for them. The focus is given to two studied enzymes – DpcA from *Psychobacter cryohalolentis* K5 and DmxA from *Marinobacter* sp. ELB17. The former is characteristic by narrow substrate specifity profile and the latter is one of the most enantioselective haloalkane dehalogenases. The reference given there (p. 18) even unpublished should have a counterpart in the list of references. The last part of this section is devoted to extremozymes, i.e. the activity at low temperatures and to thermal stability. In part 2.5 methods of protein determination are listed and since the main method is X-ray diffraction requiring crystals, significant part of the thesis was crystallization of the studied enzymes and different crystallization methods are described and compared. This part is followed by a brief description of principles of XRD crystal structure determination and individual steps as preparation of samples, X-ray data collection and structure solution by solving of the phase problem where a short characterization of the methods used is given. In next shorter sections other methods are described – NMR, modern and popular cryo-electron microscopy and small-angle X-ray scattering.

All parts of Introduction are relatively brief sometimes simplifying things but always the most important points are sticked out well.

Chapter – Materials and methods is very short showing the methods and conditions used crystallization X-ray data collection and structure determination. Since there are not unique for all studied proteins and described also in the main part – published papers, the part could be omitted.

The main fourth part consists of copies of two papers published and the third one prepared.

The first paper in *Acta Cryst F* is devoted to the crystallization and preliminary diffraction analysis of the two studied enzymes. The second paper that appeared recently deals with structural analysis of cold-adapted DpcA enzyme that is useful for environmental applications at relatively low temperatures. Typical feature is that it posseses the short access tunnels and widely open main tunnels. The third paper deals with the second studied enzyme, its structure, analysis of tunnels and construction of mutants. I think that i tis highly probable that the paper will be accepted for publication perhaps after minor revisions.

Main results of the thesis are summarized in Conclusions, the goals described in the above three papers.

The form of citations by reference to the author names was chosen and seems to be consistent. There are only a few misprints in the thesis. English is not perfect but acceptable.

I would have only some general questions. It is written that almost all crystallization techniques were used successfully. Can we conclude that the crystallization of the studied enzymes is relatively easy? What was the probability that the crystals selected after optical, mechanical and other tests gave also satisfactory diffraction patterns? What the author considers as the most difficult part of the thesis and what results she takes as the best achievements?

I recommend to the Faculty of Science at the University of South Bohemia in České Budějovice acceptance of the doctoral thesis of Katsiaryna Doleželová, if successfully defended. There is no doubt about its quality and novelty as well as about significant contribution of the student to all individual parts of the research mentioned in the thesis. The thesis is well supported by two publications. The thesis could be submitted after the acceptance of the third paper for publication. However, I assume that it will also be accepted.

In Prague 30. 5. 2019

prof. RNDr. Radomír Kužel, CSc.