Kratek opis usposabljanja mladega raziskovalca (*Short description of the Young Researcher's training)*

1. Raziskovalna organizacija (*Research organisation)*:

Univerza v Ljubljani, Biotehniška fakulteta

2. Ime, priimek in elektronski naslov mentorja (*Mentor's name, surname and email*):

Matej Skočaj, matej.skocaj@bf.uni-lj.si

3. Šifra in naziv raziskovalnega področja (*Research field*):

1.05 (Biokemija in molekularna biologija)

4. Kratek opis usposabljanja mladega raziskovalca (*Short description of the Young Researcher's training)*:

Navedite tudi morebitne druge zahteve, vezane na usposabljanje mladega raziskovalca (npr. znanje tujih jezikov, izkušnje z laboratorijskim delom, potrebne licence za usposabljanje…).

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Egerolizini so relativno majhni proteini (15 kDa), ki v zadnjem desetletju postajajo izjemno biotehnološko zanimivi (1,2). Njihova poglavitna lastnost, ki opredeljuje njihov biotehnološki potencial je visoka afiniteta do specifičnih membranskih lipidov ali lipidnih mešanic (1-3). Poleg tega so sami po sebi netoksični, razviti pa so tudi že protokoli za njihovo izolacijo v rekombinantni obliki (4,5). Egerolizini so razširjeni predvsem v kraljestvu gliv in bakterij, najdemo pa jih tudi v drugih skupinah organizmov (3). V nekaterih produkcijskih organizmih se egerolizini pojavljajo skupaj s proteini z domeno MACPF (ang., Membrane Attack Complex/Perforin). Egerolizini v kombinaciji s partnerskimi proteini, ki vsebujejo domeno MACPF, tvorijo pore v tarčnih lipidnih membranah (3,4). Do sedaj so najbolje opisani, preučeni in biokemijsko okarakterizirani egerolizini in njihovi proteinski partnerji z domeno MACPF iz glivnega rodu *Pleurotus*. Iz bukovega ostrigarja (*Pleurotus ostreatus*) so bili tako izolirani in biokemijsko opredeljeni egerolizini ostreolizin A (OlyA), ostreolizin A6 (OlyA6) in pleurotolizin A (PlyA), ter pleurotolizin B (PlyB), ki je protein z domeno MACPF, iz kraljevega ostrigarja (*Pleurotus eryngii*) pa so bili izolirani egerolizina erilizin A (EryA) in pleurotolizin A2 (PlyA2) ter erilizin B (EryB), ki je protein z domeno MACPF (4-10). Za egerolizine je značilno, da se z veliko afiniteto vežejo na lipidni receptor ceramid fosfoetanolamin (CPE), ki je značilen predvsem za nekatere žuželke in paradontalne bakterije (1). Poleg tega se nekateri egerolizini z nižjo afiniteto vežejo tudi na mešanico sfingomielina (SM) in holesterola (Hol), kar ustreza definiciji lipidnih raftov, ki so značilne lipidne mešanice v membranah vretenčarjev (4). Posledično lahko fluorescenčno označene egerolizine uporabljamo za preučevanje omenjenih lipidov ali lipidnih mešanic podobno kot protitelesa uporabljamo za preučevanje lokalizacije ali prisotnosti proteinov (5, 11).

Čeprav so egerolizini sami po sebi netoksični, postanejo lahko, ob prisotnosti proteina z domeno MACPF, del citolitičnega kompleksa, ki naluknja membrano tistih celic, ki vsebujejo ustrezen lipidni receptor, ki ga prepozna egerolizin (1,4,5). Egerolizini so torej v nekaterih organizmih del biokomponentnih citolizinov – komponenta A (egerolizin) prepozna lipidno tarčo, komponenta B (proteinski partner z domeno MACPF) pa v nadaljevanju omogoči tvorbo pore v tarčni membrani. Ta lastnost egerolizinov je tudi biotehnološka zanimiva, saj lahko ob prisotnosti ustreznega lipidnega receptorja in proteina z domeno MACPF liziramo tarčne celice (1,2).

Citolitični potencial kompleksov egerolizinov v kombinaciji s proteini z domeno MACPF je bil že pokazan na nekaterih rakavih celičnih linijah *in vitro* (ki vsebujejo lipidne rafte, oz s SM in Hol obogatene domene) in na nekaterih žuželkah, ki vsebujejo receptor CPE (koloradski in koruzni hrošč) (1,12). Slednje odkritje, da nekateri egerolizini prepoznajo lipid CPE, ki je značilen za ekonomsko pomembne kmetijske škodljivce, in v kombinaciji s proteinom z domeno MACPF delujejo insekticdno, je omogočilo razvoj mednarodne patentne prijave.

(i) Mladi raziskovalec bo v rekombinantni obliki izrazil in preučeval štiri nove egerolizine iz poletnega ostrigarja (*Pleurotus pulmonarius*) ter njihovega proteinskega partnerja z domeno MACPF, ki do sedaj še niso bili biokemijsko okarakterizirani. V doktorski nalogi bomo opredelili afiniteto vseh štirih egerolizinov do membran bogatih s SM in Chol ter do membran obogatenih s CPE ter njihov citolitični potencial v prisotnosti partnerja z domeno MACPF. Njihov biotehnološki potencial bomo opredelili s primerjavo z že znanimi egerolizini (OlyA6, EryA) in njihovimi proteinski partnerji (PlyB, EryB). Vezavo na membrane in njihovo permeabilizacijo bomo spremljali na umetnih lipidnih veziklih ter na sesalskih in žuželčjih celičnih kulturah, insekticidni potencial pa bomo ovrednotili s testi na ličinkah koloradskega in koruznega hrošča.

(ii) Hkrati bo kandidat v sklopu doktorske naloge v celokupnih vodnih ekstraktih naključno nabranih slovenskih gob iskal nove proteine, ki prepoznajo preučevane lipidne tarče: 1) kombinacijo SM in Chol ali 2) CPE, in jih poskušal nato identificirati ter pripraviti v rekombinantni različici.

(iii) Nabor preučevanih lipidnih receptorjev bomo razširili na iskanje novih lipidnih receptorjev, ki so značilni za bakterije ali žuželke. Nove proteine z afiniteto do preučevanih lipidnih receptorjev bomo prav tako iskali v naključno nabranih slovenskih gobah.

(iv) V zadnjem delu naloge bomo podrobneje raziskali mehanizem interakcije proteina Cry34Ab1, edinega egerolizinskega proteina iz družine toksinov Cry bakterije *Bacillus thuringiensis*, z lipidnimi membranami, pri čemer bomo interakcijo spremljali tako v odsotnosti kot tudi v prisotnosti njegovega neegerolizinskega proteinskega partnerja Cry35Ab1. Citolitični kompleks Cry34Ab1/Cry35Ab1 se sicer uporablja v gensko spremenjeni koruzi za zatiranje ličink koruznega hrošča, a ustrezna receptorska molekula, ki jo kompleks prepozna še ni opredeljena. V sklopu naloge bomo zato preverili, ali omenjeni citolitični kompleks prepozna lipidni receptor.

(1) Panevska, A. et al. Aegerolysins from the fungal genus Pleurotus Bioinsecticidal proteins with multiple potential applications. J. Invertebr. Pathol. **2020**.

(2) Gundner, M. et al. What can mushroom proteins teach us about lipid rafts. Membranes. **2021**.

(3) Butala, M. et al. Aegerolysins, lipid-binding proteins with versatile functions. Semin. Cell Dev. Biol. **2017.**

(4) Ota, K. et al. Membrane cholesterol and sphingomyelin; and ostreolysin A are obligatory for pore-formation by a MACPF/CDC-likepore-forming protein; pleurotolysin B. Biochimie **2013.**

(5) Skočaj, M. et al. Tracking cholesterol/sphingomyelin-rich membrane domains with the ostreolysin A-mCherry protein. PLoS ONE **2014.**

(6) Berne, S. et al. Pleurotus and Agrocybe hemolysins; new proteins hypothetically involved in fungal fruiting. Biochim Biophys Acta BBA Gen. Subj. **2002**.

(7) Sepčić, K. et al. Ostreolysin; a pore-forming protein from the oyster mushroom; interacts specifically with membrane cholesterol-rich lipid domains. FEBS Lett. **2004**.

(8) Tomita, T. et al. Pleurotolysin; a novel sphingomyelinspecific two-component cytolysin from the edible mushroom Pleurotus ostreatus, assembles into a transmembrane pore complex. **2004**.

(9) Bhat, H.B et al. Binding of a pleurotolysin ortholog from Pleurotus eryngii to sphingomyelin and cholesterol-rich membrane domains. J. Lipid Res. **2013**.

(10) Shibata, T. et al. Isolation and characterization of a novel two-component hemolysin, erylysin A and B, from an edible mushroom, *Pleurotus eryngii*. Toxicon. **2010**.

(11) Bhat, H.B., et al. Evaluation of aegerolysins as novel tools to detect and visualize ceramide phosphoethanolamine, a major sphingolipid in invertebrates. FASEB J. **2015**.

(12) Resnik, N. et al. Highly Selective Anti-Cancer Activity of Cholesterol-Interacting Agents Methyl-β-Cyclodextrin and Ostreolysin A/Pleurotolysin B Protein Complex on Urothelial Cancer Cells. PLOS one. **2015**.

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Aegerolysins are relatively small proteins (15 kDa) that have become extremely biotechnologically interesting in the last decade (1,2). Their main property that defines their biotechnological potential is their high affinity for specific membrane lipids or lipid mixtures (1-3). In addition, they are inherently non-toxic and also the protocols for their isolation in recombinant form are well established (4,5). Aegerolysins are prevalent mainly in the kingdoms of Fungi and Bacteria, but are also found in other groups of organisms (3). In some producing organisms, aegerolysins are produced together with MACPF (Membrane Attack Complex / Perforin) proteins. Agerolysins in combination with partner proteins form pores in target lipid membranes (3,4). The best described, studied in biochemically characterized aegerolysins, and their protein partners with the MACPF domain are produced by the fungal genus *Pleurotus*. From beech oyster mushroom (*Pleurotus ostreatus*), three aegerolysins: ostreolysin A (OlyA), ostreolysin A6 (OlyA6) and pleurotolysin A (PlyA) were isolated as well as one MACPF containing protein, pleurotolysin B (PlyB). From the king beech oyster (*Pleurotus eryngii*) on the other hand two aegerolysins: erylysin A (EryA) and pleurotolysin A2 (PlyA2) were isolated as well as one MACPF containing protein, erylysin B (EryB) (4-10).

The main characteristic of all aegerolysins is their high affinity for the lipid receptor ceramide phosphoethanolamine (CPE), which is characteristic for some insects and periodontal bacteria (1). In addition, some aegerolysins also bind with lower affinity to the mixture of sphingomyelin (SM) and cholesterol (Chol), which correspond to lipid rafts, which are characteristic lipid mixtures in vertebrate membranes (4). Consequently, fluorescently labeled aegerolysins can be used to study lipids or lipid mixtures similarly to antibodies that are used to study the localization or presence of proteins (5, 11).

Although aegerolysins are inherently non-toxic, they may, in the presence of a protein with the MACPF domain, become part of a cytolytic complex that perforates the membrane of cells containing the corresponding lipid receptor recognized by aegerolysin (1,4,5). Aegerolysins are therefore part of biocomponent cytolysins in some organisms. Component A (aegerolysin) recognizes the lipid target, while component B (protein partner with the MACPF domain) further enables the formation of pores in the target membrane. This property of aegerolysins is also biotechnologically interesting, since in the presence of the corresponding lipid receptor and of protein with the MACPF domain, target cells can be lysed (1,2). The cytolytic potential of aegerolysin complexes in combination with proteins with the MACPF domain has already been demonstrated in some *in vitro* cancer cell lines (which contain lipid rafts or SM and Chol enriched domains) and in some insects containing the CPE receptor (Colorado potato beetle and maize beetle) (1,12). The latter discovery that some aegerolysins recognize CPE, which is characteristic of economically important agricultural pests, and in combination with a protein with the MACPF domain exert insecticidal effects on these organisms, allowed the development of an international patent application.

(i) The young researcher will express and study four new recombinant aegerolysins from the lung oyster (*Pleurotus pulmonarius*) and their MACPF protein partner, which have not yet been biochemically characterized. In the doctoral thesis, we will define the affinity of all four recombinant aegerolysins for membranes rich in SM and Chol and for membranes enriched with CPE and their cytolytic potential in the presence of a partner with the MACPF domain. Their biotechnological potential will be defined by comparison with already known aegerolysins (OlyA6, EryA) and their protein partners (PlyB, EryB). Binding to membranes and their permeabilization will be monitored on artificial lipid vesicles and on mammalian and insect cell lines, while the insecticidal potential will be evaluated by tests on Colorado potato beetle and the western corn rootworm larvae.

(ii) At the same time, the candidate will look for new proteins in the total aqueous extracts of randomly harvested Slovenian mushrooms that either bind to combination of SM and Chol or

to CPE. The candidate will then try to identify these proteins and prepare them in recombinant form.
(iii) The set of studied lipid receptors will be extended to the search for new lipid receptors specific for bacteria or insects. We will also look for new proteins with affinity for the studied lipid receptors in randomly harvested Slovenian mushrooms.
(iv) In the last part of the research, we will investigate the mechanism of interaction of Cry34Ab1, the only aegerolysin protein from the Cry toxin family from *Bacillus thuringiensis*, with lipid membranes. The cytolytic complex Cry34Ab1 / Cry35Ab1 is used in genetically modified maize to control the western corn rootworm, but the corresponding receptor molecule recognized by the complex has not yet been identified. As part of the task, we will therefore check whether the mentioned cytolytic complex recognizes the lipid receptor.

(1) Panevska, A. et al. Aegerolysins from the fungal genus Pleurotus Bioinsecticidal proteins with multiple potential applications. J. Invertebr. Pathol. **2020**.
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