SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Peptide pattern analysis of cyanobacteria

by Milán Riba

Supervisor: Gábor Vasas, Phd, DSc

UNIVERSITY OF DEBRECEN
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By Milán Riba, biologist

Supervisor: Gábor Vasas, Phd, DSc

Doctoral School of Pharmaceutical Sciences, University of Debrecen

Head of the **Examination Committee**:	Árpád Tósaki, PhD, DSc
Members of the Examination Committee:	Györgyi Horváth, PhD
	Tamás Emri, DSc

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Head of the **Defense Committee**:	Árpád Tósaki, PhD, DSc
Reviewers:	Andrea Vasas, PhD
	István Bácsi, Phd
Members of the Defense Committee:	Györgyi Horváth, PhD
	Tamás Emri, DSc

The PhD Defense takes place on online web client (Zoom) at 11 a.m., 7th of December, 2020.
If you wish to participate in the discussion, please send an e-mail to milan.riba@gmail.com by 20:00 on the day before the discussion (6th of December, 2020). After the deadline, for technical reasons, it is no longer possible to connect to the defense.

I. INTRODUCTION AND THE AIM OF THE WORK

Cyanobacteria (blue-green algae) are ancient microorganisms which can be found throughout the world, in a variety of conditions, like in fresh waters, salt waters and on the surfaces of rocks and soil. Through their oxygen-producing photosynthesis, they have played an important role in the formation of the Earth's aerobic atmosphere and currently they have an important role in the biological cycle of carbon, nitrogen and minerals. Cyanobacteria are the basis of many aquatic and terrestrial food chains. Their use in biotechnology is growing, ranging from the production of biofertilizers to potential drugs [1]. Some of their species may be able to produce potential toxic metabolites (cyanotoxins) or other biologically relevant substances. In recent years, numerous cyanobacterial metabolites have been identified which have a wide variety of biological activities. Nowadays, plants and certain microorganisms have great medical importance, these are the most important sources of bioactive ingredients for the pharmaceutical industry. However, there is a growing interest in newly discovered cyanobacterial metabolites and the therapeutic importance of photosynthetic microorganisms is also being re-evaluated [2].

In our work we wanted to investigate the peptide metabolite production capacity of different cyanobacterial species by LC-ESI-MS/MS. In addition to detecting the toxin content of cyanobacteria, we focused on the appearance of rarely tested peptides. Our aim was to identify the *Microcystis* species observed in Lake Balaton between 2013 and 2016; to analyze their peptide metabolites; to characterize previously unidentified peptide metabolites; and to determine the spatial and temporal distribution of the different peptide chemotypes. Similarly, we wanted to investigate the peptide production

ability of some terrestrial and some endosymbiont *Nostoc* species In addition to studying the spatial distribution of the individual chemotypes, we also aimed to compare the geno- and chemotypes of the free-living and symbiotic strains. Finally, we aimed to draw attention to cyanobacteria as natural sources of bioactive substances through the identification and characterization of their potent biologically active peptide molecules.

Water samples from Lake Balaton were mixed with nitrate-containing Allen medium and incubated for 5 days, then visible *Microcystis* colonies were harvested and further cultured in nitrate-containing Allen medium at 26 ° C under constant illumination. The terrestrial cyanobacterial samples from different parts of the Great Plain were washed with sterile water 3 times, then placed in nitrate-free Allen medium and incubated at 26 ° C under constant illumination. After one week, the samples were spread onto 1.5% agar containing nitrate-free Allen plates and incubated for another week. Morphologically distinct colonies were transferred to 96-well microtiter plates containing nitrate-free Allen medium and spread onto plates 3 times in a row. *Azolla* sp. and lichen samples were surface-sterilized (10% hypo) to remove microorganisms. For lichen samples, the purified colonies were placed on 1.5% agar-solidified nitrate-free Allen medium and the cyanobacterial colonies on the periphery of the samples were collected and further cultured in nitrate-free Allen medium. *Azolla* sp. plants were chopped in a sterile mortar with scalpels, homogenized in culture medium and filtered. The filtrate was carefully centrifuged and the pellet was incubated with increasing light intensity and medium strength.

II. EXPERIMENTAL METHODS

Cyanobacterial cells grown in culture medium were harvested by centrifugation, freeze-dried and extracted with 80% methanol. The extracts were analyzed by HPLC-ESI-MS/MS. The components of the samples were separated on a Kinetex XB-C18 column (100 mm X 2.1 mm X 2.6 µm) using a water - acetonitrile gradient. MS measurement was performed in positive ion mode. Identification of metabolites from the MS/MS fragmentation patterns was based on literary data [3,4]. The identified peptides were integrated by targeted peak search, crude abundance data were filtered and log10 transformed. The data set was hierarchically grouped by the Ward method based on Minkowski and Canberra distances. For statistical testing of chemical pattern and other variables (place of origin, taxonomic position), the principal component analysis values were subjected to Kruskal - Wallis significance test.

For the phylogenetic analysis, DNA extracts were prepared by phenol-chloroform method. The concentration and purity of the DNA extracts were determined spectrophotometrically. Phylogenetic relationships between cell lines were analyzed by analysis of the 16-23S ITS region and the 16S rRNA gene sequence. The PCR products were checked on 1% agarose gels and detected by UV light. The products were purified and analyzed by Sanger sequencing. The sequences were checked and corrected as needed, then similarity searches and phylogenetic analysis were performed. Fingerprint analysis was performed using primers corresponding to the STRR (short tandemly repeated repetitive) and ERIC (enterobacterial repetitive intergenic consensus) sequences. The amplified DNA fragments were separated on 1% agarose gels and the resulting patterns were analyzed by software.

III. RESULTS AND THEIR SIGNIFICANCE

Our strains isolated from Lake Balaton showed the greatest morphological and phylogenetic similarity to *Microcystis flos-aquae*. A total of 36 anabaenopeptin, 17 microginin and 13 microcystin variants were identified from 25 Microcystis isolates and 3 bloom forming *Microcystis* community. These peptides include 32 anabaenopeptins and 15 microginins, which were previously unknown. Strains typically contained either anabaenopeptin or microginin or microcystin variants as major components but significantly lower amount of other types of peptides could be observed. Anabaenopeptin and microginin dominant strains were isolated from water samples but microcystins were also found in 10 strains. Two strains produced higher amounts of microcystins.No phylogenetic relationship was found between the chemotypes and there was no significant association between the metabolite patterns and the origin of the samples. The water bloom samples, which come from three consecutive years showed different peptide patterns.

Altogether 133 isolates were generatedfrom 65 dry field samples collected from the Great Plain. The 90% of the isolated strains belonged to the *Nostoc*genus, but were separated from the *Nostoc*, *Aliinostoc* and *Desmonostoc* reference strains in the phylogenetic tree.STRR and ERIC fingerprinting also showed differences between the strains, but no correlation was found with the results of the phylogenetic analysis.A little more than a third of our isolates produced some kind of peptide-type metabolite(s).In our study 12 nostoginin/microginin, 16 anabaenopeptin, 12 banyaside/suomilide and 1 nostopeptolide variants were found in the extracts of our isolates.The identified metabolites include 10 new nostoginin/microginin, 7 new anabaenopeptin and 9 new banyaside/suomilide variants.Most of the isolated strains produced only one type of peptides, but some produced both anabaenopeptin and banyaside-type metabolites.No correlation was found between the chemotypes and genotypes. The result of the STRR and ERIC fingerprinting showed no strict correlation with the origin of the strains nor the produced peptide type metabolites, but most of the anabaenopeptin producer strains formed a distinct group.

Our symbiotic*Nostoc*showed similarity to the *Nostoc*, *Aliinostoc* and *Desmonostoc* genus, but formed separate groups in the phylogenetic tree.Some strains were closely related to some anabaenopeptin and banyaside/suomilide producing free-living *Nostoc* strains.Lichen-derived cell lines did not produce any specific peptide-type metabolites but the *Azolla* sp. symbiont strain was able to produce a banyaside variant (to the same extent as the free-living strains).

The peptide metabolites produced by the members of this two cyanobacterial genus can be important both from a medical and health point of view. Microcystins are the most common cyanobacterial toxins and are dangerous to humans, animals and plants. Nostoginins and microginins are zinc metalloprotease inhibitors and aminoproteinase inhibitors. Due to their inhibitory effect on the angiotensin converting enzyme, these substances may be important candidates for the treatment of hypertension. Anabaenopeptins can have different effects; commonly they inhibits proteinase enzymes such as trypsin, chymotrypsin, elastase and carboxypeptidase A. Some variants are inhibitors of protein phosphatases. Previously known banyasides/suomilideshave been shown to be protease inhibitors (trypsin and thrombin), but in our preliminary studies banyaside-containing extracts inhibited the function of the angiotensin convertase enzyme. Some nostopeptolide variants are recognized as antitoxins, they inhibit the function of anion transporters responsible for the uptake of microcystins [5,6].

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List of publications related to the dissertation

1. **Riba, M.**, Kiss-Szikszai, A., Gonda, S., Parizsa, P., Deák, B., Török, P., Valkó, O., Felföldi, T., Vasas, G.: Chemotyping of terrestrial Nostoc-like isolates from alkali grassland areas by non-targeted peptide analysis.
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