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Modern Methods for Detection and Elimination of Microcystins Toxins Produced by Cyanobacteria: Mini-review

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Abstract: Microcystins are hepatotoxins produced by some cyanobacterial taxa that usually form surface blooms on water bodies world-wide. These toxins can critically influence the public health since they can cause fatal liver damage. Water treatment by lysis of toxic cyanobacterial blooms would release toxin into water and exacerbate the problem of water pollution with those toxins. Therefore, an active wise policy to face this problem is needed. In that regard, there must be a comprehensive understanding of the toxins' mechanism of action, chemical structure, biosynthetic genes, factors affecting toxin genes expression and ways of detection and elimination. Also the use of modern rapid techniques to differentiate between toxic and non-toxic strains of cyanobacteria is required. The present minireview discusses the use of advanced analyses that comprise chemical, biological, molecular and nano-techniques for the detection and removal of microcystins. The full understanding of this phenomenon would pave the way for constructing a strategy for toxic cyanobacterial bloom control and elimination of toxin from drinking water.

Key words: ELISA, HPLC, microcystins, nanotechnology, quantitative realtime-PCR and ozonation

INTRODUCTION

Microcystins are hepatotoxins produced by cyanobacteria in freshwater bodies world-wide. Microcystins are cyclic peptides, formed non-ribosomally in the cytoplasm and consist of seven amino acids including the C_{20} amino acid, Adda (3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6, dienoic acid) (Rinehart *et al*., 1994). More than seventy microcystins variants were identified whose general structural formula is [cyclo(-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-)], where X and Z are two variable L-amino acids, D-MeAsp is D-erythro-β-methylspartic acid and Mdha is N-methyldehydroalanine (Rinehart *et al*., 1994; Rouhiainen *et al*., 2004). Several cyanobacterial taxa are capable of producing microcystins including *Microcystis* (Tillett *et al*., 2000), *Planktothrix* (Christiansen *et al*., 2003) and *Anabaena* (Rouhiainen *et al*., 2004).

The toxic action of microcystins: Microcystins are potent inhibitors of two major eukaryotic signal transduction, serine phosphatase 1 and 2A enzymes. When the toxin enters the hepatocytes by anion transport bile acid carrier, it forms covalent bonds with the enzymes' catalytic units causing their inhibition (Runnegar *et al*., 1995). Inhibition results in hyperphosphorylation of proteins, leading to tumour formation (Rapala and Lahti, 2002). It may also lead to the destruction of hepatic sinusoidal endothelial cells thereby causing hemorrhages (Sturgeon and Towner, 1999). Microcystins can also cause oxidative stress, through inducing the formation of reactive oxygen species, leading to a series of mitochondrial drastic changes that would cause cell apoptosis (Mikhailov *et al*., 2003). Another target of microcystins is β-subunit of ATP synthase which can cause mitochondrial apoptotic signalling, but only if the toxin is present at high concentrations (Zaccaroni and Scaravelli, 2007).

Biological role of toxin: There were some reports on the biological roles these toxins are assumed to play. In that regard, Utkilen and Gjolme (1995) proposed that microcystin acts as an intracellular chelator of cellular available iron since microcystin is partly produced by an enzyme (synthetase) whose activity is controlled by the amount of free Fe^{2+} present. It was also suggested that microcystins may operate in gene regulation (Dittmann *et al*., 1997). Another proposed role, however, is that microcystins act as info-chemicals that alert younger cells when older cells lyse rather than a defensive tool (Schatz *et al*., 2007). In support of this, Rantala *et al*. (2004) explained that the evolution of the microcystin synthetase genes, preceeded its predators, therefore microcystin must have had different role other than deterring grazers.

Arrangement of gene operons responsible for microcystins biosynthesis: The genetic machinery of microcystin biosynthesis was deciphered in *Microcystis*

(Tillett *et al*., 2000), *Planktothrix* (Christiansen *et al*., 2003) and *Anabaena* (Rouhiainen *et al*., 2004). Two large molecular systems namely: genes encoding Non-Ribosomal Peptide Synthetases (NRPS) and genes encoding polyketide synthases (PKS) are responsible for the modification and incorporation of substrates involved in microcystin biosynthesis. In both *Microcystis* and *Planktothrix,* microcystin biosynthesis always begins with synthesis of Adda followed by the sequential addition of the other six amino acids by peptide synthetases (Christiansen *et al*., 2003). The polyketide synthase catalyses the synthesis of the fatty acid branch of the Adda (Christiansen *et al*., 2003). In addition, there are auxiliary genes which are not necessarily found in all microcystin-producing cyanobacteria (Christiansen *et al*., 2003). Moreover, there are other genes that have a combined NRPS-PKS genetic activity hybrid genes (Rouhiainen *et al*., 2004).

In *Microcystis aeruginosa*, there are ten microcystin biosynthetic genes found in two bi-directionally arranged operons. They comprise peptide synthetases genes (*mcyA, mcyB, mcyC*) polyketide synthases genes (*mcy D*), hybrid enzymes genes (*mcyE- mcyG*), methylation gene (*mcy J*), epimerization gene (*mcyF*), dehydration gene (*mcyI*) and transporter gene (*mcyH*). The variation in microcystins structure is suggested to be attributed in part to the recombination between *mcyC* and the first module of *mcyB* (Rantala *et al*., 2004). In *Planktothrix*, the microcystin synthetase gene (*mcy*) contained genes for peptide synthetases (*mcyA-mcyB-mcyC*), polyketide synthases gene (*mcyD*), hybrid enzymes genes (*mcyE-mcyG*), a putative thioesterase gene (*mcyT*), a putative ABC transporter gene (*mcyH*) and a putative peptide-modifying enzyme (*mcyJ*) (Christiansen *et al*., 2003). In *Anabaena*, the microcystin biosynthetic genes are suggested to be arranged in three operons. The first operon (*mcyA-mcyB-mcyC*) is transcribed in the opposite direction from the second (*mcyG-mcyD-mcyJ-mcyE-mcy F-mcyI*) and the third operon (*mcyH*). The peptide synthetase genes were *mcyA*, *mcyB* and *mcyC*. In the second operon, the PKS genes were; *mcyD*, *mcyJ*, *mcyF* and *mcyI*. Whereas, *mcyG* and *mcyE* are hybrid genes (Rouhiainen *et al*., 2004).

Molecular techniques for defining toxic strains and studying factors affecting toxin production: The same cyanobacterial species can have toxic and non-toxic strains which usually cannot be distinguished by morphological examination (Lyra *et al*., 2001). Therefore, molecular methods are used for defining toxic strains (Pan *et al*., 2002). In that context, Nonneman and Paul (2002) used a molecular method for detecting cyanobacteria with the potential of microcystin production. They partially amplified the *mcyB* genetic locus; one of the microcystin biosynthetic genes. They used primer pair specific for this gene to detect the potential of toxin production in *Microcystis* isolates found in environmental samples. This technique represents a rapid detection method and does not require complicated procedures or expensive tools.

Molecular techniques were also applied for defining factors affecting toxin genetic expression. El Semary (2010) used quantitative realtime-PCR (QRT-PCR) for quantifying the expression of *mcyB* genetic locus under different environmental conditions to investigate factors triggering off toxin production. The results showed that the optimum temperature for enzymatic activities is the master factor that controls toxin biosynthesis and light can only be stimulatory if not exceeding photosynthetic saturation limit. The technique proved useful in quantifying toxin biosynthetic gene expression as a function of toxin production.

Chemical analysis of microcystins: Most of microcystin variants can be extracted by several polar solvents (Harada *et al*., 1990). The reverse-phase HPLC equipped with C18 silica column and UV-detector is commonly used for toxin analysis at a wavelength of 238 nm (Harada *et al*., 1999). The eluting solvent system used is usually composed of a gradient of water and 30-70% acetonitrile to which 0.05% trifluoroacetic acid (TFA) is added (Harada *et al*., 1999; Metcalf and Codd, 2004).

Biological assays for microcystins: The Enzyme-Linked Immuno Sorbent Assay (ELISA), an immune-technique for toxin quantification, is based on raising polyclonal antibodies against microcystin conjugated to bovine serum albumin (Mouse bioassay) (Chu *et al*., 1989). Unfortunately, ELISA can show some false positives and requires rather long time to perform as well as expensive kits and training. Nevertheless, a rapid and sensitive fluorescence immunochromatography assay system was developed using monoclonal antibodies of microcystin LR (Pyo *et al*., 2005). This technique employs a fluorescence immunochromatographic strip and applies it to the water sample suspected to contain microcystins. Two chromatographic lines of fluorescence intensity curves appear in which the fluorescence intensity of the test line is inversely proportional to the concentration of microcystin, whereas, the second (control) line is related to the sample mass transport and is not affected by microcystin concentration (Pyo *et al*., 2005). Jianwu *et al*. (2007) also presented further modification in the ELISA

system as indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) to increase the technique's sensitivity.

Another biological assay which quantifies toxin at picogram level is Protein Phosphatase Inhibition Assay (PPIA). The method is based on the capacity of microcystins to inhibit the catalytic subunit of human recombinant protein phosphatase 1 enzyme expressed in *E. coli* (Carmichael and An, 1999). However, it is note worthy that reactivity of different microcystins with protein phosphatase1 is not the same and that the assay was also sensitive to other protein phosphatase inhibitors (Rapala and Lahti, 2002). In that regard, Metcalf *et al.* (2001) was able to introduce a modification in the protein phosphatase assay to make it more specific to microcystins and to overcome its sensitivity to other protein phosphatase inhibitors such as okadaic acid, calyculin A and tautomycin. They combined immunoassay-based detection of the toxins with a colorimetric protein phosphatase inhibition system in an assay called the Colorimetric Immuno-Protein Phosphatase Inhibition Assay (CIPPIA). Polyclonal antibodies against microcystin-LR were used in conjunction with protein phosphatase inhibition, which distinguished microcystin variants from okadaic acid, calyculin A and tautomycin.

Meanwhile and in an effort to device a protein phosphatase-based biosensor for microcystin detection, Campas *et al.* (2005) developed a colorimetric test based on immobilised Protein Phosphatase (PP).They compared different immobilisation matrices and supporters and found that the highest immobilisation yields were obtained with microtiter wells whereas the highest operational and storage stabilities were obtained with carbon SPEs and membranes, respectively. Those findings later lead to the development of a highly sensitive amperometric immunosensors for microcystin detection (Campas and Marty, 2007).

To evaluate the effectiveness and sensitivity of the techniques used for toxin detection and quantification, Rapala *et al.* (2002) compared the protein phosphatase inhibition assay (PPI assay), enzyme-linked immunosorbent assay (ELISA) and different HPLC methods using UV detection. They found that the former two biological methods provided not only reliable, quantitative and sensitive detection methods of microcystins in water but also they did not have the shortcomings of using HPLC such as poor sample recovery.

Microcystin elimination by natural, traditional and nanotechnological methods: When the toxin-producing cyanobacteria lyse either due to water treatments or natural cause, most of the cellular microcystins are released into water thus causing an immense toxic hazard. Therefore, lysing cyanobacterial cells by traditional chemical treatments of water does not solve the problem of toxin but rather exacerbate it. This is particularly crucial after setting the guideline value for microcystin-LR as 1.0 microg L^{-1} drinking water by the World Health Organization which means toxin concentration must stay below this value through effective water treatments (Hitzfeld *et al.*, 2000). Hence, different methods of breakdown and removal of toxin should be sought and investigated. In that regard, natural breakdown of toxin by bacteria was reported by Maruyama *et al.* (2006) who found that microystins are exposed to biodegradation by bacteria belonging to a novel genus *Sphingosinicella microcystinivorans*. Moreover, Lemes *et al.* (2008) isolated a bacterial strain belonging to *Burkholderia* sp. capable of degrading microcystin from a Brazilian lagoon. Recently, Okano *et al.* (2009) isolated a bacterial strain capable of degrading microcystin under alkaline conditions, the same condition at which toxic cyanobacterial bloom grow and flourish.

Other means of toxin breakdown and removal are also considered. Earlier, Hitzfeld *et al.* (2000) recommended ozonation as the most effective in destroying cyanobacteria and breaking microcystins but suggested it may not be so in case of massive toxic bloom situation. Also the photodegradradation of microcystin under UV light may prove effective (Metcalf and Codd, 2004). Removal of toxin using traditional adsorbents was also reported. The adsorption to bio-sand water filters especially if the filters are layered with charcoal or granulated carbon was recommended by Bojcevska and Jergil (2003).

With the emergence of nanotechnology, nano-sized adsorbents were used for toxin removal. In that regard, the use of nanocarbon tubes for adsorption of microcystins was compared to the use of traditional adsorbents by Yan *et al.* (2006). Two microcystin variants were extracted from cyanobacterial cells in China, by carbon nanotubes (CNTs) and compared with adsorption of microcystin by wood-based activated carbon and different types of clay. Carbon nanotubes were found to have a greater adsorptive ability for microcystins that was four times higher than the other two adsorbents systems tested. The decrease of CNTs diameters caused an increase in the adsorption efficiency. It is also worthy noting that the specific surface area of CNTs was a factor in the adsorption efficiency of microcystins. Thereby, the selection of a suitably-sized adsorbent is very important for achieving highest microcystins elimination efficiency from drinking water. Another use of nanoparticles for

removal of microcystin was presented by Xiaogang *et al.* (2006). They used nanofilm of titanium dioxide for photocatalytic degradation of trace levels of microcystin. The degradation efficiency of toxin was affected by the pH conditions, initial concentration and UV intensity.

From the aforementioned reports, it is noteworthy that there are only initial studies on the use of nanotechnology in toxin small-scale removal and degrdation. No large-scale, long-term studies, to the best of our knowledge, were available on massive removal of toxin from water reservoirs persistetly covered by extensive cyanobacterial blooms. Obviously, further research is needed to allow for mass-scale applications of nanotechnology in toxin removal and degradation.

CONCLUSION

The synthesis of microcystin, the cyclic hepatotoxic peptides, is perfomed non-ribosomally through the activity of two enzymatic systems (the non-ribosomal peptide synthetase NRPS and the polyketide synthase PKS). The detection of microcystins-producing cyanobacteria can be performed using rapid molecular test. The expression of the microcystin biosynthetic genes can be quantified using realtime-PCR which is useful tool in identifying factors triggering off microcystin biosynthesis. The detection of microcystin can be performed using different chemical and biological methods. The elimination of microcystin can happen by bacterial biodegradation, ozonation, photodegradation by UV exposure or by using traditional adsorbents. However, the newly-developed nano-adsorbents proved to be more efficient. More research should focus on developing this technology further to allow for massive toxin removal.

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