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Determination of microcystin-LR and its metabolites in snail (*Bellamya aeruginosa*), shrimp (*Macrobrachium nipponensis*) and silver carp (*Hypophthalmichthys molitrix*) from Lake Taihu, China

Dawen Zhang, Ping Xie *, Jun Chen, Ming Dai, Tong Qiu, Yaqin Liu, Gaodao Liang

Donghu Experimental Station of Lake Ecosystems, State Key Laboratory of Freshwater Ecology and Biotechnology of China, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, People's Republic of China

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ABSTRACT

This paper describes seasonal changes of microcystin-LR (MC-LR) and its glutathione (MC-LR-GSH) and cysteine conjugates (MC-LR-Cys) in three aquatic animals – snail (*Bellamya aeruginosa*), shrimp (*Macrobrachium nipponensis*) and silver carp (*Hypophthalmichthys molitrix*) collected from Lake Taihu, China. MC-LR, MC-LR-GSH, and MC-LR-Cys were determined by liquid chromatography electrospray ionization mass spectrum (LC–ESI-MS). The mean MC-LR concentrations in the hepatopancreas of snail and shrimp and liver of silver carp were 6.61, 0.24, and 0.027 μg g^{-1} dry weight (DW), respectively; while the average MC-LR-Cys concentrations were 0.50, 0.97, and 5.72 μg g^{-1} DW, respectively. MC-LR-GSH was usually not detectable in these samples. The above results suggest that: (1) in aquatic animals, especially fish, the main excretion form of MC-LR could be MC-LR-Cys, but not MC-LR-GSH, whereas MC-LR-Cys might play an important role in detoxication of MC-LR and (2) that efficiency of MC-LR-Cys formation differs among species. The main detoxication pathway of MC-LR in aquatic animals is suggested as follows: when MC-LR enters into liver/hepatopancreas, it firstly conjugates with polypeptide or protein (including GSH, PP-1 and 2A) containing Cys residues, perhaps also some free cysteine; subsequently, MC-LR-Cys is degraded from these polypeptide or protein; and finally is excreted from animals by the compound of MC-LR-Cys.

1. Introduction

Microcystins (MCs) are a family of cyclic hepatotoxic heptapeptides produced by some species of freshwater cyanobacteria including several strains from the genera *Microcystis*, *Oscillotoria*, *Anabaena*, *Nostoc*, and *Planktothrix* (Svrcek and Smith, 2004). Microcystins have the general structure of cyclo (-D-Ala-X-D-MeAsp-Y-Adda-D-Glu-Mdha), where X and Y are two variable amino acids, D-MeAsp, Mdha, Adda are abbreviations of D-methylaspartic acid, N-methyldehydroalanine and (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid, respectively (Carmichael et al., 1988). To date, more than 80 variants have been isolated and identified, differing primarily in the two L-amino acids at positions 2 and 4 and methylation/demethylation on MeAsp and Mdha (Dietrich and Hoeger, 2005). Among these microcystins isoforms, MC-LR is the most common and the most toxic variant (Gupta et al., 2003).

It is well known that microcystins can cause poisoning or death of fish, birds, domestic and wild animals (Carmichael, 2001), as well as illnesses and mortality in human (Azevedo et al., 2002). In 1996, a serious accident of MC-toxication resulting in more than 50 human deaths occurred in Brazil due to the use of microcystins contaminated hemodialysis waters (Carmichael, 2001; Azevedo et al., 2002). Some studies demonstrated that the high incidence of primary liver cancer in eastern China was related to the presence of microcystins in the drinking water (Yu, 1989). Recently, microcystins were identified for the first time in the serum (average 0.228 ng MC-LReq mL^{-1}) of a chronically exposed human population (fishermen at Lake Chaohu, China) together with indication of hepatocellular damage (Chen et al., 2009).

Until to now, extensive field investigations have been conducted to document bioaccumulation and distribution of MCs in various aquatic organisms (snail, mussel, and various fish) in Lake Taihu, China (Chen and Xie, 2005; Chen et al., 2006, 2007; Zhang et al., 2007, 2009a). These studies reported that microcystins can accumulate in various organs of aquatic animals and also can be transferred along the food chain, hence pose potential threats to human health (Chen et al., 2009).

Previous studies reveal that glutathione plays an important role in the metabolic pathway of MCs in both mammals and a wide

* Corresponding author. Address: Donghu Experimental Station of Lake Ecosystems, State Key Laboratory of Freshwater Ecology and Biotechnology of China, Institute of Hydrobiology, Chinese Academy of Sciences, Donghu South Road 7, Wuhan 430072, People's Republic of China. Tel./fax: +86 27 68780622.
E-mail address: xieping@ihb.ac.cn (P. Xie).

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range of aquatic organisms (Runnegar et al., 1987; Hermansky et al., 1991; Kondo et al., 1992, 1996; Pflugmacher et al., 1998; Ito et al., 2002). Hermansky et al. (1991) reported that pretreatment of mice with GSH protected them against microcystin-LR lethality. The conjugation of microcystins to glutathione and cysteine has been tested under chemical conditions and the resulting conjugates were firstly identified by the Frit-FAB LC/MS (Kondo et al., 1992). Subsequently, Kondo et al. (1996) identified the presence of microcystins glutathione and cysteine conjugates in liver of mouse and rat treated with microcystins. In vitro formation of microcystin-LR conjugate can take place enzymatically via soluble glutathione S-transferase (GST) in aquatic macrophyte, invertebrates, fish eggs, and fish (Pflugmacher et al., 1998). Ito et al. (2002) studied the distribution of MC-LR and its glutathione and cysteine conjugates in different tissues in mice by immunostaining method. However, all the above studies just provided indirect evidence for the detoxication of MCs in organisms, since no study has made quantitative determinations of microcystin glutathione and cysteine conjugates simultaneously in organisms. Hence, it is an imperative need to understand the detoxication mechanisms of microcystins in animals through quantitative determinations of MC-LR and its two metabolites (MC-LR-GSH and MC-LR-Cys).

Lake Taihu (30°5′–32°8′N and 119°8′–121°55′E), the third largest freshwater lakes in China, is located in the east part of China. It has the total surface area of approximately 2338 km^2, with a mean water depth of 1.9 m and a maximum depth of about 2.6 m. During the past decades, the lake has undergone a steady increase in eutrophication, and with a regular occurrence of cyanobacterial surface blooms in the warm seasons each year (Xie, 2008). Zhang et al. (2009b) pointed out that the toxic *Microcystis aeruginosa* was the absolute dominant species in the water column of Lake Taihu during the bloom period.

The aims of the present study were: (1) to examine seasonal dynamics of MC-LR and its conjugates (MC-LR-GSH, MC-LR-Cys) in various organisms (snails (*Bellamya aeruginosa* (Reeve, 1862)), shrimp (*Macrobrachium nipponensis*) and silver carp (*Hypophthalmichthys molitrix*)) collected from Lake Taihu and (2) to compare the difference of the detoxication pathway among the aquatic animals.

2. Materials and methods

2.1. Preparation of MC-LR and its glutathione and cysteine conjugates

2.1.1. Preparation of MC-LR

MC-LR was isolated and purified from surface blooms collected from Lake Dianchi, China, as described in a recent study (Dai et al., 2008). The content of purified MC-LR was over 95% and its identity was confirmed and determined with LC–MS.

2.1.2. Formation of MC-LR-GSH and MC-LRCys

L-Glutathione (L-GSH) and L-Cysteine (L-Cys) were purchased from Acros Organics (Geel, Belgium) and the purity of GSH and Cys was greater than 99%. MC-LR-GSH and MC-LR-Cys were prepared by the method of Dai et al. (2008) and Dai (2008). Briefly, MC-LR (4 mg) reacted with L-GSH (124 mg) in 10 mL 5% potassium carbonate aqueous solution while stirring for 2 h at room temperature. The reaction mixture was neutralized with about 15 mL 0.2 M hydrochloric acid and applied to an ODS C_{18} cartridge (5 g, Waters, Milford, MA, USA). The cartridge was rinsed with 10 mL water and eluted by 20 mL methanol to give the reaction product. The reaction product was purified further by a semipreparative reversed phase liquid chromatography (Waters 600, USA; flow rate 1 mL/min; detection, UV (238 nm)) with an ODS C18 reversed phase semipreparative column (7.8 × 300 mm, 10 μm, Waters, Milford, MA, USA) to yield 3.2 mg of the purified GSH conjugate of microcystins. The mobile phase was the mixture of methanol and water. MC-LR-Cys was formed and purified similarly. The reaction of microcystin-LR (4 mg) with L-Cys (48 mg) yielded 3 mg of the purified Cys conjugate of microcystin-LR. The content of purified glutathione and cysteine conjugates of MC-LR were over 95% and confirmed by HPLC (LC-20A, Shimadze, Kyoto, Japan) and LC–MS (Thermo Electron, Waltham, MA, USA).

2.2. Study area

Meiliang Bay, a part of Lake Taihu, with water surface area of 125 km^2, accommodates municipal and industry wastewater from Wuxi City, and acts as principal water source for the city. It is the most eutrophic part of the lake, characteristic of extremely dense accumulation of toxic *Microcystis* blooms in the summer (Xie, 2008). Gonghu Bay (31°31′–23′N, 120°16′–35′E) is located at the northeast portion of Taihu Lake (Fig. 1).

2.3. Sample preparation

Snails (*B. aeruginosa*; body weights: 4.68 ± 1.19 g; shell height: 2.80 ± 0.28 cm) and shrimp (*Macrobrachium nipponensis*; body weights: 2.3 ± 1.5 g; body weight: 5.9 ± 1.3 cm) were collected monthly from Gonghu Bay and Meiliang Bay, respectively, from November to December 2004 and from July to October 2005. The collected snails and shrimps were immediately frozen at −20 °C, and the hepatopancreas of snails and shrimps were dissected in the laboratory. To insure adequate amount of samples, we pooled separate hepatopancreas of 20 snails and shrimps into two composite samples, respectively.

Silver carp (*H. molitrix*; body weight: 1021 ± 325 g; body length: 34.5 ± 4.8 cm) was artificially stocked into a pen (Meiliang Bay, Taihu Lake, China) with a total area of 1.08 km^2 in January 2005. Two fish were collected from this pen monthly from July to December 2005. The collected fish were measured, weighed, and sacrificed immediately, and then dissected in the field into liver, kidney, and intestinal content, finally frozen at−20 °C immediately.

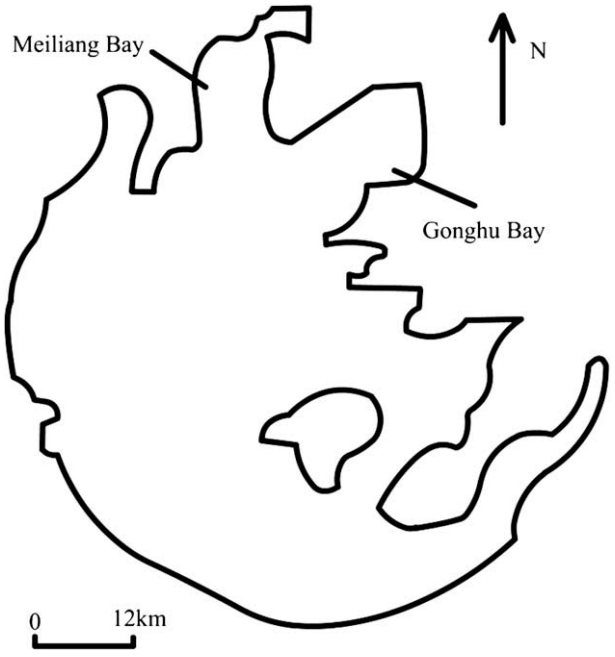

Fig. 1. Sampling locations in Lake Taihu (30°5′–32°8′N and 119°8′–121°55′E), China.