The role of cysteine conjugation in the detoxification of microcystin-LR in liver of bighead carp (*Aristichthys nobilis*): a field and laboratory study

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Abstract The role of glutathione (GSH) and cysteine (Cys) conjugates in the detoxification of microcystin-LR (MC-LR) in bighead carp (*Aristichthys nobilis*) was examined under laboratory and field conditions. Wild individuals of bighead carp were collected from 5 eutrophic lakes along the Yangtze River, while in laboratory experiment, bighead carp were injected intraperitoneally with 500 μg purified MC-LR/kg body weight (bw). Contents of MC-LR and its glutathione (MC-LR-GSH) and cysteine conjugates (MC-LR-Cys) in the liver of bighead carp were determined by liquid chromatography electrospray ionization mass spectrum (LC–ESI–MS). In laboratory experiment, low concentrations of MC-LR-GSH (mean: 0.042 μg/g dry weight (DW)) were always detectable, and the mean ratio of MC-LR-Cys to MC-LR-GSH was 6.55. While, in field study, relatively high MC-LR-Cys concentration (mean: 0.22 μg/g DW) was detected, whereas MC-LR-GSH was occasionally detectable, and the average ratio of MC-LR-Cys to MC-LR-GSH was as high as 71.49. A positive correlation was found between MC-LR-Cys concentration in the liver of bighead carp and MC-LR content in seston from the five lakes ($r = 0.85$). These results suggest that MC-LR-Cys might be much more important than MC-LR-GSH in the detoxification of MC-LR in fish liver, and that cysteine conjugation of MC-LR might be a physiological mechanism for the phytoplanktivorous bighead carp to counteract toxic cyanobacteria.

Keywords Microcystins · MC-LR-Cys · MC-LR-GSH · Bighead carp · Detoxification

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Introduction

Microcystins (MCs) are toxic monocyclic heptapetides produced by some species of freshwater cyanobacteria including several strains from the genera *Microcystis*, *Oscillatoria*, *Anabaena*, *Nostoc*, and *Planktothrix* (Svrcek and Smith 2004). They 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 nature of the two L-amino acids at positions 2 and 4 and methylation/demethylation on MeAsp and Mdha (Dittmann and Wiegand 2006). Among these MCs isoforms, microcystin-LR (MC-LR) is the most common and the most toxic variant (Gupta et al. 2003).

MCs are known to be potent hepatotoxins (Dawson 1998) and tumor promoter (Nishiwaki-Matsushima et al.

1992) by inhibiting protein phosphatases 1 and 2A in hepatocytes of animals (Eriksson et al. 1990; Falconer and Yeung 1992). They can cause poisoning or death of fish, birds, domestic and wild animals (Carmichael 2001; Malbrouck and Kestemont 2006), as well as human illnesses and even death due to their exposure to MCs contaminated water and aquatic products (Azevedo et al. 2002). For example, the high incidence of primary liver cancer in Hai-men City (Jiangsu Province) and Fusui County (Guangxi Province) in China is considered to be related to MCs in drinking water (Yu 1995). In 1996, a serious accident of MC-toxication resulting in more than 50 human deaths occurred in Brazil due to the use of MCs contaminated hemodialysis waters (Azevedo et al. 2002). Recently, MCs were detected in the serum (average 0.228 ng MC-LReq/ml) of a chronically exposed human population (fishermen at Lake Chaohu, China) together with indication of hepato-cellular damage (Chen et al. 2009).

Although MCs are rarely ingested by human in amount high enough to a lethal acute dose, chronic toxic effects from exposure through food need to be considered, especially if there is long-term frequent exposure (Magalhães et al. 2003), since previous studies verified that MCs can accumulate in various organs of aquatic animals and also can be transferred along the food chain (Song et al. 2007; Zhang et al. 2009a), hence indicating a potential threat to human health from consumption of MCs-contaminated aquatic products. Thus, the World Health Organization (WHO) established a maximum guideline for MC-LR in drinking water at 1 mg/l and a tolerable daily intake (TDI) for MC-LR in the aquatic product at 0.04 mg/kg of body weight per day (Falconer et al. 1999).

The poisoning incidents of animal and human exposure to MCs (Jochimsen et al. 1998; Kuiper-Goodman et al. 1999) emphasize the need to understand the detoxification mechanism of this compound. Previous studies reveal that glutathione plays an important role in the metabolic pathway of MCs in both mammals and a wide range of aquatic organisms (Hermansky et al. 1991; Kondo et al. 1992, 1996; Pflugmacher et al. 1998; Ito et al. 2002). The conjugations of MCs to glutathione (MC-GSH) and cysteine (MC-Cys) have 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 MC-GSH and MC-Cys in the liver of mouse and rat after intraperitoneal (i.p.) injection of MCs, and then Pflugmacher et al. (1998) detected the conjugation of MC-LR in enzyme extracts containing GST of aquatic macrophyte, invertebrates, fish and fish eggs, and concluded that the MC-LR-GSH appears to be the first step in the detoxication of cyanobacterial toxin in aquatic organisms. In addition, Ito et al. (2002) studied the distribution of MC-LR and its glutathione and cysteine conjugates in different tissues in mice by immu-nostaining method. For a better understanding of the role of MC-LR-GSH conjugate in the detoxification of MCs in animals, Dai et al. (2008) established an analytical method for the simultaneous quantitative determination of MC-LR and MC-LR-GSH in fish tissues and applied this method to quantify the MC-LR-GSH concentration in the liver of crucian carp after i.p. injection of single dose of micro-cystins (100 μg MC-LR equivalent/kg body weight). However, in our recent work, high amount of MC-LR-Cys was detected in the liver or hepatopancreas of snail, shrimp, and silver carp collected from Lake Taihu, while MC-LR-GSH was usually not detectable in these organisms (Zhang et al. 2009b). Nevertheless, so far, there has been no literature to report the simultaneous quantitative determinations of MC-LR-Cys and MC-LR-GSH in fish under laboratory conditions. Hence, it is essential to determine MC-LR and its metabolites (MC-LR-GSH and MC-LR-Cys) quantitatively in fish under field and laboratory conditions to better understand the roles of glutathione and cysteine conjugates in the detoxification of MC-LR in aquatic animals.

The main goals of the present study are to (1) examine the temporal variations of MC-LR and its two metabolites (MC-LR-GSH and MC-LR-Cys) in the liver of bighead carp (Aristichthys mobilis) after an i.p. injection of 500 μg/kg bw; (2) study the accumulation levels of MC-LR and its two metabolites (MC-LR-GSH and MC-LR-Cys) in the liver of bighead carp collected from five different eutrophic lakes with dense cyanobacterial blooms; (3) identify the roles of MC-LR-Cys and MC-LR-GSH in MCs detoxification and provide the possible information regarding the metabolic mechanisms of bighead carp to resist microcystins stress.

Materials and methods

Preparation of MC-LR and its glutathione and cysteine conjugates

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.

Formation of MC-LR-GSH and MC-LR-Cys

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%. The method

for the preparation of MC-LR-GSH and MC-LR-Cys was described by Zhang et al. (2009b). The contents of purified MC-LR-GSH and MC-LR-Cys were over 95% and confirmed by HPLC (LC-20A, Shimadze, Kyoto, Japan) and LC–MS (Thermo Electron, Waltham, MA, USA).

Laboratory study

Healthy bighead carp (*Aristichthys mobilis*) with mean weights of 899 ± 251 g were purchased from a local fish hatchery in Wuhan City, China. Fish were held for 2 days prior to experimentation in the experimental 150 l aquaria containing tap water. Water temperature was controlled at $28 \pm 1°C$, and the average dissolved oxygen concentration was 7.4 mg/l by continuously aerating. Fish were kept in a 12 h light/dark photoperiod. No food was given to the fish throughout the experiment.

The treated fish were injected intraperitoneally with a dose of 500 µg purified MC-LR (Purity > 95%) per kg bw. Three treated fish were killed at 1, 3, 6, and 12 h post-injection. For each sampling, liver samples were dissected and immediately frozen at $-80°C$ for the determination of MC-LR and its two metabolites (MC-LR-Cys and MC-LR-GSH) contents.

Field study

Study area

The field study was carried out in Huanggai, Bajiao, Maoli, Gehu, Dianshan Lakes. These five shallow lakes located at three provinces/municipality (Hunan, Jiangsu province and Shanghai) in the middle and lower reaches of the Yangtze River, and all these lakes are eutrophic or hypereutrophic with the frequent occurrence of cyanobacterial blooms in summer. The limnological parameters for the five shallow lakes were shown in Table 1.

Sample preparation

Three bighead carp (body weight: 876 ± 163 g; body length: 33.5 ± 4.8 cm) were collected in each of the five lakes during the period from July to August in 2008. The collected fish were measured, weighed, and killed immediately, and then the liver of the fish was dissected in the field, and samples were kept in cold storage and transferred from the field to the laboratory using ice box, finally frozen at $-80°C$ in the laboratory.

Meanwhile, one integrated water column sample which was a mixture of two sub-samples from 0.5 m below the surface and from 0.5 m above the bottom, respectively, was collected in each lake with Tygon tubing fitted with a one-way valve. One litre of the integrated water was filtered immediately by the glass-fiber filter ([GF/C], Whatman, Brentford, UK). The glass-fiber filter containing cyanobacterial cells was frozen at $-20°C$ and used for intracellular MCs determination. We measured the intracellular toxins according to the methods of Park and Lwami (1998).

All the fish liver samples were lyophilized by a Christ@ Alpha 2–4 freeze dryer (Martin Christ, Osterode, Germany). These lyophilized samples were extracted and purified following the methods of Zhang et al. (2009b): the lyophilized samples (~ 0.1 g DW for each sample) were extracted three times with 5 ml of 0.01 M EDTA disodium 5% acetic acid by sonicating 3 min (30% amplitude, 60 W, 20 kHz, Sonics VC130PB, Newtown, CT, USA) at 0°C and then centrifuged at 14000 r/min (BR4, Jouan, Winchester, VA, France) at 4°C. The supernatant was first applied to an Oasis HLB cartridge (500 mg, waters, Milford, MA, USA), which had been preconditioned by washing with 10 ml 100% MeOH and 10 ml distilled water. The column containing sample was washed with 20 ml water followed by 20 ml 20% MeOH, and then eluted with 20 ml 100% MeOH. The eluant was evaporated to dryness and the residue was dissolved in 100% MeOH. This solution was applied to a Sep-Pak silica gel cartridge (2 g, waters, Milford, MA, USA) which had been preconditioned by 100% MeOH. The column was washed with 20 ml 100% MeOH, and then eluted with 20 ml of 70% MeOH. This fraction was evaporated to dryness and redissolved in 100 µl of the LC mobile phase.

Table 1 MC-LR concentration in the seston and limnological parameters (Cited from Wang and Dou 1998) of 5 shallow lakes along the Yangtze River area

Lakes	Longitude	Latitude	Area (km²)	Mean depth (m)	Location	MC-LR concentration (µg/l)
Huanggai Lake	113°30′–113°38′	29°39′–29°48′	86.0	4.20	Hunan Province	5.58
Bajiao Lake	113°11′–113°15′	29°27′–29°31′	12.3	1.95	Hunan Province	2.97
Maoli Lake	111°52′–113°58′	29°23′–29°29′	36.5	3.50	Hunan Province	2.27
Gehu Lake	119°44′–119°53′	31°29′–31°42′	146.5	1.90	JIangsu Province	1.19
Dianshan Lake	120°53′–121°01′	31°04′–31°12′	62.0	2.10	Shanghai	0.24

Analysis of MC-LR and its metabolites

Qualitative and quantitative analysis of MC-LR, MC-LR-GSH, MC-LR-Cys in the liver of fish were performed using a Finnigan LC–MS system comprising a Thermo Surveyor auto sampler, a Surveyor mass spectrum (MS) pump, a Surveyor photo diode array (PDA) system, and a Finnigan LCQ-Advantage MAX ion trap mass spectrometer (Thermo Electron, Waltham, MA, USA) equipped with an atmospheric pressure ionization fitted with an electrospray ionization source (ESI) (Thermo Electron). The instrument control, data processing, and analysis were conducted by using Xcalibur software (Thermo Electron). Separation was carried out using an Agilent ZOEBAX SB-C18 Column (2.1 mm i.d. × 150 mm, 3.5 μm, Agilent Corporation, Santa Clara, CA, USA). The mobile phase consisted of solvent A [water + 0.05% (v/v) formic acid]/solvent B [acetonitrile + 0.05% formic acid]. The following linear gradient programme was used: 0 min (75% A, 25% B), 8 min (45% A, 55% B), 13 min (40% A, 60% B), 14 min (30% A, 70% B), 15 min (75% A, 25% B), 20 min (75% A, 25% B). The total flow rate was held at 0.2 mL/min at analysis stage. After the analysis stage, the percentage of solution B was adjusted to 25% and the flow rate was increased to 0.3 ml/min for 5 min before the next injection to renew the initial condition rapidly. Sample injection volume was typically 10 μl. Mass spectrum tuning and optimization were achieved by infusing microcystin-LR and monitoring the $[M + H]^{2+}$ ion at m/z 995.5. The MS analytical conditions were as follows: ESI spray voltage 4.54 kV; sheath gas flow rate 20 unit; auxiliary gas flow rate 0 unit; capillary temperature 250°C and multiplier voltage −853.19 V. The tube lens voltage was 55 V for MC-LR and 50 V for MC-LR-GSH and MC-LR-Cys. Quantification of MC-LR, MC-LR-Cys, and MC-LR-GSH was performed using the selected reaction monitoring (SRM) mode. For quantification purposes, mass chromatograms monitored the product ions at m/z 599.3 and 977.4 from the parent ion at m/z 995.5 of $[M + H]^+$ for MC-LR, and the product ions at m/z 599.6, 995.5, and 1029.5 from the parent ion at m/z 1116.5 of $[M + H]^+$ for MC-LR-Cys, while the product ions at m/z 587.1 and 1168.6 from the product ion at m/z 652.0 of $[M + H]^{2+}$ for MC-LR-GSH. Collision energy was 24, 38 and 36% for MC-LR-GSH, MC-LR-Cys, and MC-LR, respectively. The limit of detection (LOD) of MC-LR and its metabolites (MC-LR-GSH and MC-LR-Cys) are 0.005 μg/g DW.

Statistical analysis

The values below the detection limit can be represented in the data set by half of the detection limit, while the values for the target compounds that were not detected in the samples were set by zero. Pearson correlation analysis was conducted to determine the relationship between MC-LR-Cys or MC-LR-GSH concentration and MC-LR content in the liver of bighead carp using SPSS for Windows (Ver 13.0; SPSS, Chicago, IL, USA). The relationships were considered significant at $p < 0.05$.

Recovery experiment

Recovery experiments were carried out in quadruplicate spiking 100 mg of freeze-dried fish liver samples with MC-LR, MC-LR-Cys, and MC-LR-GSH solution at 0.5 μg/g DW. The extraction and analysis were performed as described above, and the recovery and the relative standard deviation of the analytical method were calculated.

Results

Characterization and relationship of MC-LR and its two conjugates

Figure 1 shows the ESI LC/MS/MS selected reaction monitoring (SRM) chromatograms for MC-LR and its two metabolites (MC-LR-GSH and MC-LR-Cys) in the liver of bighead carp 3 h post-injection of 500 μg purified MC-LR/kg bw. Based on SRM chromatogram monitored ion at m/z 652.0, and the presence of $[M + H]^+$ ions at m/z 587.1 and 1168.6, it was confirmed that peak obtained at 9.45 min was derived from MC-LR-GSH. Similarly, peaks obtained at 9.54 and 10.81 min were derived from MC-LR-Cys and MC-LR, respectively, as the peaks were detected by monitoring ions at m/z 1116.5 and m/z 995.5, respectively, and the selected ion chromatogram showed $[M + H]^+$ ions at m/z 599.6, 995.5, and 1029.5 for MC-LR-Cys and m/z 599.3 and 977.4 for MC-LR, respectively.

Laboratory study

No MC-LR, MC-LR-Cys, and MC-LR-GSH was detected in liver of bighead carp purchased from the fish hatchery and the concentration–time profiles of MC-LR and its two metabolites in liver treated with a dose of 500 μg purified MC-LR/kg bw were shown in Fig. 2. MC-LR and its two metabolites (MC-LR-Cys and MC-LR-GSH) in the liver of bighead carp were detectable during the whole study period, and the peaks of the MC-LR (2.89 μg/g DW), MC-LR-Cys (0.35 μg/g DW), and MC-LR-GSH (0.064 μg/g DW) concentrations were all occurred at 3 h post-injection. The minimum of the MC-LR-GSH and MC-LR-Cys concentrations were observed at 1 h post-injection, while the lowest content of MC-LR was found at 12 h post-injection. There were significant correlations between MC-LR-Cys
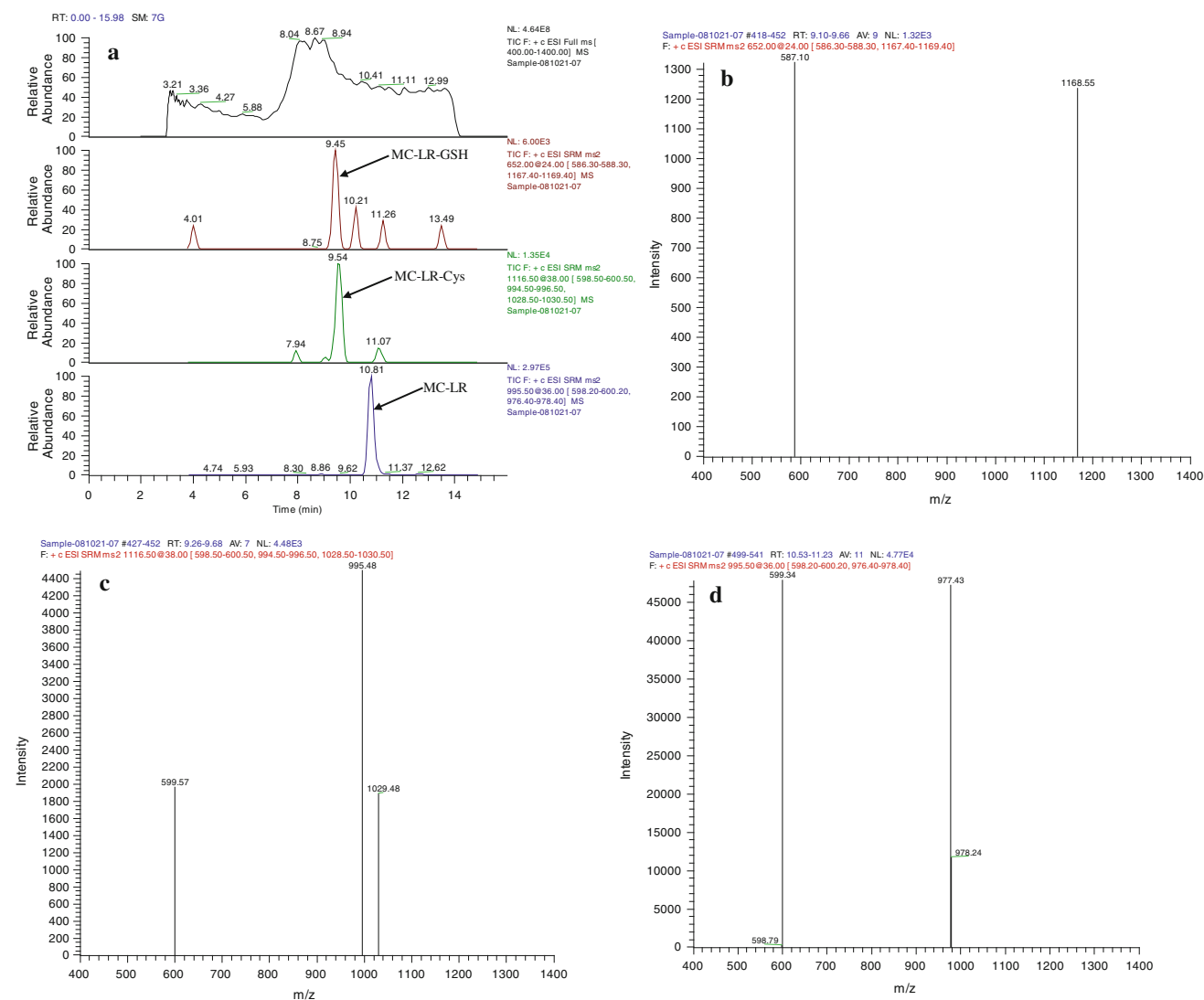
Fig. 1 ESI LC/MS/MS selected reaction monitoring (SRM) chromatograms and product ion mass spectrum for MC-LR and its two metabolites (MC-LR-GSH and MC-LR-Cys) in the liver of bighead carp 3 h after an injection of 500 µg purified MC-LR/kg bw. Shown are: **a** total ion and SRM chromatograms for MC-LR and its two metabolites (MC-LR-GSH and MC-LR-Cys); **b** product ion mass spectrum for MC-LR-GSH; **c** product ion mass spectrum for MC-LR-Cys; **d** product ion mass spectrum for MC-LR

and MC-LR ($r = 0.69$, $p < 0.01$; $n = 12$) or MC-LR-GSH ($r = 0.81$, $p < 0.01$; $n = 12$) concentrations in the liver of bighead carp, while no significant correlation was observed between MC-LR and MC-LR-GSH concentrations in the liver of bighead carp ($r = 0.48$, $p > 0.05$; $n = 12$). The average ratios of MC-LR-Cys to MC-LR, MC-LR-Cys to MC-LR-GSH, and MC-LR-GSH to MC-LR in the liver of bighead carp were 0.11, 6.55, and 0.020, respectively.

Field study

The MC-LR concentrations in seston of Maoli, Huanggai, Dianshan, Bajiao, and Gehu Lake were 2.27, 5.58, 0.24, 2.97, and 1.19 µg/l (Table 1).

The MC-LR, MC-LR-GSH and MC-LR-Cys content in the liver of bighead carp from different lakes were shown in Fig. 3. The highest MC-LR in the liver of bighead carp was found in Bajiao Lake (0.067 µg/g DW), while the maximum of MC-LR-Cys occurred in Huanggai Lake (0.41 µg/g DW). Low concentrations of MC-LR-GSH were observed in the liver of bighead carp from Dianshan (0.0063 µg/g DW), Bajiao (0.0064 µg/g DW), and Gehu Lake (below limit of detection), but no MC-LR-GSH was found in liver of bighead carp from the other two lakes. The average ratios of MC-LR-Cys to MC-LR, MC-LR-Cys to MC-LR-GSH, MC-LR-GSH to MC-LR in liver of bighead carp were 9.89, 71.49, and 0.13, respectively. No significant relationship was found between MC-LR and

Fig. 2 The mean ($n = 3$) liver concentration–time profiles for MC-LR/MC-LR-Cys, MC-LR-GSH after the intraperitoneal injection of a single dose of purified MC-LR (500 µg/kg bw) to bighead carps
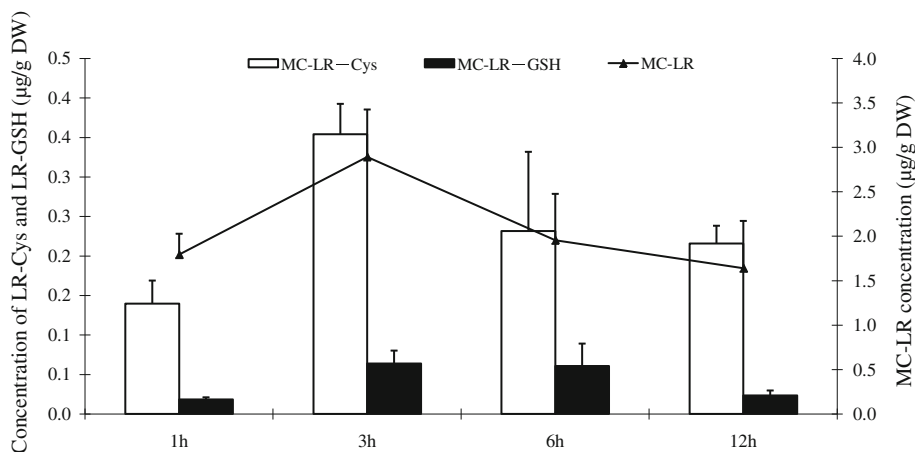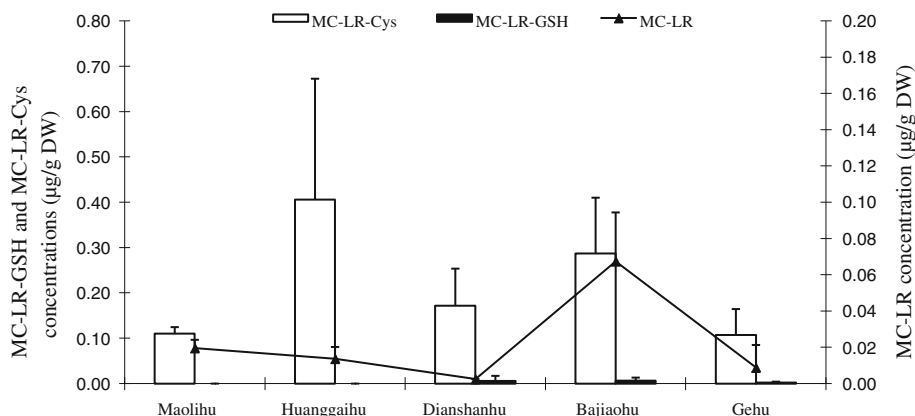

Fig. 3 The concentration of MC-LR and its conjugates (LR-Cys and LR-GSH) in the liver of bighead carp collected from the five eutrophic lakes, China

MC-LR-Cys or MC-LR-GSH concentration in liver of bighead carp ($r = 0.32$–0.40, $p > 0.05$; $n = 15$), while a positive correlation was found between the MC-LR-Cys concentration in the liver of bighead carp and MC-LR content in the seston from the five lakes ($r = 0.846$, $p = 0.085$; $n = 5$).

Recovery experiment

The average recoveries from fish ($n = 4$) liver were 60.5% (ranging from 56.8 to 68.4%), 81.6% (ranging from 79.8 to 84.2%), and 86.2% (ranging from 82.5 to 92.6%) for MC-LR-GSH, MC-LR-Cys, and MC-LR, respectively. The relative standard deviations (RSDs) of MC-GSH, MC-LR-Cys, and MC-LR were 7, 3, and 6%, respectively.

Discussion

There have been several experimental studies on the qualitative analyses of microcystins metabolites in various organisms (Kondo et al. 1996; Pflugmacher et al. 1998; Ito et al. 2002), but only one study on the quantitative determination of MCs metabolites in aquatic animals collected from Taihu Lake, China (Zhang et al. 2009b), and no study on the quantitative determination of MC-LR-Cys in animals under laboratory condition. Here, for the first time, the role of cysteine conjugation in the detoxification of MC-LR was studied quantitatively in bighead carp under both field and laboratory conditions.

GSH is well known to be present in high concentration in most living cells and is able to bind spontaneously with electrophiles. GSH conjugation with xenobiotics has been traditionally viewed as a metabolic pathway leading to detoxification (Chasseaud 1976; Ketterer et al. 1983). As for MCs, previous studies pointed out that the formation of MCs glutathione conjugate mediated by glutathione S-transferase (GST) is the first step to detoxify MCs in a wide range of aquatic organisms on the basis of in vitro experiment (Pflugmacher et al. 1998), and MC-GSH and MC-Cys were detected qualitatively in animals after acute exposure to MCs in laboratory experiments (Kondo et al. 1996; Ito et al. 2002). However, in the present study, low concentrations of MC-LR-GSH were detected in the liver of bighead carp after i.p. injection of 500 µg purified MC-LR/kg bw, while MC-LR-Cys contents in bighead carp were always much higher than MC-LR-GSH concentrations within 12 h post-injection, and the mean ratio

of MC-LR-Cys to MC-LR-GSH was 6.55. In the field condition, much higher MC-LR-Cys contents were detected in the liver of bighead carp collected from 5 eutrophic lakes, while MC-LR-GSH was occasionally detectable, and the average ratio of MC-LR-Cys to MC-LR-GSH in the liver of bighead carp reached as high as 71.49. The above results suggest that MC-LR-Cys might be much more important than MC-LR-GSH in the detoxification of MC-LR in fish liver, and that GSH conjugation with MC-LR might be triggered when fish was exposed to a high amount of MCs (especially in the acute experiment).

Similar results were observed in our recent studies, where the average MC-LR-Cys concentrations in the hepatopancreas of snail and shrimp and liver of silver carp from Lake Taihu were 0.50, 0.97, and 5.72 µg/g DW, respectively, while MC-LR-GSH was usually not detectable in these samples (Zhang et al. 2009b), and after exposure of crucian carp to a single dose of 100 µg MC-LR equivalent/kg bw, MC-LR-GSH concentration in all liver samples was near 0.05 µg/g DW, and the maximum concentration of MC-LR-GSH was just 0.083 µg/g DW (Dai et al. 2008). Furthermore, Kondo et al. (1992) confirmed that thiols of GSH and Cys add unclophilically to the α, β-unsaturated carbonyl of the Mdha moiety in microcystins, and Bagu et al. (1997) found that the inhibition of protein phosphatases 1 and 2A for MCs is through binding covalently with the Cys-273 of the PP-1 and 2A. According to the above results, we speculate a different detoxification pathway in aquatic animals: MC-LR in liver firstly conjugates with polypeptide or protein (mainly PP-1 and 2A) containing Cys residues, subsequently, MC-LR-Cys is degraded from these polypeptide or protein, and finally is excreted from animals in the form of MC-LR-Cys.

It is reasonable to think that if MC-LR conjugates with GSH and degrades to MC-LR-Cys, GSH level in aquatic organisms should be decreased after MC-LR exposure. However, the responses of GSH levels to MCs in fish and other aquatic organisms have been quite variable so far. No changes of GSH levels were observed in silver carp (Li et al. 2007) and bighead carp (Li et al. 2010) after i.p. injection of extracted MCs, in juveniles of the gold fish *Carassius auratus* injected with purified MC-LR at a dose of 125 µg/kg bw during an experiment of 96 h (Malbrouck et al. 2004), in tenca (*Tinca tinca*) after acute oral exposure (Atencio et al. 2008), and in silver carp exposed to toxic cyanobacterial blooms in Lake Taihu (Qiu et al. 2007). Furthermore, decreased GSH levels were found in the liver of goldfish following an i.p. injection of MC-LR (Xu et al. 1998), in hepatocytes of common carp exposed to 10 mg MC-LR/L (Li et al. 2003), while a significant increase in GSH level was recorded in the liver of silver carp (*Hypophthalmichthys molitrix*) exposed to a natural population of cyanobacterial bloom for 25 days (Blaha et al. 2004).

Our results together with these different responses of GSH levels doubted the importance of GSH in detoxification of MC-LR in aquatic animals. More detailed studies are needed in our future studies.

Bighead carp are phytoplanktivorous fishes that can be able to suppress and graze out toxic blooms in non-traditional biomanipulation, and have been used to control cyanobacteria blooms in eutrophic waters (Xie and Liu 2001). In the present study, there was a positive correlation between MC-LR-Cys concentration in the liver of bighead carp and MC-LR content in seston from the five lakes ($r = 0.85$), although statistically not significant ($p = 0.085$), suggesting that bighead carp can metabolize MCs efficiently. This might be a physiological mechanism for bighead carp to counteract toxic cyanobacteria.

Conclusions

Both field and laboratory experiments indicate that MC-LR-Cys might be much more important than MC-LR-GSH in the detoxification of MC-LR in fish liver, and that GSH conjugation with MC-LR might be triggered when fish was exposed to a high amount of MCs (especially in the acute experiment). We speculate a different detoxification pathway in aquatic animals: MC-LR in liver firstly conjugates with polypeptides or proteins (mainly PP-1 and 2A) containing Cys residues, subsequently, MC-LR-Cys is degraded from these polypeptide or protein, and finally is excreted from animals in the form of MC-LR-Cys. This might be a physiological mechanism for the phytoplanktivorous bighead carp to counteract toxic cyanobacteria.

References

Atencio L, Moreno I, Jos A, Pichardo S, Moyano R, Blanco A, Cameán AM (2008) Dose-dependent antioxidant responses and pathological changes in tenca (*Tinca tinca*) after acute oral exposure to *Microcystis* under laboratory conditions. Toxicon 52:1–12

Azevedo SMFO, Carmichael WW, Jochimsen EM, Rinehart KL, Lau S, Shaw GR, Eaglesham GK (2002) Human intoxication by microcystins during renal dialysis treatment in Caruaru-Brazil. Toxicology 181–182:441–446

Bagu JR, Sykes BD, Craig MM, Holmes CFB (1997) A molecular basis for different interactions of marine toxins with protein phosphatase-1, molecular models for bound motuporin,

microcystins, okadaic acid and calyculin A. J Biol Chem 272:5087–5097

Blaha L, Kopp R, Simkova K, Mares J (2004) Oxidative stress biomarkers are modulated in silver carp (*Hypophthalmichthys molitrix* Val) exposed to microcystin-producing cyanobacterial water bloom. Acta Vet Brno 73:477–482

Carmichael WW (2001) Health effects of toxin-producing cyanobacteria: "the CyanoHABs". Hum Ecol Risk Assess 7:1393–1407

Carmichael WW, Beasly V, Bunner DL, Eloff JN, Falconer I, Gorham P, Harada KI, Krishnamurty T, Min-Juan Y, Moore RE, Rinehart K, Runnegar M, Skulberg OM, Watanabe M (1988) Naming of cyclic heptapeptide toxins of cyanobacteria (blue-green algae). Toxicon 26:971–973

Chasseaud LF (1976) Conjugation with glutathione and mercapturic acid excretion. In: Arias IM, Jakoby WB (eds) Glutathione: metabolism and function. Raven Press, New York, pp 77–114

Chen J, Xie P, Li L, Xu J (2009) First identification of the hepatotoxic microcystins in the serum of a chronically exposed human population together with indication of hepatocellular damage. Toxicol Sci 108:81–89

Dai M, Xie P, Liang GD, Chen J, Lei HH (2008) Simultaneous determination of microcystin-LR and its glutathione conjugate in fish tissues by liquid chromatography-tandem mass spectrometry. J Chromaogr B 862:43–50

Dawson RM (1998) The toxicology of microcystins. Toxicon 36:953–962

Dittmann E, Wiegand C (2006) Cyanobacterial toxins—occurrence, biosynthesis and impact on human affairs. Mol Nutr Food Res 50:7–17

Eriksson JE, Toivola D, Meriluoto JAO, Karaki H, Han Y, Hartshorne D (1990) Hepatocyte deformation induced by cyanobacterial toxins reflects inhibition of protein phosphatase. Biochem Biophys Res Commun 173:1347–1353

Falconer IR, Yeung DSK (1992) Cytoskeletal changes in hepatocytes induced by microcystis toxins and their relation to hyperphosphorylation of cells proteins. Chem Biol Interact 81:181–196

Falconer I, Bartram J, Chorus I, Kuiper-Goodman T, Utkilen H, Burch M, Codd GA (1999) Safe levels and safe practices. In: Chorus I, Bartram J (eds) Toxic cyanobacteria in water—a guide to their public health consequences, monitoring and management. E & FN Spon, London, pp 155–178

Gupta N, Pant SC, Vijayarghavan R, Lakshmana Rao PV (2003) Comparative toxicity evaluation of cyanobacterial cyclic peptide toxin microcystin variants (LR, RR, YR) in mice. Toxicology 188:285–296

Hermansky SJ, Stohs SJ, Eldeen ZM, Roche VF, Mereish KA (1991) Evaluation of potential chemoprotectants against microcystin-LR hepatotoxicity in mice. J Appl Toxicol 11:65–74

Ito E, Takai A, Kondo F, Masui H, Imanishi S, Harada K (2002) Comparison of protein phosphatase inhibitory activity and apparent toxicity of microcystins and related compounds. Toxicon 40:1017–1025

Jochimsen EM, Carmichael WW, An JS, Cardo DM, Cookson ST, Holmes CE, de Antunes MB, de Melo Filho DA, Lyra TM, Barreto VST, Azevedo SFMO, Jarvis WR (1998) Liver failure and death after exposure to microcystins at a haemodialysis center in Brazil. New Engl J Med 338:873–878

Ketterer B, Coles B, Meyer DJ (1983) The role of glutathione in detoxification. Environ Health Persp 49:59–69

Kondo F, Ikai Y, Oka H, Okumura M, Ishikawa N, Harada KI, Matsuura K, Murata H, Suzuki M (1992) Formation, characterization and toxicity of the glutathione and cysteine conjugates of toxic heptapeptide microcystins. Chem Res Toxicol 5:591–596

Kondo F, Matsumoto H, Yamada S, Ishikawa N, Ito E, Nagata S, Ueno Y, Suzuki M, Harada K (1996) Detection and identification of metabolites of microcystins formed in vivo in mouse and rat livers. Chem Res Toxicol 9:1355–1359

Kuiper-Goodman T, Falconer I, Fitzgerald J (1999) Human health aspects. In: Chorus I, Bartram J (eds) Toxic cyanobacteria in water—a guide to public health consequences, monitoring and management. E&FN Spon, London, pp 113–153

Li XY, Liu YD, Song LR, Liu J (2003) Responses of antioxidant systems in the hepatocytes of common carp (*Cyprinus carpio* L.) to the toxicity of microcystin-LR. Toxicon 42:85–89

Li L, Xie P, Li SX, Qiu T, Guo LG (2007) Sequential ultrastructural and biochemical changes induced in vivo by the hepatotoxic microcystins in liver of the phytoplanktivorous silver carp *Hypophthalmichthys molitrix*. Comp Biochem Physiol C Toxicol Pharmacol 146:357–367

Li L, Xie P, Guo LG (2010) Antioxidant response in liver of the phytoplanktivorous bighead carp (Aristichthys nobilis) intraperitoneally-injected with extracted microcystins. Fish Physiol Biochem 36:165–172

Magalhães VF, Marinho MM, Domingos P, Oliveira AC, Costa SM, Azevedo LO, Azevedo S (2003) Microcystins (cyanobacteria hepatotoxins) bioaccumulation in fish and crustaceans from Sepetiba Bay. Toxicon 42:289–295

Malbrouck C, Kestemont P (2006) Effects of microcystins on fish. Environ Toxicol Chem 25:72–86

Malbrouck C, Trausch G, Devos P, Kestemont P (2004) Effect of microcystin-LR on protein phosphatase activity in fed and fasted juvenile goldfish Carassius auratus L. Toxicon 43:295–301

Nishiwaki-Matsushima R, Ohta T, Nishiwaki S, Suganuma M, Kohyama K, Ishiwaka T, Carmichael WW, Fujiki H (1992) Liver tumour promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J Cancer Res Clin Oncol 118:420–424

Park HD, Lwami C (1998) Temperal variabilities of the concentrations of intra- and extracellular microcystin and toxic Microcystis species in a hypereutrophic lake, Lake Suwa, Japan (1991–1994). Environ Toxicol Water Qual 13:61–72

Pflugmacher S, Wiegand C, Oberemm A, Beattie KA, Krause E, Codd GA, Steinberg CEW (1998) Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. Biochim Biophys Acta 1425:527–533

Qiu T, Xie P, Ke ZX, Li L (2007) In situ studies on physiological and biochemical responses of four fishes with different trophic levels to toxic cyanobacterial blooms in a large Chinese lake. Toxicon 50:365–376

Song LR, Chen W, Peng L, Wan N, Gan NQ, Zhang XM (2007) Distribution and bioaccumulation of microcystins in water columns: a systematic investigation into the environmental fate and the risks associated with microcystins in Meiliang Bay, Lake Taihu. Water Res 41:2853–2864

Svrcek C, Smith DW (2004) Cyanobacteria toxins and the current state of knowledge on water treatment options: a review. J Environ Eng Sci 3:155–185

Wang SM, Dou HS (1998) Lake in China. Science Press, Beijing, pp 179–299

Xie P, Liu JK (2001) Practical success of biomanipulation using filter-feeding fish to control cyanobacteria blooms: a synthesis of decades of research and application in a subtropical hypereutrophic lake. Sci World J 1:337–356

Xu LH, Chen GS, Chen JP, Xu JM, Zhang YY (1998) Toxic effects of microcystin on fish liver. Acta Hydrobiol Sin 22:378–379 (in Chinese)

Yu SZ (1995) Primary prevention of hepatocellular carcinoma. J Gastroenterol Hepatol 10:674–682

Zhang DW, Xie P, Liu YQ, Qiu T (2009a) Transfer, distribution and bioaccumulation of microcystins in the aquatic food web in Lake

Taihu, China, with potential risks to human health. Sci Total Environ 407:2191–2199

Zhang DW, Xie P, Chen J, Dai M, Qiu T, Liu YQ (2009b) Determination of microcystin-LR and its metabolites in snails (*Bellamya aeruginosa*), shrimps (*Macrobrachium nipponensis*) and silver carp (*Hypophthalmichthys molitrix*) from Lake Taihu, China. Chemosphere 76:974–981