The effect of cyanobacterial crude extract on the transcription of GST mu, GST kappa and GST rho in different organs of goldfish (*Carassius auratus*)

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ABSTRACT

The glutathione S-transferases play important roles in the detoxification of microcystin. Core-sequences of three classes of GST (mu, kappa and rho) were cloned from goldfish (*Carassius auratus* L.) i.p. injected with cyanobacterial crude extract at two doses (50 and 200 μg MC-LReq kg^{-1} BW). The relative changes of the mRNA abundance in liver, kidney and intestine were analyzed by real-time PCR. The transcription of GST mu was inhibited in intestine at both doses and the transcription of GST kappa was inhibited from 12 to 48 h in kidney at both doses. The decreased transcription of GST rho was detected in all three organs at the high dose. It is suggested that transcription inhibition of GST rho might be significant in MCs toxicity at higher toxin concentration in omnivorous freshwater fish. Alteration in transcription of GSTs stimulated by MCs implicates an increased health risk to fish.

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1. Introduction

With significant increase of cyanobacterial blooms in eutrophic water bodies worldwide during the past decades, the cyanotoxins have become a great threat to the health of human and aquatic animals (Hallegeff, 1993). Microcystins (MCs), primarily produced by *Microcystis aeruginosa* (Dawson, 1998) are the most widespread cyanotoxins (WHO, 2003), and involved in a variety of mammalian, avian and teleost poisonings. There are over 80 structural analogues of microcystins, among which, microcystin-LR (MC-LR), microcystin-RR (MC-RR) and microcystin-YR (MC-YR) are three most common and extensively studied forms (Fastner et al., 2002).

The toxic effects of MCs on fish and mammals have been investigated in a number of studies including histological, biochemical, behavioral and physiological examination (Fischer et al., 2000; Malbrouck et al., 2003; Baganz et al., 2004; Malbrouck and Kestemont, 2006). The presence and bioaccumulation of MCs in different fish tissues have been reported from both experimental and field studies. There have been several reports on bioaccumulation of MCs in liver (Malbrouck et al., 2003; Mohamed et al., 2003; Chen et al., 2006), intestine (Mohamed et al., 2003; Xie et al., 2004, 2005) and kidney (Mohamed et al., 2003). When MCs are absorbed and distributed into different organs, it might be biotransformed, products of which can be transported via the gallbladder to the intestine

(Tencalla and Dietrich, 1997; Bury et al., 1998), or via the blood to the kidneys for excretion (Cazenave et al., 2006).

MCs inhibits the serine/threonine protein phosphatases type 1 and type 2A (PP1 and PP2A) specifically (Honkanen et al., 1990), due primarily to toxin uptaken by the bile acid carrier, which is found in liver and, to a lesser extend, in intestinal epithelia (Landsberg, 2002) and promotes skin and liver tumour in laboratory animals (Nishiwaki-Matsushima et al., 1992).

Glutathione-S-transferases (GSTs; EC 2.5.1.18), also known as glutathione transferases, belong to phase II detoxification enzyme family and are a family of dimeric multifunctional enzymes. The functions of GSTs include catalysis of tripeptide glutathione (GSH) conjugation with diverse electrophilic substrates (Jakoby, 1978; Armstrong, 1997) including MC-LR (Pflugmacher et al., 1998), metabolism of toxic substances (Sheehan et al., 2001), and protection of cells from oxidative damage (Mannervik, 1985). Previous studies showed the existence of a MC-LR-glutathione conjugate formed enzymatically via soluble GST in various aquatic organisms ranging from plants (*Ceratophyllum demersum*), invertebrates (*Dreissena polymorpha* and *Daphnia magna*) up to fish eggs and fish (*Danio rerio*) (Pflugmacher et al., 1998). This conjugation appears to be the first step in the detoxification of cyanobacterial toxin.

GSTs have been documented in virtually every living species examined, including animals, plants and bacteria, since their first discovery in the early 1970s (Eaton and Bammler, 1999; Sheehan et al., 2001; Pearson, 2005). In mammals, three major families of glutathione transferase have been identified: the cytosolic GSTs (including seven classes, namely alpha, mu, pi, theta, sigma, omega,

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and zeta), the mitochondrial GST (kappa class) and microsomal GSTs (Hayes et al., 2005).

Studies using rodent models confirmed an increased level of GST gene transcription in rat and mice after MC-LR exposure, suggesting microcystins induced a de novo synthesis of GST protein (Bulera et al., 2001; Gehringer et al., 2004). Some classes of GSTs were found in several fish species (Leaver et al., 1997; Melgar Riol et al., 2001; Doi et al., 2004; Lee et al., 2005; Konishi et al., 2005b; Fan et al., 2007; Trute et al., 2007). A distinct GST isoform, which has no homologues in mammals, was found in fish and named as rho class in red sea bream (*Pagrus major*) (Konishi et al., 2005a). Recently, nine GST genes (alpha, rho, mu, theta, pi, kappa, mGST1, mGST2 and mGST3) and three GST genes (alpha, theta and pi) were cloned from common carp (*Cyprinus carpio*) (Fu and Xie, 2006) and goldfish (*Carassius auratus*) (Li et al., 2008), respectively, at the same time the relative changes after stimulation with microcystins were analyzed. However, information on relationships between fish GST expression and microcystins exposure is still limited.

The present study was undertaken to find the relationship between the GST isoenzymes and MCs stimulation, based on alteration of gene transcription in three organs (liver, kidney and intestine) of goldfish i.p. injected of cyanobacterial crude extract at two doses (50 and 200 μg kg^{-1} BW MC-LReq) by using real-time PCR. Three GST isoforms were chosen for investigation, among which, the mu class cytosolic GST was seldom studied, GST kappa belongs to mitochondrial class, and GST rho was found only in fish. Furthermore, the alterations of the transcription levels also allow assessment of whether these three GSTs perform roles distinct from other soluble transferases. Unlike GST activity analysis, which might be influenced by microcystins as a competitive substrate for the standard substrate of GST activity assay, GST mRNA expression analysis using real-time PCR would be a more reliable way to determine the expression alteration of GSTs in fish.

2. Materials and methods

2.1. Materials

Healthy goldfish (mean body weight 265 ± 22.6 g) were purchased from a local fish hatchery in Wuhan City, China, and were transported to the laboratory in College of Fishery, Huazhong Agricultural University. After being acclimated for 14 days in 150 l aquarium (95 cm L × 55 cm W × 40 cm H) containing dechlorinated tap water in temperature-controlled room with 12L:12D photoperiod, the experiment was conducted with a water temperature of 25 ± 1 °C and a dissolved oxygen concentration between 6.0 and 7.1 mg l^{-1} by continuously aerating. Feeding was terminated 48 h before initiation of the experiment, and no food was supplied to fish throughout the experiment.

2.2. Toxin

The cyanobacterial material (mainly composed of *Microcystis* spp. *Microcystis aeruginosa*) used in the experiment was collected from Lake Dianchi, Yunnan Province of China. Prior to use, the material was analyzed for toxin content via reverse-phase high performance lipid chromatography (HPLC, LC-20A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan) according to the method of Fastner et al. (1998). The MC content was 1.41 mg g^{-1} dry weight (DW), with MC-LR, -RR, and -YR being 0.50, 0.84, and 0.07 mg g^{-1} DW, respectively. Crude algae were extracted with methanol, and finally suspended in distilled water.

2.3. Experimental protocol

The doses of approximately 1.0 ml suspension of extracted solution of microcystins in physiological saline solution, amounting to 50 and 200 μg kg^{-1} MC-LReq body weight (BW), respectively, were given i.p. under ventral fin into the peritoneum by syringe. Thus, the low dose group ($n = 60$) and high group ($n = 60$), 50 and 200 μg kg^{-1} BW, respectively, were designed in the experiment. Fish ($n = 60$) injected i.p. with physiological saline solution served as control. The 60 fish in each dose group and the control were respectively divided equally into 12 aquariums, where water was continuously aerated but not circulated.

No mortality of fish in the low dose group was observed during a period of 7 days. However, fish in the high dose group only had a mean survival time of 50 ± 5 h. Three fish were collected for total RNA isolation samples at 1, 3, 12, 24, 48 h and 7 d (low dose groups) post-injection in each group. Each fish was killed and the livers, kidneys and intestines were quickly removed, minced, and stored frozen at liquid nitrogen until RNA isolation.

2.4. Total RNA isolation

Total RNA was isolated from 30 to 50 mg sections of goldfish liver, kidney and intestine using Trizol reagent (Invitrogen) and quantified by determination at OD$_{260}$. RNA was extracted as manufacturer's protocol, resuspended in 50 μl RNase-free water, and stored at -80 °C. Quantification was done using Eppendorf Biophotometer (Hamburg, Germany).

2.5. Cloning of partial cDNA sequences of goldfish sGST

Reverse transcription was performed with oligo(dT) primer using First Strand cDNA Synthesis Kit (toyobo, Japan). PCR was performed using primer GSTM-F/GSTM-R, GSTK-F/GSTK-R and GSTR-F/GSTR-R, with Taq polymerase (TaKaRa, Japan). PCR products of the expected length (512, 552 and 482 bp for GSTM-F/GSTM-R, GSTK-F/GSTK-R and GSTR-F/GSTR-R, respectively) were purified from 1.5% agarose gel, and an aliquot of the PCR mixture was used for subcloning of GST mu, GST kappa and GST rho cDNA fragment into the pGEM-T Easy vector (Promega). The ligation mixture was used to transform *E. coli* JM109 using heat shock transformation. Positive transformants were selected, and inserts were sequenced. Primers used in the PCR are listed in Table 1.

Table 1
Primer sequences in this study

Name of primer	Sequence of primer
GSTM-F	5′-TGGGATATACGCGGGCTTGC-3′
GSTM-R	5′-TCGTAGTCATCCAGGCACTC-3′
GSTK-F	5′-GTTTCTCCGTATTCCTGGATGGC-3′
GSTK-R	5′-CTCAGCCTTCCCGTTCACATG-3′
GSTR-F	5′-TAAGCGGTCATGGCGCAG-3′
GSTR-R	5′-AGCCTTTACCCATCTTCTCC-3′
muRT-F	5′-TGCCTTACTTAGAGGATGGAGACACG-3′
muRT-R	5′-AGCAAACCACTTCCTGTCACCAAGG 3′
kappaRT-F	5′-TCAATGAGGGAGGCAGGCTGGGTA-3′
kappaRT-R	5′-GGCAATCAGCCTCCTGGAATCGTCC-3′
rhoRT-F	5′-GCATGGAGACATCGTCGTGAACG-3′
rhoRT-R	5′-CCATCTTCTCCAAGTATCCATCCC-3′
GAPDH-F	5′-GCCAGTCAGAACATTATCCCAGCCT-3′
GAPDH-R	5′-GGTCCTCAGTGTATCCCAGAATGCC-3′

2.6. Real-time fluorescent quantitative PCR to determine the levels of gene expression

SYBR Green qPCR kit (Finnzymes) was used as the fluorescent dye for real-time quantitative PCR on a Chromo4 96-well reactor with optical caps (MJ Research, Cambridge, MA). The housekeeping gene GAPDH was analyzed in samples, and the level of it was stable in the present experiments. Therefore, GAPDH was used as the endogenous assay control. Primes used muRT-F/muRT-R, kappaRT-F/kappaRT-R, rhoRT-F/rhoRT-R and GAPDH-F/GAPDH-R for GST mu, GST kappa, GST rho and GAPDH, respectively, and length of the real-time PCR products are 266, 275, 281 and 235 bp for GST mu, GST kappa, GST rho and GAPDH, respectively. The specification of each pair of primers was confirmed by randomly sequencing six clones, and further confirmed by the melting curve analysis using real-time PCR. The amplification efficiency of each pair of primers was tested by constructing corresponding plasmid and only primers with similar amplification efficiency were used in this experiment. The reactions were performed in a 20 μl volume mix containing 10 μl SYBR Green I mixture (Finnzymes), 1 μl primers, 1 μl cDNA and 7 μl DEPC-treated water. The thermal cycling program was: 3 min denaturation at 94 °C; for amplification and quantification 45 cycles of 30 s at 94 °C, 30 s at 68–78 °C (the annealing temperature varied depending on the gene specific primers), 30 s at 72 °C, 1 s at 76–81 °C (temperature varied depending on the gene specific primers, plate read for targeted gene), 1 s at 78 °C (plate read for GAPDH), a 10 min extension at 72 °C, and then a melt curve from 70 to 90 °C with a heating rate of 0.3 °C s^{-1}. Real-time PCR reactions were performed in triplicate for each cDNA sample, and melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected data were analyzed with the Option Monitor software 2.03 version (MJ Research, Cambridge, MA). The relative expression levels (fold induction) of the genes, calculated using the relative expression software tool (MJ Research, Cambridge, MA), were based on mean threshold cycle differences between the sample and the control group (Pfaffl et al., 2002). All the primers used in the real-time PCR are listed in Table 1.

2.7. Statistical analysis

All values are expressed as mean ± standard deviation for each treatment group. Statistical analysis of differences between treatment groups and control groups was done using The SPSS (Chicago, IL, USA) for Windows (Ver 13.0) by one-way analysis of variance (ANOVA) and the post hoc test. Differences were considered significant if $P \leq 0.05$.

3. Result

3.1. Multiple alignment and phylogenetic tree (Fig. 1)

The corenucleotide of GST isoforms of mu, kappa and rho were cloned (The Genbank accession numbers of these sequences were EU527004, EU527004 and EU527006). These three classes of GST isoforms have not been reported in goldfish before. In order to investigate the phylogenetic relationship of goldfish GST mu, kappa and rho with different classes of GST enzymes from other vertebrates, a phylogenetic tree was constructed with deduced amino acid sequences (Fig. 1). Sequences of three goldfish GSTs have the highest similarity with those from common carp. The GenBank accession numbers of sequences used were listed in Table 2.

3.2. GST mu (Fig. 2)

In liver, the transcription of GST mu isoform increased significantly in the first 12 h, and then decreased till the 7th day at the low dose. While at the high dose it was depressed in the first 3 h, and significantly increased at 12 and 24 h. The transcriptions of mu class of GST at both doses in kidney were depressed from 1 to 48 h, except for a significant induction at 3 h in the low dose group. In intestine, the transcription level of mu class GST was decreased at the low dose, among which, the depressed transcription at 1, 12, and 48 h was significant relative to control. At the high dose in intestine, the transcription of mu class GST decreased in the first 12 h, then increased at 24 and 48 h. In the low dose groups, the transcription of mu class GST recovered on the 7th day in all three organs.

3.3. GST kappa (Fig. 2)

Decreased transcription of GST kappa isoform was detected at the first 12 h post-injection, while increased transcription was detected from 24 to 48 h in liver at both doses. The transcript of GST kappa was at a relatively high level in kidney at 1 h and then suppressed till 48 h except the puniness rebound at 24 h in the high dose groups. In the low dose groups in kidney, an increased transcription was detected at the first 3 h and then suppressed. In intestine, a significantly induced transcription was found at 1 and 24 h in the low dose groups and 12 h in the high dose groups. While significant suppression was found at 12 h in the low dose groups and 24 h in high dose groups. The transcription level of kappa class of GST recovered on the 7th day in all three organs at the low dose.

3.4. GST rho (Fig. 2)

The transcription of GST rho was inhibited in all three organs at both doses except for the first 3 h in liver at the low dose, and recovered on the 7th day at the low dose. In liver, the GST rho expression level was the highest among the three organs, with a 1.5-fold increase at 1 h relative to control, and then decreased to 48 h at the low dose. At the high dose in liver, the transcription of GST rho was inhibited with the lowest level (about 0.3-fold relative to control) at 1 h. In kidney, the transcriptional level of GST rho was inhibited in the first 2 days after administration, with significant depression in the first 12 h at the low dose and from 3 to 48 h at the high dose. In intestine, the transcription of GST rho was lower than control at both doses and at 12 h the transcription reached the lowest level in the low dose groups.

4. Discussion

Among the eight classes of soluble GST in mammals, class mu and kappa enzymes are less studied, and another soluble cytosolic GST rho which only present in fish is much less investigated. Actually, almost nothing is known about the transcription alteration responding to MCs stimulating in the omnivorous freshwater fish. Because class kappa enzymes are only distantly related to the other soluble GST families in mammalian (Ladner et al., 2004), and class rho GST have no homologue molecules in mammals, it is likely that these classes GST perform unique biological functions.

In the present study, some fish of the high-dose group died with soft and pale livers and kidney when we dissected the carcases (Zhang et al., 2007) and the highest concentration of MCs were found in the liver (Lei et al., 2008). Meanwhile, GST rho, which plays important roles in reducing the harmful effects of xenobiotic exposure (Martinez-Lara et al., 2002), was the only gene whose transcription was inhibited in all three organs at the high dose. It revealed a unanimous depressive effect on the transcription of GST
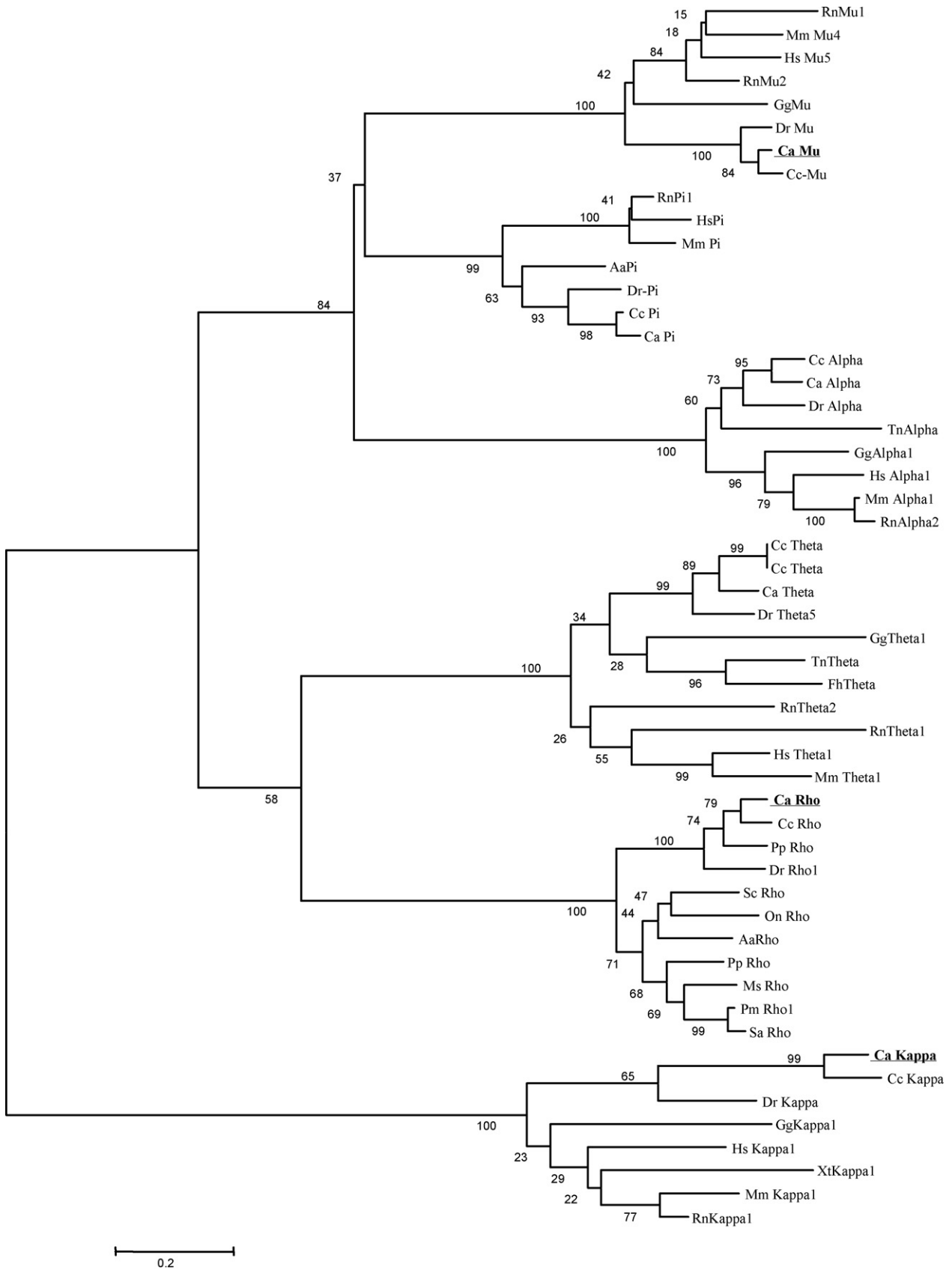
Fig. 1. Phylogenetic tree of piscine GSTs and their homologue molecules from mammals. Multiple sequence alignments were performed using the CLUSTALX program. The phylogenetic tree based on the results of alignments was obtained by using the Mega 3.1 program. A bootstrap analysis was performed using 1000 replicates to test the relative support for particular clades. The GenBank accession numbers of sequences used were listed in Table 2.

Table 2
GST sequences used for phylogenetic tree construction

Species	Protein	Accession number	Species	Protein	Accession number
Homo sapiens	Hs Alpha1	AB169530	*Danio rerio*	Dr Alpha	AAH60914
	Hs Kappa1	NM_015917		Dr Pi	AAH83467
	Hs Mu5	CAI13812		Dr Theta	NP_956878
	Hs Pi	AAV38753		Dr Mu	BC057526
	Hs Theta1	NP_000844		Dr kappa	XM_001346502
Mus musculus	Mm Alpha1	AAH61134		Dr rho1	NM_001045060
	Mm Kappa1	NM_029555	*Cyprinus carpio*	Cc Alpha	DQ411310
	Mm Mu4	BC030444		Cc Kappa	DQ411311
	Mm Pi	AAH61109		Cc Mu	DQ411312
	Mm Theta1	AAH12254		Cc Pi	DQ411313
Rattus norvegicus	Rn Alpha2	NM_017013		Cc Rho	DQ411314
	Rn kappa1	NM_181371		Cc Theta	DQ411315
	Rn mu1	NM_017014	*Carassius auratus*	Ca Alpha	EU182590
	Rn mu2	NM_177426		Ca Pi	EU183358
	Rn pi1	NM_012577		Ca Theta	EU183359
	Rn Theta1	BC097423	*Tetraodon nigroviridis*	Tn Alpha	CAG09409
	Rn Theta2	NM_012796		Tn Theta	CAG09655
Gallus gallus	Gg Alpha	NM_001001777	*Xenopus tropicalis*	Xt kappa1	BC087819
	Gg kappa1	XM_416525	*Fundulus heteroclitus*	Fh Theta	AY725219
	Gg Mu	X58248	*Pagrus major*	Pm rho	AB158412
	Gg theta1	NM_205365	*Micropterus salmoides*	Ms rho	AY335905
Siganus canaliculatus	Sc Rho	ABV24477	*Pleuronectes platessa*	Pp Rho	X63761
Anguilla anguilla	Aa Rho	ABY47336	*Oreochromis niloticus*	On Rho	ABV24480

rho in the high dose groups. In the study of Fu and Xie (2006), the transcription of GST rho was also inhibited from 12 to 72 h in common carp. Therefore, it was likely that transcription inhibition of GST rho could be significant in MCs toxicity at higher toxin concentration in omnivorous freshwater fish.

GST catalyzes the formation of microcystin-glutathione conjugate, which is the first and key step in the detoxification process (Pflugmacher et al., 1998; Takenaka, 2001; Beattie et al., 2003). Many studies examined enzyme activities of GSTs after exposure to MCs in various organisms. Elevated GST activity is observed in aquatic organisms (Pietsch et al., 2001) and mice (Gehringer et al., 2004), and decreased activity is found in juvenile goldfish *C. auratus* (Malbrouck et al., 2003). It is also reported that only minor activation of the soluble GST was found in zebrafish (Wiegand et al., 1999) and even no change of GST activity was recorded during 6 h exposure to MC-LR (Li et al., 2003). The GSTs are homodimers or heterodimers (for example, alpha and mu classes can form heterodimers). However, due to broad and often overlapping sub-
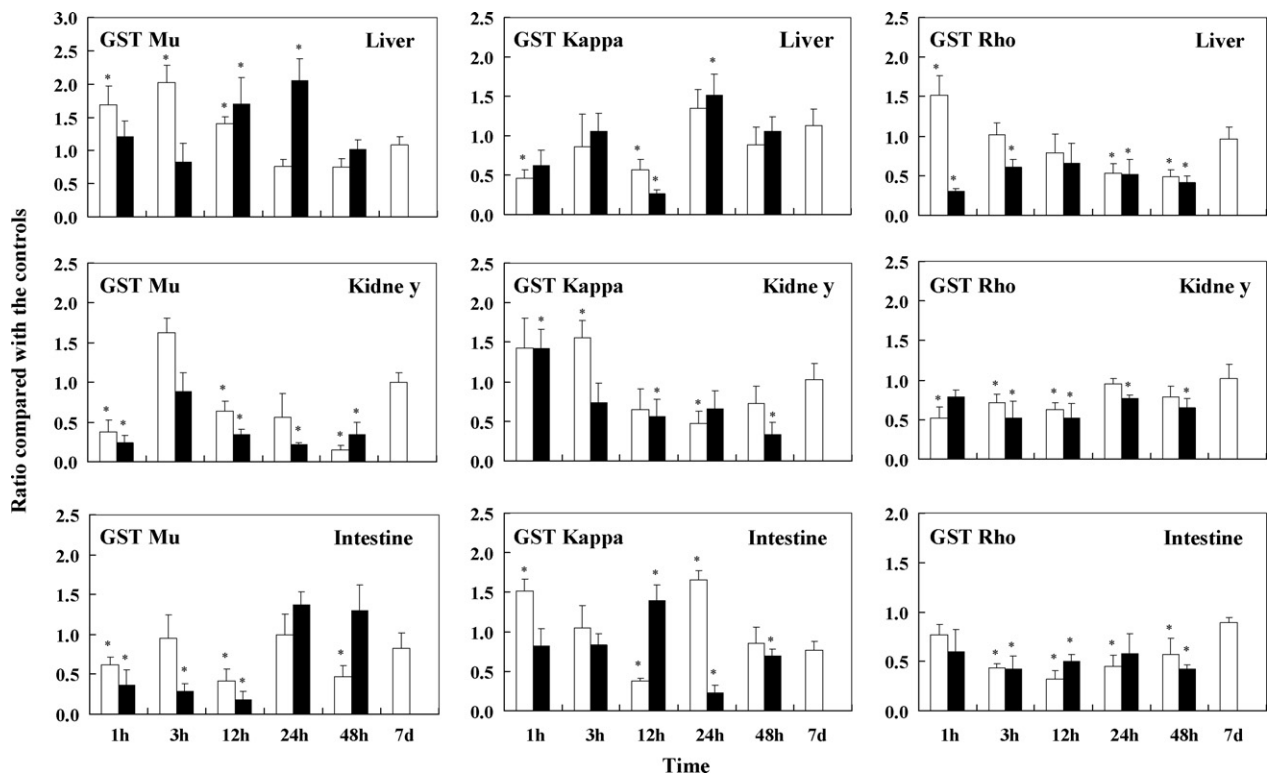

Fig. 2. The temporal changes of GSTs transcripts after microcystin stimulation compared with controls. GSTs control transcription remained stable throughout the time and showed negligible inter-individual variations during our experiment. Black columns represent high dose group and white columns represent low dose group. (* indicates significant change at $P < 0.05$).

strates, it is difficult to definite a distinction between GST classes by specific substrates (Sheehan et al., 2001). In this experiment, real-time PCR was used to detect the expression of three GST isoforms, which would be more reliable than the activity analysis.

In the present study, transcription of mu class of GST was inhibited in the first 12 h after treatment in intestine at both doses, while the expression kappa class of GST was inhibited from 12 to 48 h in kidney at both doses. The present results are not consistent to that of early studies. The transcription of mu, theta and mGST2 class of GST was relatively stable, and it of kappa pi and mGST1 was similar to the control at 6 h in common carp liver treated with MC-LR (Fu and Xie, 2006). Increased liver transcription of rho class GST was detected in silver carp and Nile tilapia at 8 h post-injection of MC-LR, while no increase was detected in grass carp either at 8 h or 24 h post-injection (Liang et al., 2007). Such diverse results may be due to difference in test animals, toxin level, exposure route and sampling interval. In our study, cyanobacterial crude extract was used, which is more close to the toxin in natural water. Some compounds like lipopolysaccharide (LPS) might also affect the activity or transcription of GSTs in some way (Pietsch et al., 2001; Best et al., 2002; Wang et al., 2006).

In this study, the transcriptional level of the three GST isoforms varied in different ways within an organ and among organs, and there are two possible explanations for this. First, GST isoforms were tissue-specific and variably expressed in different organs; at the same time in one organ the abundance of different GST isoforms was also variable. GST kappa is localized within the mitochondria (Strange et al., 2001); though a human GST has been found in peroxisomes (Morel et al., 2004). Western blotting analysis of mouse tissue homogenates demonstrated that mGSTK1 is expressed at relatively high levels in liver. Moderate expression was observed in kidney, sparse or essentially no mGSTK1 protein was detected in small intestine (Thomson et al., 2004). GST rho is the predominant one in red sea bream liver (Konishi et al., 2005a, b). In common carp liver the copy number of GST rho was only less than GST-alpha in nine classes investigated (Fu and Xie, 2006). The abundance of GSTs protein or the copy numbers in different organs before administration could influence the balance of the transcription. Second, the concentration of toxin varied in different organs in the process of the study. Many studies have identified the presence of MCs in liver, kidney and intestine (Mohamed et al., 2003; Xie et al., 2004, 2005) with variation of toxin concentration in a time-dependent mode. A decrease tendency in microcystin concentration was observed in liver, while an increase tendency was observed in kidney and intestine at the dose of 200 μg MC-LReq kg^{-1} BW (Lei et al., 2008). The variation in microcystin concentration could be a factor influencing the transcription of GST isoforms.

In this study, the expression of different GST isoforms (mu, kappa and rho) was stimulated by cyanobacterial crude extract at both doses. The toxicity of MCs depends on the balance between accumulation and metabolism (Ito et al., 2002). Meanwhile GSTs play important roles in the detoxification of MCs (Pflugmacher et al., 1998). Our results indicate that the transcription of GST rho was inhibited throughout the study, suggesting that transcription inhibition of GST rho might be a key mode of MC toxicity. Nowadays, cyanobacterial blooms are common in freshwater all around the world and long-lasting MC-exposure of fish is getting more and more ordinary, and the consequent alteration in transcription of GSTs stimulated by MCs implicates an increased health risk to fish.

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