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The acute effects of microcystin LR on the transcription of nine glutathione *S*-transferase genes in common carp *Cyprinus carpio* L.

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Abstract

The glutathione *S*-transferases play important roles in the detoxification of microcystin. In this experiment, nine glutathione *S*-transferase genes including cytosolic GSTs (rho, mu, theta, alpha and pi), mitochondrial GST (kappa) and microsomal GSTs (mGST1, mGST2 and mGST3) were cloned from common carp *Cyprinus carpio*. The mRNA abundance of each carp GST isoform in liver was analyzed by real time PCR. The relative changes after stimulation with microcystin LR were also analyzed: increased levels of transcription of GST alpha, rho and mGST3 isoforms were detected at 6 h post stimulation; the transcription of mu, theta and mGST2 isoforms were relatively stable; and all the GST isoforms except GST kappa and rho recovered to original levels compared with controls at 72 h. It is suggested that MC-LR showed different effects on the transcription of nine carp GST isoforms.
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Keywords: Microcystin; Glutathione *S*-transferases; Real time PCR; Common carp; *Cyprinus carpio*

Microcystins are a family of potent hepatotoxins produced by cyanobacteria. Since cyanobacterial blooms increased significantly in eutrophic water bodies worldwide during the past decades, cyanotoxins have become a threat to the health of human and aquatic animals (Hallegeff, 1993). Microcystin-LR (MC-LR), the most commonly encountered toxic type, specifically inhibits the serine/threonine protein phosphatases (PP1 and PP2A) (Honkanen et al., 1990; Matsushima et al., 1990; Yoshizawa et al., 1990), resulting in the disruption of many cellular processes and alteration of the cytoskeletal structures (Gehringer, 2004). The effects of MC-LR on fish embryo, larvae, juvenile and adult fish have been investigated, and the physiological parameters of exposed fish such as growth rate, serum biochemistry, iron regulation, heart rate would be influenced (Malbrouck and Kestemont, 2006). Compared with mammals, fish (especially phytoplanktivorous species) are more resistant to MC-LR (Xie et al., 2004). Previous studies indicated that the detoxification of MC-LR in the liver begin with a conjugation reaction to glutathione catalyzed by glutathione *S*-transferases (GSTs) (Kondo et al., 1996; Pflugmacher et al., 1998; Takenaka, 2001).

The glutathione *S*-transferases are a multiple gene family of dimeric enzymes that are ubiquitously distributed, and are involved in the detoxification of reactive electrophilic compounds including various intracellular metabolites, pollutants, drugs and pesticides (Hayes et al., 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 and zeta), the mitochondrial GST (kappa class) and microsomal GSTs (Hayes et al., 2005). However, information of fish GSTs is limited, although fish are a large heterogeneous group of lower vertebrates. The alpha, pi and theta GSTs were found in several fish species (George, 1994; Melgar Riol et al., 2001), and a special GST isoform, which have no homologue molecules in mammals was found in fish (Konishi et al., 2005). This special GST was named as rho class in red seabream *Pagrus major*, although its homologue molecules was found previously in the plaice *Pleuronectes platessa* and the largemouth bass *Micropterus salmoides* (Leaver et al., 1997; Martinez-Lara et al., 2002; Doi et al., 2004). The substrate activity of recombinant place GST rho isoforms (GST-A and GST-A1) had been tested, and the results indicate that these enzymes play important roles in reducing the harmful effects

Abbreviations: GST, glutathione *S*-transferase; MC-LR, microcystin LR; mGST, microsomal GST

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Table 1
PCR conditions used in this paper

RT-PCR	RACE-PCR	Real-time PCR
94 °C for 3 min	94 °C for 3 min	94 °C for 4 min
94 °C for 30 s (30 cycles)	94 °C for 30 s (30 cycles)	94 °C for 20 s (40 cycles)
58 °C for 30 s (30 cycles)	64 °C for 30 s (30 cycles)	60 °C for 20 s (40 cycles)
72 °C for 1 min (30 cycles)	72 °C for 1 min (30 cycles)	72 °C for 25 s (40 cycles)
72 °C for 5 min	72 °C for 5 min	72 °C for 5 min

of lipid peroxidation products generated naturally or exacerbated by xenobiotic exposure (Martinez-Lara et al., 2002). Some piscine GST isoenzymes have been utilized as valuable biomarkers for exposure to environmental pollutants (Pérez-López et al., 2002). More information is needed to clarify the relationship of microcystin and fish GST family. In this paper, we report molecular cloning of nine GST isoforms from common carp, and analyze their relative transcription in liver and their changes after stimulation with MC-LR.

The mRNA sequences of mammal GSTs were used to search in zebrafish and pufferfish genome and database for homologous genes, and degenerate primers were designed from conserved regions. RNA from carp liver was isolated using Trizol reagent (Invitrogen) and reverse transcribed into cDNA by Powerscript II reverse transcriptase with CDS primer (SMART RACE cDNA amplification kit, Clontech). The PCR cycling conditions were listed in Table 1. PCR products were cloned and sequenced. To recover the full length of cDNA sequence, 3′ RACE and 5′ RACE were performed by using the gene specific primers and adaptor primers. The sequence identity of carp GST genes with its homologues from other animals was analyzed by using the DNAstar software.

Common carp, each weighing about 20 g, were obtained from fish farm and acclimatized in aerated fresh water tanks for 1 month before being used in the experiment. Hepatic RNA was isolated from six fish, respectively using Trizol reagent, and treated with RNase-free DNase (Takara). Total RNA was quantified by determination at OD_{260}. The purified total RNA (2 μg) was then reverse transcribed into cDNA. All the primers used in the real time PCR were listed in Table 2. 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 the similar amplification efficiency were used in this experiment. The plasmid concentration of each GST isoform was measured at OD_{260} and the corresponding copy numbers were calculated based on the formula that 1 μg of 1000 bp DNA equivalent to 9.1×10^{11} molecules.

Quantitative real-time RT-PCR was conducted by amplifying 1.0 μl of cDNA with the SYBR Green qPCR kit (Finnzymes) on a Chromo4 Real-Time Detection System (MJ Research). Amplification conditions were listed in Table 1. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. Each sample was run in three tubes, and PCR reactions without the addition of the template used as blanks. The absolute standard curve of each isoform was constructed in the range of 10^5 to 10^9 plasmid molecules. After completion of the PCR amplification, data were analyzed with the OpticonMonitor software 2.03 Version (MJ research), and the molecular copy of each GST isoform was derived from corresponding threshold value compared with standard curve.

To study the kinetic changes of some carp GST gene transcription temporal changes of carp GST transcripts after MC-LR (HPLC: 95.8%, Calbiochem) stimulation, a group of fish were injected intraperitoneally with 100 μg MC-LR /kg body weight in sterile saline, and another group injected with sterile saline used as controls. Livers were removed and RNA isolated at 6, 12, 24, 48 and 72 h post-injection. RNA isolation, cDNA synthesis and real time PCR were the same as mentioned above. 18S RNA was used as a control gene. All values are expressed as mean ± S.D. One-way ANOVA were performed to determine whether treatment group were significantly different from control group ($P < 0.05$).

The full open reading frame of one GST isoform from alpha, mu, pi, theta, rho, kappa and three microsomal class have been cloned (The Genbank accession numbers of these sequences were DQ411310–DQ411318). Kappa and three microsomal GST isoforms have not been reported in fish before. As shown in Fig. 1, the carp GST sequences have the highest similarity with those from zebrafish, and the fish rho GSTs have no homo-

Table 2
Real time PCR primers used in this paper

Target gene	Primer sequence (5′–3′)		Size (bp)
	Forward	Reverse	
alpha	AGTTGAGCCGTGCTGACGTTCAC	CGGCTGGAGGAACTTGCTGA	146
kappa	GTCTCTCGGCCAGTGAGTTGGA	AAGAGCTCAGCCTTCCCGTTCA	154
mu	GGAAACTGATGAAGCGCAGATGAG	ATCTTGTCCCCAGCAAACCACTT	180
pi	GATCTGCCCAACCACCTCAAACC	CCGGGCAGCAATCTTATCCACAT	192
rho	GTCATGGTCGCGCTGGAGGAGAA	AGATACAGACACGCGGCGAACGA	176
theta	TATGCTGTACCTGACTGAGAAGTT	TCCTCTACTGCATTGTCCATCTT	208
mGST1	GATGTGTTCCTGGCCTTCTCCACA	GAAGACCAATCACCACAAAGGGAA	247
mGST2	CGGGGCTGATTCCAAAACAGAAG	CGAGACTGCTGCGAGGAGAACTG	168
mGST3	GTATCCTATTGCCGCCTCTGTGTT	GAATGACTCCAAACAGCCCGATGT	137
18sRNA	CCGTGGGTGACATCGTTACA	TCAGGACATTGAACCTCACTGTCT	69

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 3.0 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 3.

logues in mammals. Since only one isoform from each class GST was found in carp as yet, further research is need to find more members of GST family.

The copy number of each carp GST isoform (molecules/ng total RNA) was calculated by each standard curve and the result is shown in Fig. 2. The copy numbers of GST alpha, rho and pi isoforms were above 3000 molecules/ng RNA, and mGST2 is the scarcest type GST with about 200 molecules/ng RNA. Doi et al. (2004) demonstrated that there were mainly three class GST proteins in large mouth bass liver analyzed by HPLC, and the rho class GST was the predominant one. However, the mRNA abundance of each GST isoform has not been investigated before, and our experiment demonstrated that the transcription of all the found carp GST isoforms could be detected in liver.

The temporal changes of carp GST mRNAs are shown in Fig. 3. The transcription of these molecules showed different temporal kinetics. Increased transcription of GST alpha, rho and mGST3 isoforms were detected at 6 h post injection, which may indicate that the transcription of these GST isoforms could be induced by MC-LR at early time. However their transcription was different from 12 to 72 h post injection: alpha reverted to the original level; rho was suppressed following time and showed no recovery tendency even at 72 h; the transcription of mGST3

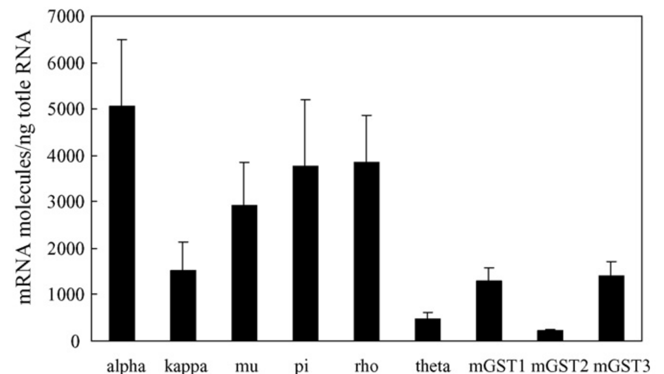

Fig. 2. The absolute abundance of transcripts of nine glutathione *S*-transferase isoforms in carp liver.

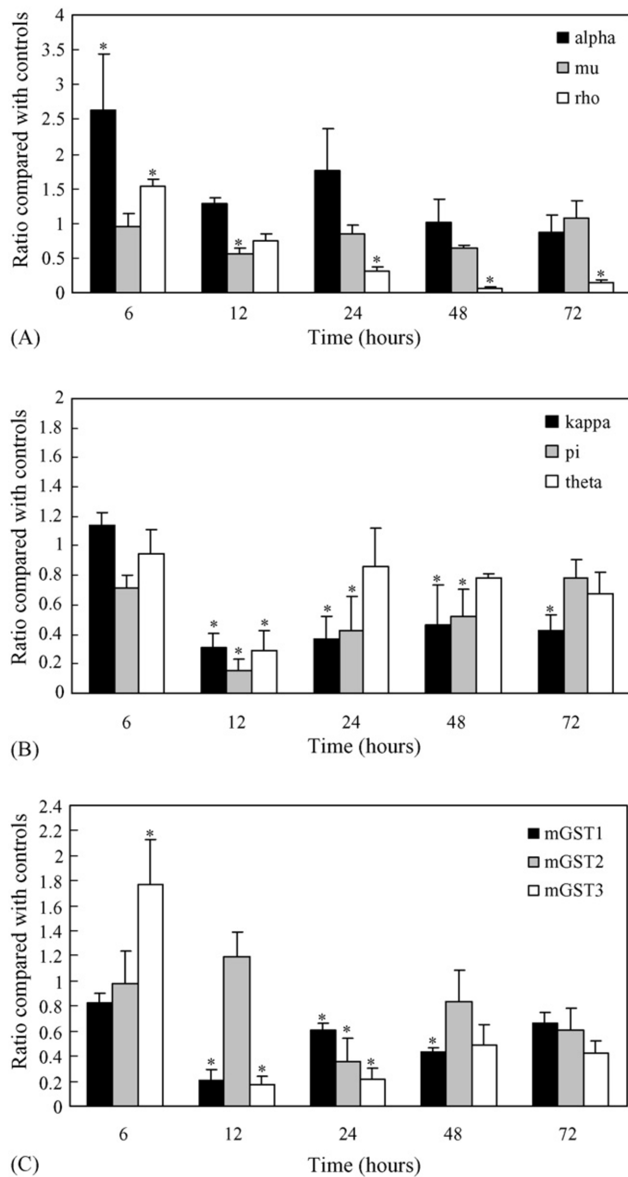

Fig. 3. The temporal changes of GSTs transcripts after microcystin stimulation compared with controls. (A) black column represents alpha GST; gray column represents mu GST; white column represents rho GST; (B) black column represents kappa GST; gray column represents pi GST; white column represents theta GST; (C) black column represents mGST1; gray column represents mGST2; white column represents mGST3. *Significant change ($P < 0.05$).

decreased at 12 and 24 h but showed a recovery tendency at 48 h. The transcription of the mu, theta and mGST2 was relatively stable with only slightly decreased transcription detected at 12 or 24 h. The transcription of kappa, pi and mGST1 were similar to the control at 6 h and then decreased gradually. Pi and mGST1 isoforms showed a recovery tendency at 48–72 h. Different effects of MC-LR on the transcription of GST genes were also found in mammals. In a micro-array analysis using rat exposed to MC-LR at 50 μg/kg, the transcription of GST alpha and mu was elevated at 3 h and mGST1 decreased at 6 h, however kappa-1 and pi had no change at 3 and 6 h post injection (Bulera et al., 2001). In another micro-array analysis, when mice were exposed to MC-LR at 75% LD_{50}, transcription of

Table 3
GST sequences used for phylogenetic tree construction

Species	Protein	Accession no.
	Hs Alpha1	CAI13812
	Hs Alpha2	AAH02895
	Hs Alpha3	NP_000838
	Hs Alpha4	AAH15523
	Hs Kappa1	AAH01231
	Hs Mu1	AAV38750
	Hs Mu2	AAI05067
Homo sapiens	Hs Mu3	NP_000840
	Hs Mu4	AAI08730
	Hs Mu5	AAH58881
	Hs Pi	AAV38753
	Hs Theta1	NP_000844
	Hs Theta2	AAG02373
	Hs mGST1	AAH05923
	Hs mGST2	NP_002404
	Hs mGST3	AAH00505
	Mm Alpha1	AAH61134
	Mm Alpha2	AAH30173
	Mm Alpha3	AAH09805
	Mm Alpha4	AAH12639
	Mm Kappa1	NP_083831
	Mm Mu4	AAH30444
	Mm Mu6	AAH31818
Mus musculus	Mm Pi	AAH61109
	Mm Theta1	AAH12254
	Mm Theta2	Q61133
	Mm Theta3	AAH03903
	Mm mGST1	AAQ93322
	Mm mGST2	NP_778160
	Mm mGST3	AAH29669
	Dr Alpha	AAH60914
	Dr Kappa1	XP_698521
	Dr Kappa1	XP_686115
	Dr Kappa1	XP_708257
	Dr Mu	NP_997841
	Dr Mu1	XP_690427
	Dr Pi	AAH83467
Danio rerio	Dr Pi2	NP_001018349
	Dr Theta	NP_956878
	Dr Theta3	XP_692427
	Dr Rho	CAK10882
	Dr Rho	XP_693045
	Dr mGST1	AAH74022
	Dr mGST2	XP_700815
	Dr mGST3	XP_695658
	Tn Alpha	CAG09409
	Tn Kappa1	CAF97858
	Tn Mu	CAG07510
Tetraodon nigroviridis	Tn Theta	CAG09655
	Tn mGST1	CAF97117
	Tn mGST2	CAG04538
	Tn mGST3	CAG09920
Pleuronectes platessa	Pl Rho	CAA64493
	Pl Rho	CAA64496
Pagrus major	Pm Rho	BAD98443
Pimephales promelas	Pr Rho	AAF78081
Micropterus salmoides	Ms Rho	AAQ91198
Carassius auratus	Ca Pi	ABF57553
Anguilla anguilla	Aa Pi	AAS01601

the GST pi was elevated at 8 h, and GST mu (mu 2 and mu 5 isoform) increased during 8–24 h (less than two fold compared with controls) (Gehringer et al., 2004).

The abundance of most GST mRNAs decreased at 12–24 h post injection, and there are two possible explanations for this. First, prolonged exposure and high concentration of microcystin might influence some transcriptional regulating factors that bind to the promoter region of some GST genes, and the transcription of some GST isoenzymes was then influenced. Second, the accumulation of GSH conjugate of MC-LR might be an inhibitor of GST, since previous studies have shown that GSH conjugate of (−)-*anti*-BPDE [(−)-*anti*-BPD-SG] is an inhibitor of GST mu in human and GST pi in mice (Srivastava et al., 1998, 1999).

There have been studies to examine enzyme activities of GSTs after exposure to microcystin in various organisms. Elevated GST activity are observed in aquatic organisms (Pietsch et al., 2001) and mice (Gehringer et al., 2004), however decreased activity is also found in juvenile goldfish *Carassius auratus* (Malbrouck et al., 2003). It is also reported that only minor activation of the soluble GST was found in zebrafish, and the reaction of the microsomal GST was not so obvious (Wiegand et al., 1999). Such different results may be due to difference in test animals, toxin level, exposure route and sampling interval. In this experiment, real time PCR was used instead of enzyme kinetic analysis for the following reasons: the GSTs are homodimers or heterodimers (for example, alpha and mu classes can form heterodimers), due to broad and often overlapping substrates, whereas it is difficult to definite a distinction between GST classes by specific substrates due to broad and often overlapping substrates (Sheehan et al., 2001), while the real time PCR method, however, can be used to accurately quantify gene transcripts at low copy number in small samples.

The results of this study suggest that MC-LR showed different effects on the transcriptions of nine carp GST genes. Although the GSTs play important roles in the first step to detoxify microcystin, detailed evaluation on the role of each member in microcystins detoxification awaits future research.

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