Chemical Proteomic Analysis of the Potential Toxicological Mechanisms of Microcystin-RR in Zebrafish (*Danio rerio*) Liver

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ABSTRACT: Microcystins (MCs) are common toxins produced by freshwater cyanobacteria, and they represent a potential health risk to aquatic organisms and animals, including humans. Specific inhibition of protein phosphatases 1 and 2A is considered the typical mechanism of MCs toxicity, but the exact mechanism has not been fully elucidated. To further our understanding of the toxicological mechanisms induced by MCs, this study is the first to use a chemical proteomic approach to screen proteins that exhibit special interactions with MC-arginine-arginine (MC-RR) from zebrafish (*Danio rerio*) liver. Seventeen proteins were identified via affinity blocking test. Integration of the results of previous studies and this study revealed that these proteins play a crucial role in various toxic phenomena of liver induced by MCs, such as the disruption of cytoskeleton assembly, oxidative stress, and metabolic disorder. Moreover, in addition to inhibition of protein phosphate activity, the overall toxicity of MCs was simultaneously modulated by the distribution of MCs in cells and their interactions with other target proteins. These results provide new insight into the mechanisms of hepatotoxicity induced by MCs. Environ Toxicol 31: 1206–1216, 2016.

Keywords: microcystin-RR; chemical proteomics; toxicological mechanisms; zebrafish; liver

INTRODUCTION

In recent years, the outbreak of the cyanobacterial blooms in freshwaters has become a worldwide concern because of the release of cyanotoxins. Among these cyanotoxins, microcystins (MCs) are the most common and dangerous group. MCs contain five D-amino acids and two variable L-amino acids, with molecular weights between 900 and 1000 Da (Dietrich and Hoeger, 2005). To date, over 80 different MC structural variants have been identified, among which, MC-LR, MC-RR, and MC-YR are the three most common (Dietrich and Hoeger, 2005). MCs can get absorbed by and bioaccumulate in organisms, including humans, through the food web, and they can cause poisoning, illnesses, and even death (Jochimsen et al., 1998; Soares et al., 2006; Chen et al., 2009). In humans, MCs were first identified in the serum of fishermen, and they were associated with hepatocellular damage (Chen et al., 2009). Acute exposure to MCs could lead to more serious consequences. In 1996, 76 patients died at two dialysis centers in Caruaru of Brazil because the water used for hemodialysis contained MCs (Pouria et al., 1998).

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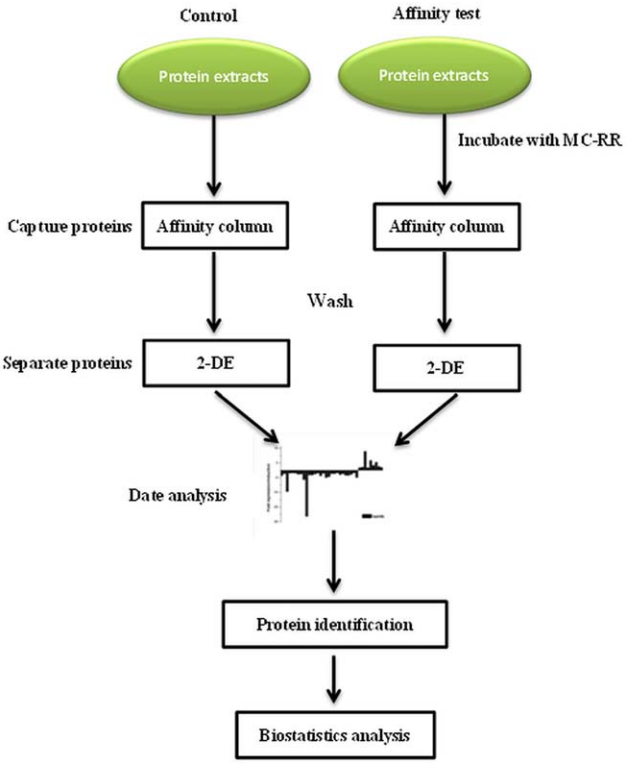

Fig. 1. Diagram of the chemical proteomic approach used to explore the potential toxicological mechanisms of MC-RR. Proteins that bind directly to the MC-RR are captured from cell lysates by affinity column chromatography. The captured proteins are then separated, analyzed by 2-DE and identified by biological mass spectrometry. Finally, the function of each identified protein was analyzed using bioinformatics tools. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The canonical mechanism of action for MCs is inhibition of protein phosphatases 1 and 2A (PP1 and PP2A) (Yoshizawa et al., 1990), followed by destruction of the hepatic cytoskeleton, which leads to liver hemorrhage, necrosis, and apoptosis. (Fischer and Dietrich, 2000; Gehringer, 2004). However, other toxic effects have been widely reported, including oxidative damage (Ding et al., 2001; Wang et al., 2010), endoplasmic reticulum stress (Christen et al., 2013; Menezes et al., 2013), and mitochondrial damage (Chen et al., 2013). For instance, MC-LR activates all of the endoplasmic reticulum stress response pathways and plays a crucial role in the hepatotoxic, inflammatory, and tumorigenic action of MC-LR (Christen et al., 2013). Additionally, Blom and Juttner (2005) reported that MCs toxicity and PP inhibition did not correlate. Taken together, these results indicate that unknown target proteins mediate MCs toxicity.

Aquatic animals, such as zebrafish (*Danio rerio*) and medaka fish (*Oryzias latipes*), are excellent models for investigating the cellular responses to toxins; proteomic-based approaches have been used to investigate the effects of MCs in such model organisms (Mezhoud et al., 2008b; Wang et al., 2010; Malecot et al., 2011; Zhao et al., 2012). These studies revealed that many proteins involved in multiple pathways, such as oxidative stress, metabolism, and signal transduction, are deregulated by MCs. However, the information from these studies is generally indirect, as distinct from toxins directly interacting with their target proteins, which may provide direct evidence regarding a toxin's mode of action (Katayama and Oda, 2007). Chemical proteomics, a systematic process of affinity chromatography and proteomics approaches, provides a direct and effective method for elucidating the mechanism of action of bioactive small molecules in cells or tissues of interest (Bantscheff et al., 2009; Sato et al., 2010). In the study of MCs, such approaches have only been used to purify protein phosphatases (PPs) or demonstrate the association of signaling proteins with PPs from some components of rat liver proteins, such as hepatic glycogen-proteins particles (Moorhead et al., 1995), nuclear protein extracts (Tran et al., 2004), and cytosolic proteins (Boudrez et al., 1999). However, due to the incomplete and limited nature of these studies, other MCs-interacting proteins have not been investigated.

To gain insights into potential mechanisms of MCs virulence, we screened proteins that exhibited specific interactions with MC-RR from total protein extracts of zebrafish (*D. rerio*) liver (Fig. 1). Utilizing MC-RR affinity chromatography combined with two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer (MS), 17 proteins were identified as target proteins by an affinity ligand blocking experiment. The function of each identified protein was analyzed using bioinformatics tools. The purpose of this study was to explore potential MC target proteins and provide new insights into the potential risk of MCs on human health using a model animal.

MATERIALS AND METHODS

Chemicals

The cyanobacterial toxin used in this experiment, MC-RR, was extracted and purified from freeze-dried surface blooms collected from Lake Dianchi in China, according to the methods of Ramanan et al. (2000) and Dai et al. (2008). H_2N-MC-RR was synthesized using the method of Wu et al. (2010). Briefly, MC-RR was reacted with 1000-fold molar excess of 2-aminoethanethiol in 5% potassium carbonate aqueous solution for 2 h at room temperature while stirring. The reaction mixtures were neutralized by the addition of acetic acid, and the product was purified from the reaction mixture using an ODS C18 cartridge (2 g/12 mL, Waters, Milford, MA). H_2N-MC-RR was stored at -80 °C until use.

The purity of MC-RR and H_2N-MC-RR were identified by liquid chromatography-electrospray ionization-mass spectrometry (Thermo Electron Corporation, Waltham, MA) and quantified by high performance liquid chromatography (purity was above 95%). An MC-RR standard with >95%

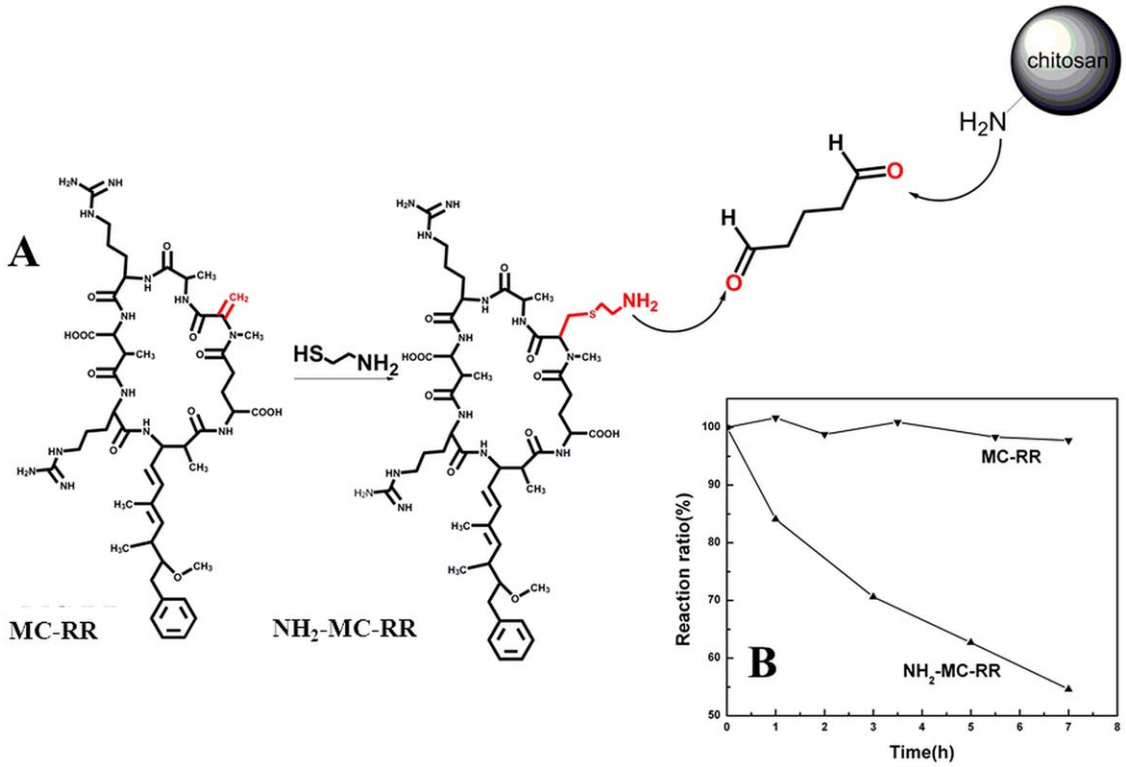

Fig. 2. Preparation of MC-RR affinity column. (A) The coupling procedure of MC-RR to chitosan microspheres (For simplicity, only one MC-RR molecule is shown, although in theory, several MC-RR molecules could be coupled to chitosan.). (B) Effect of time on immobilization of MC-RR or H_2N-MC-RR to chitosan microspheres. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

purity (Pure Chemical Industries, Wako, Osaka, Japan) was used for qualitative and quantitative analysis. All other chemical reagents were of the highest grade, and they were obtained from Sigma-Aldrich (St. Louis, MO) or Shanghai Chemical Reagents (Shanghai, China).

Preparation of MC-RR Affinity Chromatography Columns

MC-RR affinity chromatography columns were prepared by modifying the synthesis of the complete antigen of MCs (Sheng et al., 2006). MC-RR was immobilized on chitosan microspheres to prepare the affinity column (Fig. 2). Briefly, chitosan microspheres were prepared following the method described in the work by Jiang et al. (2005). Chitosan microspheres were previously incubated with phosphate buffer (pH 7.0) for 20 h. After filtration, the chitosan microspheres were added to 100 mL of phosphate buffer (0.05 M, pH 9.2) containing excess H_2N-MC-RR. The mixture was stirred in a shaker at 25 °C for 17 h. After the reaction was completed, the chitosan microspheres were packed into a column (Φ 0.8 cm × 25 cm) and washed with MilliQ water (Millipore, Bedford, MA) until H_2N-MC-RR was no longer detected.

Extraction of Total Protein from Normal Zebrafish Liver Tissue

Healthy adult zebrafish (*D. rerio*) were obtained from the Institute of Hydrobiology, Academy of Science, China. Zebrafish livers were dissected, pooled, and homogenized in nondenaturing lysis buffer (50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% NP-40, 1 mM PMSF, and 0.1 mM leupeptin) on ice. The homogenates were centrifuged at 10 000 × *g* (BR4, Jouan, Winchester, VA, France) at 4 °C for 10 min. The supernatant was collected and stored at −80 °C until use. Protein concentration was determined using the Bradford assay, and bovine serum albumin was used as a standard.

MC-RR Affinity Blocking Test

The affinity chromatography column was equilibrated with buffer A (50 mM Tris-HCl (pH 7.5) and 0.15 M NaCl) before use. Two milliliter of lysis solution was incubated with 1 mL buffer A at 4 °C for 1 h. When affinity ligand blocking experiments were carried out, free MC-RR was added (0.2 mg) to the lysate. Then, each protein solution was subjected to MC-RR affinity

chromatograph column. The columns were washed with buffer A until essentially no protein was eluted from the column. The captured proteins were then released with buffer B (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 3 M NaSCN), and the eluted fraction was collected. The eluted fraction was dialyzed with Milli Q water at 4 °C and freeze-dried prior to electrophoresis.

Proteomics Analysis

2-DE was performed in accordance with the manufacturer's instructions. The protein samples were mixed with rehydration buffer and then loaded onto 7-cm IPG trips (pH 4–7, liner). This pH range allowed proteins with similar isoelectric point (pI) values to be separated with higher resolution. Isoelectric focusing was performed at 20 °C, and the total Vh of isoelectric focusing reached 20 000 Vh. Then, the focusing strips were subjected to a second dimension of electrophoresis using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. In the second dimension, proteins were separated based on their molecular weight. Finally, sliver staining or Coomassie Brilliant Blue G-250 staining were used to visualize the proteins. 2-DE was performed in triplicate for each group.

The 2-D gel maps were analyzed using PDQuest software (BioRad, Hercules, CA). Spots that changed conspicuously in the control groups or affinity blocking groups (fold change $\geq$ 2-fold, $p < 0.05$) were manually excised from 2-DE gels. The gel pieces were destained twice with 200 mM ammonium bicarbonate in 50% acetonitrile/water (45 min at 37 °C), then dehydrated using acetonitrile, and spun dried. The dried gel bands were rehydrated with a trypsin solution (Promega, Madison, WI) (10 ng/L) and incubated overnight at 37 °C. The trypsin-digested samples were mixed with the matrix ((α)-cyano-4-hydroxycinnamic acid dissolved in 0.1% trifluoroacetic acid of 50% acetonitrile aqueous solution) and analyzed by MALDI-TOF/TOF MS (4800 Proteomics Analyzer, Applied Biosystems, Foster City, CA). GPS explorer software (v3.6, default parameters, Applied Biosystems) was used to generate the peak lists, and the resulting data were searched against the NCBInr 20130120 zebrafish (*D. rerio*) protein database (38 935 sequences) using the Mascot search engine. The identification parameters were set as follows: species, zebrafish; enzyme, trypsin; allow for one missed cleavage site; fixed modification, carbamidomethyl (C); variable modification, oxidation (M); peptide charge, 1+; monoisotopic; and mass tolerance, ±50 ppm for the precursor ions and ±0.5 Da for fragment ions.

Bioinformatics Analysis

The specific processes or functions of the identified proteins were matched by searching Gene Ontology (www.geneontology.org). Pathway analysis was performed using a molecule annotation system (MAS 3.0) (http://bioinfo.capitalbio.com/mas3/). Further analysis of the subcellular localization of the identified proteins was predicted using YLoc (http://abi.inf.uni-tuebingen.de/Services/YLoc/webloc.cgi) (Briesemeister et al., 2010).

RESULTS

Intermediate Product Identification

The intermediate product, H_2N-MC-RR, was analyzed by mass spectrometry using the ion trap. As shown in Figure 3, the double precursor ion was at *m/z* 558.2, which was consistent with the $[M+H]^{2+}$ for H_2N-MC-RR (theoretical calculated value of molecular weight: 1115.4) formed via the addition of 2-aminoethanethiol to MC-RR. These results indicate that the modification of MC-RR was successful.

Preparation of MCs-Binding Proteins from Zebrafish Livers Using Affinity Chromatography

The first step in the isolation of MCs-binding proteins from zebrafish livers with affinity chromatography was the selection of lysis buffer for homogenization. The ideal lysis would leave proteins in their native conformation and minimize the denaturation of ligand binding sites from samples. Certain nondenaturing lysis buffers have been widely used for affinity enrichment, such as NP-40 (Xu et al., 2007) and Triton X-100 (Cuatrecasas, 1972) lysis buffers. Second, to obtain more information about proteins that exhibit specific interactions with MCs, the affinity column should be washed with a low concentration salt buffer because MCs-protein complexes are sensitive to salt, and some will dissociate at high salt concentrations (Moorhead et al., 2007). Therefore, we selected NP-40 lysis buffer to release proteins from zebrafish livers, and 0.15 M NaCl was used for MCs affinity chromatography research.

A major problem associated with affinity-based target identification is the existence of nonspecific binding proteins (Bantscheff et al., 2009). Efforts were made to overcome this obstacle by performing an affinity ligand blocking experiment where free MC-RR was added to the lysates in the affinity blocking group. When the lysate mixture was treated with an affinity carrier, the free MC-RR competed with MC-RR on the carrier, affecting the recovery of proteins that selectively bound to the immobilized compound. Hence, proteins with different recovery in the competition experiment were likely target candidates.

In this study, 27 mg of total zebrafish liver protein were loaded on MC-RR affinity columns, and normal liver proteins were separated into MC-RR unbound and bound fractions. The bound fractions were dialyzed with Milli Q water at 4 °C and freeze-dried prior to proteomics analysis. The

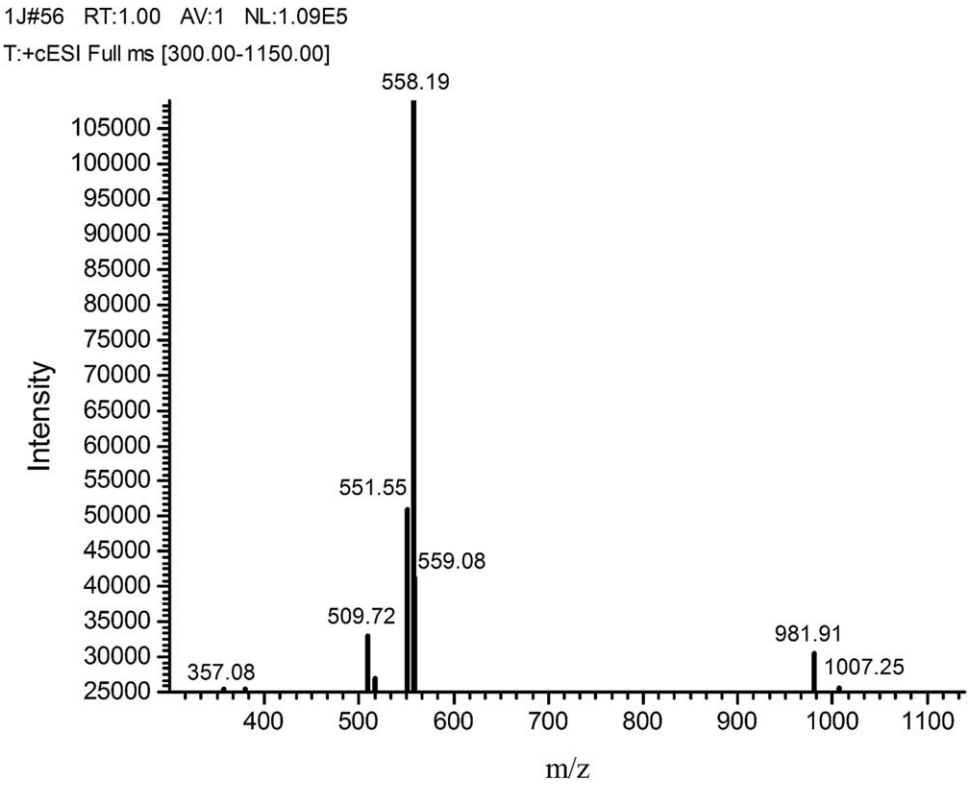

Fig. 3. Mass spectrum of H_2N-MC-RR. The double precursor ion is at *m/z* 558.2, which is consistent with the theoretical calculated value.

chromatography yield of MC affinity proteins was 1.3% compared with the total liver protein loaded.

The Proteomic Profiles of MCs-Binding Proteins from Zebrafish Livers

In general, previous studies used sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels to analyze proteins captured from affinity columns (Mikhailov et al., 2003; Tran et al., 2004; Mori et al., 2012). Notably, staining caused by nonspecific binding proteins overlaps with the region corresponding to target proteins (Yang et al., 2007). Therefore, to improve the efficiency of screening the target proteins of MC-RR, the captured proteins from the affinity column were analyzed by 2-DE in this study; this approach represents a mature technique for the analysis of several thousand proteins in a single sample.

In this study, a cutoff value of 2-fold was used as the criterion for a significantly altered protein ($p < 0.05$). Compared with 2-DE gels of control group, 36 protein spots were significantly altered in the affinity blocking experiment (Fig. 4). These spots were excised and submitted for identification using MALDI-TOF/TOF MS, followed by database searching using the NCBInr database for zebrafish. Thirty-one spots were successfully identified with CI values greater than 95%. The identified proteins were distinguished into 17 different proteins, eight of which increased and nine of which decreased. These proteins include the acknowledged target protein (PP1 catalytic subunit 7, A05) and the newly identified target protein (ATP beta catalytic subunit, B02) (Fig. 4). The specific functions of the identified proteins were analyzed using bioinformatics tools. Several representative proteins were selected, and magnified images from 2-DE gels are shown in Figure 5.

Bioinformatic Analysis

The molecular functions of the identified proteins were analyzed using the molecule annotation system (MAS 3.0), and the results indicated that MC-RR affects several crucial cellular pathways. We determined the functions of these proteins using the results of this study combined with GO annotation (Table I). Six of the identified proteins were characterized as metabolism-related proteins (spots A05, A15, A16, A18, B36, and B38), three proteins were related to the cell cytoskeleton (spots B06, B10, and B16), four proteins were involved in the response to oxidative stress (spots A21, A02, B02, and B25), and four proteins (spots A07, A09, A14, and B03) were categorized as other functional proteins.

To gain more information about the mechanism of MCs virulence, these proteins were further classified according to their molecular function and biological processes using

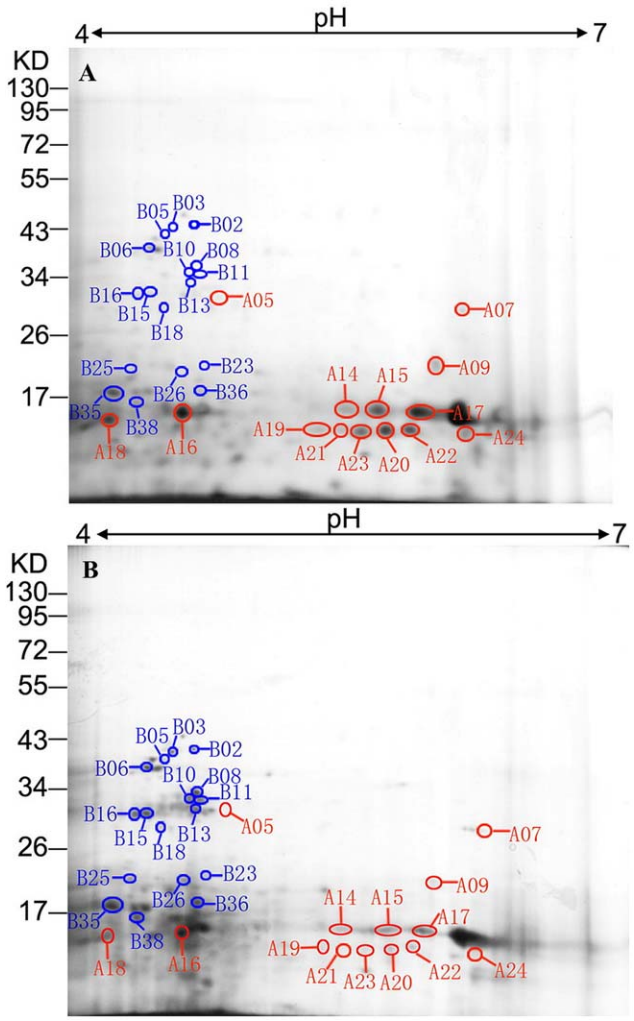

Fig. 4. 2-DE gel image of the captured proteins from control (A) and affinity blocking (B) experiments. Zebrafish liver total proteins were incubated with buffer A (with or without MC-RR) before affinity chromatography. The captured proteins were separated by 2-DE and visualized by silver staining. The protein spots altered in the affinity blocking test are labeled with numbers. Each gel is representative of three independent replicates. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

MAS 3.0 [Fig. 6(A,B)]. Grouping proteins according to their biological process revealed that a majority of the identified proteins were involved in physiological process (28%), cellular process (26%), and metabolism (21%). According to the functional classification, 47% of the identified proteins had catalytic activity, 35% had binding activity, 12% had structural molecule activity, and 6% had antioxidant activity. Further analysis of the predicted subcellular localization revealed that the identified proteins were allocated to the cytoplasm (35%), extracellular space (23%), mitochondria (18%), nucleus (12%), endoplasmic reticulum (6%), and lysosome (6%) [Fig. 6(C)].

DISCUSSION

With the goal of studying the potential mechanisms of MCs, proteins that exhibited special interactions with the MC-RR from zebrafish livers were first screened using chemical proteomics. The protein spots significantly altered in affinity blocking experiments were investigated, and 17 types of proteins were identified. These proteins were apparently or potentially related with cytoskeleton organization, oxidative damages, and metabolism disorder.

Cytoskeleton disruption was a striking cytotoxic effect caused by MCs. The most extensively studied mechanisms of action for MCs is the inhibition of PP activity, modifying the phosphorylation state of cytoskeleton-associated protein (Yoshizawa et al., 1990; Fu et al., 2005; Li et al., 2012a). For example, the disruption of intermediate filaments could be attributed to MC-induced hyperphosphorylation of keratin 8 and keratin 18 (Toivola et al., 1997; Mezhoud et al., 2008b). In this study, PP1 regulatory subunit 7 was identified as a target protein, which was consistent with previous studies. Another interesting finding of this study was that three intermediate filaments proteins, keratin 18, keratin type I cytoskeletal 18, and keratin type II cytoskeletal 8, exhibit specific interactions with MCs, but no microfilament or microtubules proteins were observed. These results suggest that MCs directly bind to the intermediate filaments, and intermediate filaments are disrupted by MCs prior to the effects on microfilaments and microtubules. Actually, the previous studies have compared the effects of MC-LR on the organization of intermediate filaments, microfilaments, and microtubules in hepatocytes, fibroblasts, and kidney cells (Wickstrom et al., 1995; Khan et al., 1996). These studies indicated that alterations occurred first in intermediate filaments, followed by microtubules and microfilaments. These data indicate that the molecular mechanisms by which MCs induce cytoskeleton disruption likely involves the synergistic effect of protein phosphatase inhibition and direct binding with intermediate filaments, which influence microfilaments and microtubules.

Liver metabolism is disturbed by MCs. Several studies have reported that MCs influence the proteolysis process in rat and zebrafish liver (Claeyssens et al., 1995; Lankoff and Kolataj, 2001; Wang et al., 2010). In this study, we found that the disturbance of the proteolysis process in zebrafish liver was related to zgc:66382 and zgc:92590. These proteins are trypsin-like serine proteases that cleave peptide bonds following a positively charged amino acid (lysine or arginine). Serine proteases play crucial roles in many physiological processes, including digestion, apoptosis, signal transduction, and the immune response (Neurath and Walsh, 1976; Shi et al., 1992; LeMosy et al., 1999; Gorman and Paskewitz, 2001). Furthermore, Wang et al. (2010) also demonstrated that the expression of zgc:66382 in zebrafish liver was significantly reduced after chronic exposure to MC-LR concentrations (2 or 20 μg/L MC-LR) for 30 days.

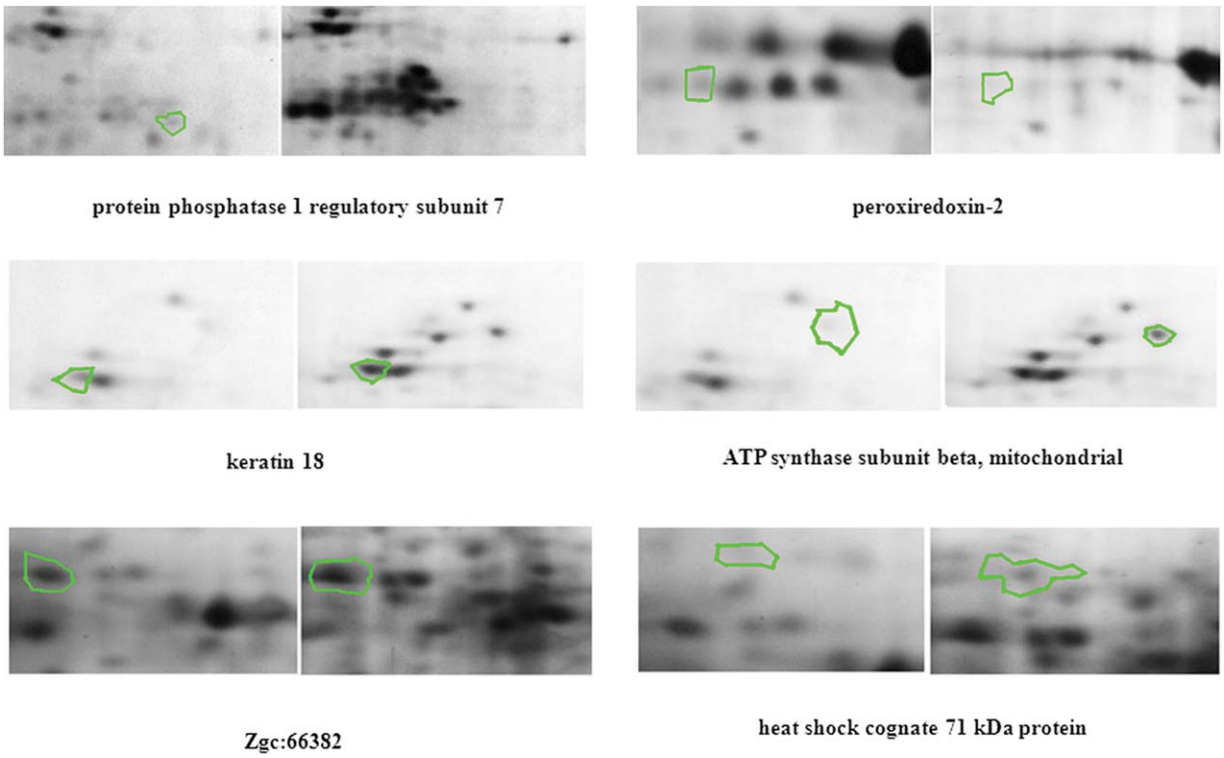

Fig. 5. Magnified images of representative proteins spots from 2-DE gels. Protein phosphatase 1 regulatory subunit 7 and ATP synthase subunit beta were identified as MCs target proteins in previous studies, zgc:66382 was characterized as metabolism-related protein, peroxiredoxin 2 and heat shock cognate 71-kDa protein are involved in response to oxidative stress, and keratin 18 was classified as being involved in cytoskeleton assembly. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Therefore, zgc:66382 plays a crucial role in the toxic effects of MCs, and it might be a biomarker for MCs-induced liver injury.

A large number of studies, including histological, metabolomic, and biochemical investigations, reported that lipid mechanisms were also disrupted by MCs in cells and organisms (Moreno et al., 2005; He et al., 2012; Zhao et al., 2012). Here, we found that three apolipoproteins (apoa1b, apoa1, and apoa4) exhibit specific interactions with MCs, among which, apoa1 is the major protein component of high-density lipoprotein particles and apoa4 is a major apolipoprotein constituent of rat HDL (Mahley et al., 1984). Apolipoproteins are synthesized in the liver and transport lipids through the blood stream and lymphatic systems. Previous proteomic studies using 2-D gels and iTRAQ revealed that the expression of apolipoproteins (apolipoprotein A1, Apolipoprotein A-IV/V, Apolipoprotein E, and 14-kDa apolipoprotein) in fish liver were enriched by the effect of MCs (Mezhoud et al., 2008a; Malecot et al., 2009, 2011). Moreover, components of hepatic lipids were altered after chronic exposure to MC-LR (Sedan et al., 2010). These studies indicate that the lipid transport mechanism was altered. The observed up-regulation of apolipoproteins may be a compensatory effect in response to changes in lipids induced by MCs in fish species.

Our results demonstrate that peroxiredoxin 2 (Prdx2), ATP synthase subunit beta (ATP5b), heat shock cognate 71-kDa protein (hspa8), and NADH dehydrogenase iron-sulfur protein 3 exhibit special interactions with MCs. These proteins are related to oxidative stress, which was consistent with the previous studies that MCs induce reactive oxygen species (ROS) production, followed by oxidative stress, leading to potential molecular damage (Zegura et al., 2011). Peroxiredoxin 2 belongs to a ubiquitous family of antioxidant enzymes that act as antioxidants, and it reduces hydrogen peroxides and alkyl hydroperoxides with the help of reducing systems. Several studies demonstrated that the expression levels of the peroxiredoxin family proteins were remarkably altered after MCs exposure, and Prdx2 seems to be up-regulated (Chen et al., 2005; Fu et al., 2005; Li et al., 2012b). Our results indicate that MCs can bind Prdx2, suggesting that the activity of Prdx2 was inhibited. Hence, it was conceivable that up-regulation of Prdx2 was the cellular response to MCs exposure, protecting the cell from destruction by oxidative stress. Mikhailov et al. (2003) and our data demonstrate that MCs can bind to ATP5b, which could reduce ATP synthesis and affect cellular processes that depend on ATP, such as glutathione (GSH) synthesis. GSH plays a critical role in cells during the response to oxidative stress, and previous studies indicate that the level of GSH in cells or organs was

TABLE I. A list of differential protein recoveries in affinity blocking test

Spot Id.	Protein Identity	Accession Number	Fold Change	MW (kDa)/pI Experimental	Score	SC[a]	Function Category
Metabolism							
A05	Protein phosphatase 1 regulatory subunit 7 (ppp1r7)	gil82658210	−10000[b]	39.42/4.94	274	40	Phosphatase activity
A15	Apolipoprotein A-Ib precursor (apoa1b)	gil424036615	−2.9	30.18/6.05	110	52	Lipid metabolism
A17			−3.1		223	52	
A20			−5.2		152	41	
A22			−6.2		196	49	
A23			−3.7		139	36	
A24			−10000		80	35	
A16	Apoa protein (apoa1)	gil53733942	−2.5	19.74/5.31	136	47	Lipid binding
A18	Apolipoprotein A-IV (apoa4)	gil49902823	−2.3	30.05/4.82	229	33	Lipid transport, lipoprotein metabolism
B35	Zgc:66382 (zgc:66382)	gil33585756	2.7	27.05/4.94	86	28	Proteolysis
B36			3.8		113	32	
B38	Uncharacterized protein LOC474322 precursor (zgc:92590)	gil55742601	3.0	27.80/4.86	80	35	Proteolysis
Cytoskeleton							
B05	Keratin 18(krt18)	gil29335504	10000[c]	41.25/5.07	356	42	Intermediate filament
B06			2.6		149	33	
B10	Keratin, type I cytoskeletal 18 (krt18)	gil30410758	10000	48.57/5.53	77	12	Intermediate filament
B11			4.8		111	14	
B15	Keratin, type II cytoskeletal 8 (krt8)	gil41056085	4.7	57.78/5.15	108	16	Intermediate filament keratin filament
B16			5.6		91	14	
Response to oxidative stress							
A21	Peroxiredoxin-2 (prdx2)	gil50539996	−5.2	21.95/5.93	196	54	Antioxidant activity, oxidoreductase activity
A19	NADH dehydrogenase (ubiquinone) iron-sulfur protein 3, mitochondrial (ndufs3)	gil62955483	−6.3	29.44/5.92	150	32	Oxidation reduction
B02	ATP synthase subunit beta, mitochondrial (ATP5b)	gil366039974	2.3	55.21/5.25	208	33	ATP synthase activity
B08			10000		210	32	
B13			10000		67	14	
B18			2.1		252	27	
B23			10000		194	28	
B26			10000		248	32	
B25	Heat shock cognate 71-kDa protein (hspa8)	gil160333682	4.1	71.36/5.18	95	12	Response to stress
Other functions related							
A07	Calnexin precursor (canx)	gil47087435	−10000	67.83/4.37	141	15	Calcium ion binding unfolded protein binding
A14	Rplp0 protein (rplp0)	gil29124460	−4.1	34.55/5.73	63	7	Structural constituent of ribosome
A09	Uncharacterized protein LOC100126125 precursor (zgc:174237)	gil157954494	−8.8	28.71/6.78	71	21	Unknown
B03	Uncharacterized protein LOC796447 precursor (LOC796447)	gil153792718	10000	40.36/5.09	118	28	Liver development

[a]SC indicates the sequence coverage of the protein in percentage obtained by MS/MS identification.
[b]−10000, the spot disappeared in the affinity test group.
[c]10000, the spot disappeared in the control group.

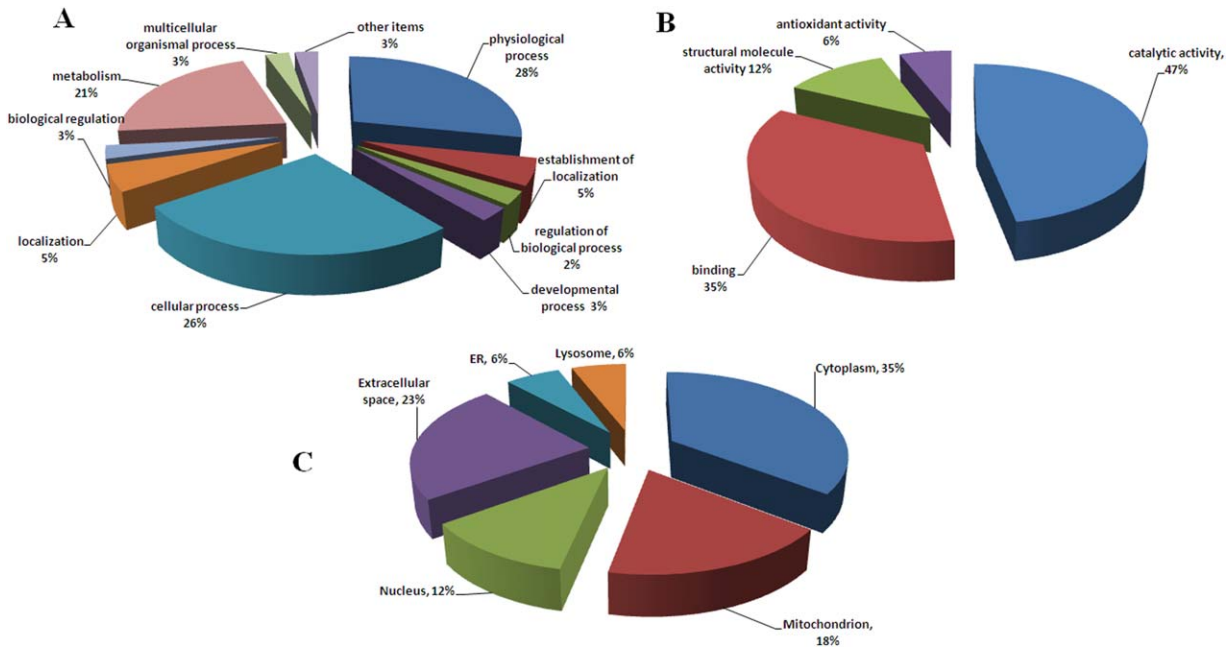

Fig. 6. Classification of identified proteins. The total amount of 17 distinct proteins was set to 100%. (A) Biological process of identified proteins according to database entries. (B) Molecular function of identified proteins according to database entries. (C) Subcellular localization of the identified proteins was determined according to YLoc prediction. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

remarkably altered in response to MCs exposure (Gehringer et al., 2004; Zegura et al., 2006; Zhao et al., 2012). Additionally, inhibition of ATP synthase might increase the intensity of mitochondrial membrane depolarization, disrupt the mitochondrial electron transport chain, and favor ROS generation (Ding et al., 2002). Furthermore, the induction of heat shock proteins is considered an early marker of oxidative stress (Peng et al., 2000). In this study, the heat shock cognate 71-kDa protein was shown to be one of the proteins that specifically interact with MC-RR. This protein is a fascinating chaperone protein, and it is involved in many cellular processes, including ATPase function (Stricher et al., 2013). Our group reported that hspa8 levels were strikingly decreased by microinjecting 14.4 μM MC-RR directly into zebrafish embryos at 72 h postfertilization (Zhao et al., 2011). This result also suggested that MC-RR might impair ATP production. In fact, MCs decreased ATP content in a time- and dose-dependent manner in fish lymphocytes and rat hepatocytes (Zhang et al., 2007; Shimizu et al., 2013). Together with the present results, these reports suggest that inhibition of ATP synthesis plays a pivotal role in MC-RR-induced oxidative damage, and hspa8 may serve as an earlier marker.

Among the 17 proteins identified in this study, prediction of subcellular localization indicated that these proteins are localized in different cellular components. These findings revealed that in addition to inhibition of protein phosphate activity, the overall toxicity of MCs was simultaneously modulated by the distribution of MCs in cells, and the presence of other target proteins will lead to various toxic mechanisms under different conditions. For example, it has been reported that the chronic toxicity of MCs in zebrafish might act through the ROS pathway instead of the protein phosphate pathway (Wang et al., 2010). The BID-BAX-BCL-2 and ROS pathways play a major regulatory role in MC-LR-induced apoptosis when BALB/c mice were treated with different doses of MC-LR for 24 h (Chen et al., 2005).

In summary, we applied MC-RR affinity chromatography, 2-DE, and MALDI-TOF/TOF MS for the first time to screen for proteins that exhibit special interaction with MC-RR from zebrafish livers. An affinity blocking experiment identified 17 proteins, which involved in multiple functions and participated in a variety of biological processes, such as cytoskeleton assembly, proteolysis process, lipids transport, and oxidative stress. By integrating the results of previous proteomic studies, we believe that some of these proteins may serve as early markers for further MCs toxicity research. This work could help us fully understand the effects of MC-RR and provide novel and substantial chemical/biochemical evidence on the toxicological mechanisms of MC-RR.

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