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# Proteomic study of the effects of microcystin-LR on organelle and membrane proteins in medaka fish liver

Mélodie Malécot[a], Karim Mezhoud[a], Arul Marie[b], Danièle Praseuth[c], Simone Puiseux-Dao[a], Marc Edery[a,*]

[a] CNRS, FRE 3206 Molécules de communication et adaptation des microorganismes, and MNHN, USM 505 Cyanobactéries, cyanotoxines et environnement, Département Régulations, développement et diversité moléculaire, Muséum national d'Histoire naturelle, CP 39, 57 rue Cuvier, F-75231 Paris Cedex 05, France
[b] Plateforme de spectrométrie de masse et de protéomique and CNRS, FRE 3206, Molécules de communication et adaptation des microorganismes, Département Régulations, développement et diversité moléculaire, Muséum National d'Histoire Naturelle, CP 54, 57 rue Cuvier, F-75231 Paris Cedex 05, France
[c] INSERM, U 565 and MNHN, USM 503, Département Régulations, développement et diversité moléculaire, Laboratoire des Régulations et dynamique des génomes, CNRS, UMR 5153, Acides nucléiques: dynamique, ciblage et fonctions biologiques, Muséum national d'Histoire naturelle, CP 26, 57 rue Cuvier, F-75231 Paris Cedex 05, France

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ABSTRACT

The microcystin-leucine-arginine toxin (MC-LR) is produced by cyanobacteria that sometimes bloom in water reservoirs. It targets the liver, thus posing potential health risks to human and animals. Microcystin inhibits the protein phosphatases PP1 and PP2A, leading to diverse cellular deregulation processes. A proteomic approach was applied to the medaka fish (*Oryzias latipes*) to obtain an overview of the effects of MC-LR on the liver. As membrane and organelle proteins are major structural and functional components of several cell signalling pathways, we decided to investigate here the membrane and organelle-enriched fractions from the livers of control and MC-LR treated medaka fish. Seventeen proteins were identified by proteomic analysis as being modulated in response to MC-LR treatment. This is the first time for eight of them to be reported as being involved in MC-LR effects: prohibitin, fumarylacetoacetase, protein disulfide isomerase A4 and A6, glucose regulated protein 78 kDa, 40S ribosomal protein SA, cytochrome b5, and ATP synthase mitochondrial d subunit. These proteins are involved in protein maturation or in the response to oxidative stress highlighting the role of organelles in protein processing and the complex cooperation associated with oxidative stress.



## 1. Introduction

Some cyanobacteria can produce harmful toxins such as microcystins (Dawson, 1998; Codd et al., 1999; Falconer and Humpage, 2005). These cyanotoxins have been demonstrated to be fatal to animals or humans mainly by targeting the liver (Teixeira et al., 1993; Pouria et al., 1998; Codd et al., 1999; Carmichael et al., 2001; Fleming and Stephan, 2001; Azevedo et al., 2002; Falconer and Humpage, 2005). So far, this toxin family is known to include 70 variants and one of them, the leucine-arginine variant (LR), is also suspected of being a tumor promoter in liver (Codd et al., 1999; Sekijima et al., 1999; Zhou et al., 2002). As microcystins are widespread throughout the world and sometimes occur in drinking water reservoirs, they have been assessed as a health risk by WHO (World Health Organization). The maximal acceptable level of microcystin-LR in drinking water has been set at 1 μg/L (WHO, 2004).

Microcystins are heptapeptides that are non-ribosomally synthesized (Dittmann and Borner, 2005). Variants of this family of toxins differ from each other by two amino acids and by the methylation of 3-methylaspartic acid and N-methyl-dehydroalanine. Microcystin-LR, the most toxic variant, inactivates the protein phosphatases PP1 and PP2A (Gehringer, 2004), and this in turn leads to deregulation of several cellular processes, since phosphorylation of amino acids in specific protein sequences, is a mechanism for regulating the activity of proteins (Garcia et al., 2003; Trinkle-Mulcahy and Lamond, 2006). Inactivation of these phosphatases is followed by disruption of cytoskeleton, oxidative stress, MAPK deregulation and DNA damages (Ding et al., 2000; Batista et al., 2003; Bouaïcha and Maatouk, 2004; Bouaïcha et al., 2005; Zhu et al., 2005; Jayaraj et al., 2006; Lankoff et al., 2006; Zegura et al., 2006; Komatsu et al., 2007). Thus, this toxin disrupts the physiology of the target cell and can induce two cellular processes: cell death or proliferation.

As cyanobacteria that produce microcystins such as *Microcystis* spp. or *Planktothrix* spp. live in freshwater, fish species at the top of food chains in aquatic ecosystems are considered as natural vertebrate models to study the effects of the toxin and could provide the appropriate bio-indicators to reveal the presence of toxin. Medaka, *Oryzias latipes*, is a small freshwater fish native to South East Asia. It is easy to breed and is commonly used in carcinogenesis and developmental biology (Boorman et al., 1997; Wittbrodt et

* Corresponding author. Tel.: +33 140793126/3195; fax: +33 140793594.
*E-mail address:* medery@mnhn.fr (M. Edery).

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al., 2002). It is also a recommended model in environmental toxicology of cyanotoxins (Jacquet et al., 2004; Huynh-Delerme et al., 2005; Escoffier et al., 2007; Lecoz et al., 2008) and is therefore used in this study.

One way to obtain an integrated view of a response pathway to a toxin is to use global methods such as proteomics. Proteomics provides a complete analysis of all the expressed proteins in a particular cell type or tissue of a species following exposure to a stressor. The most widely used technique in proteomics is two-dimensional (2D) electrophoresis (Freiburghaus, 1994; Fey and Larsen, 2001). This method permits to separate and study the expression of hundreds of proteins in a single experiment.

Only a few proteomic studies have been performed that analyze the effects of microcystin-LR and in most cases mammals have been used as the model organisms (Imanishi and Harada, 2004; Tran et al., 2004; Chen et al., 2005; Fu et al., 2005; Mezhoud et al., 2008a,b). As the main target of microcystin-LR is the liver, we studied the changes in protein expression in this tissue. The complexity of the total protein expression of a specific organ or tissue presents numerous challenges for a comprehensive investigation of the many attributes of protein expression (such as protein sequence identity, quantity, post-translational modification) in a single analysis (Wetmore and Merrick, 2004). To reduce its complexity, the protein extract was subjected to subcellular prefractionation. This step yielded cystosolic, membrane and organelle, nuclear and cytoskeleton fractions. Our previous proteomic studies had focused on the cytosolic fraction of the medaka liver (Mezhoud et al., 2008a,b) and revealed that several proteins involved in different pathways such as the MAPK pathway, oxidative stress, cell structure regulation and metabolism, were all modulated by this toxin. As membrane and organelle proteins are the major structural and functional components of these pathways, mechanism of action of microcystin-LR in the liver of treated medaka was undertaken using 2D gel analysis particularly with the fraction corresponding to membranes and organelles. Changes induced by microcystin-LR in this subcellular fraction were investigated using one-dimensional (1D) electrophoresis gel in a previous study (Mezhoud et al., 2008b). In the study reported here, 17 proteins that display different changes in phosphorylation and expression in response to microcystin exposure were identified using nanoESI-MS/MS and nanoLC-ESI-MS/MS. Most of these proteins are implicated in the oxidative stress response or protein maturation and degradation.

## 2. Materials and methods

### 2.1. Medaka (O. latipes) breeding and gavage

*O. latipes* fish were bred in aquaria filled with 1/3 of tap water and 2/3 of reverse osmosis filtered water. The fish were raised under a 12h/12h L/D cycle in order to prevent them from entering the reproductive state.

The adult fish were separated and anaesthetized in water containing 100 mg/L ethyl 3-aminobenzoate methanesulfonate (Sigma). The medakas then underwent gavage either with 5 μL of water containing phenol red (control fish) or 5 μL of water containing 5 μg microcystin-LR (Alexis® biochemicals) and phenol red (treated fish). Ten fish (control or treated) were included in each batch and were killed 2 h later in ice-cold water in order to analyze their early response to the toxin. This contamination period had been selected after experiments with radiolabelled microcystin indicating that, under the conditions used here, the maximum radioactivity in liver was observed 3 h after gavage. At the high dose used (1000× WHO recommended threshold), the experiments focused on the early hepatocyte response to acute contamination and the proteome of the cytosolic fraction displayed the greatest number of changes 2 h after gavage (Mezhoud et al., 2008b). All experimental procedures complied with European Union regulations concerning the protection of experimental animals.

### 2.2. Subcellular fractionation of fish liver

Fish livers were dissected out in ice-cold Yamamoto's rearing medium (10% NaCl, 0.3% KCl, 0.4% $CaCl_2$, 1.6% $MgSO_4$and 0.01% methylene blue in distilled water). Livers from the same batches were pooled. They were then homogenized with a Dounce homogenizer in extraction buffer I containing protease inhibitor of ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem®). Afterwards, the subcellular fractionation was performed with ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem®) following the manufacturer protocol. The 1D and 2D gels patterns were significantly different between the cytosolic-enriched extracts (Mezhoud et al., 2008a) and membrane/organelles-enriched fractions (the present study) and the proteins that were identified in each study were shown to belong to the corresponding fraction (Table 5).

### 2.3. Preparation of samples for 2D electrophoresis

The proteins present in membrane and organelle fraction were precipitated with trichloroacetic acid at 12%. The samples were incubated at 4° C and centrifuged. The pellets were washed with acetone and resuspended in TUC buffer (2 M thiourea, 7 M urea and 3% CHAPS).

Protein concentration was measured according to Bradford assay and 200 μg of protein was prepared for isoelectrofocalization.

### 2.4. 2D gels

The first dimension was performed on Immobiline™ DryStrip pH 4–7, 7 cm (GE Healthcare) in a Multiphor II electrophoresis unit (GE Healthcare) using the following program: 0–50 V in 2 min, 50 V for 30 min, 50–200 V in 15 min, 200 V for 30 min, 200–2000 V in 30 min, 2000 V for 3 h (maximum current per strip: 25 μA). After equilibration in buffers (6 M urea, 50 mM Tris–HCl pH = 8.8, 2% SDS, 30% glycerol) supplemented with 1% DTT and then 2.5% iodoacetamide, the second dimension was performed on 11% SDS-PAGE gels (acrylamide : bisacrylamide = 29 : 1). PeppermintStick™ phosphoprotein molecular weight standards (Molecular Probes™) and Perfect Protein™ marker (Novagen®) were also loaded on gels as molecular weight markers.

After migration, gels were stained successively with Pro-Q® Diamond and SYPRO® Ruby (Molecular Probes™) to detect phosphorylated proteins or all the proteins, respectively according to the manufacturer's protocol. Gels images were acquired with a Typhoon 9410 (GE Healthcare) at the appropriate wavelength (532 nm and 457 nm).

2D gels images were analyzed with ImageMaster™ 2D Platinum software (version 5.0, Amersham Biosciences). There were four gels for samples from control fish and three gels for samples from treated fish. Spot normalization was done by analyzing the relative volume (volume percentage). For each spot, the mean percentage volume of the spots for control and treated samples were compared by statistical tests with a threshold of 5% (Student and Mann–Whitney tests) in order to identify spots showing a significant change in percentage volume after treatment with microcystin-LR (Mezhoud et al., 2008a). A *F*-test was done before the Student test to check the homogeneity of variance between the control and treated samples.

### 2.5. Protein identification and bioinformatics

Protein spots from the gels corresponding to microcystin-treated samples that had statistically different volumes as