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Microcystin-LR bioconcentration induces antioxidant responses in the digestive gland of two marine bivalves *Crassostrea gigas* and *Mytilus edulis*

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ABSTRACT

Microcystins (MCs) are a major group of potent cyanobacterial toxins found in freshwater and even brackish waterbodies. To understand the putative correlation between bioconcentration of MCs and antioxidant responses of the digestive gland of bivalves, Pacific oyster *Crassostrea gigas* and blue mussel *Mytilus edulis* were exposed to different concentrations (0.1, 1, 10 and 20 $\mu g\,L^{-1}$) of MC-Leucine-Arginine (LR) for seven days. MC-LR bioconcentrated in the digestive glands of both bivalves during exposure period. The levels were slightly reduced when the bivalves were exposed to seawater during depuration (7 days), while approximately 0.1 $\mu g\,L^{-1}$ of MC-LR was observed in the 10 and 20 $\mu g\,L^{-1}$ exposed bivalves at the end of depuration. Intracellular malondialdehyde (MDA) and glutathione (GSH) levels were significantly elevated in the 10 and 20 $\mu g\,L^{-1}$ exposed bivalves at 7 day, and the levels were maintained during depuration in both bivalves. Overall, significant higher levels of enzymatic activities of antioxidant defense systems such as glutathione *S*-transferase (GST), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) were observed in the 10 and 20 $\mu g\,L^{-1}$ exposed bivalves. Interestingly, most of higher levels of Pacific oyster were detected at exposure period, while blue mussel showed higher levels at depuration phase, suggesting a species-specific sensitivity upon MC-LR. These patterns were correlated with the bioconcentration patterns of MC-LR as Pacific oyster was highly accumulated by MC-LR during exposure period, but blue mussel showed prolonged high levels of MC-LR for depuration phase. Our results will be useful to understand species-specific bioconcentration of MC-LR in bivalves and their effects on intracellular oxidative status via accumulation.

1. Introduction

Cyanobacterial blooms have been observed with increasing frequency worldwide in the eutrophic aquatic systems of fresh, brackish and marine waters. Accumulation of cyanobacterial biomass can produce secondary metabolites as a harmful naturally occurring toxin (Carmichael, 1992). Although only about 40 of nearly 2700 described cyanobacterial species were highlighted as being toxic (Hitzfeld et al., 2000; Nabout et al., 2013), the quality of drinking water and risk assessment of emerging cyanotoxins have become critical global public health issues in recent years. Some cyanotoxins are even considered as the most dangerous toxin. Among cyanotoxins, microcystins (MCs) are the most abundant cyclin heptapeptides and the most studied cyanotoxin as the chemically characterized hepatotoxins (Neilan et al., 1999). To date, mainly two variable L-amino acids result in approximately 100 MC congeners with a range of molecular weights from 900 to 1100 Da (Rantala et al., 2004; Zastepa et al., 2015). Of main isoforms such as MC-LR (Leucine-Arginine), MC-RR (Arginine-Arginine), and MC-YR (Tyrosine-Arginine), a hydrophobic variant MC-LR is most common and toxic isoform (WHO, 2003). MC-LR is environmentally stable and can be bioaccumulated in the food chain due to relatively high resistance to biological or chemical degradation (Malbrouck and Kestemont, 2006). MC-LR exerts toxicity by inhibition of protein phosphatases (PP) 1 and 2A, and induction of oxidative stress in the hepatocyte ultrastructure via cytoskeletal dysfunction (MacKintosh et al., 1990; Amado and Monserrat, 2010).

Until recently, the frequent occurrence of cyanobacterial blooms in freshwater ecosystems (e.g. rivers, lakes, reservoirs and aquaculture) has been widely reported due to the detrimental potentials on human health risk. However, environmental surveillance of cyanotoxins and

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food monitoring are scarce in coastal and marine ecosystems yet, even though information on outflows of cyanotoxins to the estuary and coastal regions has been continuously reported (Malbrouck and Kestemont, 2006; Ibelings and Chorus, 2007; Smith et al., 2008; Alvarenga et al., 2015). Thus, the increased observations of MCs demand better understanding of their potential effects on molecular and biochemical mechanisms for sensitivity/toxicity, accumulation, detoxification, metabolism and adaptation of marine animals. Accumulation and/or physiological effects of cyanotoxins were reported in brackish or marine copepods, corals, mussels, fish and mammal (DeMott and Moxter, 1991; Williams et al., 1997; Magalhães et al., 2001; Malbrouck and Kestemont, 2006; Ibelings and Chorus, 2007; Richardson et al., 2007; Smith et al., 2008; Miller et al., 2010). Despite these concerns, sensitivity and toxicity of MC-LR in marine bivalves have received little attention as yet, even though freshwater invertebrates including several mollusks have been used in numerous studies to evaluate the adverse health effects of MC-LR.

Marine bivalves are economically important as major aquaculture species worldwide. As filter-feeders, bivalves ingest suspended nutrients and food particles from water bodies. Subsequently, they can be directly impacted by numerous environmental and/or anthropogenic factors. In fact, bivalves have very complex molecular and biochemical systems in response to excretable water-soluble metabolites and bioaccumulated compounds (Zhang et al., 2016a). The digestive gland is the key metabolic organ for intracellular digestion, digestive enzyme secretion and cellular immunity in mollusks. Also, the digestive gland has important roles for detoxification of both endogenous metabolites and exogenous xenobiotics and for their excretions. In general, the digestive gland is the target organ for main detoxification processes in bivalves (Livingstone et al., 1992). Biochemical responses of antioxidant defense system in the digestive glands are relatively well-defined in bivalves (Viarengo et al., 1991; Livingstone et al., 1992; Doyotte et al., 1997). The primary objective of the present study is to investigate the MC-LR bioconcentration and its effects on the lipid peroxidation and antioxidant defense system in the digestive gland of two bivalves, Pacific oyster *Crassostrea gigas* and blue mussel *Mytilus edulis*. Of bivalves, the genus *Crassostrea* and *Mytilus* are model organisms for biomonitoring of the coastal areas of the world via their huge biomass in both size and density. Thus, numerous studies have conducted from molecular to physiological and ecological levels of individuals and populations of these species.

As one of strong biomarkers for sublethal stress, analysis of functional oxidative modifications of redox-sensitive metabolites (i.e. molecules of the antioxidant system) or proteins is considered to be a promising tool upon diverse environmental changes and anthropogenic threats (Lesser, 2006; Zhang et al., 2016a). For this reason, our study focused on potential oxidative effects of MC-LR accumulation in two model bivalves by analyzing the response of systematic antioxidant defense system. Our results will provide important information to better understand the intracellular dynamics of sub-lethal concentration of MCs on the biochemical and physiological metabolism in marine bivalves.

2. Materials and methods

2.1. Culture and chemical exposure

The Pacific oyster *C. gigas* and the blue mussel *M. edulis* used in this study was collected from the Integrated Multi-Trophic Aquaculture (IMTA) system of Southeast Sea Fisheries Research Institute (National Institute of Fisheries Science; NIFS) in Tongyeong, Gyungnam, South Korea. This IMTA site is leased to Southeast Sea Fisheries Research Institute of National Institute of Fisheries Science (NIFS), Korea. No permission was required for sample collections. Both bivalve spp. are neither endangered nor protected species. Adult oysters (≈95.4 ± 16.2 mm in length) and mussels (≈104.4 ± 14.5 mm in length) were reared in filtered seawater with aeration at the automated culture system of Southeast Sea Fisheries Research Institute. The environmental conditions were maintained at 30 practical salinity unit (psu), 20 °C and 16 h: 8 h L:D photoperiod. All the experiments using bivalves were approved by the animal care and use committee of NIFS.

MC-LR was purchased from Cayman Chemical (MI, USA; purity > 95%) and was dissolved in miliQ water (10 mg L^{-1}). Although there is very limited information available on environmental concentration of MC-LR in estuary or coastal regions, MC-LR can be detected up to μg L^{-1} level by MC-contaminated runoff from freshwater (Miller et al., 2010; Gibble and Kudela, 2014; Paldavičienė et al., 2015). In this study, two bivalve species were exposed to different concentrations of MC-LR (0.1, 1, 10 and 20 μg L^{-1}) in 60 L filtered seawater (100 L glass aquaria; ≈2 L per animal) at 20 °C with constant aeration for seven days. In each exposed oyster or mussel group, four specimens were collected at regular intervals (1, 3, 5 and 7 days) during exposure and depuration periods, and dissected for digestive gland preparation. Each bivalve sample was not pooled, and four samples for each concentration were individually analyzed as biological replicates. During the experiment, half of the filtered seawater with an addition of equivalent concentration of MC-LR was changed every 24 h. They were fed daily with a microalgae suspension (4×10^6 cellules mL^{-1} containing *Dunaliella* sp., *Isochrysis* sp. and *Tetraselmis* sp.) in both exposure and depuration periods.

2.2. MC-LR analysis

Free MC-LR contents in the digestive gland of each bivalve per dry weight (dw) were measured using High-Performance Liquid Chromatography (HPLC; Agilent 1260 Infinity, Palo Alto, CA, USA) analysis in the Advanced Science & Technology Center of Yonsei University (Seoul, South Korea). Overall methods were followed to the previous study with slight modifications (e.g. buffer volume, employed basic instruments) (Xie and Park, 2007). Lyophilized digestive gland samples were sonicated in 10 mL of HPLC grade BuOH:MeOH:H_2O (1:4:15, v/v/v) on an ice bath at 3000 rpm for 90 s. After homogenization, the samples were centrifuged at 10000 rpm at 4 °C for 15 min. Free MC-LR was extracted three times with 10 mL of the same solution, and the supernatant was transferred to an Oasis HLB cartridge (60 mg, Waters Corp., Milford, MA, USA) which was pre-activated with 3 mL of 100% MeOH and 10 mL of ultrapure water. The MC-LR was eluted from the cartridges with 10 mL of 100% aqueous methanol containing 0.1% trifluoroacetic acid (TFA). The eluent was then evaporated to dryness using N_2, and the residue was re-dissolved in 100% MeOH. Subsequently, the total supernatant was isolated by a silica gel cartridge (2 g)/plus silica gel (0.69 g) tandem cartridge (Waters Corp.) that was pre-activated by 100% MeOH. The column containing the MC-LR was washed with 100% MeOH and eluted with 70% MeOH. This elution fraction was also evaporated to dryness and the residue then dissolved in 100% MeOH for HPLC analysis according to the technical team of the Advanced Science & Technology Center of Yonsei University.

2.3. Malondialdehyde (MDA) analysis

Approximately 43 ± 4.5 mg of digestive gland tissue was sampled for each bivalve specimen. We followed a previously reported MDA analysis assay for oyster (*C. gigas*) and mussel (*M. edulis*) tissues (Geret et al., 2002). Tissues were homogenized in 5 vol of buffer (20 mM Tris, 150 mM NaCl, 10 mM β-mercaptoethanol, 20 μM leupeptin, 2 μM aprotinin and 100 μM benzamidine). After centrifugation for 30 min at 30,000*g* (4 °C), supernatants were heat-denatured for 15 min at 75 °C. Thiobarbituric acid reactives (TBARs) were measured at 535 nm with a Thermo Varioskan Flash spectrophotometer (Thermo Fisher Scientific, Tewksbury, MA, USA) using malonaldehyde bis (tetrametoxypropan, Sigma-Aldrich, Inc.) as standard. The concentration of lipid