Comparative Biochemistry and Physiology, Part C 157 (2013) 54–62

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Transcriptional response of lysozyme, metallothionein, and superoxide dismutase to combined exposure to heavy metals and bacteria in *Mactra veneriformis*

Yan Fang [a,b], Hongsheng Yang [a,*], Baozhong Liu [a], Libing Zhang [a]

[a] Institute of Oceanology, Chinese Academy of Sciences, Qingdao, 266071, China
[b] Shandong Marine Fisheries Research Institute, Shandong Provincial Key Laboratory of Restoration for Marine Ecology, Yantai, 264006, China

ARTICLE INFO

Article history:
Received 31 July 2012
Received in revised form 3 October 2012
Accepted 3 October 2012
Available online 8 October 2012

Keywords:
Clam
Mollusk
Lysozyme
Metallothionein
Superoxide dismutase
Heavy metals
Bacteria
Mactra veneriformis

ABSTRACT

The response of the defense components lysozyme (LYZ), metallothionein (MT), and superoxide dismutase (SOD) to combined exposure to heavy metals and bacteria was assessed at transcriptional level in the surf clam *Mactra veneriformis*. First, the full-length LYZ cDNA containing 808 nucleotides and encoding 194 deduced amino acids was identified from the clam. Multiple alignments revealed that MvLYZ had a high identity with invertebrate-type LYZs from other mollusks. Next, clams were exposed to *Vibrio parahaemolyticus* and a mixture of cadmium and mercury, alone or in combination, for 7 days. Cumulative mortality of clams and mRNA expressions of the three defense components were analyzed. The highest cumulative mortality took place in the combined treatment on day 7. The expression of the three genes was up-regulated in response to treatments compared to the control with different response times and transcriptional levels; the response to combined exposure occurred earlier than to single exposure. Among the experimental groups, MvLYZ expression and MvSOD expression peaked in the combined treatment on day 3, whereas MvMT expression peaked in heavy metals treatment on day 5. Furthermore, interactive effects of heavy metals and *Vibrio* on transcriptional response changed over the exposure time. Therefore, transcriptional regulation of the three genes under combined exposure was more complex than under single exposure.

1. Introduction

Costal environments are imperiled by anthropogenic inputs of waste products from agricultural, chemical, and industrial processes. Heavy metals, which are dominant pollutants, have increased in the marine environment due to their wide usage in industry and manufacturing (Paul-Pont et al., 2010). They can accumulate within the tissues of marine organisms and consequently lead to adverse effects. In addition to anthropogenic stressors, organisms in coastal waters are exposed to a variety of natural stressors. As one type of natural stressors, pathogens cause infectious diseases in aquatic organisms. In recent years, the combined effects of pathogens and pollutants on the health of marine organisms have raised concern (Morley, 2010).

Bivalves are used worldwide as sentinel species for monitoring coastal environments due to their ease of sampling, sessile habits, high filtering rate, and great efficiency in bioaccumulating pollutants (Kwan et al., 2003; Santovito et al., 2005). Certain bivalves, such as clams, oysters, and scallops, are important aquaculture species with high economic value. However, mass mortalities of bivalves have been reported in China, Japan, Korea, and the European Atlantic (Gómez-León et al., 2005; Yue et al., 2010), resulting in large economic losses. Pollutants can impair the defense system of organisms and thus increase host susceptibility to pathogens (Pipe and Coles, 1995). Thus, the prevalence of pathogens and pollutants is an important suspected cause for mass mortalities (Zhang et al., 1999). Accordingly, identifying the defense mechanisms of the bivalves in response to combined exposure to pathogens and pollutants is essential for health management and disease control.

Defense effectors in marine animals, such as lysozyme (LYZ, EC 3.2.1.17), metallothionein (MT), and superoxide dismutase (SOD, EC 1.15.1.1), can respond to stressors and protect organisms from serious damage. LYZ is involved in innate immunity against infection by bacterial pathogens and in digestion of marine bacteria (Cheng et al., 1975; Prager and Jollès, 1996; Xue et al., 2007). It has been reported that the expression of bivalve lysozymes can be regulated in response to some pathogens (Li et al., 2008; Zhao et al., 2010). The activities of LYZs in bivalves and polychaetes have been proposed as biomarkers of aquatic pollution because LYZs can respond sensitively to xenobiotics (Marcano et al., 1997; Oliver and Fisher, 1999). However, few studies have examined the transcriptional response of LYZ to xenobiotics and especially to combined exposure to pathogens and pollutants.

MT is a cysteine-rich, low-molecular-weight protein with high affinity for metals, thus it plays an important role in metal metabolism,

* Corresponding author at: Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Nanhai Road 7, Qingdao 266071, Shandong Province, China. Tel./fax: +86 532 82898582.
E-mail addresses: hshyang@ms.qdio.ac.cn, hshyang@126.com (H. Yang).

1532-0456/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cbpc.2012.10.002

detoxification of heavy metals, immune response, and the antioxidant process (Davis and Cousins, 2000; Serafim and Bebianno, 2009). SOD acts as the first defense against oxidative stresses. Many factors, such as exposure to heavy metals, bacteria challenge, and temperature, can modify the expression of MT and SOD. Hence, researchers have suggested the use of MT and SOD levels in organisms as early warning bioindicators of aquatic pollution (Amiard et al., 2006; Kim et al., 2011).

Despite the common co-existence of pathogens and pollutants in coastal waters, many studies have concentrated on an organism's response to a unique stressor, and studies of combined exposure are rare. Because interactions between pathogens and pollutants are synergistic, antagonistic, or additive depending on the nature of the pollutant and the host–pathogen system (Desclaux-Marchand et al., 2007), extensive work is needed to elucidate the defense mechanisms used by organisms under conditions of combined exposure.

The most commonly used bivalves in biomonitoring programs are mussels and oysters, which are not widely distributed in tidal flats as the lack of hard substrates necessary for attachment. There are large areas of tidal flats in the world, especially in China. Hence, exploring new species is needed for monitoring environment in tidal flats. The surf clam *Mactra veneriformis* is an infaunal suspension-feeding bivalve that is ubiquitous and abundant in tidal flats in China, Korea, and Japan. The previous study has shown that a large quantity of heavy metals could accumulate in the tissues of *M. veneriformis* (Wang et al., 2005). Additionally, MT and SOD levels in this clam have been proposed as biomarkers of heavy metal pollution in the marine environment (Fang et al., 2010, 2012). Therefore, studies of the response of *M. veneriformis* to multiple stressors would benefit our national monitoring programs and the current state of health management of bivalves.

Considering the recent cadmium (Cd) and mercury (Hg) contamination along the Chinese coast (Meng et al., 2008; Wang et al., 2009a) and the importance of the genus *Vibrio* as a cause of disease in cultured bivalves, the present study focused on the response of *M. veneriformis* to combined exposure to Cd, Hg, and *Vibrio*. Specifically, the full-length cDNA of MvLYZ was cloned, and mRNA expressions of MvLYZ, MvMT, and MvLYZ under combined exposure were examined in order to assess the transcriptional response of defense components in relation to multiple stressors.

2. Materials and methods

2.1. Clams and exposure

Surf clams (*Mactra veneriformis*, Bivalvia: Mactridae) (average shell length: 3.33 ± 0.34 cm; mass: 13.21 ± 1.93 g) were obtained from a local market and acclimated in our laboratory for 1 week. The water temperature of the experiment was increased from 14 °C to 20 °C step-by-step with a 2 °C daily increase using a thermostatic control. The clams were then acclimated at 20 °C for 5 days.

After acclimatization, 50 clams were transferred to plastic aquaria filled with 20 L of filtered natural seawater (control) or treated seawater (treatments). The three treatments were *Vibrio* exposure, heavy metals exposure, and combined exposure to *Vibrio* and heavy metals. Six replicates were carried out for each experimental condition including both the treatment and the control group. Three of the replicates of each experimental group were used to calculate clam mortality and the rest three were used to analyze mRNA expression.

Vibrio parahaemolyticus (MM21) used in the present study was isolated from the digestive gland of the clam *Meretrix meretrix* by Yue et al. (2010). This bacterial strain was incubated in liquid TSAYE broth at 28 °C until the OD value of the colonies at 600 nm reached 0.7. The bacteria then were centrifuged (10 min, 2000 *g*) and resuspended in seawater. The clams used in the *Vibrio* treatment were exposed to bacteria via immersion by adding MM21 to the seawater at a final concentration of 1.2×10^{10} CFU·L^{-1}.

For the heavy metals exposure, $CdCl_2 \cdot 2.5H_2O$ and $HgCl_2$ were added to the seawater at final concentrations of 200 μg·L^{-1} and 20 μg·L^{-1}, respectively. These dosages have been used as sublethal concentrations in previous studies of *M. veneriformis* (Fang et al., 2010, 2012).

For the combined exposure, clams were exposed to 1.2×10^{10} CFU·L^{-1} MM21, 200 μg·L^{-1} Cd, and 20 μg·L^{-1} Hg. The concentration of each stressor was the same as that used for the single exposure treatment.

Throughout the experiment, the water was constantly aerated (dissolved oxygen 7.7–8.3 mg/L; pH 7.9–8.1; salinity 32; temperature 20 °C) and changed daily. The clams were fed 2 h before the water change, and the corresponding concentrations of heavy metals and bacteria were added after the water change.

After 0, 1, 3, 5, and 7 days of exposure, the digestive glands of the clams for mRNA expression analysis were collected from three replicates of each experimental group. In each replicate per experimental group, three clams were pooled as one sample replicate, and there were three sample replicates for each time point. Thus, in the end of the experiment, 45 clams (3 calms × 3 sample replicates × 5 time points) were used in each replicate per experimental group. In addition to the digestive gland, the gill, mantle, foot, and adductor muscle were also isolated from the clams in the control group at day 0 for analysis of tissue-specific MvLYZ expression. All the samples were frozen in liquid nitrogen, and stored at −80 °C.

2.2. Cloning the full-length cDNA of MvLYZ

The full-length cDNA of MvLYZ was identified by reverse transcription PCR method, homology-based cloning strategy, and rapid amplification of cDNA 3′ and 5′ ends (3′ and 5′ RACE) technique. Briefly, total RNA was extracted from the digestive gland using a Trizol kit (Invitrogen, Carlsbad, CA, USA). Then, cDNAs were synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA) with total RNA as a template. PCR amplification was performed to obtain the fragment of MvLYZ cDNA with degenerate primers (LYZF1 and LYZ R1, Table 1), which were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) based on the highly conserved regions from other bivalves. The PCR program was set at 94 °C for 5 min, followed by 35 cycles of 94 °C for 20 s, 47 °C for 30 s, 72 °C for 45 s, and the final extension step at 72 °C for 10 min in a 25 μL reaction volume. Subsequently, the full-length cDNA of MvLYZ was obtained using 3′ and 5′ RACE with four gene-specific primers (LYZF2, LYZF3, LYZR2, LYZR3) designed based on the obtained fragments. Details are provided in our previous study (Fang et al., 2010).

Table 1
Primers used in this study.

Primer name	Sequence(5′→3′)
LYZF1 (forward)	GTTGGYTCNBTGTCSTGCGG
LYZR1 (reverse)	CCHCTTGGDCCDCCRTTATG
LYZF2 (forward)	GCTTACTTCAAAGATTGTGGCAT
LYZR2 (reverse)	TTCCCTGGCATATCCTTCACAT
LYZF3 (forward)	GGGAAAGAGTGGAAATCCTGTG
LYZR3 (reverse)	CACATGTAGCAGGACATTTATTACG
LYZF4 (forward)	GGGAAAGAGTGGAAATCCTGTG
LYZR4 (reverse)	TTCCCTGGCATATCCTTCACAT
MTF (forward)	ATTGTGCAGGAAAGTGTGATTGT(Fang et al., 2010)
MTR(reverse)	AGGAGCAGTCGGTTCCACAT(Fang et al., 2010)
SODF (forward)	CACATTTCAATCCCGGTGGTA(Fang et al., 2010)
SODR(reverse)	AGCATGCACCACAACTGTCCT(Fang et al., 2010)
ActinF (forward)	TCGGCAGTGGTTGTGAAAGAGT(Fang et al., 2010)
ActinR (reverse)	GATTGGATTTGGCTGGTAGAGAT(Fang et al., 2010)

Y = CT; R = AG; W = AT; V = ACG; H = ACT.