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Proteomic and metabolomic responses in D-shape larval mussels *Mytilus galloprovincialis* exposed to cadmium and arsenic

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ABSTRACT

Cadmium (Cd) and arsenic (As) are the main metal/metalloid contaminants in the coastal environments of the Bohai Sea, China. In this work, a combined proteomic and metabolomic approach was applied to investigate the biological effects of Cd and As (V) in the early life stage (D-shape larvae) of mussel *Mytilus galloprovincialis*. Results indicated that Cd was a potential immune toxicant to D-shape larval mussel because of the numerous proteomic responses related to immune system. Additionally, Cd induced oxidative stress, cellular injury and disturbance in nucleic acid metabolism in D-shape larval mussels. However, only two identified proteins were significantly altered in As (V)-treated group, suggesting that D-shape larval mussel was less sensitive to As (V) than to Cd at protein level. These two proteins in response to As (V) suggested that As (V) influenced anti-oxidative system and cell proliferation in D-shape larval mussels. Metabolic responses indicated that Cd and As (V) induced disturbances in osmotic regulation and energy metabolism in D-shape larval mussels via different metabolic pathways. In addition, Cd reduced lipid metabolism as well. This work demonstrated that a combination of proteomics and metabolomics could provide an insightful view in the biological effects of pollutants in mussel *M. galloprovincialis* at an early life stage.

1. Introduction

As it is known, there are numerous metal (e.g., zinc, gold) mining and smelting factories along the Bohai coast, which has led to severe metal pollution in the Bohai marine and coastal environments. Mu et al. (2009) found that Cd and As were the dominant metal pollutants in the sediments from the Laizhou Bay [1]. In the mussels collected from the Bohai Sea in China, a survey reported that the mussels from some of the sampling sites were polluted by Cd and As with average concentrations at ~2 and 5 μg/L, respectively [2]. In our recent study, the Cd and As pollution have been confirmed by the high accumulations of Cd and As in marine shrimp *Crangon affinis* collected from the Yellow River Estuary and Laizhou Bay along the Bohai coast, respectively [3]. Although Cd is relatively rare in marine environment, the concentration of Cd has reached 50 μg/L in some heavily polluted estuaries or harbors and ports [4]. In open ocean seawater, the concentration of total arsenic is usually around 1 μg/L and As (V) is the dominant form of inorganic arsenic [5].

Both Cd and As are known carcinogens and environmental toxicants inducing multiple toxicities in animals. Cd may inactivate many metabolic enzymes by competing for the catalytic sites with other metals [6,7]. In addition, both Cd and As can induce oxidative stress in animals by producing excessive reactive oxygen species [8]. Since Cd and As have become severe metal/metalloid contaminants in the Bohai coast, it is necessary to characterize the biological effects of Cd and As in marine animals.

As two main -omic approaches in system biology, proteomics and metabolomics, have been well-established and successfully applied in marine biology [9,10]. Since proteomics and metabolomics can directly characterize the minor metabolite and protein responses, these two −omic techniques and their combination have represented their practicability to elucidate the biological effects and mechanisms of pollutants in environmental bioindicators. Especially, a combination of proteomics and metabolomics could validate and complement one another, when testing the biological effects of environmental pollutants [11,12].

Marine mussels are widely used as bioindicators in

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environmental monitoring and assessment programs because of their sessile filter, feeding lifestyle and high accumulation for organic and inorganic pollutants in the marine environment [13]. Usually, the adult mussels are applied in either ecotoxicology or environmental monitoring programs. Due to the immature defense system, however, the early life stages of animals are more sensitive than adults, which may present differential responsive profiles to environmental pollutants [14]. To our knowledge, the responses of early life stages of mussels under Cd and As (V) stresses were not fully characterized. Consequently, it is necessary to elucidate the biological effects and responsive mechanisms in the early life stage of mussel exposed to the environmentally relevant concentration of Cd and As (V). In this work, we planned to use the early life stage, D-shape larval mussel *M. galloprovincialis*, as the experimental animal to elucidate the biological effects of Cd and As at an environmentally relevant concentration (5 μg/L). Both two-dimensional gel electrophoresis (2-DE)-based proteomics and proton nuclear magnetic resonance (^{1}H NMR)-based metabolomics were conducted on the D-shape larval mussel samples to elucidate the biological effects of Cd and As (V) in the early life stage (D-shape larvae) of mussel *M. galloprovincialis*.

2. Materials and methods

2.1. Larvae breeding and experimental design

Adult mussels *M. galloprovincialis* reached sexual maturity were collected in April 2015 from a less disturbed site (Yantai, China). All mussels were transported to the laboratory and acclimatized in aerated natural seawater (salinity 31 psμ) at 21 °C for 7 days. After acclimatization, these mussels were kept in the air in dark place for 2 h. Then each mussel was put into a beaker containing warmer seawater (24 °C). After 2 h, the majority of mussels came to ovulation and spermiation. The 500 mesh sieve screen was used to filter impurities. Then the sperms and eggs were quickly collected to fertilize. All the fertilized eggs were then transferred into normal filtered seawater (50 L) in a bucket. Continuous aeration was conducted during incubation. After approximately 48 h, the majority of fertilized eggs developed into D-shape larvae (Fig. 1). Then the D-shape larval mussels were divided into three groups (control, Cd (as $CdCl_2$) and As (V) (as Na_3AsO_4) exposures) each containing 8 buckets (50 L) with a density of ~25 D-shape larval per milliliter. The environmentally relevant concentration (5 μg/L) of Cd and As was used for the exposures of D-shape larval mussels *M. galloprovincialis*. During the acclimatization and exposure periods, all the D-shape larvae were kept under a photoperiod of 12 h light and 12 h dark, and fed with the *Chlorella vulgaris* daily. In control and treatment groups, all the D-shape larval mussels were not significantly different in morality and morphology. After exposure for 48 h, all the larvae (~1.0–1.3 × 10^6 individuals) from each bucket were immediately filtered out by 500 mesh sieve screen and collected into two larvae samples for proteomic and metabolomic analysis, respectively. These D-shape larval mussel samples were quickly snap-frozen in liquid nitrogen and stored at −80 °C.

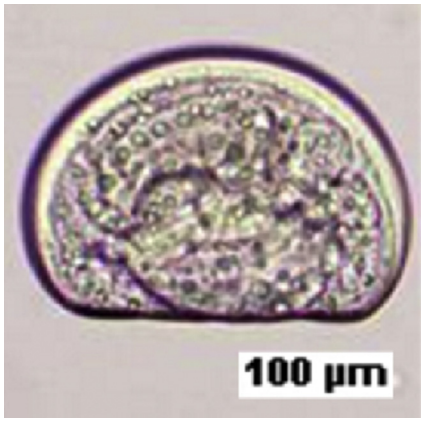

Fig. 1. A representative image of a D-shape larval mussel *M. galloprovincialis* from control group.

2.2. Metabolite and protein extraction

Polar metabolites in D-shape larval mussels (n = 8 for each treatment) were extracted by the extraction protocol with some modifications [15]. All the D-shape larval mussel samples (ca. 100 mg wet weight) were homogenized and extracted in 4 mL/g of methanol, 5.25 mL/g of water and 2 mL/g of chloroform. The methanol/water layer with polar metabolites was transferred to a glass vial and then dried in a centrifugal concentrator. The extracts of soft tissue were then re-suspended in 600 μL of phosphate buffer (100 mM Na_2HPO_4 and NaH_2PO_4, including 0.5 mM TSP, pH 7.0) in D_2O. The mixture was vortexed and then centrifuged at 3000*g* for 5 min at 4 °C. The supernatant substance (550 μL) was then pipetted into a 5 mm NMR tube prior to NMR analysis.

Total protein extraction was performed based on previous studies with some modifications [16,17]. Briefly, each D-shape larval mussel samples was homogenized quickly on ice with 1 mL of TRIzol reagent and centrifuged at 12000*g* for 5 min at 4 °C. The supernatant was added with 200 μL of chloroform before shaking vigorously for 3 min and precipitating for 3 min, then the mixture was centrifuged at 12000*g* for 15 min at 4 °C, and its upper aqueous layer was discarded. A volume of 300 μL of absolute ethyl alcohol was added and the mixture was allowed to stand for 3 min at room temperature before being centrifuged at 2000*g* for 5 min at 4 °C. The phenol/ethanol supernatant was precipitated for 30 min at room temperature by the addition of 750 μL of isopropanol prior to centrifugation at 14000*g* for 10 min at 4 °C. Pellets obtained were washed with 1 mL of ethanol (v/v 95%) and centrifuged at 14000*g* for 10 min at 4 °C. This procedure was repeated twice. The pellets were solubilized in the lysis buffer (7 M urea; 2 M thiourea; 4% m/V CHAPS; 65 mM DTT and 0.2%W/V Bio-lyte buffer) and then incubated for 3 h at room temperature. The homogenate was centrifuged at 15000*g* for 10 min and the supernatant was applied to electrophoresis. The total concentrations of proteins were determined by Protein Assay Kit of TianGen.

2.3. ^{1}H NMR spectroscopy

Metabolite extracts of D-shape larval mussel samples were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 25 °C) as described previously [18]. All ^{1}H NMR spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin (version 2.1, Bruker).

2.4. Spectral pre-processing and multivariate analysis

All one dimensional ^{1}H NMR spectra were converted to a data matrix using the custom-written ProMetab software in Matlab version 7.0 (The MathsWorks, Natick, MA) [18]. Each spectrum was segmented into bins with a width of 0.005 ppm between 0.2 and 10.0 ppm. The bins of residual H_2O between 4.70 and 5.20 ppm were excluded from all the NMR spectra. The total spectral area of the remaining bins was normalized to unity to facilitate the comparison between the spectra. All the NMR spectra were generalized