



Differential biological effects in two pedigrees of clam *Ruditapes philippinarum* exposed to cadmium using iTRAQ-based proteomics

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ABSTRACT

Due to the industrial discharges, cadmium (Cd) has been one of typical heavy metal pollutants in the Bohai Sea. Manila clam *Ruditapes philippinarum* is frequently used for pollution biomonitoring and consists of several pedigrees, of which White and Zebra clams are the dominant pedigrees along the Bohai Sea coast. However, limited attention has been paid on the differential biological effects in different pedigrees of clam to heavy metals. In this work, the proteome profiling analysis was performed to reveal the differential proteomic responses in White and Zebra clams to Cd exposure (200 µg/L) for 48 h, followed by bioinformatical analysis. The proteomic investigations showed that Cd treatment induced more differentially expressed proteins (DEPs) in White clam samples than in Zebra clam samples. Based on the DEPs, we found that some key biological processes consisting of immune response and metabolism were commonly induced in both two pedigrees of clam. Uniquely, some processes related to cellular signaling, proteolysis and energy production were enhanced in Cd-treated White clam samples. Comparatively, the depletion in some unique processes on proteolysis and energy production was elicited in Cd-treated Zebra clam samples, as well as disorder in gene expression. Moreover, Cd exposure caused increases in CAT and POD activities in White clam samples and decreases in SOD and CAT activities in Zebra clams samples, which were consistent with the proteomic responses. Overall, these findings confirmed the differential biological effects of White and Zebra clams to Cd treatment, suggesting that the pedigree of animal should be taken into consideration in ecotoxicology studies.

1. Introduction

Due to the anthropogenic activities, metal pollution has been a major threat to the health of biota in marine and coastal ecosystems. With the rapid development of industry surrounding the Bohai Sea, cadmium (Cd) has become one of the most serious metal pollutants in the Bohai marine environment (Luo et al., 2013; Ji et al., 2016b). As a nonessential element, Cd has been reported to endanger the growth and development of aquatic life (Fernandez-Leborans and Herrero, 1999; Wang and Rainbow, 2006; Qu et al., 2013), which has raised great

concern over marine ecosystem health.

Marine invertebrates, especially marine bivalves, are frequently used as test species for conducting toxicity experiments since they are mostly in benthic habitat and filter feeders (Rittschof and McClellan-Green, 2005). As one of the most important economic species in marine aquaculture in China, Manila clam *Ruditapes philippinarum* is widely distributed along the Bohai Sea coast and has a high tolerance to environmental changes. Furthermore, *R. philippinarum* is a preferred bioindicator in the “Mussel Watch Programs” for pollution biomonitoring. Therefore, this species is often used as an experimental animal

Abbreviations: DEPs, differentially expressed proteins; CAT, catalase; POD, peroxidase; SOD, superoxide dismutase; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; BP, biological process; MF, molecular function; CC, cellular component; iTRAQ, isobaric tags for relative and absolute quantification; ICP-MS, inductively coupled plasma mass spectrometry; MAPK, mitogen-activated protein kinase

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model on pollutant-induced biological effects (Moschino et al., 2012; Wu et al., 2013; De Marchi et al., 2017). It is worth noting that there are several pedigrees (White, Zebra, Liangdao Red and Marine Red) of clam distributed in the marine environment in China, of which White and Zebra clams are two dominant pedigrees and always found to be co-existed in the Bohai Sea region, showing no significant geographical distribution (Zhang et al. 2008). In studies on stress response, the responses of animals to pollutants treatment may differ depending on its size, sex and strains or pedigrees (Wu et al., 2011; Soffker et al., 2012; Musasia et al., 2013; Ji et al., 2016a; Xu et al., 2016). Previous studies have revealed that tolerances to environmental stressors varied in species or pedigrees (Masood et al., 2016; Liu et al., 2013a,b). With regard to the multiple pedigrees of clams, Yan et al. (2005) reported that the Zebra clam had higher survival rate and tolerance to high temperature than other pedigrees of clam. Moreover, differential metabolic responses to heavy metals between White and Zebra clam were also found. Liu et al. (2011) utilized metabolomics to investigate the differential responses of the gill tissue from different pedigrees of clam to mercury treatment, and concluded that White clam was the preferable bioindicator for marine mercury monitoring.

Traditional ecotoxicology studies on stress response often focused on a bottom-up approach to understand the effects of environmental stressors (Kultz, 2005). Selected biomarkers, such as a few genes, proteins, or biochemical reactions, are studied at a time, which fails to explore the global profiles related to the biological perturbations induced by environmental stressors in organisms (Garcia-Reyero and Perkins, 2011). With the development of system biology, the -omic techniques, including genomics, transcriptomics, proteomics and metabolomics, enable researchers to comprehensively profile one type of molecules such as genes, proteins and metabolites and their alterations to characterize the biological responses with high-throughput analysis. Among these -omic techniques, proteomics is known to provide a means to study the changes occurring at the level of the proteome including the protein homeostasis and responses to both the ontogenetic events and external environment in organisms (Tomanek, 2011). Hence, proteomic methodologies can be used not only to unravel mechanisms underlying toxicological effects of stressors but also to identify new candidate biomarkers (Lemos et al., 2010). To date, however, no attention has been paid to the differential biological effects in response to heavy metals at protein level between different pedigrees of clam.

In this study, we used iTRAQ-based proteomics to compare the proteomic profiling of White and Zebra clams exposed to Cd for 48 h, followed by bioinformatical analysis consisting of gene ontology (GO) annotation, Kyoto encyclopedia of genes and genomes (KEGG) pathway and related enrichment analysis, aiming to elucidate the differential mechanisms of responses of these two pedigrees of clam to Cd treatment at protein level.

2. Materials and methods

2.1. Experimental animals and conditions

A hundred and sixty adult clams *R. philippinarum* consisting of White and Zebra clams (shell length: 3.67 ± 0.10 cm and each pedigree consisting of 80 individuals) were purchased from a local culturing farm (Qingdao, China). After transported to the laboratory, the animals were acclimatized in aerated natural seawater (25 °C, 33 psu) in the laboratory for 1 week and fed with the *Chlorella vulgaris* at a ration of 2% tissue dry weight daily. After acclimation, clams of each pedigree ($n = 80$) were randomly divided into 2 groups (control and Cd-treatment groups). Each group was carried out 4 replicates in 4 independent tanks, and 10 individuals were included per replicate tank. The clams in the Cd-treatment group were exposed to one sublethal concentration (200 µg/L) of Cd. After exposure for 48 h, the digestive gland tissues were dissected quickly and flash-frozen in liquid nitrogen, and then stored at -80 °C. For each pedigree, the individuals used for the follow-

up assays were randomly selected from 4 replicates of each group.

2.2. Cadmium concentrations in digestive gland tissues

The digestive gland samples ($n = 5$) of clams were dried at 80 °C to constant weights, followed by being digested in concentrated nitric acid (70%, Fisher Scientific) using a microwave digestion system (CEM, MAR5). Then, the samples were heated in the microwave oven (heating to 200 °C and holding at 200 °C for 15 min). All completely digested samples were diluted with ultrapure water for the quantification of Cd using inductively coupled plasma mass spectrometry (ICP-MS) technique (Agilent 7500i, Agilent Technologies Co. Ltd, Santa Clara, CA, USA). GBW08571 Marine mussel tissue (State Bureau of Technical Supervision, People's Republic of China) was employed as a certified reference material for metal analysis in clams (Li et al., 2012). The recovery of target elements, as tested by three individual spiking experiments, was restricted within 95.5%–104.3% for Cd. Student's *t*-test was conducted on the data of Cd concentrations in digestive gland samples from control and Cd treatment groups, and a *p* value < 0.05 was considered statistically significant.

2.3. Measurement of antioxidant enzyme activities

The digestive gland samples of clams ($n = 6$) were firstly homogenized by adding phosphate buffer solution (0.1 M, pH 7.4). Then the antioxidant enzyme activities in the digestive gland samples were measured using a multimode reader (Infinite M200, TECAN, Switzerland) according to the manufacturer's protocols for enzyme kits (Jiancheng, Nanjing, China) with some modifications. In this work, the reaction system was minimized by 5 times and all the samples loaded on the 96-well plate were simultaneously determined using a multimode reader. The antioxidant enzymes for the activity measurement consisted of superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6). In details, the SOD activity was assayed spectrophotometrically at 550 nm by use of the xanthine and xanthine oxidase system. One unit of SOD activity was defined as the amount of SOD required for 50% inhibition of the xanthine and xanthine system reaction in 1 mL reaction volume per milligram of protein at 37 °C. The POD activity was measured by evaluating the rate of oxidation of pyrogallol, indicated by increase in absorbance at 420 nm. One unit of POD was defined as the amount that formed 18 µM purpurgallin from pyrogallol per milligram protein per minute at 37 °C. The CAT activity was determined by measuring the residual H₂O₂ after incubation with enzyme extract, indicated by the decrease in absorbance at 405 nm. One unit of CAT activity was defined as the amount of CAT required for decomposing 1 µM H₂O₂ per milligram protein per second at 37 °C. All the enzyme activities were expressed as U mg⁻¹ protein. Protein concentration was determined by the Coomassie brilliant G-250 dye-binding method with bovine serum albumin as standard (Bradford, 1976). Student's *t*-test was conducted on the data of antioxidant enzyme activities in digestive gland samples from control and Cd treatment groups, and a *p* value < 0.05 was considered statistically significant.

2.4. Quantitative proteomic analysis

Each pooled digestive gland sample (from 10 individuals) was ground into powder in liquid nitrogen and then dissolved in lysis buffer (9 M urea, 4% CHAPS, 1% w/v DTT and 1% IPG buffer) with protease inhibitor (Roche Applied Science, Mannheim, Germany) to extract proteins. The concentrations of the protein extracts were determined using the Bradford method (Bradford, 1976). iTRAQ technique was applied to quantitative proteomic analysis. Briefly, 100 µg of protein extracted from each sample was dissolved in a dissolution buffer (AB SCIEX, MA, USA). After being reduced, alkylated and trypsin-digested, the protein samples were labeled using iTRAQ 8-plex reagents (AB

SCIEX, MA, USA) according to the manufacturer's protocol. Eight samples (two biological replicates per treatment) were labeled with the iTRAQ tags as control group of White clam (113 tag and 117 tag), Cd-treated group of White clam (114 tag and 118 tag), control group of Zebra clam (115 tag and 119 tag) and Cd-treated group of Zebra clam (116 tag and 121 tag). The peptides were then purified from excess labeling reagent by strong cation exchange (SCX) chromatography using Agilent 1200 HPLC (Agilent, CA, USA), followed by being separated using Eksigent nanoLC-Ultra 2D system (Eksigent, CA, USA). The LC fractions were analyzed by MS/MS using a Triple TOF 5600 mass spectrometer (AB SCIEX, MA, USA). More detailed information on iTRAQ analysis was described in the Supplementary material S1.

Protein identification and relative quantification were performed with ProteinPilot Software 4.0 (AB SCIEX). MS/MS data were searched against a mollusc protein database (translated from *R. philippinarum* transcriptome database with 38,224 sequences, built in 2013) and a decoy database for false discovery rate (FDR) analysis. The database search parameters were the followings: iTRAQ 8-plex quantification, cysteine modified with iodoacetamide, methionine modifications of oxidation, trypsin digestion, one missed cleavage allowed, peptide tolerance was set as 0.05 Da, and MS/MS tolerance was set as 0.05 Da. Proteins with unused score > 1.3 (FDR < 0.05), matching reliable peptides (≥ 2 unique peptides with FDR < 0.05) and p value < 0.05 were considered as positively identified proteins. Protein ratios were calculated by averaging the signal intensity of -113 and -117 for White clams in control group, -115 and -119 for Zebra clams in control group, -114 and -118 for the White clams in Cd-treated group, and -116 and -121 for Zebra clams in Cd-treated group. The protein with a fold change ratio > 2.0 or < 0.5 was considered as differentially expressed proteins (DEPs). More detailed information on protein identification and quantification was described in the Supplementary material S1.

2.5. Bioinformatics analysis

Evaluation of function of DEPs was performed with the Database for Annotation, Visualization and Integrated Discovery (DAVID) tools for GO annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation, together with functional enrichment analysis based on the Fisher's exact test. Briefly, the homology search was firstly conducted for all query DEPs using blastp against the modal species *Homo sapiens*. The top 10 blast hits with E-value less than $1e-10$ for each query sequence were retrieved, and the hit with the optimal identity to the query was selected as homologous. Then, GO annotation and KEGG pathway analysis were performed using DAVID tools (<https://david.ncifcrf.gov/>). GO annotation consists of three ontologies, biological process (BP), molecular function (MF) and cellular component (CC). To further explore the impact of DEPs in cell physiological process and discover internal relations between DEPs, enrichment analysis was performed by Fisher's exact test at the background of identified proteins. Only functional categories and pathways with p value under a threshold of 0.05 were considered as being significant.

3. Results

3.1. Cadmium concentrations in clam digestive gland tissues

After Cd treatment for 48 h, accumulations of Cd in digestive gland tissue samples were observed in both White and Zebra clams. In details, the average Cd contents in Cd-exposed White and Zebra clam samples were 13.59 and 15.16 $\mu\text{g/g}$ dry weight, respectively, which were significantly higher ($p < 0.05$) than those of clam samples in control groups (Fig. 1). However, Zebra clam samples showed slightly higher accumulation of Cd than White clam samples without statistical significance.

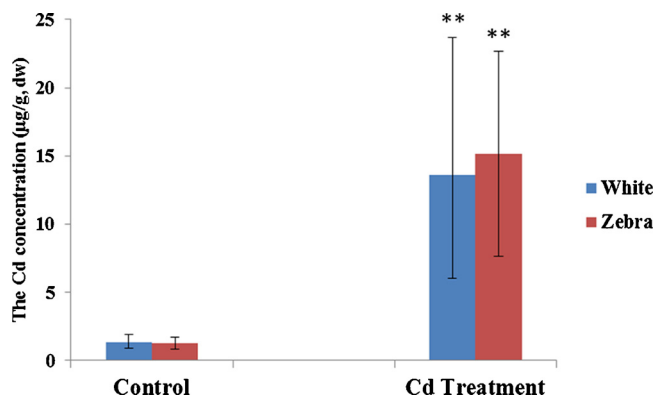


Fig. 1. The accumulations of Cd in digestive glands from control and Cd-treated clams. Values are presented as mean \pm standard deviation. ** represents p value < 0.01.

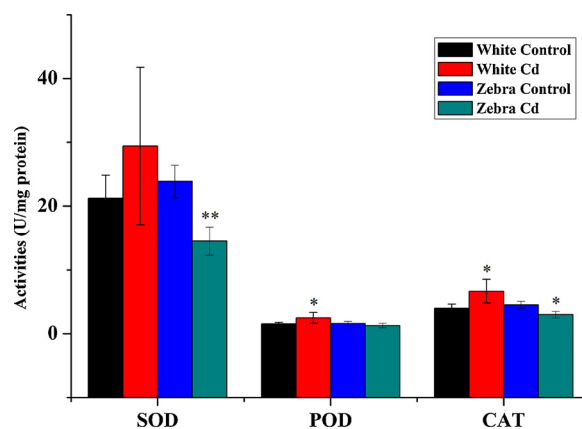


Fig. 2. The antioxidant enzyme activities in digestive glands from control and Cd-treated clams. Values are presented as mean \pm standard deviation. * and ** represent p value < 0.05 and p value < 0.01, respectively.

3.2. The responses of antioxidant enzyme activities to Cd treatment

Fig. 2 represents the alterations of antioxidant enzyme activities in clam digestive gland samples. After Cd treatment for 48 h, the SOD activities and CAT activities in digestive gland samples were significantly decreased in Zebra clams. For White clams, Cd treatment induced significant increases in both POD and CAT activities in digestive gland samples. Moreover, other alterations such as elevation of SOD activities in Cd-treated White clam samples and depletion of POD activities in Cd-treated Zebra clam samples were also observed with non-significant trends.

3.3. Overview of proteome in White and Zebra clam samples in response to Cd treatment

In order to compare the proteome responses of the two pedigrees of clam to Cd treatment, the proteomic analysis based on iTRAQ was conducted to identify the DEPs in White and Zebra clam samples. In total, 1250 proteins were identified from the combined data of two biological replicates with 862 shared proteins. In this research, any protein changed with > 2.0 or < 0.5 fold difference and a p value < 0.05 would be considered as significant DEPs (Supplementary material S2). As shown in Fig. 3, apparently, Cd treatment induced a larger number of DEPs in White clam samples than in Zebra clam samples. In details, 128 proteins were significantly changed in Cd-treated White clam samples. Of these, 71 proteins (55.5%) were increased (fold change > 2.0, $p < 0.05$) and 57 proteins (44.5%) were decreased (fold change < 0.5, $p < 0.05$). For Zebra clams, a total of 97 proteins

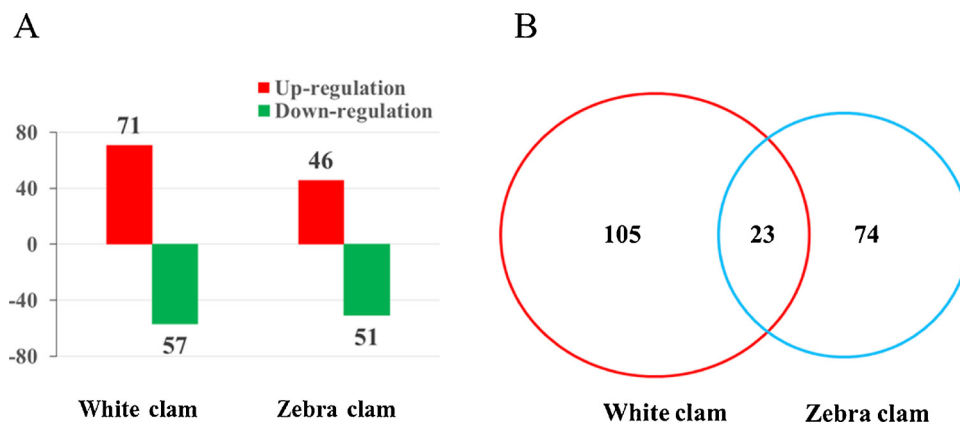


Fig. 3. Statistics of differentially expressed proteins in White and Zebra clams exposed to Cd. (A) Numbers of up-regulated and down-regulated proteins. (B) Venn diagram showing the numbers of uniquely and commonly expressed proteins.

were differentially expressed in digestive gland samples after Cd treatment, with 46 proteins (47.4%) up-regulated (fold change > 2.0, $p < 0.05$) and 51 proteins (52.6%) down-regulated (fold change < 0.5, $p < 0.05$). Further comparison displayed that only 23 out of 202 DEPs (11.4%) were commonly responded to Cd treatment in both Cd-treated pedigrees, and 105 and 74 DEPs were uniquely altered in Cd-treated White and Zebra clam samples, respectively (Fig. 3B).

3.4. Bioinformatical analysis

GO annotation and enrichment analysis were successfully conducted on the DEPs. Based on their involvements, second-level GO terms were applied to classify proteins into three categories, BP, MF and CC categories (Fig. 4). Basically, a total of 42 s-level GO terms were enriched in Cd-treated White and Zebra clam samples, among which 31 terms, consisting of 20 BP categories, 9 CC categories and 2 MF categories, were common to the two pedigrees of clam treated by Cd, while 4 and 7 terms were unique to Cd-treated White and Zebra clams, respectively (Fig. 4). The top enrichment terms of BP, MF and CC based on the counts in Cd-treated White clams were cellular process, binding and cell part, respectively, which were also mostly enriched in Cd-

treated Zebra clams. Furthermore, four terms consisting of membrane, catalytic activity, electron carrier activity and metallochaperone activity were uniquely enriched in Cd-treated White clams. Another seven terms, consisting of cell aggregation, cell killing, rhythmic process, extracellular matrix, extracellular matrix component, molecular function regulator and structural molecule activity, were unique to Cd-treated Zebra clams.

Based on the DEPs, KEGG pathway enrichment analysis revealed that a total of 24 pathways (details in Supplementary material S2) were enriched in the two pedigrees of clam treated by Cd. For White clams, 21 pathways were significantly enriched in digestive gland samples, and the top three pathways were focal adhesion ($p = 7.39e-07$, 12 proteins), regulation of actin cytoskeleton ($p = 7.67e-06$, 12 proteins) and mitogen-activated protein kinase (MAPK) signaling pathways ($p = 6.79e-03$, 11 proteins). By contrast, 8 pathways were significantly enriched in Cd-treated Zebra clam samples, of which focal adhesion ($p = 0.00702$, 7 proteins), regulation of actin cytoskeleton ($p = 0.0295$, 7 proteins) and carbon metabolism ($p = 0.0047$, 6 proteins) were the top three pathways. Further comparison showed that 5 of the 21 KEGG pathways were commonly enriched in the two clam pedigrees resulting from Cd treatment. Moreover, sixteen and three KEGG pathways were

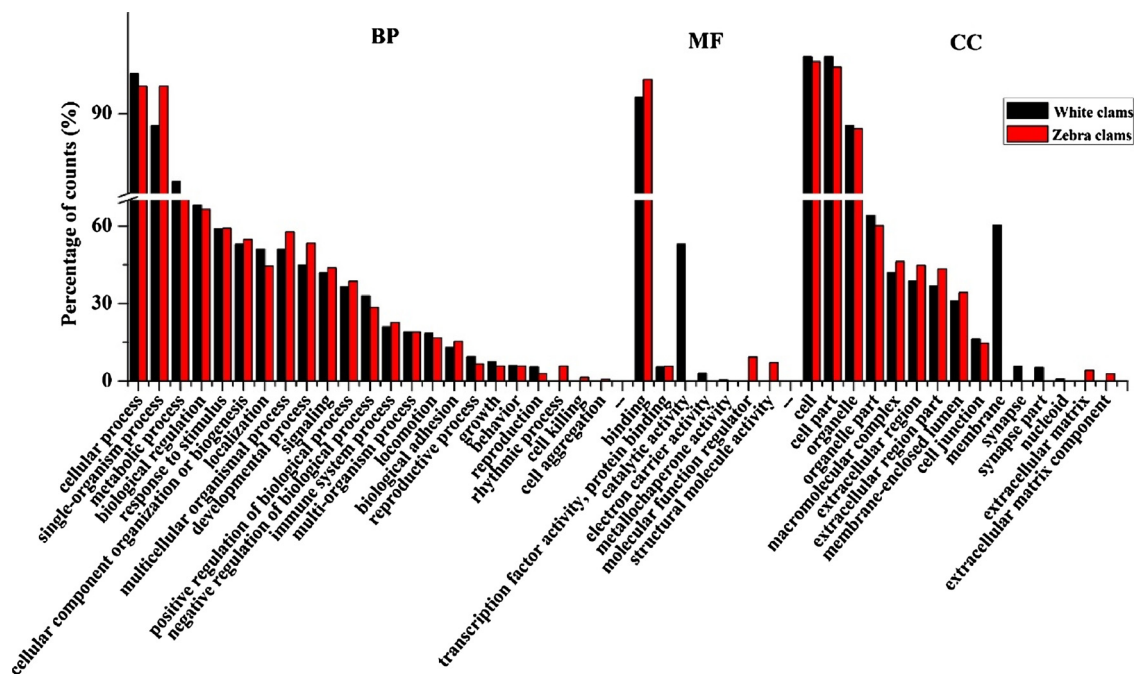


Fig. 4. The second-level terms of GO enrichment in Cd-treated White and Zebra clams. BP, biological process; MF, molecular function; CC, cellular component.

Table 1
KEGG pathways enriched in Cd-treated White and Zebra clams.

Pathway Name	Pathway ID	P value ^a	Count
Commonly enriched in both White clams and Zebra clams (White / Zebra)			
Focal adhesion	hsa04510	0.00000739 /0.00702	12 /7
Regulation of actin cytoskeleton	hsa04810	0.00000767 /0.0295	12 /7
Endocytosis	hsa04144	0.00782 /0.0276	8 /4
Carbon metabolism	hsa01200	0.00103 /0.0047c	6 /6
Biosynthesis of amino acids	hsa01230	0.0117 /0.00373	5 /4
Uniquely enriched in White clams			
MAPK signaling pathway	hsa04010	0.00679	11
Glycolysis / Gluconeogenesis	hsa00010	0.000203	6
Fc gamma R-mediated phagocytosis	hsa04666	0.00692	6
HIF-1 signaling pathway	hsa04066	0.0112	6
Fatty acid degradation	hsa00071	0.000133	5
Ubiquitin mediated proteolysis	hsa04120	0.0125	5
ErbB signaling pathway	hsa04012	0.0221	5
Spliceosome	hsa03040	0.0357	5
Fc epsilon RI signaling pathway	hsa04664	0.0354	4
Ascorbate and aldarate metabolism	hsa00053	0.0127	3
Lysine degradation	hsa00310	0.0147	3
beta-Alanine metabolism	hsa00410	0.0185	3
Pentose and glucuronate interconversions	hsa00040	0.0275	3
Glycine, serine and threonine metabolism	hsa00260	0.0361	3
Pyruvate metabolism	hsa00620	0.0361	3
Toll-like receptor signaling pathway	hsa04620	0.0443	3
Uniquely enriched in Zebra clams			
2-Oxocarboxylic acid metabolism	hsa01210	0.0000922	3
Proteasome	hsa03050	0.0228	3
mRNA surveillance pathway	hsa03015	0.0386	4

^a P value represents the significance of enrichment to the pathway.

uniquely enriched in Cd-treated White clams and Zebra clams, respectively (Table 1).

4. Discussion

In this study, we applied proteome profiling to evaluate the differential responses of two pedigrees of clams to Cd treatment, together with Cd accumulation and antioxidant enzyme activities. It is worth noting that digestive gland tissues were selected as target organ because of its capability of heavy metal accumulation and detoxification (Choi et al., 2007). Though there was no significant difference in the capability of Cd accumulation in digestive gland samples between the two pedigrees of clam, at least during an acute Cd treatment for 48 h, the responses of antioxidant enzymes and proteome profiles to Cd treatment differed between White and Zebra clam samples.

4.1. Antioxidant enzyme responses of White and Zebra clams to Cd treatment

As it is known, environmental stressors may lead to an overproduction of ROS, which are byproducts of aerobic metabolism and would be harmful to organisms if overproduced (Schieber and Chandel, 2014). To minimize cellular damage caused by ROS, organisms have evolved a scavenging system composed of antioxidants and antioxidant enzymes (Nimse and Pal, 2015). Therefore, antioxidant enzyme activities are routinely used for determining oxidative stresses induced by toxicants. In general, a mild increment in ROS generation can invoke cells to increase the antioxidant enzymes and overcome stress. However, cells will fail to protect themselves and even antioxidant enzymes could be degraded if oxidative damages persist for a long time (Yu et al., 2009). Of these antioxidant enzymes, SOD is the major O₂^{•-}

scavenger and its enzymatic process results in H₂O₂, which can be decomposed by CAT and POD (Sabharwal and Schumacker, 2014). In White clams, Cd treatment induced significant increases in POD and CAT activities ($p < 0.05$), indicating that Cd treatment might have induced excessive H₂O₂ in digestive gland samples. Comparatively, the SOD and CAT activities in Cd-treated Zebra clam samples presented significant decrease trends ($p < 0.05$), which might be accounted for the overloaded ROS beyond the antioxidant capacity of Zebra clam. Moreover, another detoxification enzyme glutathione S-transferase pi-class was found to be up- and down-regulated at protein level in Cd-treated White and Zebra clams, respectively. Interestingly, the glutathione S-transferase pi-class was consistently altered with other antioxidant enzymes like SOD, POD and CAT in response to Cd treatment, which confirmed the alterations of antioxidant systems in Cd-treated clams. Taken together, the responses of clams to Cd treatment differed in antioxidant enzymes, depending on pedigrees, which were also found in plants exposed to heavy metals (Gratao et al., 2005).

4.2. Proteome characterization of White and Zebra clam samples responsive to Cd treatment

iTRAQ-based proteomic analysis has been widely used to characterize the proteome responses to multiple stress factors (Ji et al., 2014; Zhang et al., 2015; Masood et al., 2016). In this work, proteome profiling based on iTRAQ was firstly conducted to investigate the proteome responses of White and Zebra clam samples to Cd exposure. Basically, Cd treatment induced significant proteomic alterations in both White and Zebra clam samples. However, the altered proteomic profiles significantly differed between White and Zebra clam samples. Firstly, more DEPs and a larger percentage of up-regulated DEPs were triggered in Cd-treated White clam samples than those in Cd-treated Zebra clam samples. Moreover, the two pedigrees shared a very low ratio of DEPs (11.4%) that were commonly responsive to Cd treatment. These findings confirmed the distinctive proteomic responses to Cd treatment between the two pedigrees of clam samples. To reveal the differential toxicological mechanisms of Cd on the two pedigrees, bioinformatical analysis were subsequently conducted on the DEPs.

4.3. Bioinformatic analyses revealed the differential toxicological mechanisms of Cd in two pedigrees

By comparing the proteomes from control and treated group, we could get the detailed information on proteomic responses caused by treatment. However, analysis of high-throughput proteome data typically yields a list of DEPs, which contains multiple information but fails to provide insights into the underlying mechanisms of organisms under different conditions. Fortunately, GO annotation and KEGG pathway analysis enable us to group a large amount of proteins by annotation or pathway to reduce the complexity and identify active pathways differing between two conditions, which contributes to the biological interpretation of high-level systematic functions (Camon et al., 2003; Khatri et al., 2012).

In this work, GO annotation, KEGG pathway and enrichment analysis were conducted on the DEPs in Cd-treated White and Zebra clam samples, respectively. In general, GO enrichment analysis exhibited the BP, CC and MF categories that were significantly enriched in Cd-treated White and Zebra clams. Apparently, more GO annotations were enriched in Cd-treated White clam samples than in Cd-treated Zebra clam samples, which could be accounted for the larger number of DEPs in Cd-treated White clam samples. Further comparisons, however, showed that a large percentage of GO annotation terms (73.8%) were common to the two pedigrees of clam with close percentages of counts, despite a small part of DEPs shared as mentioned above (11.4%), indicating that Cd treatment led to similar GO annotations enriched in White and Zebra clam samples. The BP category response to stimulus as an example here, was enriched in Cd-treated White clam samples (59.0%) and Zebra clam

samples (59.1%) with extremely close percentages of counts. However, the DEPs contributed to the percentages were almost completely different between the two pedigrees of clams. For instance, thioredoxin, cytochrome c oxidase, cytochrome P450 and heat shock protein were uniquely observed in Cd-treated White clam samples, while stress induced phosphoprotein 1 and acyl-CoA dehydrogenase were unique in Cd-treated Zebra clam samples. Therefore, we concluded that Cd treatment elicited response to stimulus in both White and Zebra clam samples with differential mechanisms. Furthermore, a total of four GO terms including membrane, catalytic activity, electron carrier activity and metallochaperone activity were uniquely enriched in Cd-treated White clam samples. Seven terms containing cell aggregation, cell killing, rhythmic process, extracellular matrix, extracellular matrix component, molecular function regulator and structural molecule activity were uniquely enriched in Zebra clams. Overall, these results of GO enrichment analysis implied the differential strategies of White and Zebra clams in response to Cd treatment.

In order to ascertain the molecular networks responsive to Cd treatment, KEGG pathways analysis was conducted on the DEPs from Cd-treated White and Zebra clam samples, respectively. A total of 24 pathways were enriched in the two pedigrees of clam, of which only 5 pathways, consisting of focal adhesion, regulation of actin cytoskeleton, endocytosis, carbon metabolism and biosynthesis of amino acids, were shared. As it is known, focal adhesion cooperated with action cytoskeleton plays key roles in signal transduction, which contributes to the cell migration, growth, differentiation and other biological processes (Carragher and Frame, 2004; McKay and Simpson, 2013; Pieri et al., 2014). As the key proteins involved in focal adhesion pathway, calcium-binding protein, guanine nucleotide exchange factor VAV2 and integrin have been commonly altered in Cd-treated White and Zebra clams samples in this work, which were also found in organisms in response to other stress factors like organic pollutant and changes of living environment (Liu et al., 2013a,b; Ji et al., 2014; Varney et al., 2015). In general, endocytosis plays key roles in nutrient uptake, cell signaling and immune response (Miaczynska and Stenmark, 2008; Dannevig et al., 1994). In this work, Cd treatment triggered endocytosis in both pedigrees of clam samples, supported by the significant up-regulation of Ras-related protein, Rab-21, which plays a positive role in endocytosis (Subramani and Alahari, 2010). It is not surprising that Cd treatment could induce endocytosis in clam samples, since endocytosis have been regarded as a new feature of stress response (Vega et al., 2010). Moreover, carbon metabolism and biosynthesis of amino acids were also found to be commonly responsive to Cd treatment. As fundamental metabolism pathways that are consistently responsive to diverse stressors and conserved in all organisms (Kultz, 2005), carbon metabolism and biosynthesis of amino acid pathways have been frequently reported to be influenced by environmental stress factors (Harding et al., 2003; Yang et al., 2013; Lahtvee et al. 2016). The negative regulation of carbon metabolism and biosynthesis of amino acids, marked by the decrease of related DEPs, implied that Cd treatment have depleted some process of carbon utilization or fixation and biosynthesis of amino acid in the two pedigrees of clams.

Apart from the commonly enriched KEGG pathways in the two pedigrees of clam, there were still KEGG pathways that uniquely enriched in either White or Zebra clam samples. For White clams, Cd treatment induced the up-regulation of ubiquitin mediated proteolysis, fatty acid degradation and pyruvate metabolism, evidenced by the up-regulated proteins related to these processes, indicating that Cd treatment could significantly enhance proteolysis and energy production. Interestingly, the positive regulation of energy production and proteolysis were consistent with the elevation of POD and CAT activities in Cd-treated White clam samples, since both energy production and proteolysis have been reported to be involved in oxidative stress (Mehlase and Grune, 2002). Meanwhile, a variety of pathways related to cellular signaling, consisting of MAPK signaling pathway, HIF-1 signaling pathway, ErbB signaling pathway and Fc epsilon RI signaling

pathway, were also involved in the response to Cd treatment. Activation of signaling transduction pathways is always the first response to stress factors, which leads to changes in the metabolism indispensable for adaptation to unfavorable conditions (Nemmiche, 2017). By contrast, however, no signaling pathway was enriched in Cd-treated Zebra clam samples. Instead, only mRNA surveillance pathway and two metabolic pathways, 2-oxocarboxylic acid metabolism and proteasome, were unique to Cd-treated Zebra clam samples. The mRNA surveillance pathway is a quality control mechanism that detects and degrades abnormal mRNA, ensuring that the multistep process of gene expression is accurately executed and adapted to cellular needs (Wagner and Lykke-Andersen, 2002). Based on the DEPs, we conclude that mRNA surveillance pathway was significantly enhanced in Cd-treated Zebra clams samples, indicating that Cd treatment have produced more abnormal mRNA and dramatically affected the process of gene expression in Zebra clams samples. Furthermore, 2-oxocarboxylic acid metabolism that plays vital roles in energy production was significantly down-regulated in Cd-treated Zebra clam samples, indicated by the decreases of citrate synthase and isocitrate dehydrogenase. Moreover, proteasome involved in the proteolysis process was down-regulated in Cd-treated Zebra clam samples. Also, the depletion of energy production and proteolysis could be confirmed by decrease of SOD and CAT activities as a result of long-time stress in Cd-treated Zebra clams.

5. Conclusions

In the present study, proteome profiling analyses based on iTRAQ were performed on White and Zebra clam samples to investigate the differential responses to Cd treatment. We found that the responses of proteome profiles and antioxidant enzymes to Cd treatment remarkably differed between the two pedigrees of clams. In details, Cd treatment induced more DEPs in White clam samples than in Zebra clam samples. Based on the DEPs, we concluded that some key biological processes consisting of immune response and metabolism were commonly induced in both two pedigrees of clam. Uniquely, Cd treatment elicited an enhancement in signal transduction, proteolysis and energy production in White clam samples. For Zebra clam samples, the depletion in proteolysis and energy production as well as disorder in gene expression were elicited in response to Cd treatment. Moreover, Cd exposure caused elevations in CAT and POD activities in White clam samples and decreases in SOD and CAT activities in Zebra clams samples, which were consistent with the proteomic responses. Overall, these findings confirmed the differential biological effects of White and Zebra clams to Cd treatment, suggesting that the pedigree of animal should be taken into consideration in ecotoxicology studies.

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.etap.2018.12.002>.

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