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Gonad differential proteins revealed with proteomics in oyster (*Saccostrea cucullata*) using alga as food contaminated with cadmium

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ABSTRACT

As mercury and lead, cadmium (Cd) is one of the highly toxic metals in both the ocean and land environments, but its toxicological mechanism in organisms including human is still unclear because of the complex toxicological pathways *in vivo*. Here, the alga *Chlorella vulgaris* were cultivated at room temperature under the stress of cadmium (1 mg L^{-1}) to obtain a toxic food, and then the contaminated food were directly supplied to oyster (*Saccostrea cucullata*) in seawater. After feeding with *C. vulgaris* contaminated with Cd (*C. vulgaris*–Cd), the differential proteins in the oyster gonad (OG) were effectively separated and identified with proteomic approaches. Eleven protein spots were observed to be significantly changed in the OG feeding with *C. vulgaris*–Cd, which seven spots of these differential proteins were down-regulated while four spots were up-regulated. These altered spots were further excised in gels and identified by a combined technique of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS) and database searching. A portion of these differential proteins were further proofed by real-time PCR and Western blotting. The results indicate that the major functions of these differential proteins were described as follows: binding, protein translocation, catalysis, regulation of energy metabolism, reproductive function and skeleton structure. These differential proteins in part may effectively provide a few novel biomarkers for the evaluation of Cd pollution level *via* a food pathway for harming halobios, mammal and human health, and for understanding the complex mechanisms of Cd toxicity *in vivo*.

1. Introduction

Cadmium (Cd) is a nonessential element to living organisms in nature, which can cause highly toxicity to most organisms. Presently, a variety of responses have been attributed to Cd toxicity, but the exact molecular mechanism responsible for the toxic effects of Cd is far from being completely understood. Its toxicity is caused by its strong binding capacity to sensitive groups in proteins, such as thiols and histidyls, thus leading to the deterioration of biologically important molecules (Vallee and Ulmer, 1972). In addition, Cd may induce oxidative stress by producing hydroxyl radicals (O'Brien and Salasinski, 1998), superoxide anion radicals, nitric oxide, and hydrogen peroxide (H_2O_2) (Stohs et al., 2001; Waisberg et al., 2003), resulting in lipid peroxidation, DNA damage, and S-glutathionylation of proteins (Risso-de Faverney et al., 2001; Geret et al., 2002; Bebianno and Serafim, 2003).

Fossil fuels, batteries, fertilizers (Schutze et al., 2003) and sewage sludge were normally determined to micro-metal Cd (Bliefert and Perraud, 2001). As terrestrial organisms are mainly exposed to Cd complex by ingestion food (Robards and Worsfold, 1991), it transfers along food chains constitutes an important route of exposure that must be taken into account for ecotoxicological risk assessment. It was reported that significant amounts of Cd can be transferred from contaminated soil to plants and grass (Zhu et al., 2007), causing accumulation of the potentially toxic metal in grazing ruminants (Wilkinson et al., 2003), particularly in cattle (Miller et al., 2004). Accumulation of Cd in ruminants causes toxic effects in cattle (Blanco-Penedo et al., 2006), but also in humans consuming meat contaminated with toxic metals (De Vries et al., 2007).

When Cd contaminates the aquatic ecosystem, it can enter the aquatic food chain through direct consumption of water or biota; and through non-dietary routes such as absorption through epithelia (Ruangsomboon and Wongrat, 2006). Both shellfishes and fishes are often part of the aquatic food chain and may enrich large amount of Cd by consuming algae or the water, subsequently, human may further amass larger quantity of Cd through diet. Oyster is one of the most important shellfishes consumed by human, the presence

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of Cd in oyster may constitute a serious health hazard. For example, a high amount of Cd accumulated in the human body can injure the kidney or liver, with symptoms of chronic toxicity including impaired kidney function, tumors, and hepatic dysfunction (Mansour and Sidky, 2002).

It is known that cadmium affects human and animal reproductive functions (Henson and Chedrese, 2004; Thompson and Bannigan, 2008), probably through interference with endocrine system. Data suggest that cadmium may interfere directly with hormone production and is therefore classified as an endocrine disrupting chemical (EDC) (Henson and Chedrese, 2004; Iavicoli et al., 2009). In mammals, cadmium exposure is associated with ovulation failure (Rehm and Waalkes, 1988; Lienesch et al., 2000), defective steroidogenesis (Piasek et al., 2002), suppressed oocyte maturation (Leoni et al., 2002), and implantation failure (Yu et al., 1985). Exposure of pregnant women to cadmium is related to an increased incidence of premature delivery (Nishijo et al., 2002). It has also been demonstrated that cadmium exposure is associated with endometrial cancer (Akesson et al., 2008).

The previously mentioned studies were all mammals related, and the effects of Cd induction on the gonad of aquatic organisms, especially marine organisms, have seldom been mentioned. Here, we emphasize particularly on proteomic alterations of the gonad in oyster using *Chlorella vulgaris*–Cd as food. As a result, a few differential proteins might be established to shed new light on the mechanisms underlying Cd toxicity, to assist in the development of biomarkers for evaluating the Cd pollution level in the aquatic environment, and to evaluate the risk of Cd to aquatic organism survival as well as human health.

2. Materials and methods

2.1. Chemicals, alga and animals

Analytical grade of Cd (II) chloride was purchased from Sigma (USA) for experiments. *C. vulgaris* was obtained from State Key Laboratory of Marine Environmental Science, Xiamen University.

Oysters (*Saccostrea cucullata*) were purchased from a commercial supplier in Xiamen, China, and raised in a stock tank at a temperature of 25 ± 2 °C in the laboratory. Both salinity and pH in the experimental seawater for oyster maintenance were controlled to be 2.8% and 8.0 respectively. The seawater for oyster maintenance was replaced in 24 h. *C. vulgaris* was divided into two groups for breeding in the presence or absence of 1 mg L^{-1} $CdCl_2$ (*C. vulgaris*–Cd), respectively. Both *C. vulgaris* and *C. vulgaris*–Cd as feedstuff were collected to feed the oysters, respectively. After being acclimated for 1 week, five oysters being fond of moving (approximately 45 ± 5 g), without bacterial infection and pollutant exposure were unceasingly fed by *C. vulgaris*–Cd against the control group in the presence of *C. vulgaris* for 48 h. Both the control and Cd-treated groups were divided into three parallel groups respectively, and five oysters were maintained in each parallel group.

2.2. Determination of Cd content by ICP-MS

To measure the Cd content in *C. vulgaris*–Cd and its enrichment in OG by food pathway, ICP-MS method was performed for Cd analysis according to Luo et al. (2011). 0.2 g both *C. vulgaris*–Cd and *C. vulgaris* were subjected to analyze the content of Cd by ICP-MS approach. In addition, after being fed the feedstuff of *C. vulgaris* and *C. vulgaris*–Cd [under the stress of $CdCl_2$ (1 mg L^{-1}, 2 mg L^{-1}, 4 mg L^{-1})], the Cd content of the gonad (0.5 g) were determined by ICP-MS method in oyster, respectively. Each measurement was carried out for three experimental replicates.

2.3. Protein sample preparation

Five oysters (*S. cucullata*–Cd) were fed with *C. vulgaris*–Cd exposed to 1 mg L^{-1} $CdCl_2$ for 48 h in each parallel group. After the Cd stress, gonad samples (about 50 mg of tissue) from each five oysters of both *C. vulgaris*–Cd and control groups were obtained and homogenized in Lysis Buffer (7 M urea, 2 M thiourea, 4% CHAPS, 60 mM DTT, 10 mM Tris, 1 mM EDTA, 0.5% ampholyte 3–10, 1% protease inhibitor cocktail), then placed on ice for 2 h. After sonication, the homogenates were centrifuged to remove debris at 100 000*g* for 30 min. The remaining supernatants were collected for proteome separation by 2-DE separation or protein identification by Western blotting. Total protein content in the samples was measured using the Bradford assay (Bradford, 1976). The protein samples were kept at −80 °C until used.

2.4. 2-DE

2-DE was performed as Huang and Huang (2011) reported previously. For each protein sample (control or Cd-treatment), three experimental replicates were subjected to 2-DE.

2.5. Silver-staining and image analysis

Proteins on the gels were visualized using silver-staining as described previously (Shen et al., 2007). The silver-stained gels were scanned with an Image Scanner II apparatus (GE Healthcare, Italy). Digitized images of the gels were analyzed using Image Master 2D Platinum software (Version 5.0, GE Healthcare, Italy). Protein spots were detected and matched between different samples, and individual spot volume values were obtained according to the program instructions. To eliminate gel-to-gel variation, the individual spot volume of each gel was normalized relative to the total valid spot volume, and the proteins differentially expressed with statistical differences were selected for identification.

2.6. In-gel digestion and MALDI-TOF MS/MS analysis

Protein spots were manually excised from the 2D gels and washed with water. After being destained, the proteins were in-gel reduced, alkylated and digested with trypsin as reported (Chen and Huang, 2011). The peptide extracts were redissolved in 5 μL of 0.5% trifluoroacetic acid (TFA) and 1 μL of the peptide mixtures was then mixed with an equal volume of matrix α-cyano-4-hydroxy-cinnamic acid (CHCA) saturated with 50% acetonitrile/0.05% TFA, spotted onto a MALDI target plate and analyzed using a Bruker Autoflex III® MALDI-TOF/TOF 200 mass spectrometer (Bruker, Germany) and an ABI 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, USA). Laser shots of 200 per spectrum were used to acquire spectra with a mass range of 800–3000 Da. Spectra were calibrated using trypsin autodigested ion peaks (m/z = 842.510 and 2211.1046) as internal standards. The PMF data were used to search for candidate proteins using MASCOT (http://www.matrixscience.com) software. The database (NCBInr) searches were performed using the following parameters: all entries, trypsin digest, allow up to 1 missed cleavage, fixed modification of carbamidomethylation, variable modification of methio nine oxidation and maximal mass tolerance of 100 ppm. Individual ions score greater than 32 were defined as significant ($p < 0.05$).

2.7. Western blotting

Proteins (50 μg) from gonad samples were separated on 12% SDS–PAGE. After electrophoresis, there solved proteins were transferred to PVDF membrane. The membrane was then blocked for 1 h in TBST (50 mmol L^{-1} Tris–Cl, pH 7.6, 150 mmol L^{-1} NaCl and 0.1%

Tween 20) containing 5% (w/v) nonfat milk, and then incubated with primary antibody anti-prohibitin (Abnova, Taiwan) (1:2000 dilution) overnight. The membrane was washed three times with TBST and incubated with HRP-conjugated goat anti-rabbit IgG (Pierce, USA) (1:10000 dilution) for 1 h. The membrane was again washed three times with TBST, and the blots were developed using ECL. The signals from each protein band were normalized against the β-actin content using the polyclonal antibody anti-actin (Santa Cruz Biotechnology, USA) (1:2000 dilution). The expression level of control was designated value "1" and thereby the expression ratio of treatments was expressed in relation to the control.

2.8. Quantitative real-time PCR

Total RNAs were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA) from gonad of five oysters, and cDNAs were synthesized using ImProm-II™ Reverse Transcription (Promega, USA). Real-time PCR reactions were performed in a Rotor-Gene™6000 real-time rotary analyzer (Corbett Life Science, Australia) following the manufacturer's instructions (TaKaRa, Japan) (1 cycle at 95 °C for 10 min and 40 amplification cycles at 95 °C for 5 s, 56 °C for 15 s and 72 °C for 20 s). In brief, 10 μL of reaction mixture contained 1 μL of reverse transcribed product template, 5 μL of SYBR Premix Ex Taq™ II and the gene-specific primer pair at a final concentration of 500 nM for each primer. All primer sets are described in Table S1. Oyster gonad DNA was used to construct standard curves (CT versus log copy number) for target amplicons. Relative quantification of each gene expression level was normalized according to the β-actin gene expression. The PCR data of treatments was calibrated to the control values (control = 1).

2.9. Statistical analysis

The data are expressed as means ± SD of triplicate experiments. Significant differences among groups were determined using a one-way ANOVA followed by the LSD post hoc test. Probabilities of $p < 0.05$ and $p < 0.01$ were considered as statistically significant.

3. Results

3.1. Accumulation of Cd by C. vulgaris and oyster

ICP-MS was carried out to examine if Cd can be accumulated by *C. vulgaris* and oyster through food chain. According to Fig. 1, we found that the numbers of Cd absorbed by *C. vulgaris* and OG have a positive correlation with the concentration of $CdCl_2$. Although the levels of Cd are somewhat higher in OG than in *C. vulgaris* between the two control groups, total number of Cd in OG is much more than that in the *C. vulgaris* when exposed to 4 mg L^{-1} $CdCl_2$. In addition, total number of Cd in OG is still more than that in *C. vulgaris* at the same concentration of $CdCl_2$ respectively. Therefore, it is obvious that Cd has been accumulated by food chain. We chose 1 mg L^{-1} $CdCl_2$ as the treatment concentration considering the Cd^{2+} concentration in aquatic ecosystem and the significant accumulation results.

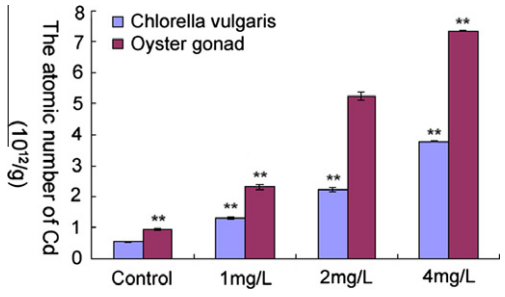

Fig. 1. The atomic number of Cd (10^{12} g^{-1}) in *Chlorella vulgaris* and OG under different concentration of Cd treatment (control, 1 mg L^{-1}, 2 mg L^{-1}, 4 mg L^{-1}) measured by ICP-MS. $^{**}p < 0.01$.

3.2. Cd altered protein profiles of OG

To detect the differentially expressed proteins induced by Cd-treatment, samples of control and Cd-treated were subjected to 2-DE analysis. Silver staining revealed most of the protein spots in the pH 5–8 interval and in a molecule weight range of 25–66.2 kDa on the gels (Fig. 2). Quantitative image analysis revealed 11 protein spots showing statistically significant changes (decrease or increase, $p < 0.05$) in the Cd-treated versus the control. These 11 spots were named Spots 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 as shown in Fig. 1, with four spots (Spots 1, 2, 4 and 9) being up-regulated (Fig. 3A) and seven spots (Spots 3, 5, 6, 7, 8, 10, 11) down-regulated (Fig. 3B).

3.3. Identification of differentially expressed proteins

The differential protein spots shown in Fig. 2 were further identified using MALDI-TOF MS/MS and database searching. The nature of each spot and the Cd-treated/control abundance ratio are summarized in Table 2. A reduced abundance was observed for ATP-dependent Clp protease, Mitochondrial import inner membrane translocase, 4Fe–4S ferredoxin, alphaTub84B, ribonuclease R, prohibitin and serine/threonine-protein kinase.

As a result of the search of the UniProt database (http://www.uniprot.org/), the functional properties and subcellular localization of these differential proteins were further classified, and they were found to be involved in binding, protein translocation, catalysis, cell structure and metabolic regulation. The multiple types and functions of these differential proteins indicate the complexity of Cd toxicology. As to the subcellular localization and functions of these proteins, it can be referred to Tables 1 and 2.

3.4. Validation of differential proteins using Western blotting

To verify the reliability of the proteomics analysis, prohibitin was selected as representative protein and subjected to Western blotting (Fig. 4A). The results of triplicate Western blots for prohibitin, whose abundance decreased significantly in OG with Cd treatment, were in good agreement with those of 2-DE, thus confirming a lower expression level for prohibitin in the Cd-treated samples. The results further verified that prohibitin was a Cd-responsive protein.

3.5. mRNA expression level of differential proteins

To examine whether the expression changes of prohibitin, GH11448, alphaTub84B, 5-oxoprolinase, sfrs17a protein, Os07g0576100, and mitochondrial import inner membrane translocase (MIIMT) proteins were attributable to transcriptional regulation, we extracted RNA from OG and carried out Quantitative Real-time PCR analysis (Figs. 4B and 5). In all cases, the results of PCR analysis were consistent with those of 2-DE. Similar to the Western blotting data, the mRNA expression level of prohibitin decreased under Cd-treatment (Fig. 4B).

4. Discussion

Toxicoproteomics is the use of global protein expression technologies to identify critical proteins and pathways in biological systems that are affected by and respond to adverse chemical

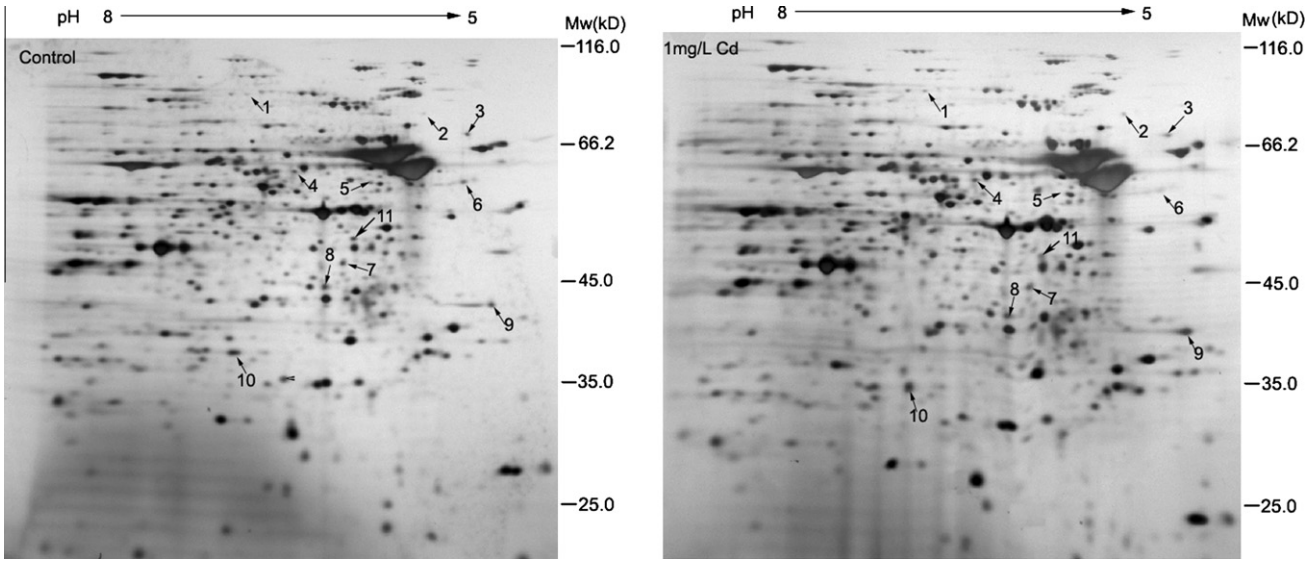

Fig. 2. 2-DE maps indicating protein spots which changed in volume after 1 mg L^{-1} Cd^{2+} exposure in OG. Total gonad proteins (150 μg) were separated by 2-DE (pH 5–8; T = 11%) and visualized by silver staining. Arrows indicate the proteins with a significantly modified expression level after Cd^{2+} exposure. (A) Control; (B) 1 mg L^{-1} Cd.

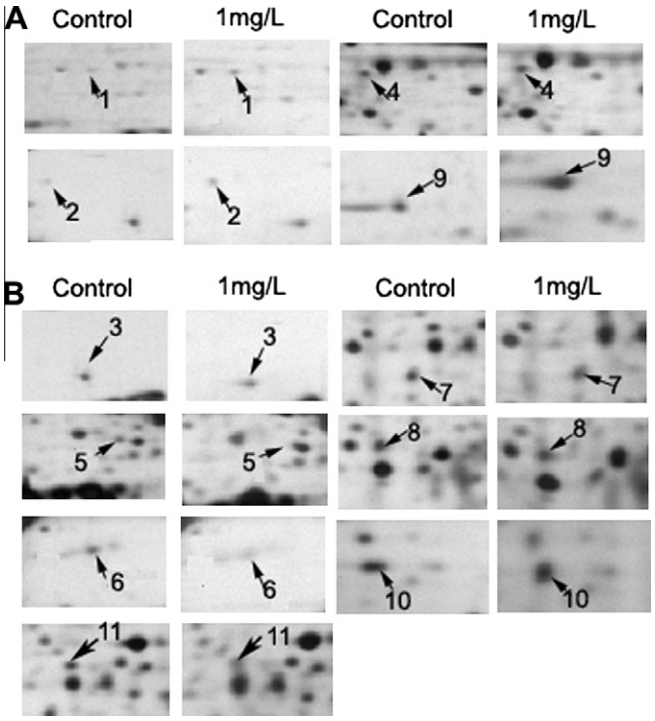

Fig. 3. Magnified images of protein spots that showed significantly different changes between control and Cd-treatment groups. (A) Up-regulated proteins found in Cd-treatment groups with respect to control; (B) down-regulated proteins found in Cd-treatment groups with respect to control.

and environmental exposures (Wetmore and Merrick, 2004). It compares the protein expression of a control versus an altered condition and reveals a set of biomarkers indicative of that altered state. Selection of biomarkers that reflect not only exposure but also provide information about potential physiological effects will generate data that are more valuable (Benninghoff, 2007). However, many classic biomarkers such as AChE, vitellogenin, metallothionein and many others can almost function as singular indicators of specific types of exposure and seem inadequate to this purpose. Instead, multiple markers that function as a "molecular signature" or "metabolic fingerprint" may be needed for classifying and describing mechanisms of toxicant action. Therefore, proteomics is proposed as an efficient tool for screening biomarkers of environmental toxicants such as Cd.

Exposure to Cd is believed to affect human and animal reproductive functions (Thompson and Bannigan, 2008), and alter the expression profiles of target proteins in the reproductive system. In the present study, proteomic approaches were used to reveal the changes in the global proteome of OG following Cd exposure. Five proteins (prohibitin, MIIMT, 4Fe–4S ferredoxin, serine/threonine-protein kinase, alphaTub84B), which are of importance in maintaining the normal status of the reproductive system were found to be responsive to Cd toxicity. Among these proteins, prohibitin and serine/threonine-protein kinase were associated with reproductive function in OG closely.

Prohibitin, a member of the stomatin/prohibitin/flotillin/HflK/C (SPFH) family of proteins (Browman et al., 2007), play a role in the maintenance of structure and function of mitochondria, the regulation of cell cycle, as well as in protection against oxidative damage in various cells and tissues types (Ande and Mishra, 2011). In addition, prohibitin also can play a part in the regulation of sperm metabolism (Sutovsky, 2003) and uterine development in mammals (He et al., 2011), indicating that it can take a part in keeping the reproductive function normally in human. Studies indicated that prohibitin protein acts as a binding site of ubiquitin. As ubiquitinated protein is degraded through proteasome, it suggests a relationship between prohibitin and protein degradation. Therefore, prohibitin participates in the sperm quality control (Ou et al., 2010). As mentioned previously, Cd may induce oxidative stress by producing hydroxyl radicals, superoxide anion radicals, nitric oxide, and hydrogen peroxide (H_2O_2), the study showed that PHB expression is decreased during oxidative stress (induced by H_2O_2) in cultured intestinal epithelial cells, IBD, and experimental models of colitis (Theiss et al., 2007). In addition, some studies have reported that hydroxyl radical induced by Cd^{2+} can cause oxidative damage in liver of *Carassius auratus* (Shi et al., 2005). Thus, the down-regulation of prohibitin in OG (Figs. 3B and 4A) may also be attributed to the oxidative toxicity of Cd^{2+} in our current study. According to the previous studies, the degradation of prohibitin protein induced by Cd^{2+} might have a great effect on the reproductive function in OG.

Table 1
Summary of differentially expressed proteins in OG exposed to 1 mg L^{-1} Cd.

Spot no.	Accession number	Protein name	Score	Subcellular location	Change	Relative intensity ratio[a]
1	gi|197245917	sfrs17a protein	64	Unknown	↑	1.92
2	gi|297607504	Os07g0576100	59	Unknown	↑	2.01
3	gi|300767811	ATP-dependent Clp protease ATP-binding subunit ClpC	69	Cytoplasm	↓	0.86
4	gi|195034694	GH11448	111	Nucleus	↑	1.22
5	gi|225718282	Mitochondrial import inner membrane translocase	62	Mitochondrion	↓	–
6	gi|303327364	4Fe–4S ferredoxin	67	Cytoplasm	↓	0.39
7	gi|38047815	alphaTub84B	329	Cytoplasm	↓	0.83
8	gi|255066353	Ribonuclease R	65	Nucleus	↓	0.81
9	gi|288791753	5-Oxoprolinase	75	Mitochondrion	↑	1.18
10	gi|241065293	Prohibitin	136	Mitochondrion	↓	0.84
11	gi|168705239	Probable serine/threonine-protein kinase	60	Cytoplasm	↓	0.67

[a] The ratio of the relative spot volume (Cd-treated/control), where a ratio above 1 indicates up-regulation and a ratio below 1 indicates down-regulation in Cd-treatment. "–" Indicates that the spots could not be detected in 2-DE gel.

Table 2
Functional classification of the proteins identified.

Spot no.	Protein name	Molecular function
1	sfrs17a protein	Unknown
2	Os07g0576100	Unknown
3	ATP-dependent Clp protease	ATP binding, ATP-dependent helicase activity, nucleic acid binding
4	GH11448	Helicase, hydrolase
5	Mitochondrial import inner membrane translocase	Proteins translocation
6	4Fe–4S ferredoxin	Electron transport in redox reactions
7	alphaTub84B	The major constituent of microtubules
8	Ribonuclease R	RNA hydrolysis
9	5-Oxoprolinase	Catalyze ATP hydrolysis
10	Prohibitin	Regulation of transcription, mitochondrial protein folding, sperm metabolism and uterine development
11	Probable serine/threonine-protein kinase	Signal induction, germ cell development

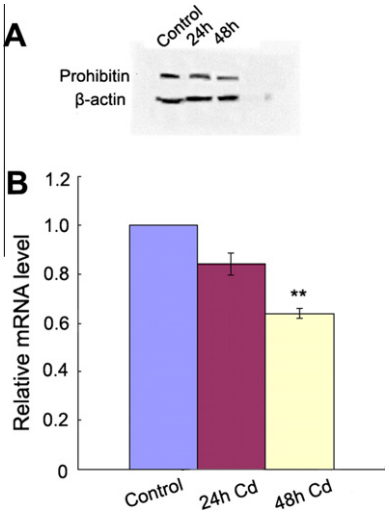

Fig. 4. (A) Western blot analysis of prohibitin expression level in OG. Protein (50 μg) was loaded onto a 12% SDS–PAGE and probed with antibody against prohibitin. β-Actin was also measured as loading control and was used for data normalization; (B) quantitative RT-PCR analysis of prohibitin mRNA level. Quantitative RT-PCR was performed on cDNA using gene specific primers for prohibitin and β-actin. Relative quantification of prohibitin mRNA level was normalized to β-actin level (mean ± SD, $n = 3$), $^{*}p < 0.05$; $^{**}p < 0.01$.

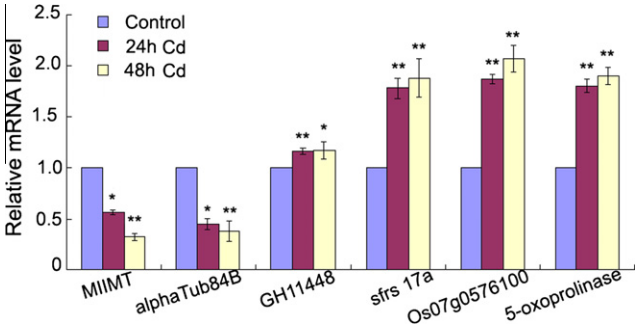

Fig. 5. Quantitative RT-PCR analysis of MIIMT, alphaTub84B, GH11448, sfrs17a protein, Os07g0576100, 5-oxoprolinase mRNA level in OG. Quantitative RT-PCR was performed on cDNA using gene specific primers for MIIMT, alphaTub84B, GH11448, sfrs17a protein, Os07g0576100, 5-oxoprolinase and β-actin. Relative mRNA levels of MIIMT, alphaTub84B, GH11448, sfrs17a protein, Os07g0576100, 5-oxoprolinase were normalized to that of β-actin (mean ± SD, $n = 3$), $^{*}p < 0.05$; $^{**}p < 0.01$.

Mitochondrial import inner membrane translocase (MIIMT) is localized in the inner membrane of mitochondria, which participates in the translocation of proteins across the mitochondrial inner membrane (van der Laan et al., 2010). It is known that the process of translocation is ATP-driven, thus, MIIMT should cooperate with ATPase tightly. Many studies have suggested that Cd affects ATPase activity in aquatic organisms. The gill and intestine $Na^{+}K^{+}$–ATPase and muscle Ca^{2+}–ATPase activities are inhibited by Cd in *Oreochromis niloticus* according to the reference (Atli and Canli, 2007). $Na^{+}K^{+}$–ATPase enzymatic activities, as measured in intestinal and branchial of eel (*Anguilla anguilla*), are inhibited by Cd in a dose-dependent manner (Lionetto et al., 2000). Therefore, Cd can affect MIIMT activity indirectly by inhibiting ATPase activities. In our work, a decreased expression of MIIMT was observed in response to Cd (Fig. 5), which is likely caused by the decrease of ATPase activities, indicating proteins translocation may be hampered in mitochondria and metabolism in the OG by Cd.

Ferredoxins are small, acidic proteins involved in the electron transport in a wide variety of redox reactions (Daas et al., 1994). The proteins typically contain non-heme iron and acid-labile sulfur

coordinated by cysteines, which can sustain cyclic as well as linear electron flow. The cyclic electron flow creates proton gradient across the photosynthetic membrane and allow ATP synthesis independent of photosystem II activity. During linear electron flow, reduced ferredoxins provide the electrons necessary for $NADP^+$ reduction in a reaction catalyzed by ferredoxin: $NADP^+$-reductase (FNR) (S'etif, 2001). Ferredoxins serve also as an electron donor to a number of soluble enzymes involved in nitrogen metabolism, sulfur metabolism and the regulation of carbon metabolism (Knaff, 1996). It was reported that Cd^{2+} might inhibit $NAD(P)^+$-linked GPDH activity in skeletal muscles of jerboa (*Jaculus orientalis*) (Berrada et al., 2002), consequently, Cd^{2+} might inhibit ferredoxins synthesis by blocking the electron transport chain. Almeida et al. (2002) reported that oxidative stress induced by Cd^{2+} can change the expression of some proteins. In our current research, ferredoxins showed a lower level of expression in the OG induced by Cd^{2+} exposure, whether the oxidative stress induced by Cd^{2+} is another reason for the ferredoxins activity decline needs further studies. The decreased expression of ferredoxins suggests the adverse effects of Cd on energy metabolism in organisms.

Protein kinases play an important role in signal transduction by phosphorylating specific amino acids of downstream substrates (Josso and di Clemente, 1997). The level of phosphoserine and phosphothreonine of intracellular protein substrates is determined in part by the balance of phosphorylation by mitogen-activated protein kinases (MAPK), a family of serine/threonine kinases, and dephosphorylation by serine/threonine protein phosphatase 2A (PP2A) (Sugiyama et al., 2003). Recent studies have shown that male reproductive function is modulated via the mitogen-activated protein kinase (MAPK) cascade (Li Michelle et al., 2009). The MAPK pathway is involved in many stages of germ cell development. These include spermatogenesis, germ cell cycle progression, germ cell apoptosis, acquisition of motility in the epididymis, sperm capacitation and acrosome reaction before the fertilization of oocytes (Wong and Cheng, 2005; Almog and Naor, 2008). Some studies have demonstrated that MAPKs could also affect germ cell development indirectly through their effects on Sertoli cells. Any perturbation in Sertoli cell function would impair spermatogenesis and thus reduce the semen quality (Li Michelle et al., 2009). In addition, studies have demonstrated that the toxicity of Cd is related to its induction of oxidative stress, e.g., reactive oxygen species (ROS), in various types of cells (Kim et al., 2005; Monroe and Halvorsen, 2006). The activities of creatine phosphokinase were reported to be reduced by Cd-induced oxidative stress (Almeida et al., 2002). In our work, a decreased expression of serine/threonine-protein kinase was observed in response to Cd (Fig. 3B), indicating that the oxidative stress caused by Cd may also decrease the activity of kinase in shellfish, which can make a severe damage in OG by disturbing the reproductive function.

Microtubules are polymers of α/β-tubulin heterodimers with complex dynamic structures and multiple protein-interacting surfaces (Nogales et al., 1999). Microtubules are cylindrical structures that participate in a variety of diverse functions in eucaryotic cells including mitosis, morphogenesis, and vesicle transport (Soifer, 1986). A molecular understanding of these functions will involve in part detailed knowledge of the properties of the major constituent protein of the microtubule, tubulin, and it two homologous subunits, α- and β-tubulin. When incubated with micromolar concentrations of Cd^{2+} or Ca^{2+} alone, or a mixture of both ions, microtubules in the extracted cytoskeletons disassembled (Perrino and Chou, 1989), indicating that Cd^{2+} can disrupts tubulin polymerization, and hypothesized that the toxicity of Cd^{2+} may be linked to disruption in microtubule function. In our study, the expression of alpha-tubulin was down-regulated by Cd treatment (Figs. 3B and 5), which was consistent with the results of Silvestre et al. (2006). In addition, some studies demonstrated that Cd induction of microtubule depolymerization by binding to the abundant sulfhydryl groups present on tubulin (Liliom et al., 2000). Therefore, we proposed that the down-regulation of alpha-tubulin may be another significant factor for tubulin depolymerization, subsequently, hindering the functions of microtubules in OG.

In this study, Western blotting and quantitative PCR were utilized to validate the 2-DE results. Western blotting directly reflects the protein expression level, while quantitative PCR displays mRNA level. It is well known that changes in rates of gene transcription do not necessarily correlate with protein expression or protein activity, because there are various levels of regulation during protein synthesis, e.g., translational, post-translational and post-transcriptional regulation (Hegde et al., 2003). Interestingly, the Western blot and PCR results both correlated well with the 2-DE data in the current study (Figs. 4 and 5). These results further confirm our confidence in the protein alterations revealed by proteomic approaches, and may indicate that the expression changes of these proteins are attributable to transcriptional regulation.

5. Conclusions

The comparative analysis in this study revealed eleven proteins, whose expression levels were significantly altered by Cd. Identification and functional analysis of these proteins indicated that disruption of protein transport, reproductive dysfunction, energy homeostasis and skeleton structure may be the factors contributing to Cd-exposure damage in the OG. Prohibitin and serine/threonine protein kinase are two promising indicative proteins for the reproductive dysfunction evaluation in OG. Therefore, they also might be as the biomarkers indicative of Cd contamination in mammals and human after accumulation of Cd by food chain. This study aims to develop useful biomarkers indicative of Cd toxicity. However, considerable validation work is still required before these proteins can be accepted as reliable biomarkers. In addition, it should be mentioned that the changes of protein profiles described here may reflect only a part of the entire change taking place in OG exposed to Cd, for the analysis was performed under specific Cd concentrations and exposure times. We hope that the data obtained in this work may provide positive insights for a better understanding of Cd toxicology as well as the development of novel candidate biomarkers of exposure to environmental Cd.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2011.12.032.

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