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RESEARCH ARTICLE

Proteomic analysis of dimethoate-responsive proteins in the oyster (*Saccostrea cucullata*) gonad

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Abstract
Introduction The organophosphorus pesticide dimethoate (DM) has been widely used in agriculture, and its extensive use could still have left many environmental problems.
Methods In the present study, the oyster (*Saccostrea cucullata*) was subjected to acute DM toxicity (2 mg/L), and gas chromatographic analysis revealed and quantified residues of DM in the oyster gonad.
Results Two-dimensional gel electrophoresis showed 12 differentially expressed proteins in the DM-exposed oyster gonad in comparison to the control. Among these 12 protein spots, nine were down-regulated, and three were up-regulated. Both matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry and database searching were utilized to identify these differential proteins, and revealed five proteins previously described as being related to DM toxicity. In addition, the levels of mRNA expression corresponding to these differential proteins were further proved in part by real-time PCR. The functions of these proteins were summarized as: carrying out energy metabolism, DNA repair, DNA transcriptional regulation, and oxidative protection. The remaining seven protein spots were of particular interest in terms of their responses to DM, which have seldom been reported.
Conclusion These data might point to a number of novel and significant biomarkers for evaluating the contamination levels of DM and provide useful insight into the mechanisms of DM toxicity in vivo.

Keywords Dimethoate · Oyster · Gonad · Reproductive toxicity · Proteomics · Biomarker

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1 Introduction

Organophosphorus pesticides (OPs) are deliberately released on farms for the control of low levels of agricultural pests because of their high insecticidal efficiency. However, extensive use of OPs can result in enhancing the level of environmental contamination and toxicity on targeted organisms leading to a number of pathological or disturbed biochemical processes (Dogan et al. 2011; Kumar et al. 2010). Among common OPs, dimethoate (DM) is widely used in agriculture against a wide range of insects and mites as both a systemic and a contact pesticide (Sayim 2007). However, its extensive use may also induce various adverse effects on health because of its persistence in soil, crops, and water (WHO/IPCS 1996). DM can also influence the health of the general population, as well as pesticide producers, workers, and farm owners etc. It causes diminished carbohydrate metabolism (Soler et al. 2011), histopathological changes (Rodrigues and Fanta

1998), respiratory disturbance (Shereena et al. 2009), endocrine disruption (Astiz et al. 2009), and carcinogenic effects (Reuber 1984). Many studies (Verma and Mohanty 2009; Farag et al. 2007; Abdallah et al. 2010) prove that DM can induce reproductive and developmental toxicity. DM can produce adverse effects on fertility, reproductive performance, and sperm parameters in male rats at 15 to 28 mg/kg/day; and a main reason for the reproductive toxicity is the reduction of testosterone caused by DM (Farag et al. 2006). DM exposure can also increase incidences of resorptions, decrease fetal body weight, and result in developmental toxicity. Exposure of female mice to 28 mg/kg/day DM during days 6–15 of gestation produces maternal fetotoxicity (Mahadevaswami and Kaliwal 2004).

Until now, almost all studies were mammal related, and the effects of DM induction on the gonad of aquatic, especially marine, organisms have seldom been reported. Various OPs can be exported to other places, particularly the sea, by inflows from rivers, lakes, and underdrainage (Zhang et al. 2002). Oysters live on the inshore beach and come into contact with various contaminants such as OPs from the water flowing into the ocean (Collin et al. 2010; Tang and Wang 2009). As a result, DM can be accumulated in the gonad and damage the reproductive system of the oyster, in that the normal function of the reproductive system is impaired and this reduces the production of oysters. In addition, cadmium and zinc, similar to DM, can accumulate quickly in humans through the contaminated food chain, and then hamper directly the human reproductive system (Cai et al. 2009; Green et al. 2010). Thus, it is of great significance to study the accumulation mechanism of OPs (especially DM) in vivo to reveal their harmful pathways.

In recent years, considerable interest has been shown in linking toxic responses to various gene and protein expression patterns (Ulrich and Friend 2002; Furness 2002; Yamagata et al. 2002), and proteomics serves as a useful tool to search for biomarkers indicating toxic responses (Yamagata et al. 2002). Therefore, using proteomic approaches, we analyzed alterations in the gonads of the exposed oyster, *Saccostrea cucullata*. As a result, significant changes were characterized in the oyster versus the control gonads. These proteins might play a biomarker role in monitoring the level of DM contamination in the sea.

2 Materials and methods

2.1 Chemicals and animals

We used analytical grade DM in our experiments. Oysters (*S. cucullata*) were purchased from a commercial supplier in Xiamen City, China, and held in a stock tank filled with seawater at a constant temperature of 25±2°C. The salinity of the water was approximately 2.8%. The medium pH was controlled to 8.0 and dissolved oxygen content was approximately 8 mg/L. The water was filtered and sterilized by ultraviolet radiation approach before used. After being acclimated for 1 week, six healthy oysters, of approximately the same size, were exposed to seawater containing 2 mg/L DM for 48 h. Meanwhile, another six oysters remained in seawater in the absence of DM as the control. Ternate parallel experiments of both control and DM exposure groups were carried out to get an average data with statistical analysis, respectively.

2.2 Protein sample preparation

One group of six oysters was held in 2 mg/L DM for 48 h against another group that lives in the absence of DM. After treatment, both the experimental and control gonad groups were quickly obtained using normal anatomical methods and homogenized at 4°C 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) for 5 min. Next, the homogenized sample was held at 4°C overnight for protein release and centrifuged at 100,000×*g* for 10 min, collecting both the supernatant and removing the cell debris. The protein concentration in the supernatant was measured using Bradford assay. All experimental protein samples were kept at −80°C until use.

2.3 CAT activity assay

After 12, 24, 48, 72, 96 h of exposure to 2 mg/L DM, the gonad tissue of oyster were excised under the condition of 4°C. The tissues were homogenized in 0.05 M PBS (pH 7.2) at 1:10 (*w*/*v*). Then, the homogenate were centrifuged at 15,000×*g* for 20 min. The supernatant was collected for analysis of enzyme activity, which was performed as reported previously (Zhu et al. 1996).

2.4 Residual weight analysis of DM using gas chromatography

The experimental oysters were randomly divided into four groups. Three groups were exposed to 1, 2, and 5 mg/L DM for 48 h, while the fourth, in the absence of DM, was considered to be the control. The experimental gonads tissue from the oyster was quickly transferred into 1.5 mL centrifuge tubes at 4°C. Then, the gonad samples were washed four times with 1 mL 0.9% NaCl (pH 9.0), and then 200 μL acetone accurately added. Next, the experimental samples were homogenized in a microhomogenizer at 2,000 rpm/min for 10 s with five repeats. Then, 1 mL of acetone was added into each tube to extract the DM. After being placed on ice for 15 min, these tubes were centrifuged at 12,000×*g*

at 4°C for 10 min. The supernatants were carefully collected, removed to 5 mL disposable glass tubes, and the same extraction procedure repeated three times. All the DM extracts for each experimental sample were mixed and then evaporated under a stream of nitrogen at 40°C. Finally, the collected DM residue of each sample was stored at −20°C before analysis. This involved the residue being dissolved in 100 μL *n*-hexane, vortexed, centrifuged, and the supernatant collected for GC analysis of the DM residue weight in the oyster gonad, using an Agilent 6890 series gas chromatograph. The DM analytes were then separated on a DB-5MS capillary column (60 M×0.32 mM×0.25 μm) by injecting 1 μL of extraction sample into the injection port, and the temperature was held at 230°C. The GC oven temperature was programmed at 70°C for 1 min, increased to 230°C at 20°C/min, and held for 3 min, and the FDP detector temperature was kept at 300°C. Three determinations were carried out for each sample to obtain the average weight of DM residue in the gonad. A standard regression curve was made using the DM concentration as the abscissa axis and characteristic peak volume as the ordinate. The weight of DM residue in the gonads was calculated based on the formula obtained from the standard curve.

2.5 Two dimensional gel electrophoresis

Two dimensional gel electrophoresis (2D-GE) was performed as reported (Feng et al. 2008). In brief, 120 μg protein sample was loaded onto 13 cm strips, and isoelectric focusing (IEF) was performed using carrier ampholyte (pH 5.0 to 8.0). A complete process for protein separation has been carried out by accumulative total of 10,000 Vh. Once the electrophoresis was completed, it will take 15 min to equilibrate the first dimension strip (equilibrium reagent, 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and 1% DTT). Next, it will take 30 min for SDS-PAGE (T=12%) as second dimension at 10 mA per gel, then at 25 mA per gel until the bromophenol blue arrives in the front of the bottom edge in the gel. For each protein sample (control or DM-treatment), three experimental replicates were subjected to 2-DE.

2.6 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), and 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 based on 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.

2.7 In-gel digestion and matrix-assisted laser desorption/ ionization time-of-flight tandem mass spectrometry 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 (Chen and Huang 2011). The peptide extracts were redissolved in 5 μL of 0.5% trifluoroacetic acid (TFA) and 1 μL matrix α-cyano-4-hydroxy-cinnamic acid added to 1 μL of the mixture. This was saturated with 0.05% TFA, spotted onto a MALDI target plate, then analyzed using a Bruker AutoflexIII® MALDI-TOF/TOF 200 mass spectrometer (Bruker, Germany) and an ABI4700 proteomics analyzing MALDI-TOF/TOF mass spectrometer (Applied Biosystems, USA). Database searches, including peptide mass fingerprinting and MS/MS, were performed using the MASCOT searching engine (http://www.matrixscience.com). The NCBInr database searches were performed using the following parameters: trypsin digest; using all entries; allow up to one missed cleavage; fixed modification of carbamidomethylation; variable modification of methionine oxidation; and maximal mass tolerance of 100 ppm. The results with confidence interval percent (C.I.%) values greater than 95% were considered to be a positive identification.

2.8 Quantitative real-time PCR

Total RNA was extracted from the gonads of six oysters using TRIzol reagent (Invitrogen, Carlsbad, CA), 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, 55°C for 15 s, and 72°C for 30 s). In brief, 10 μL of reaction mixture contained 1 μL of reverse transcribed product template (diluted to 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}), 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 1. 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 β-actin gene expression. The PCR data of treatments were calibrated with the control values.

Table 1 Specific primer pairs for the six oyster genes used in this study

Gene name	Primer 5′-3′
Cavortin	TTATGCTCAATGCGAGATGGA
	GTCTCCCCGTTGATGCATTT
Malate dehydrogenase	CTGTACCAATCGCAGAGGAAG
	CCTGGGAGAGCAGAGGAATA
Ribosomal S 2	GATTCGGCTCTGGTGGTAGA
	CCTTCAGAGATGGTCCAAGG
DNA alkylation protein	ACTTCCAAAGGCTCACGTTC
	CACCCTACCAACAATAGAAAAGG
FAD-dependent reductase	GCCATGTTTGTGGATGAGAA
	CCCAACTTTTCGGTGATGTC

The sequences were derived from the EST database

2.9 Statistical analysis

The data are all expressed as the mean±SD of triplicate experiments. Significant differences among multiple groups were determined using a one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered statistically significant at $p<0.05$ and $p<0.01$.

3 Results

3.1 The variation of CAT activity induced by DM

CAT is responsible for degrading hydrogen peroxide into water and oxygen, which works to convert ROS to water. The studies have showed that DM can trigger the CAT enzyme activity increased as an oxidative stress response (Dogan et al. 2011; Saafi et al. 2010). We also detected the CAT enzyme activity to reveal the oxidative stress that caused by DM. At the beginning, CAT activity varied in a typical dose–time–response fashion as exposure time increased. CAT enzyme activity of gonad raise to the maximum when exposure in DM for 48 h. However, its activity somewhat decreased as the time extended. It indicated that the biological responses of oysters to DM in 48 h were very significant. Thus, we chose the 2 mg/L DM exposure for 48 h as an experimental factor for selecting functional proteins. The results were shown in Fig. 1.

3.2 Residual weight of DM in the gonad

Gas chromatography analysis was carried out to detect the quantity of residual DM in both the oyster gonad control and the DM-treated groups. As shown in Fig. 2, a chromatography peak in 9.8 min represented the residual DM content in the gonad. However, this peak was not found in the control group using the same analytical and separating conditions. The DM residue content increment in the gonad matched with the DM concentration increment in seawater (Fig. 3), giving a positive correlation between them, and the residual quantity of DM was approximately 0.69 mg/kg in the gonad exposed to 2 mg/L DM. The results suggested that DM could be accumulated by the oyster gonad, and this could directly affect its physiological functioning.

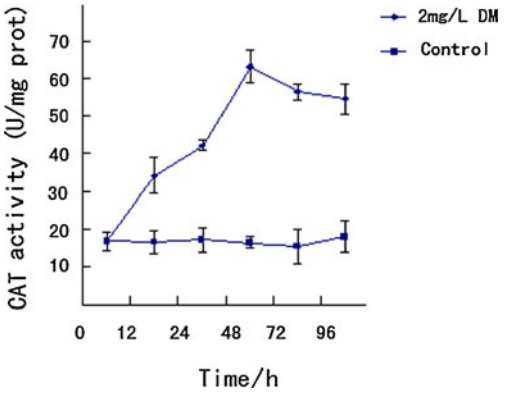

Fig. 1 CAT activity in the gonad of oyster at different times

3.3 Finding DM altered protein sequences of oyster gonad using PROWL

To detect the differentially expressed proteins induced by DM treatment, both control and DM-treated samples were subjected to 2-DE analysis. Silver staining revealed that most protein spots in the sample were located in the pH range 5.0 to 8.0 and the molecule weight of the protein subunit in the gels ranged from 25 to 66.2 kDa. Only the relatively abundant proteins in the gels were selected for identification, and 12 protein spots showed statistically significant differences in intensity between the two groups (the spots labeled no. 1 to 12 in Figs. 4 and 5). Of these, nine were down-regulated and three up-regulated. The 12 spots were excised from the gels, digested with trypsin, and the resultant peptide fragments analyzed using MALDI-TOF/TOF MS. Ion masses derived from the mass spectra were submitted to the MASCOT online database for identification. The identified protein, whose CI % is above 95%, has a high reliability, and we have also searched the homologous protein using the BLAST. Some protein have their homologous sequence in Bivalvia, but some proteins that can't be searched has homologous sequence in Bivalvia. These may be due to the protein database in Bivalvia which is not intact. The results are shown in Table 2.

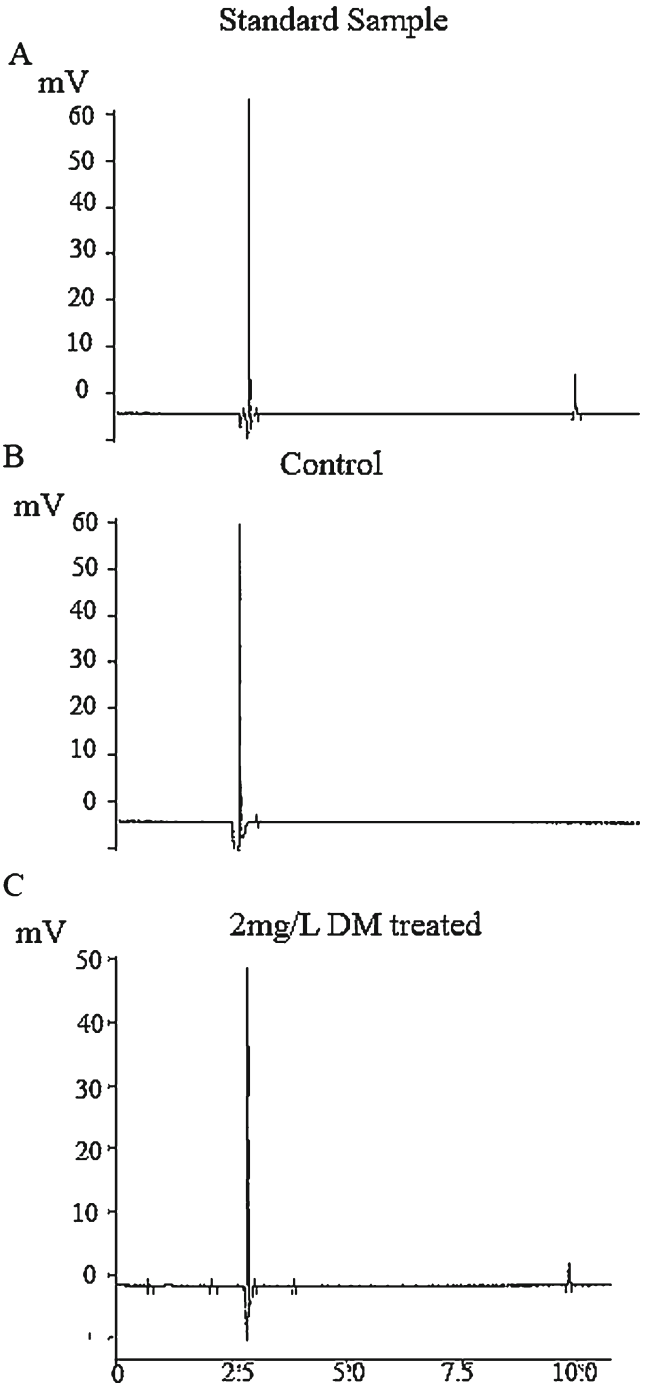

Fig. 2 Chromatograms of standard DM sample (**a**) , oyster gonad extract without DM treated (**b**), and oyster gonad extract after exposure for 48 h to 2 mg/L DM (**c**)

The functional properties and subcellular localization of some of these differential proteins were further classified by searching the Uniprot database (http://www.uniprot.org/). These differential proteins were localized in the cytoplasm, mitochondria, ribosome, and nucleus. Their main functions involved DNA repair, transcriptional regulation, and energy metabolism (Table 3).

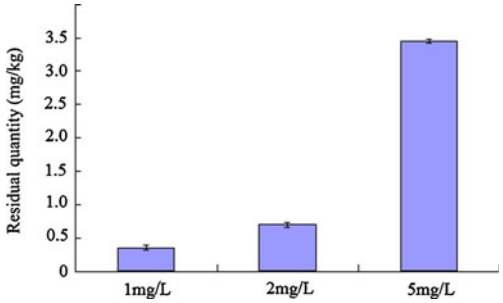

Fig. 3 The DM residual level in the oyster gonad affected by the concentration of DM added to the seawater

3.4 Real-time PCR analysis

To investigate whether the differential level of proteins was related to the different mRNA level, we used real-time PCR analysis. A total of five genes that had their sequences in the oyster database were chosen for this analysis. The real-time PCR results were closely consistent with the results of 2-DE (Fig. 6). This result further confirmed our confidence in the proteins indentured using the proteomic approach.

4 Discussion

The five proteins identified are reported to be related to DM toxicity. The quantitative PCR, utilized to study the mRNA level of the differential proteins and to validate the 2-DE results, showed good correlation between the two. Our present work further confirmed that DM can directly impair the biological functions of cell through affecting the expression level of these proteins.

The differential expression of these proteins suggested that DM could impair the oyster reproductive system through several pathways as follows:

Oxidative stress: Two differential proteins, whose functions are related to oxidative stress, were identified in our study. Cavortin, the major membrane protein of the Pacific oyster, is a non-pigmented, glycosylated protein with serine protease inhibitor activity (Itoh et al. 2011). The main role of cavortin is assumed to be as an oxygen storage and transport protein in oyster (Huvet et al. 2004). Cavortin may protect organisms from oxidative stress and, when bound to *Escherichia coli* 026:B6 LPS, lipid A and bacteria, it possesses superoxide dismutase activity (SOD). Cavortin is also associated with iron, which indicates that it may function as a chelator to prevent free iron from generating highly toxic reactive oxygen species (Gonzalez et al. 2005). Needed to be studied further is whether cavortin has a direct SOD enzyme activity; and it is unclear as to how the oxidative protection process by this enzyme participates (Gonzalez et al. 2005; Itoh et al. 2011). In addition, there is an

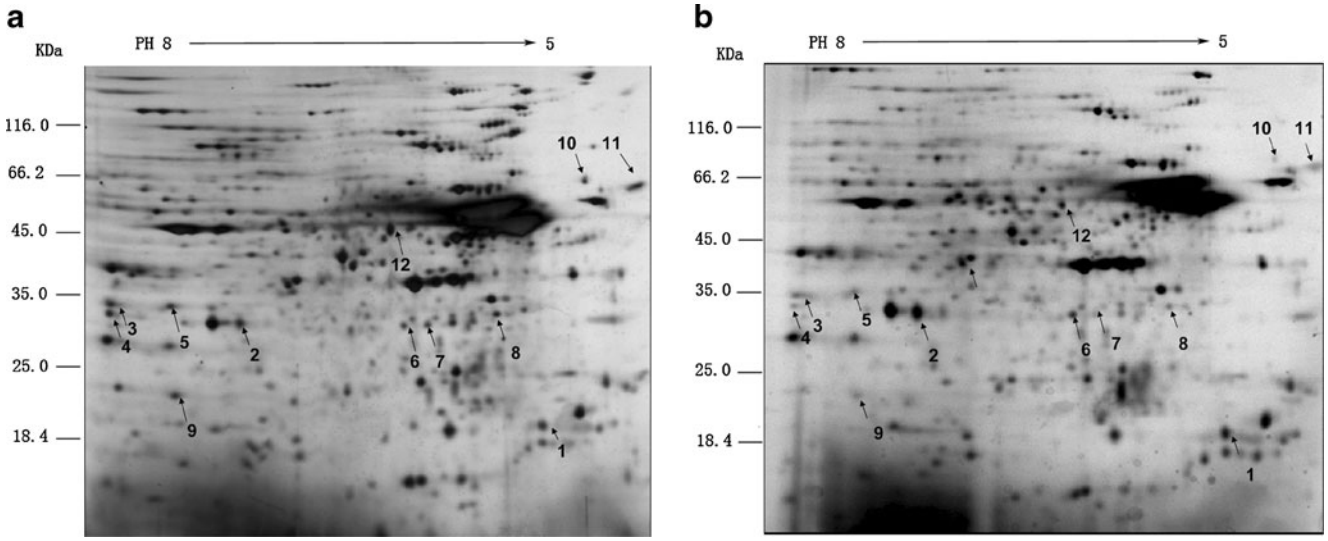

Fig. 4 2-DE maps of gonads of control group (**a**) and gonads of oysters which was exposed in 2 mg/L DM for 48 h (**b**), indicating protein spots which changed in volume after exposure to different concentrations of DM in the oyster gonad. Total proteins (120 μg) were separated using 2-DE and visualized using silver staining. *Arrows* indicate the proteins with a significantly modified expression level after DM exposure

indication that cavortin has a host defense role in oysters. (Huvet et al. 2004) have shown that the carvotin gene upregulation for resistence to summer morality. The studies also showed differential expression of the cavortin gene from *C. virginica* in response to infection of the oysters with the protozoan parasite *Perkinsus marinus* (Tanguy et al. 2004).

FAD-dependent oxidoreductase is a large type of oxidoreductase, whose function requires the cofactor FAD. Studies show that many types of FAD-dependent oxidoreductase have an anti-oxidative function. For example, NQO1, which is a widely distributed FAD-dependent oxidoreductase, catalyzes the reduction of quinones, quinoneimines, nitroaromatics, and azodyes in mouse (Dinkova-Kostova and Talalay 2010). It has multiple antioxidant activities through both its catalytic role in the reduction of quinones and its ability to scavenge superoxide directly (Siegel et al. 2004). In addition, FAD-dependent oxidoreductase Frm2p enhances resistance to oxidative stress by regulating the antioxidant enzymatic activities of SOD, CAT, and GPx in *Saccharomyces cerevisiae* (de Oliveira et al. 2010). Our results showed that both cavortin and FAD-dependent oxidoreductase were down-regulated at both the mRNA level and protein level in the treated group, indicating that the oxidative protection mechanism was hampered and that the ROS level in the gonad might be overproduced. Such overproduction of the ROS level is detrimental to sperm, being associated with male infertility (Guo et al. 2005; Turner

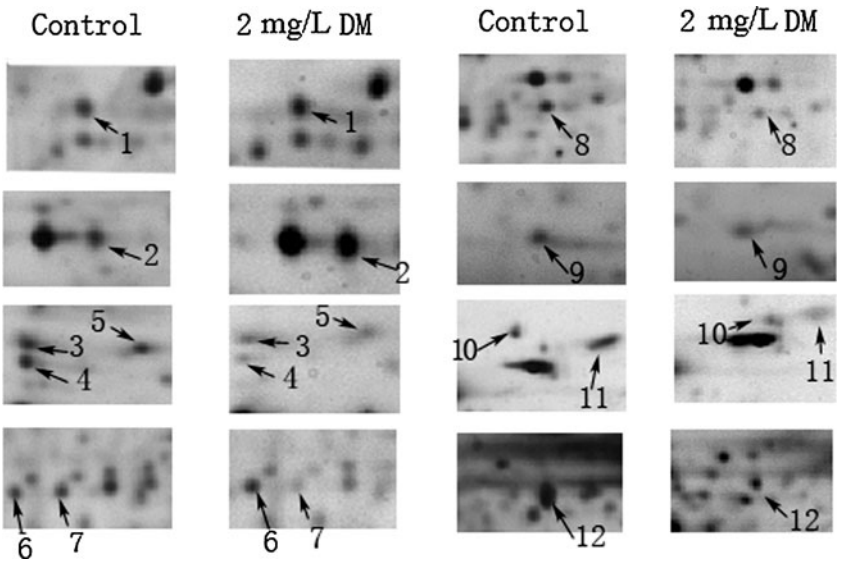

Fig. 5 Magnified images of protein spots that showed significantly different changes between control and DM treatment groups

Table 2 Identification of proteins differentially expressed in response to acute exposure for 48 h of DM at 2 mg/L

Spot no.	Accession number[a]	Organism	Protein name	Protein score	C.I.%[b]	Homolog protein (accession and organism)	Change	Individual intensity/total intensity (control)[c]	Individual intensity/total intensity (2 mg/L)
1	gi|227504122	*Corynebacterium striatum ATCC 6940*	DNA alkylation repair protein	65	96%	*Pinctada fucata* (AAX56337.1)	↑	0.273	0.381
2	gi|281210696	*Polysphondylium pallidum PN500*	DNA repair and recombination protein rad22	73	96%	*Pinctada fucata* (BAF73720.1)	↑	0.283	0.532
3	gi|85090420	*Neurospora crassa OR74A*	Malate dehydrogenase	61	100%	*Crassostrea angulata* (ACU33972.1)	↓	0.218	0.121
4	gi|191170944	*Escherichia coli F11*	Hypothetical protein EcF11_0472	52	97%	*Pinctada fucata* (ABL63470.1)	↓	0.201	0.085
5	gi|256767690	*Streptomyces sp. C*	Protein involving differentiation	66	100%	*Mytilus galloprovincialis* (BAJ15435.1)	↓	0.186	0.153
6	gi|20129563	*Drosophila melanogaster*	CG17331	56	95%	*Crassostrea gigas* (ACL31211.1)	↑	0.143	0.254
7	gi|194289459	*Cupriavidus taiwanensis LMG 19424*	Transcriptional accessory protein	79	100%	*Ruditapes philippinarum* (ACU83230.1)	↓	0.196	0.112
8	gi|134096962	*Saccharopolyspora erythraea NRRL 2338*	LysR family transcriptional regulator	61	100%	*Crassostrea virginica* (BAF30874.1)	↓	0.22	0.137
9	gi|30039400	*Crassostrea gigas*	Cavortin	101	100%		↓	0.265	0.173
10	gi|118496840	*Francisella tularensis subsp. novicida U112*	Ribosomal protein S2	84	100%	*Crassostrea gigas* (AAS93901.1)	↓	0.223	0.132
11	gi|152983626	*Pseudomonas aeruginosa PA7*	Hypothetical protein PSPA7_3329	93	99%	*Crassostrea gigas* (BAG12303.1)	↓	0.564	0.403
12	gi|114049845	*Streptomyces ambofaciens*	Putative FAD-dependent oxidoreductase	58	100%	*Crassostrea gigas* (CAE51045.1)	↓	0.602	0.341

[a] Protein accession numbers came from the SWISS-PROT database or NCBInr Data Bank

[b] Percentage of the protein sequence covered by the matched peptides

[c] Relative volumes (individual/total) of corresponding spots in 2D-GE gel of control and DM-treatment groups

Table 3 Subcellular localization and biological function of representative proteins associated with DM toxicity

Spot no.	Protein name	Subcellular location[a]	Biological function[b]
1	DNA alkylation repair protein	Cytoplasm/nuclear	DNA repair[b]
2	DNA repair and recombination protein rad22	Nuclear	DNA replication/DNA recombination
3	Malate dehydrogenase	Cytoplasm/mitochondria	Glycolysis/TCA cycle
4	Hypothetical protein EcF11_0472	Unknown	Unknown
5	Protein involving differentiation	Unknown	Unknown
6	CG17331	Cytoplasm/nucleolus/ proteasome complex	Threonine-type endopeptidase activity
7	Transcriptional accessory protein	Cytoplasm	DNA binding
8	LysR family transcriptional regulator	Nuclear	Regulation of transcription, DNA-dependent
9	Cavortin	Membrane/cytoplasm	Oxidative protection
10	Ribosomal protein S2	Ribosome	Structural constituent of ribosome
11	Hypothetical protein PSPA7_3329	Unknown	Unknown
12	Putative FAD-dependent oxidoreductase	Mitochondria	Flavin-containing monooxygenase activity

[a]Cellular component in which the protein was located

[b]The functions of protein which have been reported

The functional properties and subcellular localization of these differential proteins in part were further classified by searching the Uniprot database (http://www.uniprot.org/)

and Lysiak 2008). Here, we indicate that the host defense mechanism may also be hampered when exposed to DM because the expression of the cavortin is down-regulated.

DNA damage: O^6-alkylguanine-DNA alkylansferase (AGT) is a widely studied 21-kDa DNA repair protein that normally catalyzes the transfer of alkyl substituents from the O^6-position of guanine to an active cysteine (Cys145) acceptor site within the protein (Niture et al. 2005; Mitra and Kaina 1993). It is found in the cytoplasm as well as the nucleus and has a protective effect on both tumor cells and normal cells against the mutagenic, carcinogenic, and toxic effects of O^6-alkylating agents in humans (Pegg 2000). As an organophosphate, DM can induce alkylation of DNA and result in DNA damage. The electron-rich atoms in DNA are readily attacked by electrophile-caused alkylation, resulting in mutagenic or clastogenic effects (Dogan et al. 2011). Our research showed that AGT was up-regulated in the treated group. It is assumed to be a self-protection mechanism against DNA alkylation, suggesting that DM can cause DNA damage and fetal variation in oyster.

Transcriptional and translational disturbance: Ribosomal proteins are integral components of the basal cellular machinery involved in protein synthesis. Ribosomal protein S2, which is loosely associated with the 40S subunit, spans the head–body hinge region of the 40S subunit. It participates in aminoacyl-transfer RNA binding to the ribosome, potentially affecting the fidelity of mRNA translation in fission yeast. (Moll et al. 2002). Moreover, many ribosomal proteins are also involved in cell growth regulation and transformation (Bachand and Silver 2004). Thus, ribosomal proteins appear to be important for metabolic adjustments. Our results showed that ribosomal protein S2 was down-regulated in the oyster gonad. This indicated that DM can hamper the translation and transcription process in the cell and affect the normal development of oyster larvae.

Energy metabolism: Malate dehydrogenase, which catalyzes the interconversion of oxaloacetate to malate, exists in two isozymic forms viz, cytoplasmic (cMDH) and mitochondrial (mMDH). The latter participates in the tricarboxylic acid cycle inside mitochondria and catalyzes the generation of protons, while cMDH is a cytoplasmic protein involved in anaerobic glycolysis and catalyzes the inter conversion of pyruvate to lactate. As a result, malate dehydrogenase is a key enzyme in both aerobic and anaerobic energy metabolism (Mishra and Shukla 1997; Samuel and Sastry 1989). The specific activity of cMDH and mMDH declines significantly in response to treatment with the organophosphate pesticide phorate (Tripathi et al. 2009). The reduction of enzyme activities (cMDH, mMDH) could be caused by binding to phorate or its metabolites. Our results also showed that the protein level of MDH was reduced in the DM-treated oyster. Likewise, the mRNA level of MDH decreased as a function of increasing DM exposure concentration. Since this enzyme was inhibited, the TCA cycle in gonad cells must be hampered, and thus, we propose that

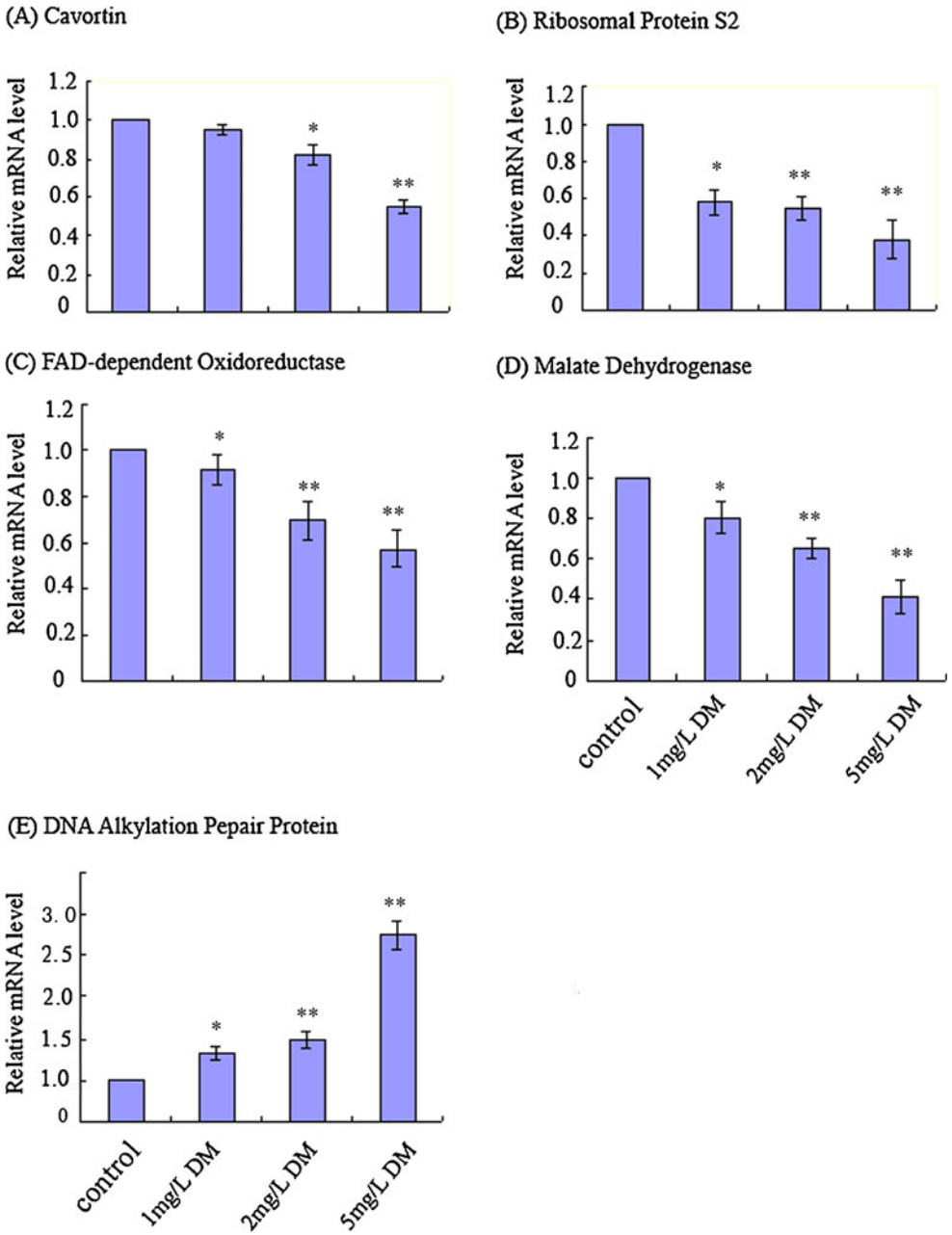

Fig. 6 Quantitative RT-PCR analysis of cavortin (**a**), ribosomal protein S2 (**b**), FAD-dependent oxidoreductase (**c**), malate dehydrogenase (**d**), and DNA alkylation repair protein (**e**) mRNA level. Quantitative RT-PCR was performed on cDNA using genes specific for cavortin, ribosomal protein S2, FAD-dependent oxidoreductase, malate dehydrogenase, and DNA alkylation repair protein. Relative mRNA levels were normalized to that of β-actin (mean±SD, $n=3$), *$p<0.05$; **$p<0.01$

DM represses aerobic energy metabolism in the oyster gonad.

5 Conclusion

In the current study, we detected the changes of protein expression in oyster gonad treated with DM (2 mg/L). As a result, 12 proteins were found to significantly change when exposed to DM. Biological functional analysis indicate that these proteins will be connected with oxidative stress, disturbance of energy metabolism, DNA alkylation, dysregulation of transcription and translation, which might be the important factors contributing to DM-exposure damage. These differentially expressed proteins might also serve as potential biomarkers of reproductive toxicity to oysters, but further work is still required before these proteins can be accepted as reliable biomarkers. However, the data obtained in this work might provide positive insights for a better understanding of DM toxicology as well as the development of novel candidate biomarkers of exposure to environmental DM.

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