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Proteomic analysis of methyl parathion-responsive proteins in *Sparus latus* liver

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ABSTRACT

The contamination of marine ecosystems by organophosphate pesticides is of great concern. The use of protein expression profiles may provide a good method to help us understand the methyl parathion (MP) toxicity to aquatic organisms. In this study, *Sparus latus*, was selected as the target organism. The toxicological effects of MP were investigated after 24 h exposure using proteomics to analyze their liver tissues. Certain enzyme activity parameters of the liver extracts were also examined, including CAT. After analyzing the proteomic profile of the liver using 2D gel electrophoresis, we found that the protein expression levels of 25 spots increased or decreased significantly in the exposed groups. Sixteen of the 25 protein spots were successfully identified using MALDI-TOF MS/MS. These proteins were roughly categorized into diverse functional classes such as cell redox homeostasis, metabolic processes and cytoskeleton system. These data demonstrated that proteomics was a powerful tool to provide valuable insights into the possible mechanisms of toxicity of MP contaminants in aquatic species. Additionally, these data may provide novel biomarkers for evaluation of MP contamination in the environment.

1. Introduction

In recent decades, many populations of aquatic species have declined in terms of the number of geographical locations worldwide, particularly in Southest Asia [1]. The causes are assumed to be human alterations to the environment and exposure to environmental pollutants, such as organophosphate (OP) pesticides. Environmental pollution from OP pesticides is an important issue that has attracted widespread public concern. Residual amounts of OP pesticides have been detected in the soil, water bodies, vegetables, grains and other foods products [2,3]. OP pesticides are known to cause inhibition of acetycholinesterase and pseudocholinesterase activity in the target tissues [4]. Other systems that are could be affected by OP intoxicants are the immune system [5], pancreas [6] and liver [7] and also hematological changes may result.

Methyl parathion (*O*,*O*-dimethyl *O*-4-nitro-phenylphosphorothioate, MP) is one of the most widely applied OP insecticides in agriculture [8]. A recent study indicates that traditional farmers in developing countries are highly exposed to MP [9]. Furthermore, its low cost and high efficacy have led to commercial and illegal domestic use. MP has been reported to cause DNA damage [10], and a significant increase in the percentage of abnormal sperm is observed in mice treated orally with MP [11], but few reports were found showing proteome change in halobis under MP stress.

Oxidative stress arises due to an imbalance between the formation of reactive oxygen species (ROS) and clearance of the ROS by components of the antioxidant defense system [12].Oxidative stress is implicated in the pathogenesis of disease and ageing in the liver and for example, liver damage caused by excessive acute and chronic ethanol ingestion1 [13] and iron overload [14] are attributed to increase ROS. During ageing, ROS and oxidative damage to the mitochondria increase [15]. MP induces oxidative stress by the generation of ROS, such as the superoxide anion radical ($O_2^{-}\bullet$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (•OH), resulting in lipid peroxidation, DNA damage and protein oxidation in organisms [16–18]. The sensitivity of the cell to ROS is attenuated by an array of non-enzymatic and enzymatic antioxidants. Catalase (CAT), which is regarded as enzymatic antioxidant is implicated in the defense of toxic effects of ROS.

Direct monitoring of protein expression levels is a useful tool to achieve a potentially overall view in understanding the mechanisms of toxicity of MP. Proteins are the actual functional molecules in the cell and therefore, proteomic analysis may provide more direct insight into the mechanisms of the MP effects [19,20]. The use of the proteomics technique in vertebrate ecotoxicology has increased greatly in recent years, for example in rat [21,22], frog

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[23], and fish [24]. Recently, environmental proteomics has also been applied to marine organisms at the laboratory and field level, such as sheepshead minnows [25] and mussel [26]. However, a proteomic approach to assess the OP effects including MP toxicity has seldom been reported. A significant work is necessary to find effective biomarkers of OP level and study their toxicology in both terrestrial animals and halobios for food safety.

For these reasons, we analyzed the proteomic alterations in *Sparus latus* liver after continuous exposure to 2 mg/L MP over 24 h, using 2-DE and MALDI-TOF MS/MS approaches. These approaches may identify proteins altered by MP that, once validated, can be used as new unbiased biomarkers which are used in MP pollution monitoring by providing a full and prior knowledge of MP toxicity.

2. Materials and methods

2.1. Experimental animals

Houttuyn (*Sparus latus*) were purchased from a commercial supplier in Xiamen, China, and raised in a stock tank at a temperature of 10 °C in the laboratory. After being acclimated for one week, ten healthy animals, approximately the same size (12 cm length), were exposed to 2 mg/L of MP (Sigma, USA) prepared using freshwater. Meanwhile, ten fish were maintained in freshwater without MP as control.

2.2. CAT activity assay

After 4, 8, 12, 24 h of exposure to 2 mg/L MP, the liver tissues of *Sparus latus* were excised. The tissues were homogenized in 0.05 M PBS (pH 7.2) at 1:10 (w/v) using an IKA® T10 basic Disperser (IKA, Germany). The homogenates were centrifuged at 15000 × *g* for 20 min, and the supernatant was used as the enzyme source for the estimation of CAT activity. The activity assay was performed as reported previously [27].

2.3. Protein sample preparation

After being acclimated for one week, houttuyn were randomly divided into a treated and a control groups. The treated group was exposed to 2 mg/L MP for 24 h before used. The control group was bred in freshwater without MP under the same condition of breed aquatics.

After being ground with liquid nitrogen, liver samples of each group (about 40 mg of tissue from 5 fish) were dissolved for 4 h 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). Next the homogenates were centrifuged at 100,000 × *g* for 30 min to remove debris and the supernatants were collected for 2-DE or Western blotting, and the protein content measured using the Bradford assay [28].The protein samples were stored at −80 °C before used.

2.4. 2-DE

2-DE was carried out as previously reported [29].

2.5. Silver-staining and image analysis

Silver-staining was performed as described previously [30]. The silver-stained gels were scanned using a GDS8000pc gel image formation analysis system, and the differential protein spots were analyzed and counted using ImageMaster 2D Platinum v5.0.

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

The protein spots of interest were excised from the 2D gels and washed with water before digestion. Digestion and peptide extraction were performed according to reference methods [31]. 2.5 μL 0.5%TFA (v/v) was added to dissolve the peptides. The proteins were analyzed with a Bruker Autoflex III® MALDI-TOF/TOF 200 mass spectrometer (BRUKER, Germany). Laser shots of 200 per spectrum were used to acquire spectra with a mass range of 800–3000 Da. pectra 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. Individual ions scores greater than 32 were defined as significant ($P < 0.05$).

2.7. Western blotting

Proteins (40 μg) from each liver sample were separated on 12% SDS-PAGE. After electrophoresis, the resolved proteins were transferred to PVDF membrane. The membrane was then blocked for 1 h in TBST (50 mmol/L Tris–HCl, pH 7.6, 150 mmol/L NaCl and 0.1% Tween 20) containing 5% (w/v) nonfat milk, and then incubated with primary antibody anti-methionine adenosyltransferase I/II/III (H-300) (Santa Cruz, USA) (1:2000 dilution) overnight. The membrane was washed three times with TBST and incubated with HRP-conjugated goat anti-mouse IgG (Pierce, USA) (1:10000 dilution) for 1 h. The membrane was again washed three times with TBST, and the blots were developed with ECL. The signals from each protein band were normalized against the β-actin content using the polyclonal anti-actin antibody (Santa Cruz Biotechnology, USA). 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

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) (one 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 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. The 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. Result and discussion

3.1. The variation of CAT activity induced by MP

MP induces oxidative stress by the generation of ROS. The cellular response to the formation of oxygen radicals involves many defense mechanisms that including an increased activity of a free radical scavenger enzyme, CAT [32,33]. CAT is responsible for degrading hydrogen peroxide into water and oxygen, which works to convert ROS to water. Therefore, we focused on the variation in