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Proteomic changes in response to acute cadmium toxicity in gill tissue of *Paralichthys olivaceus*

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ABSTRACT

In the present study, we developed a two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) technique for examining the response of the proteome from gill tissue of *Paralichthys olivaceus* (POGT) to acute cadmium (AC) toxicity. Approximately 700 protein spots were detected from the gill sample when applying a 600 μg protein 2D-PAGE gel in the pH range 5.0–8.0, and approximately 400 of these were identified by peptide mass fingerprinting (PMF) and database search. Compared to a control sample, significant changes were visualized in 18 protein spots exposed to seawater cadmium acute toxicity at 10.0 ppm for 24 h. Among these spots, two were up-regulated, one was down-regulated, seven showed low expression, and eight showed high expression. The collected spots were further identified by PMF and database search. Ten of the 18 proteins identified on the 2D-PAGE gel, including heat shock protein 70 and calcium-binding protein, demonstrated a synchronous response to AC, and we suggest that the variable levels and trends of these spots on the gel might be utilized as biomarker profiles to investigate cadmium contamination levels in seawater and to evaluate the degree of risk of human fatalities. The experimental results emphasize that the application of multiple biomarkers has an advantage over single biomarkers for monitoring levels of heavy metal contamination in seawater.

1. Introduction

The pollution of aquatic ecosystems with metals is a serious threat to the environment due to their persistent nature, long distance transport, and toxicity to aquatic organisms (Bench et al., 2001; Huang et al., 2005). The level of heavy metal pollution in aquatic ecosystems (water and sediment) is usually monitored by chemical methods such as atomic absorption spectrometry (AAS) or inductively coupled plasma mass spectrometry (ICP-MS). Although measurements of total metal concentration provide useful information concerning the pollution status of the environment by these chemicals, they do not show more direct evidence of bioavailability, bioaccumulation, or toxicity of metals to aquatic organisms (Glynn, 2001; Huang et al., 2004; Redeker et al., 2004). In fishes, the gill is an important target organ for the toxic actions of water-borne Cd, Zn, Cu, etc., mainly due to disturbance of ion-transport in the gill epithelium (Glynn, 2001; Redeker et al., 2004), so that heavy metal ions such as Cd^{2+} are readily taken up from the water into the gill epithelium. Several studies indicate that calcium-transporting chlorides are important sites of apical Cd^{2+} and Zn^{2+} uptake. Moreover, these results show that the influx of both Cd and Zn ions from the water to the circulatory system are similarly influenced by Ca^{2+}, suggesting that interactions between the ions may occur at sites involved in Ca^{2+} transport through the gill (Devos et al., 1998). Despite these findings regarding interaction between Cd and Zn ions with Ca^{2+} binding sites *in vivo*, little is known about the direct Cd influx into the circulatory system in the fish gill while exposed to variable levels of Cd pollution.

More specifically, inside the gill epithelium, so-called chloride cells are implicated in ion transport. The structure of these cells is characterized by their content of numerous small mitochondria and an extensive tubular network (Takaki et al., 2004; Uriu et al., 2000), and so changes in cadmium pollution on the surface of chloride cells can be observed by transmission electron microscopy. In summary,

Abbreviations: AAS, atomic absorbance spectrometry; AC, acute cadmium; CaM, calmodulin; CBB, Coomassie brilliant blue; CBP, calcium-binding protein; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; Hsp, heat shock protein; ICP-MS, inductively coupled plasma mass spectrometer; IEF, isoelectric focusing; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PMF, peptide mass fingerprinting; POGT, gill tissue of *Paralichthys olivaceus*; SCAT, seawater cadmium acute toxicity; Sp1, specificity protein 1.

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fish gill cells can provide biomarkers for monitoring contaminating levels of Cd and Pb ions in the water because the gill acts as an accumulative tissue for heavy metal ions and is always exposed to the flowing water.

The most common stress proteins are in the heat shock protein (Hsp) 60-, Hsp70- and Hsp90-kDa families, which show structures very homologous between species (Madden et al., 2002). Hsp70 can accumulate in renal epithelial cells during prolonged cadmium exposure, and the cadmium induces differential expression of Hsp in the cells, so that protein expression patterns in epithelial cells are specific to the cadmium concentration and degree of cellular injury (Bonham et al., 2003). A potential role for Hsp70 in the cellular response to sublethal cadmium-induced injury is also indicated by these authors.

Calmodulin (CaM) is important in many processes involved in cell proliferation. Cd can replace calcium and, by inducing a similar conformational change in CaM, can remove the control of CaM from calcium, and so disturb many cellular functions (Jaren et al., 2002). In functional analysis, cadmium retention and signs of toxicity are enhanced by a low Ca diet and increased calcium-binding protein (CBP) activities due to Ca restriction are responsible for the increased Cd uptake observed.

The toxicity of cadmium to animals including fishes has been extensively studied and is well known. Most studies have focused on the physiological affect of acute exposure to various heavy metal ions. Though there are a large number of research reports regarding the crucial physiological effects of cadmium on the liver, gill and brain, its *in vivo* toxicity mechanisms are still unclear or in dispute. Since different studies focus on measuring heavy metal accumulation in the tissues of different organisms, the effects of heavy metal toxicity at the cellular level are still not yet adequately recognized. Although the levels of various biomarkers are known to indicate heavy metal intoxication in organisms and their different tissues, the mechanisms of only a few markers are understood (Almedia et al., 2002; Hollis et al., 1999; Torre et al., 2000).

In the present work, the toxic effects of Cd on the fish gill were first evaluated, by a combined off-line approach involving two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), peptide mass fingerprinting (PMF) and database search. Protein changes were then analyzed by means of their expression levels since these act as common mechanisms for those fishes tolerant to cadmium. Here, we suggest that these specific changes show more sensitivity and dependability than current analytical techniques such as AAS or ICP-MS.

2. Materials and methods

2.1. Chemicals and materials

The fish *Paralichthys olivaceus* was purchased from the Xiamen Fish Company, China. Feeding was suspended 24 h prior to and during the experiment in order to avoid any effect resulting from the feed containing heavy metals. Ampholines with pH grads covering pH 5.0–8.0 or pH 3.5–10.0 were obtained from the Amersham Biosciences Co. (Sweden). Acrylamide, bisacrylamide, and matrix α-cyano-4-hydroxy-cinnamic acid (HCCA), and other electrophoresis reagents were obtained from Sigma Co. (Cleanse, USA). Inorganic chemicals were analytical grade reagents which came from commercial sources (Shang Hai Yi Yao Co.) in China.

2.2. Sample preparation

After 1 week's acclimatization, five healthy, 3-month-old fish of approximately the same weight (500 g) were exposed to concentrations of cadmium (as $CdCl_2$) at 1.0, 5.0 and 10.0 ppm prepared, using stock seawater. Here, it was noted that Cd ions could be directly absorbed onto the filter which resulted in a shift in the ionic concentration of seawater. For this reason Cd levels of acute exposure (10.0 ppm) were monitored in the seawater tank using AAS or ICP-MS with reference to the desired experimental time. Three replicate experiments were analyzed for the control and for each test concentration (1.0, 5.0 and 10.0 ppm), but the experimental results described here are only for 10.0 ppm, because this concentration represented the toxicity of acute cadmium (AC) rather than that of chronic cadmium (where the differential proteins showed minor changes when exposed to cadmium at 8.0 ppm or lower for 24 h). The whole gill was excised and promptly dissected free from the arches for sample preparation. It was then placed in Tris–HCl (pH 7.5) and maintained at −80 °C before use. Gill tissue was carefully homogenized using a handcraft triturator fitted on quartz sand in the presence of 4 °C seawater for 12 min. The mixture of gill plasma and sand was transferred into a 5.0 mL polypropylene tube packed with Tris–HCl buffer (pH 7.5) for further protein release while held at 4 °C overnight, and then extracted twice for 6 min using sonication. After homogenization and extraction, the gill samples were centrifuged for 5 min at 10,000 × *g* to completely remove the sand. Finally, the gill tissue protein suspension was collected carefully from the tube using a pipet.

2.3. Two-dimensional electrophoresis

For each ecotype sample, 2D-PAGE was performed in triplicate using 13 cm linear pH 5.0–8.0. The protein samples were dissolved in sample buffer (7 M urea, 4% CHAPS, 2 M thiourea, 60 mM DTT, 10 mM Tris, 1 mM EDTA, 0.002% bromophenol blue) supplemented with 1% (w/v) carrier ampholytes (Amersham Bioscience, Sweden), at pH 5.0–8.0. To optimize solubilization of proteins, we saturated the protein solution with urea by the addition of solid urea. Isoelectric focusing (IEF) of protein samples with the carrier ampholytes for first dimensional electrophoresis was performed at 11,200 Vh in a stepwise fashion (2 h at 300 V, 2 h at 500 V, 16 h at 600 V) with an acrylamide concentration of 4.0%T/0.1%C (add 3.896 g of acrylamide and then 0.03 g bisacrylamide to 100 mL of H_2O) in 1.5 mm × 15 cm tube gels. The gel buffer contained 2% NP-40, 0.1% CHAPS, 0.01% APS and 1% carrier ampholytes. SDS-PAGE for second dimensional electrophoresis was carried out using an Investigator 2D Gel System (PerkinElmer Co.). The gels were stained using colloidal Coomassie brilliant blue (CBB) G-250. Images from stained gels were digitalized at 800 dpi with a flatbed scanner (PerkinElmer Co.) and analyzed using the Melanie 4 software for numbers analysis of protein spots. Gel triplicates were matched to create an average gel with spots present at least on three of the four gels for protein identification using MALDI-TOF-MS analysis. In addition, in order to compare the protein resolution separated using a typical 2D-PAGE with that described here, the first dimension (IEF) of the current high resolution 2D-PAGE packed with non-linear wide-range immobilized pH gradients (pH 4–9 range/18.0 cm, Amersham Pharmacia Biotech) with a horizontal electrophoresis apparatus (PerkinElmer Co.) was carried out according to the reference method described by Gorg et al. (2000). IPG dry strips were rehydrated directly with the same solution (in-gel rehydration) and were run according to the manufacturer's instructions. The experimental results indicated that the two methods (that is, both the traditional 2D-PAGE and the current 2D-PAGE packed with IPG strips (2D-$PAGE_{IGP}$)) showed similar protein resolution and a close number of protein spots on the gels. Based on the large number of optimized conditions here, we have successfully established a 2D-PAGE approach to separate the proteome of gill tissue of *Paralichthys olivaceus* (POGT) at high resolution for proteomic analysis.

2.4. Protein identification by PMF

Protein identification by PMF was carried out as previously described (Simpson, 2003) with minor modification. After visualization with CBB staining, pieces of gel were then washed using a solution consisting of ammonium carbonate and acetonitrile. Different protein spots were excised and destained with a solution of 50 mM ammonium bicarbonate/50% acetonitrile, and then dehydrated with acetonitrile. Proteins were digested in the gel overnight at 37 °C with trypsin (sequencing grade; Promega, Madison, WI). Supernatants were mixed with equal volumes of HCCA matrix [50 mM HCCA in 4 vol. acetonitrile and 6 vol. 0.1% (v/v) TFA] and spotted onto targets. Peptide mass fingerprints were acquired using a Reflex™III mass spectrometer (Burker Daltonics, Bremen, Germany). Spectra were recorded in the reflector mode and calibrated using acid peptide [residues 26, $M(H^+) = 2961.51$] synthesized by an automated peptide synthesizer (model PS3) and then purified by HPLC in order to remove other proteins/peptides. Accuracy of molecular ion mass determination was better than ±0.1 Da up to a mass of 6000 Da.

PMF of each appointed protein was performed using the MASCOT network. Peak harvesting was done automatically using XACQ and XMASS software from the MALDI-TOF mass spectrometer. Maximum tolerance for mass was adjusted to 50 ppm after an internal calibration using autolysis products of trypsin, and only one missed cleavage for tryptic peptides was allowed. M_r and p*I* values of each analyzed spot were taken from the 2D-PAGE gel, and SWISS-PORT, MSDB, and NCBInr databases were used for proteomic searches, but the latter database was the first choice for the search. In order to obtain consistent results for further analysis of the physiology of these differential proteins we emphasize use of the LOCtree method as recently described by Nair and Rost (2005). Current indications are that LOCtree can achieve sustained levels of 74% accuracy for non-plant eukaryotes, 70% for plants, and 84% for prokaryotes.