The Identification of HSC70 as a Biomarker for Copper Exposure in Medaka Fish

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송사리 모델계에서 구리 노출에 대한 생물지표로서 HSC70의 동정

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요 약

구리는 환경에 광범위하게 존재하며, 생물체에게 필수적인 무기질이지만 고농도로 존재할 경우 독성을 발휘한다. 본 연구는 프로티옴 기술을 응용하여 수서태계에 구리와 같은 중금속의 존재 여부를 신속하게 평가하기 위한 생물지표를 발굴하기 위하여 수행되었다. 즉, 송사리 (*Oryzias latipes*)를 이용하여 여러 농도의 구리용액 (0.1, 1, 5 mg/L)에 24시간 노출시킨 다음, 머리부분에서 선택적으로 발현이 증가되는 단백질을 동정하고자 시도하였다. 본 시스템에서 유의적으로 발현이 증가하는 것으로 나타난 단백질은 beta-tubulin, heat shock cognate 70 (hsc70)이었으며, 이 결과의 일부를 semi-quantitative RT-PCR를 이용하여 확인하였다. 이와 같이 구리 처리에 특이적으로 발현이 증가된 송사리 단백질들은 노출평가를 위한 생물지표로서 개발을 위하여 더 연구할 가치가 있는 것으로 평가된다.

Key words : copper, medaka fish, proteomics, hsc70, biomarker

INTRODUCTION

The pollution of aquatic ecosystems by heavy metals has assumed serious proportion due to their toxicity and accumulation behavior (Jain, 2004). Copper is released into the environment by anthropogenic activities, such as from pesticides, fungicides and industrial wastes (Yruela, 2005). Copper is an essential redox-active transition metal, which acts as a structural element in regulatory proteins and participates in photosynthetic electron transport, mitochondrial respiration, oxidative stress responses, cell wall metabolism and hormone signaling (Yruela, 2005). However, at elevated concentrations of higher than $20 \sim 30$ µg/g dry matter (Marschner, 1995), copper becomes toxic to organisms and alters membrane permeability, chromatin structure, protein synthesis, enzyme activities, photosynthesis and respira-

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tory processes and may induce senescence (Fernandes and Henriques, 1991; Yruela, 2005). Development of monitoring tool for aquatic contamination with copper is of essential importance for environmental assessment.

Proteomics can increase the speed and sensitivity of toxicological screening by identifying protein markers for toxicity. Proteomics studies can also provide insights into the mechanisms of action of a wide range of substances including heavy metals (Kennedy, 2002).

In this study we attempted to identify proteins differentially expressed in medaka fish exposed to copper since the protein selectively regulated by copper treatment could be useful as a tool for assessing environmental contamination by the heavy metal.

MATERIALS AND METHODS

1. Experimental animals and chemical exposure

Medaka fish (*Oryzias latipes*) developed by Bioscience Center were obtained from Korea Institute of Toxicology, Korea Research Institute of Chemical Technology (KRICT: Daejeon, S. Korea). One-year old fish were fed a commercially prepared flake diet (TetraMin, USA) once daily. Fish were held at $22 \pm 2°C$ and 16L/8D light cycle to minimize reported seasonal variation in xenobiotic-metabolizing enzymes. Fish were held in a square glass chamber $(45 \times 24 \times 30$ cm$)$ containing 30-liter of dechlorinated water (pH=6.5~7.3) with aeration. Copper sulfate (purity: 99.9%) was obtained from Aldrich Chemical (Milwaukee, MI, USA) and dissolved in deionized water. Fish were exposed to various concentrations of copper for 24 h in a static environment. A vehicle control was run for each replication.

2. Preparation of tissue sample for proteome analysis

Fish were quickly anaesthetized by submersing in chilled water and dissected into head and body.

Tissues were homogenized (approximately 20 mg of tissue per ml of phosphate buffer (pH 8.0, 0.1M)) in a Polytron homogenizer and were centrifuged at $20,000 \times g$ for 25 min at 4°C. The supernatant was collected in a 50 mL centrifugal tube. The protein was precipitated by adding trichloroacetic acid at one tenth of the total volume and incubation for 1 h. The pellet was washed with 10 mL twice and 1 mL of cold ethanol three times. After washing, each pellet was dried in a Speed-Vac (Centrifugal evaporator CVE-2000, Eyela, Japan) for 50 min. The protein pellet was redissolved in a rehydration buffer II (6 M Urea, 2 M thiourea, 4% CHAPS, 130 mM DTT, 0.2% Ampholyte, 0.001% bromophenol blue). The protein concentration was determined in the final supernatant using a Bio-Rad protein assay dye reagent (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The sample was then stored in Eppendorf tubes at −70°C.

3. 2-DE

The procedure for 2-DE was as previously described (Oh *et al.*, 2004). Briefly, proteins were dissolved in a rehydration buffer II and about 1.5 mg of proteins were loaded on to a ReadyStrips™ IPG Strip and then the IPG strips were rehydrated passively for 10 min at 22°C and actively for 13 h at 50 V, followed by isoelectric focusing at 250 V for 15 min; ramping to 10,000 V for 4 h; and focusing at 10,000 V up to 70,000 V. The IPG strips were equilibrated in a 500 µL equilibration buffer I and II containing 0.375 M of Tris-HCl buffer, pH 8.8, with 130 mM of DTT (buffer I) or 135 mM of iodoacetamide (buffer II), 2% (w/v) SDS, 20% (v/v) glycerol, and 6 M of urea.

The equilibrated IPG strips, after the first-dimensional electrophoresis, were placed on to a second-dimensional disc gel that consisted of 12% acrylamide/bis, pH 8.8, for the separating gel and 4% acrylamide, pH 6.8, for the stacking gel. Second dimensional separation was run at 10 mA per gel at 15°C overnight. After 2-D SDS PAGE, the gel was rinsed with distilled water for a minute and stained

with 0.1% CBB R-250 in methanol/acetic acid/water (40 : 10 : 50, v : v : v) for 3 h. The stained gel was destained with methanol/acetic acid/water (40 : 10 : 50, v : v : v) in order to become a desirable background. To perform subsequent analyses, the gels were washed with distilled water and stored in a refrigerator.

4. Image analysis

The CBB-stained gels were scanned with a GS-800 densitometer (Bio-Rad), and image files were exported to the PDQuest 2-D gel analysis software (Bio-Rad, USA). Spots over certain levels of intensity were detected, and the detected spots were counted by automatic spot-detection. The intensity of spots on the gel was compared in terms of molecular mass and isoelectric point values.

5. In-gel protein digestion

The protein spots of interest were excised from CBB-stained gels, minced with a scalpel, destained for 30 min using the following destaining solutions; 30% methanol (10 min), 50% ACN (10 min) and 100% ACN (10 min). Destained gels were dried in a Speed-Vac for a hour and subjected to in-gel digestion (37°C, overnight) with a 20 µL trypsin solution (10 ng/mL in 50 mM of NH_4HCO_3). Peptides were extracted for a total of 40 min with the following solutions; 50 mM of ABC (20 min), 50 mM of ACN (10 min) and 50% of ACN (10 min), consecutively, and dried in a Speed-Vac for 12 h. Dried peptides were re-dissolved in a resuspension solution (50% ACN in 0.5% TFA) and the solution was mixed with a matrix solution (5 mg of CHCA in 50% ACN in 0.5% TFA) at a ratio of 1 : 1, and spotted on the MALDI plate, and dried entirely in the clean-bench.

6. MALDI-TOF MS

Measurements were performed on a Voyager DE-STR MALDI-TOF mass spectrometer and MALDI-TOF/TOF 4700 proteomics analyzer (Applied Biosystems, Framingham, MA, USA), equipped with a reflectron. The spectra were acquired in the delayed extraction, reflector mode, under optimized conditions (20 kV acceleration voltage, 200 ns delay time). The mass scale was internally calibrated with the trypsin autolytic products of a known amino acid sequence; m/z 842.51 (angiotensin I), 1045.56 (bradykinin), 2211.10 (neurotensin).

7. Target identification using database search

Mass values of analyzed peptides were queried to search protein databases using MS-fit from the Protein Prospector at the University of California, San Francisco (UCSF) (http://prospector.ucsf.edu/ ucsfhtml 4.0/msfit.htm) and the Mascot search engine, which uses raw MS/MS data to search the NCBI protein database. Protein identification was considered accurate when the MS/MS results from three or more peptides, in a given sample, identified the same protein. A maximum of one missed trypsin cleavage was allowed, although most matches did not contain any missed cleavages. A mass tolerance of 50 ppm was allowed for matching of peptide mass values.

8. Semi-quantitative RT-PCR

For total RNA extraction the treated fish were immediately frozen in the liquid nitrogen and preserved in a deep freezer (-70°C) until use. Total RNA was extracted according to a protocol accompanied in RNA extraction kit (RNeasy mini kit, QIAGEN, Germany). Total RNA purified with RNeasy kit was checked for the purity by Spectrophotometer (V-530, Jasco, Japan). Total RNA (5 µg) were used for reverse transcription (RT), and 1/10 of the resultant cDNAs were used for each PCR reaction. The primer sequences for heat shock cognate 70 (hsc70) were as follows; forward 5′-atc ctc atg ggc gac acc tct g-3′ and reverse 5′-tgt cgg cct ctt gca cca ttc tc-3′. The hsp90 β primer sequences were as follows: forward 5′-ctc cgc cgc tcc tga aga cat t-3′ and reverse 5′-cac aac aca ggc cag cac tca gg-3′. The beta -actin primer sequences were as follows: forward 5′-

ccc cat tga gca cgg tat t-3′ and reverse 5′-agc ggt tcc cat ctc ctg-3′. The RT-PCR reagents were purchased from Promega Corporation (Madison, MI, USA). The reaction mixture contained 2 μL of 10 × reaction buffer, 2.5 μL of 2.5 mmol/L deoxy-ribonucleoside triphosphate (dNTP) mixture, 2 μL of 10 μmol/L of each primer, Taq polymerase (5 U/uL), 0.3 μL deionized water. The components were mixed by tapping and put into directly thermal cycler (HBP × 220, Thermohybaid, UK). The first-strand cDNA was synthesized at 37°C for 90 min, followed by denature of the template at 94°C for 5 min. Amplification was achieved with 40 cycles of denaturing (94°C, 15s), annealing (60°C, 30s), and extension (72°C, 1 min). The final extension was performed at 72°C for 5 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis, and visualized under UV transillumination of ethidium bromide-stained gel. Beta-Actin was used as a loading control.

RESULTS

In order to screen protein (s) selectively regulated by copper, medaka fish were treated with 0.1, 1, 5 mg/L copper for 24 h, dissected into head and body parts, and subjected to proteomic analysis. The separated protein spots of the cellular proteins on two-dimensional electrophoresis were visualized by

staining with CBB R-250. Scanned images were analyzed by an image analysis program, PDQuest. When medaka fish were treated with copper for 24 h, total 100 spots were differentially expressed. Most of them were distributed within 5.0～7.0 of isoelectric points (pI) (Fig. 1). Tryptic digestion was performed and protein identification was conducted on the differentially expressed spots using MALDI-TOF MS and a database search. Selected lists of the proteins differentially expressed in medaka fish treated with copper are summarized in Table 1. The proteins quantitatively up-regulated in the fish
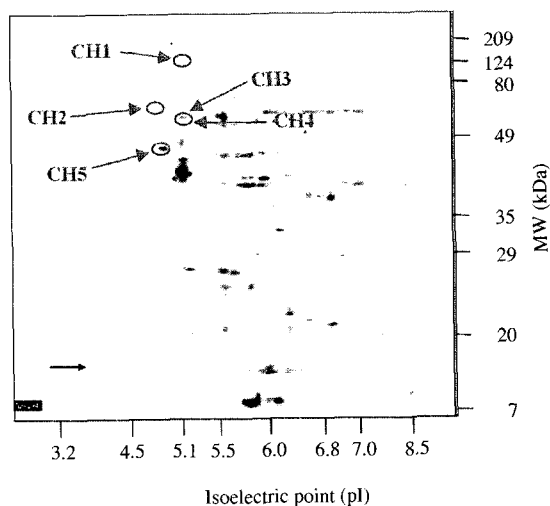

Fig. 1. Spot ID numbers of specific proteins differentially expressed in Medaka fish treated with copper.

Table 1. 2-DE and MALDI-TOF MS identification of proteins that are differentially expressed in head of medaka fish exposed to copper*

Spot No.	Proteins identified	Protein MW (Da)/pI	Accession #	# (%) Masses matched	Peptide coverage (%)	Mowse score
CH1	DNA-directed RNA polymerase, beta subunit	128481/4.9	23335342	4 (8)	7	62.4
CH2	78 kDa glucose-regulated protein-precursor	131531/5.9	2506545M	16 (32)	24	6.73E+07
CH3	Heat shock cognate 70 kDa protein	71259/5.3	28569550	15 (30)	27	3.13E+08
CH4	Heat shock cognate 70 kDa protein	71284/5.2	232285 M	17 (34)	31	4.62E+08
CH5	Tubulin beta	49808/4.8	20809886	23 (46)	44	2.31E+11

*Medaka fish were exposed to 0, 0.1, 1, 5 mg/mL copper for 24 h, and head part was subjected to proteomic analysis.
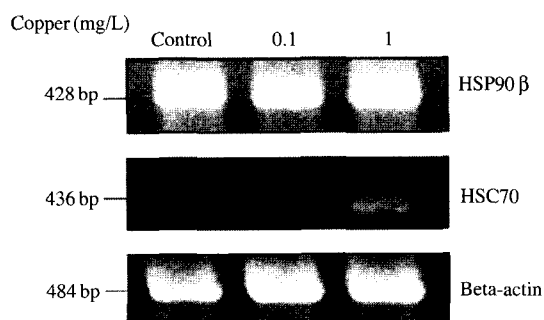
Fig. 2. Effect of copper concentration on the expression of hsp90 β and hsc70 genes expression in medaka fish treated with copper for 24 h.

exposed to copper were identified to be grp78, hsc70, and beta-tubulin.

DISCUSSION

The aim of this study was to identify proteins selectively up-regulated in medaka fish treated with copper. As shown in Fig. 1, approximately 100 spots from protein extract of medaka fish head were shown to be changed by 3-fold in their expression by copper treatment. However, the identification of proteins differentially expressed by the treatment was limited due to insufficient database for medaka proteins. The proteins identified with good reliability (high MOWSE score) were beta-tubulin and heat shock proteins including grp78 and hsc70.

The regulation of heat shock cognate 70 (hsc70) expression by copper was confirmed by RT-PCR (Fig. 2) although the regulation of other proteins including beta-tubulin and hsp90beta could not be confirmed. Tubulins, the cytoskeleton proteins, are abundantly present in the most kinds of cell and are relevant to the movement, reproduction, and death of cell. These proteins are also reported to be regulated by toxic chemicals such as heavy metal in fish (Silvestre *et al.*, 2006).

Heat shock proteins (hsp) shown to be regulated by copper treatment in this study are known to help maintain the animal's normal or homeostatic state

(Iwama *et al.*, 1999; Iwama *et al.*, 2004). There have been several efforts to validate the use of the hsp response as an indicator of stressed states in fish. Indeed, it has been shown that several forms of environmental stressors can induce the hsp response in fish. For example, increased levels of various hsps have been measured in tissues of fish exposed to industrial effluents (Vijayan *et al.*, 1998), polycyclic aromatic hydrocarbons (Vijayan *et al.*, 1998), several metals such as copper, zinc and mercury (Sanders *et al.*, 1995; Williams *et al.*, 1996; Duffy *et al.*, 1999), pesticides (Hassanein *et al.*, 1999) and arsenite (Grosvik and Goksoyr, 1996). These studies and others revealed that the use of hsp as an indicator of stressed states in fish is a very complex issue (Duppy *et al.*, 1999; Iwama *et al.*, 2004; Lee *et al.*, 2006).

The overall results suggest that the expression of hsc70 gene in medaka fish could give useful information for diagnosing general health conditions in fresh water ecosystem. Further research that will elucidate the relationship between heavy metal stress and regulation of heat shock proteins is needed prior to use of heat shock proteins as an indicator of stress in fish.

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