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The Identification of Alpha-Tubulin as a Biomarker for Diazinon Exposure in Medaka Fish

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송사리 모델계에서 다이아지논 노출에 대한 생물 지표로서 알파 튜블린의 동정

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요 약

환경오염을 신속하게 모니터링하기 위한 생물지표의 개발은 증가하고 있는 오염의 심각성에 비추어 매우 중요한 과제로 여겨지고 있다. 본 연구에서는 독성물질처리에 의하여 선택적으로 발현이 조절되는 단백질의 동정을 통하여 독성물질에 대한 단백질 생물지표를 발굴하고자 시도하였다. 즉, 송사리(*Oryzias latipes*)를 유기인계 살충제인 다이아지논(diazinon)에 0, 0.1, 1, 5 mg/L 농도로 24시간 노출시킨 후, 머리와 몸통부분으로 나누어 단백질 발현패턴을 분석하였다. 본 시스템에서 다이아지논 처리에 의하여 유의적으로 발현이 증가된 단백질로서 alpha-tubulin, ribonuclease pancreatic precursor, protein hfq 등을 동정하였으며, 이 가운데 alpha-tubulin과 hsp90β의 발현이 다이아지논 농도에 의존적으로 증가하는 것을 semi-quantitative RT-PCR 방법으로 확인하였다. 이와 같이 다이아지논 처리에 특이적으로 발현이 증가된 송사리 단백질들은 노출평가를 위한 생물지표로서 개발에 응용될 수 있을 것으로 평가된다.

Key words : diazinon, medake fish, proteomics, alpha-tubulin, biomarker

INTRODUCTION

Proteomics has been applied to various fields of studies in toxicology including biomarker discovery and drug development. Proteomics can increase the

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speed and sensitivity of toxicological screening by identifying protein markers for toxicity. Proteomics studies have also provided insights into the mechanisms of action of a wide range of substances, from metals to peroxisome proliferators (Kennedy, 2002). In this study we attempted to identify proteins differentially expressed by the treatment of diazinon, one of the orgnanophosphate insecticides, since the protein selectively regulated by the insecticide treatment could be useful as a tool for assessing environmental contamination.

Diazinon [O, O-diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate], is an organophosphorous insecticide widely used for the control of agricultural and household pests, the toxic effects of which are mainly due to the inhibition of cholinesterase (ChE) (Prijono and Leighton 1991; Kim *et al.*, 1999). Although diazinon undergoes fairly rapid degradation in the environment, it may cause toxic effect on fish due to prolonged exposure with repeated input into the aquatic environment. This insecticide has a highly acute toxicity and can result in numerous poisonings in non-target species. Diazinon is relatively highly toxic to fish, and it is well known that it causes vertebral malformation and behavioral change of fish at relatively low concentrations (Dutta *et al.*, 1992; Pan and Dutta, 1998). Behavioral change caused by pesticides is most likely related to changed levels in neurotransmitters such as acetylcholine, dopamine, serotonin, and norepinephrine, whereas physical distortion may be caused by abnormal expression or modification of skeletal muscle proteins. In this study, we investigated the change of protein expression profile caused by diazinon treatment in medaka fish.

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± 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 × 24 × 30 cm) containing 30-liter of dechlorinated water (pH 6.5~7.3) with aeration. Diazinon (purity: 99%) was obtained from Wako pure chemical industry, LTD (Osaka, Japan). Fish were exposed to diazinon (0.1, 1 and 5 mg/L) dissolved in dimethylsulfoxide for various times in a static environment. A vehicle control was run for each replication (Kim *et al.*, 1999; Shin *et al.*, 2001).

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, 0.1 M)) in a Polytron homogenizer and were centrifuged at 20,000 × 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-dimensional electrophoresis (2-DE)

The procedure for 2-DE was as previously described (Oh *et al.*, 2004). Briefly, proteins were dissolv-

ed 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 NC-BI 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 alpha-tubulin were as follows; forward 5′-act aca ccg tcg gca agg a-3′ and reverse 5′-aga tgt cgt aga tgg cct cgt-3′. The hsp90β primer sequences were as follows: forward 5′-gcc gac gac aag gac aac tac-3′ and reverse 5′-ttg ccg tcc aac tcc ttc ag-3′. The beta-actin primer sequences were as follows: forward 5′-gtc cac cgc aaa tgc ttc ta -3′ and reverse 5′-aac tga agc cat gcc aat ga-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/μL), 0.3 μL deionized water. The components were mixed by tapping and put into directly thermal cycler (HBPX220, Thermohybaid, UK). The first-strand cDNA was synthesized at 37°C for 90 min, followed by denaturation of the template at 95 °C for 2 min. Amplification was achieved with 30 cycles of denaturing (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 1 min). The final extension was performed at 72°C for 5 min. The PCR products were analyzed by 1.2% 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 diazinon, medaka fish were treated with 0.1, 1, 5 mg/L diazinon 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 diazinon for 24 h, 47 and

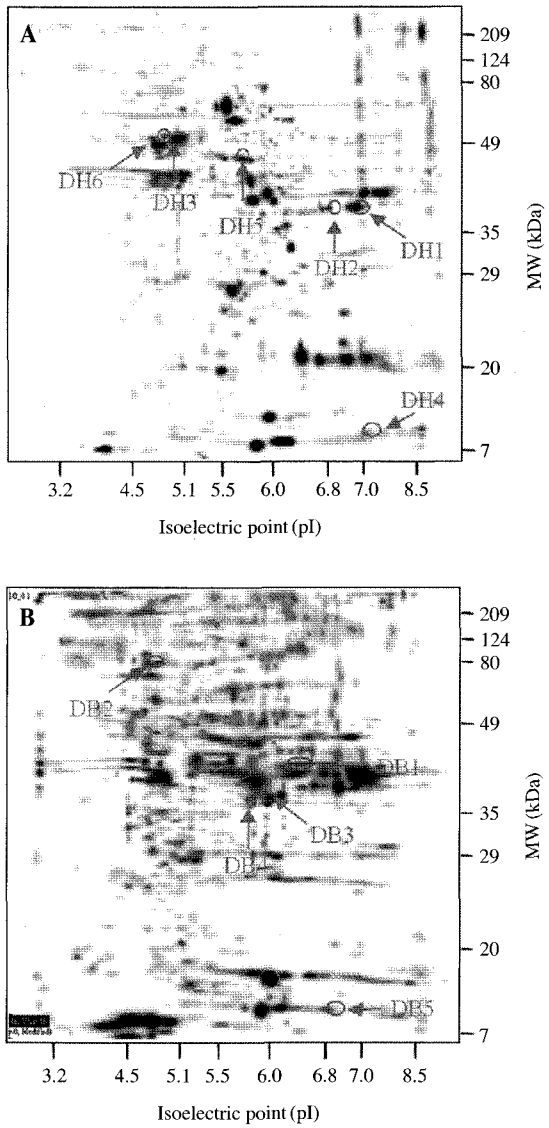

Fig. 1. Spot ID numbers of specific proteins differentially expressed in medaka fish treated with diazinon.

92 spots were differentially expressed in head and body, respectively. Most of them were ranged from 30~40 kdal in molecular weight and showed 5.0~7.0 of isoelectric points (pI). Typical imaginary picture of 2-DE protein expression pattern with differentially expressed proteins with arrows was shown in 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 diazinon are summarized in Table 1. Most proteins could not be identified due to insufficient database for medaka genes and proteins.

The proteins quantitatively up-regulated in head section by treatment included alpha-tubulin, ribonuclease pancreatic precursor, protein hfq, UDP-N-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase, 60 kDa chaperonin 2, and vacuolar protein-sorting protein BRO1. The proteins up-regu-

Table 1. 2-DE and MALDI-TOF MS identification of proteins that are differentially expressed by diazinon treatment in medaka fish*

Spot No.	Proteins identified	Protein MW (Da)/pI	Accession #	#(%) masses matched	Peptide coverage (%)	Mowse score
[Head]						
DH1	UDP-N-acetylmuramoylalanyl-D-glutamate-2, 6-diaminopimelate ligase	53705/5.6	Q81WC7	6(12)	16.3	810,029
	60 kDa chaperonin 2	56718/4.8	Q00768	6(12)	15.6	409,612
DH2	Pyruvate-flavodoxin oxidoreductase	131531/5.9	Q3M8N0	4(8)	5.3	5,740
DH3	Rab GTPase-binding effector protein 1	99291/4.9	Q15276	7(14)	10.7	8,432
	hydrolase 38	116547/5.8	Q8NB14	5(10)	5.6	7,604
DH4	UPF0102 protein HI1656	13812/6.1	P45300	5(10)	19.3	14,502
DH5	Ribonuclease pancreatic precursor	17528/8.8	Q8SQ04	6(12)	14.1	2.41E+06
	Protein hfq	9045/7.9	Q2K8U6	4(8)	27.5	1.06E+06
DH6	Tubulin alpha-1 chain	50136/4.9	P68369	13(26)	37	1.12E+10
[Body]						
DB1	Polyphosphate kinase	81646/8.7	Q9KU07	8(16)	15.5	4,452
DB2	Homoserine kinase	33293/5.2	33293/5.2	4(8)	29.6	7,777
DB3	DNA-directed RNA polymerase II largest subunit	204691/5.8	P18616	10(20)	5.9	197,516
	Phenylalanyl-tRNA synthetase beta chain	87348/5.0	Q65TL3	9(18)	17.1	161,051
DB4	DNA-directed RNA polymerase beta chain	149771/5.2	Q4QN33	7(14)	11.4	151,688
DB5	DNA-directed RNA polymerase II largest subunit	204691/5.8	P18616	10(20)	5.9	197,516
	Phenylalanyl-tRNA synthetase beta chain	87348/5.0	Q65TL3	9(18)	17.1	161,051
	DNA-directed RNA polymerase beta	149771/5.2	Q4QN33	7(14)	11.4	151,688

*Proteins were extracted from head (DH) of medaka fish exposed to 0.1, 1, 5 mg/L for 24 h and were subjected to 2-DE (pH 3~10) and detected by CBB staining. Proteins from body (DB) of medaka fish were also isolated in 2-DE (pH 3~10).

lated in body part of medaka exposed to diazinon include DNA-directed RNA polymerase II largest subunit, heme/hemopexin utilization protein C precursor, 3-isopropylmalate dehydratase small subunit, THO complex subunit RLR1, polyphosphate kinase, and vacuolar protein-sorting protein BRO1.

DISCUSSION

The aim of this study was to identify proteins selectively up-regulated in medaka fish treated with diazinon. As shown in Fig. 1, approximately 100 spots were shown to be changed by 3-fold in their expression by diazinon treatment. However, the identification of proteins differentially expressed by the treatment was limited due to insufficient database for medaka proteins. The only protein identified with good reliability (high MOWSE score) was alpha-tubulin. The regulation of alpha-tubulin expression by diazinon was confirmed by RT-PCR (Fig. 2). Alpha-Tubulin, one of the cytoskeleton proteins, is abundantly present in the most kinds of cell and is relevant to the movement, reproduction, and death of cell. Recent studies have indicated that microtubule trafficking in neuronal cell is also adversely affected by exposure to the OP pesticide. For instance, exposure of in vitro rat hippocampal slice to chlorpyrifo produced a progressive decrease in neuronal viability that may be associated with impaired microtubule synthesis and/or function (Prendergast *et al.*, 2007). Thus increased synthesis alpha-tubulin in medaka fish exposed to diazinon might be compensatory mechanism for impaired microtubule function caused by OP pesticide diazinon. It has also been shown that cisplatin, one of the most effective drugs for treatment of solid tumors, induced the expression of alpha-tubulin in rat cochlear, suggesting its usefulness as an early biomarker for the drug-induced cochlear damage. There are more evidences that alpha-tubulin may be biomarker for monitoring toxicity by various toxic compounds (Yi *et al.*, 2006; Greene *et al.*, 2007; Prendergast *et al.*, 2007). Microtubules (MTs) are dynamic, polarized structures that are formed by the polymerization of heterodimeric complexes of α- and β-tubulin. Its polarity is relevant to its transport processes and kinesins, direct transport processes to the plus-end, and dyneins to the minus-end. Recent studies suggested that both actin and MTs were key regulators of many cell morphological events and most likely played important roles in neurite initiation. Abnormal behavior and physical deformation caused by diazinon (Shin *et al.*, 2001; Chon *et al.*, 2005) might be associated with altered expression of microtubule components such as alpha-tubulin.

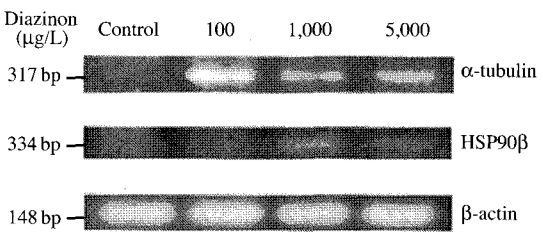

Fig. 2. Increased expression of alpha-tubulin and hsp90β gene in medaka fish exposed to diazinon as analyzed by semi-quantitative RT PCR.

There are several lines of evidences that stress proteins such as heat shock proteins were regulated by various toxic compounds or environments (Rendell *et al.*, 2005; Eder *et al.*, 2007; Gravel *et al.*, 2007; Hansen *et al.*, 2007; Maradonna *et al.*, 2007). We, therefore, tested whether diazinon treatment regulates the level of some heat shock proteins such as hsp70, hsc 70, and hsp90. In medaka fish only hsp90β turned out to be slightly induced upon exposure to diazinon at 1,000 μg/L or higher (Fig. 2). Recently, several families of heat shock proteins (hsps) have been proposed as indicators of a generalized stress response at the cellular level. Recent findings that hsp levels, in various fish tissues, respond to a wide range of stressors have supported the use of these proteins as indicators of stressed states in fish. Hsp90 (80 ~ 90 kD) is active in supporting various components of the cytoskeleton, enzymes and steroid hormone receptors (Iwama *et al.*, 2004). Although heat shock proteins are recognized as stress proteins upregulated in res-

ponse to various kinds of toxicants or stress environments, hsp90 seems to have relatively high specificity in responding to toxicants. For example, common forms of hatchery-related stressors (exposure to anesthesia, formalin, hypoxia, hyperoxia, capture stress, crowding, feed deprivation and cold stress) did not alter levels of gill hsp30, hsp70 and hsp90 in Atlantic salmon (*Salmo salar*) (Zarate *et al.*, 2003). But hsp90β was upregulated by morphine in rats 8 h after morphine injection. Immunoassay analysis also demonstrated that amounts of the hsp90 family increased in kidneys and liver from marine teleost *Sparus sarba* during nitrite exposure (Salas *et al.*, 2007).

In conclusion, the identification of relevant proteins in the pathogenic process by exposure to toxicant may allow the development of biomarker for early monitoring of environmental toxicants.

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