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Two-Dimensional Gel Electrophoresis Analysis of Liver Proteins Following Nonylphenol Treatment of *Bombina orientalis* Boulenger

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Chemical contamination as a consequence of pesticide application continues to be postulated as a contributing factor in the global decline of amphibians (Berrill et al. 1998; Renner 2002). While a number of studies have assessed surfactant toxicity in a range of aquatic species, they have focused predominantly on fishes and crustaceans (Lewis 1991). Evidence for abnormalities in sex differentiation in wild fish populations implies that some aquatic environments contain estrogenic compounds at concentrations high enough to be of concern (Purdom et al. 1994). There is limited data pertaining to the effects of these chemicals on amphibians (Mann and Bidwell 2000; Hayes et al. 2002; Renner 2002). Much of the amphibian toxicological literature describes studies using representatives of the genera - *Bufo*, *Rana*, or *Xenopus* (Mann and Bidwell 1999; Hayes et al. 2002; Renner 2002). The Korean red-bellied frog, *Bombina orientalis* is phylogenetically distinct from European, North American and African species. To date, however little is known about the impact of the environmental pollutants on this species.

Recently high throughput screening of gene expression at the RNA or protein level has been employed to find highly specific indicators of stress. The entire protein complement expressed at a given time, the proteome, is increasingly being studied and has mainly been concerned with isolating individual proteins as markers of disease or targets of drug action. Now it is being used to isolate protein markers in the field of environmental toxicology. Recently, techniques have been developed to analyze large numbers of proteins simultaneously to discern subtle changes in protein expression (Herbert et al. 1997). Therefore, it is promising to seek sets of proteins induced and repressed by the environment in an attempt to detect indicators of stress such as endocrine disrupting chemicals.

Nonylphenol (NP), one of the estrogenic endocrine disrupters is associated with adverse reproductive and health effects in wildlife and laboratory animals (Chapin et al. 1999). The weakly estrogenic NP has been found in sewage treatment plant (STP) effluents, river systems, estuaries, sediments and tissues and may already be ubiquitous (Kuch and Ballschmiter 2001). In the present study, we have investigated the protein changes in liver tissues of *B. orientalis* following administration of nonylphenol.

MATERIALS AND METHODS

B. orientalis was collected from the Myungji Creek at Kapyung-Gun, Kyunggi-Do in Korea on August 2003. Frogs transported to the laboratory were put in a tank with water and fed the mealworm at 18°C for two weeks. Male frogs having similar body weight (10 ± 0.1g) were selected and given an intraperitoneal (IP) injection of NP (10 μg/g body weight) or vehicle (sesame oil). Following NP dosing, frogs were reared for 48h and subjected to protein analysis.

Briefly, frogs were killed by decapitation, and livers were dissected and homogenated directly by mortor-driven homogenizer (PowerGen125, Fisher Scientific), and then the protein pellet was solubilized in sample buffer composed with 7M urea, 2M thiourea containing 4% (w/v) 3-[(3-cholamidopropy) dimethyammonio]-1-propanesulfonate (CHAPS) (Sigma, St), 1% (w/v) dithiothreitol (DTT) (Sigma) and 2% (v/v) pharmalyte (Amersham Biosciences, UK) and 1mM benzamidine (Sigma). Proteins were extracted for one hour at room temperature with vortexing. After centrifugation at 15,000xg for one hour at 15°C, insoluble material was discarded and the soluble fraction was used for two-dimensional gel electrophoresis. Protein loading was normalized by BioRad Protein assay kit. IPG dry strips (Amersham Biosciences) were equilibrated for 12-16h with 7M urea, 2M thiourea containing 2% CHAPS, 1% DTT, 1% pharmalyte and respectively loaded with 200ug of sample. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham Biosciences) following manufacturer's instruction. For IEF, the voltage was linearly increased from 150 to 3,500V during 3 hours for sample entry followed by constant 3,500V, with focusing complete after 96kVh. Prior to the second dimension, strips were incubated for 10 minutes in equilibration buffer (50mM Tris-Cl, pH6.8 containing 6M urea, 2% SDS and 30% glycerol), first with 1% DTT and second with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20-24cm, 10-16%). SDS-PAGE was performed using Hoefer DALT 2D system (Amersham Biosciences). 2D gels were run at 20°C for 1.7kVh. 2D gels were silver stained as described by Oakley et al. (1980) but the fixing and sensitization step with glutaraldehyde was omitted. Quantitative analysis of digitized images was carried out using the PDQuest software (version 7.0, BioRad) according to the protocols provided by the manufacturer. The quantity of each spot was normalized by total valid spot intensity. Protein spots were selected for significant expression variation deviated over two fold in its expression level compared with the control or normal sample. Each protein spot was compiled by pI value and molecular weight (MW).

RESULTS AND DISCUSSION

There was no sign of narcosis in NP-injected frogs at the end of treatment, suggesting that the NP dose of 10 μg/g body weight did not elicit a systemic toxic effect. In the 2D/E gel 709 protein spots were identified (Figure 1A and B). Analysis of the visualized protein spots allowed identification of 28 protein spots

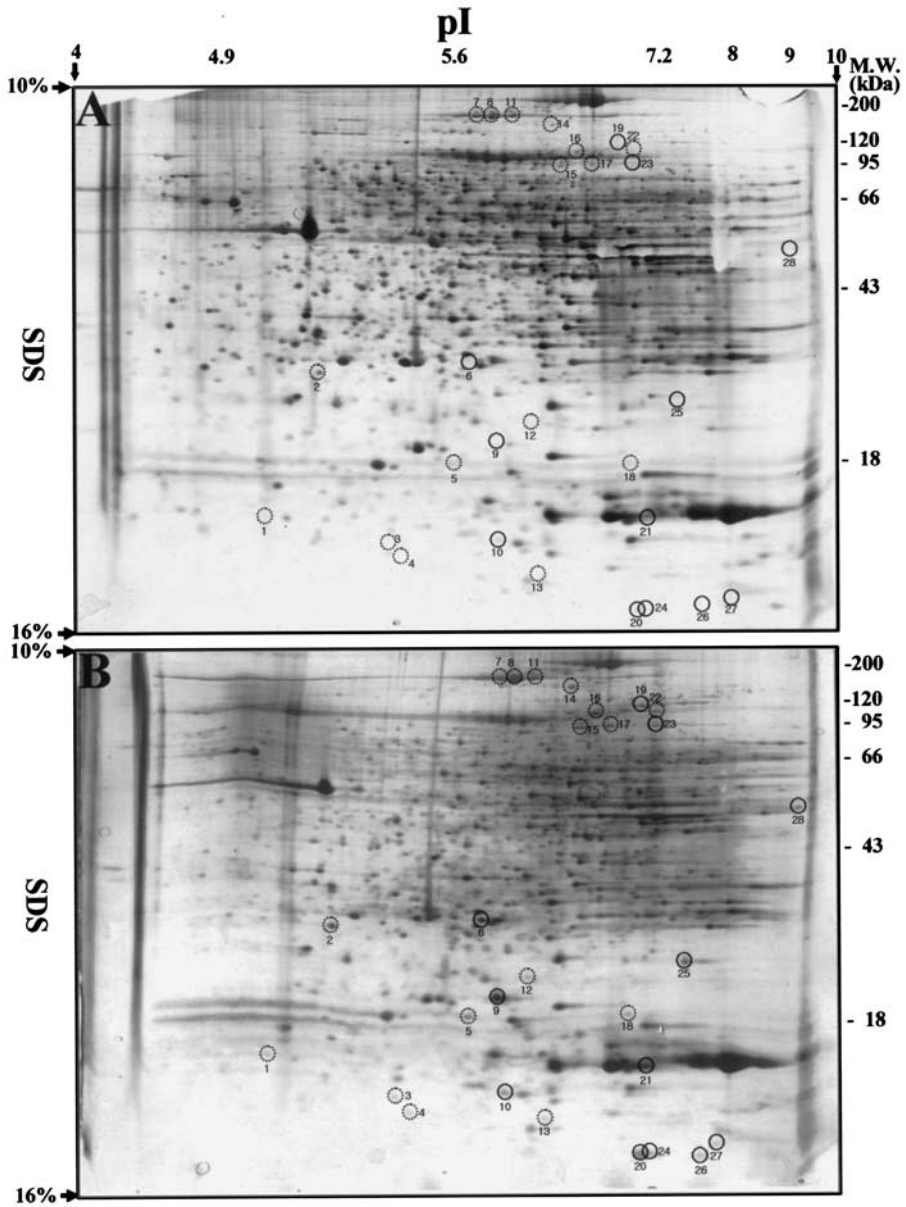

Figure 1. 2D/E of liver proteins from *B. orientalis* exposed to nonylphenol (NP). Adult male frogs were given a single intraperitoneal injection of NP (10 μg/g body weight). After 48h, liver proteins were analyzed by 2D/E. (A) Vehicle controls. (B) NP treatment. Key proteins increased (dashed circles) or newly appeared (solid circles) in NP treatment group are denoted with number.

increased more than 3 fold in NP treated animals, which was approximately 3.9% of the total protein spots. Of these, 12 protein spots were newly appeared following NP treatment (Table 1).

Table 1. The major proteins spots in liver induced following nonylphenol injection in *B. orientalis*

Spot No*	6	9	10	19	20	21	23	24	25	26	27	28
PI	5.7	5.8	5.8	6.5	6.5	6.6	6.7	6.7	7.3	7.8	8.0	9.0
Mr.(kDa)	29	20	13	115	9	15	95	9	24	9	10	50

Total number of spots visible on 2D/E gels was 709. Of these, major protein spots increased more than 10 folds for stressor compared to untreated control were presented. *, Specific number of protein spots depicted in Fig. 1.

In general, protein expression changes with the state of development, the tissues and the internal and external environmental conditions. Concomitantly, induction of different proteins is specific to stressors including polycyclic aromatic hydrocarbons, polychlorinated biphenyls and heavy metals, and osmotic stress. Basically NP is weakly estrogenic and induces estrogen-responsive marker genes such as vitellogenin in *Xenopus* liver cells (Hurter et al. 2002). Previously it was reported that the minimum detection level for responses related to the estrogenic activity of NP in reproductive and mammary tissues is about 40 mg/kg/day (Chapin et al. 1999). However, IP administration of NP appears to lower the minimum detection level of NP in the immature rat uterotrophic assay to 18 mg/kg (Lee and Lee 1996). In amphibians, the 48h EC50 values for nonylphenol ethoxylate (NPE) ranged between 1.1 mg/L for mild narcosis and 12.1 mg/L for full narcosis (Mann and Bidwell 2001). In this study, when NP was administered at 10 mg/kg body weight, no systemic response such as narcosis was found, suggesting this dose might be proper for the proteome analysis of possible estrogenic effects of NP in this species. However, it cannot be excluded that the induced proteins might be related to the detoxification or adaptation processes in the NP-exposed frog.

In summary, proteome analysis of liver proteins using 2D gel electrophoresis from NP injected *B. orientalis* revealed the induction of several proteins which are potentially useful for developing biomarkers for environmental contamination by nonylphenol in amphibians.

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