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Expression Changes of *Chironomus riparius* Proteome reflecting Ecdysterone Inducible Genes and Mitogenome exposure to Bisphenol-A

Inn-Sil Kwak

Department of Life Science, College of Natural Science, Hanyang University, Seoul 133-791, Korea

Abstract - Development of the fourth-instar larvae of *Chironomus riparius* has a sensitive to ecdysteroid hormones. The 2D/E gel analysis for polypeptide expression reflecting early-ecdysterone inducible gene has conducted the emerged female from larval phase exposure to bisphenol A (BPA). In the 2D/E gel 1108 protein spots were identified. The visualized protein spots allowed extraction of 17 protein spots differed more than 3 fold in BPA treated animals, which was approximately 1.6% of the total protein spots. However, polypeptide expression reflecting early-ecdysterone inducible gene didn't change after treatments. In addition, detection for the damages or changes in mitogenome level was observed. The conserved cytochrome oxidase I in DNA level affected exposure to BPA (1 μg L^{-1}) in this preliminary study.

Key words : *Chironomus riparius*, cytochrome oxidase I, polypeptide expression, bisphenol A

INTRODUCTION

Bisphenol A (BPA), an intermediate in the production of polycarbonate and epoxy resins (Krishnan *et al.* 1993; Alexander *et al.* 1988; Staples *et al.* 1998) is known elicit oestrogenic responses in fish via interaction with the cellular receptor, and has been reported in surface waters at concentrations well in excess of those known to cause endocrine disruption. The amounts of BPA in natural water systems are not detected in Europe and Japan (Staples *et al.* 1998). BPA manufacturing sites have 7~8 μg L^{-1} concentrations in the United States and BPA in hazardous waste landfill leachates have ranged from 1.3 to 17,200 μg L^{-1} in Japan (Yamamoto *et al.* 2001).

BPA has been reported to have estrogenic activity (Krishnan *et al.* 1993; Nagel *et al.* 1997). Also, BPA is oxidized to bisphenol-oquinone, an intermediate that binds to DNA (Atkinson and Roy 1995). BPA reduces the CYP2C11/6 and CYP3A2/1 protein levels and decreases the estradiol- 2-hydroxylase and testosterone hydroxylase activities of the rat (Hanioka *et al.* 1998). In water system, emergence time and percentage adult emergence of *Chironomus riparius* were affected by BPA or EDCs (Watts *et al.* 2001; Kwak and Lee 2005). No effects to *Daphnia magna* observed at any BPA test concentration for either mortality or reproduction (Bayer 1996). *C. riparius* (Chironomidae), is a test species which has been extensively used in environmental assessment schemes and standardized chronic assays (USEPA 1994), and has a well studied endocrine system. In this study, we used *C. riparius* which were selectively exposed to BPA.

Development of arthropods is affected metamorphosis environments, and accordingly protein expression reflecting gene of metamorphosis determined functions. The promoter of the I-18 C gene contains the ecdysterone regulatory elements and also the heat-shock regulatory elements (Lezzi *et al.* 1989). The protein coding genes in mtDNA are

* Corresponding author: Inn-Sil Kwak, Tel. 02-2220-0951, Fax. 02-2296-7158, E-mail. iskwak@hanyang.ac.kr

a wide range of levels of conservation. Mitogenome, especially cytochrome oxidase I is highly conserved that it may be difficult to detect any amino acid change. Even the most highly conserved gene at the amino acid level has as great a rate of silent change as does the most variable gene. This makes the design of universal primers easier for highly conserved genes. Amino acid evolution is faster in less conserved genes, and for phylogenetic reconstructions this type of data should be the most information. This study focused on the protein expression of I-18 C gene, early ecdysterone inducible gene, and to detect the difference of cytochrome oxidase I in DNA level after exposure to BPA.

MATERIALS AND METHODS

Test organism

Conditions were consistent with the suggestions for a standard procedure submitted by Streloke and Kopp (1995). *C. riparius* egg masses were reared in an environmental chamber, under long-day conditions with a light : dark cycle of 16 : 8 hours, and a light intensity of about 500 lx. Water temperature was maintained at 20±1°C in an incubation chamber (Sanyo MIR-553, Japan). Twenty fourth-instar larvae were introduced into each test vessel. In order to perform the toxicity test, animals were kept in 300 mL crystallizing dishes (Schott Duran, Germany) filled with 200 mL of M4 (Elendt and Bias 1990), and a 1 cm sediment layer of fine sand (< 63 μm particle size). The test vessels were aerated continuously after midge larvae were introduced. Water loss due to evaporation was negligible, but when necessary, vessels were refilled with new M4. Each vessel was provided with 10 mg of ground fish food (Tetra-Werke, Melle, Germany). To achieve an exposure to constant substance concentrations throughout the midges' pupal phase, and to ensure that excess food did not alter water quality, M4 was removed daily and replaced by new M4. To prevent the escape of adults during test periods, each vessel was covered with a 0.5 mm mesh net.

Test chemical and 2D/E

BPA (Junsei, 98%) was dissolved in analytical-grade acetone in order to create a stock concentration of 20 mg L^{-1} active ingredient. The nominal concentrations were the control, and 10 μg L^{-1}. Morphological characteristics of emerged adults, such as head capsule length, head capsule width, body length, body width and body volume, were measured with the Meta Morph 6.0 program (Universal Imaging Corporation®) under an Olympus SZX-ILLB 200. Female *C. riparius* adults were homogenated directly by a motor-driven homogenizer (PowerGen125, Fisher Scientific), and then the protein pellet was solubilized in sample buffer composed of 7 M urea, 2 M thiourea containing 4% (w/v) 3-[(3-cholamidopropy) dimethy-ammonio]-1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT) and 2% (v/v) pharmalyte and 1 mM benzamidine. Proteins were extracted for one hour at room temperature with vortexing. After centrifugation at 15,000 g 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 Bradford assays (1976). IPG dry strips were equilibrated for 12~16 hours with 7 M urea, 2 M thiourea containing 2% 3-[(3-cholamidopropy)dimethyammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), and 1% pharmalyte, and then respectively loaded with 200 μg of sample. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and an EPS 3500 XL power supply (Amersham Biosciences), according to manufacturer's instructions. For IEF, the voltage was increased in a linear fashion from 150 to 3,500 V for 3 hours for the sample entry, followed by a constant 3,500 V, with focusing complete after 96 kVh. Prior to the second dimension, strips were incubated for 10 minutes in equilibration buffer (50 mM Tris-Cl, pH 6.8 containing 6 M urea, 2% SDS and 30% glycerol), first with 1% DTT and then with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20~24 cm, 10~16%). SDS-PAGE was performed using a Hoefer DALT 2D system (Amersham Biosciences) in accordance with the manufacturer's instructions. 2D gels were run at 20°C for 1.7 kVh. The 2D gels were then 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 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 in which the expression level was 3-fold or more than the control value were selected.

Each protein was then compiled according to pI and molecular weight (MW).

Total DNA isolation and PCR amplification and sequencing

Animals were homogenized in a solution of 50 mM Tris-HCl, pH 8.0, 400 mM NaCl, 20 mM EDTA, 0.5% SDS. After homogenization, proteinase K was added to a final concentration of 150 mg mL^{-1} and the mixture was incubated for 3 h at 55°C. After incubation 5 M NaCl was added to a final concentration of 1.1 M. Debris was removed by centrifugation, and the DNA was precipitated from the supernatant by adding an equal volume of 96% ethanol. After centrifugation the DNA pellet was washed with 70% ethanol, dried at room temperature, and dissolved in water. The sequences of the primers used in this work are shown in Table 2. PCR was carried out in a volume of 30 mL using the standard technique with *Taq* polymerase at an annealing temperature of 42°C. PCR products were isolated from agarose gel using the Wizard PCR Preps DNA Purification System (Promega). Amplified fragments were sequenced directly by the dideoxy chain termination method of Sanger, for which the DNA Cycle Sequencing Version (Gibco BRL) was used. The DNA sequencing was done by direct sequencing of PCR amplification products, according to published protocols by Guryev *et al.* (2001). The homogeneity for sample sequence was calculated www.ncbi.nlm.nih.gov/blast/bl2seq.

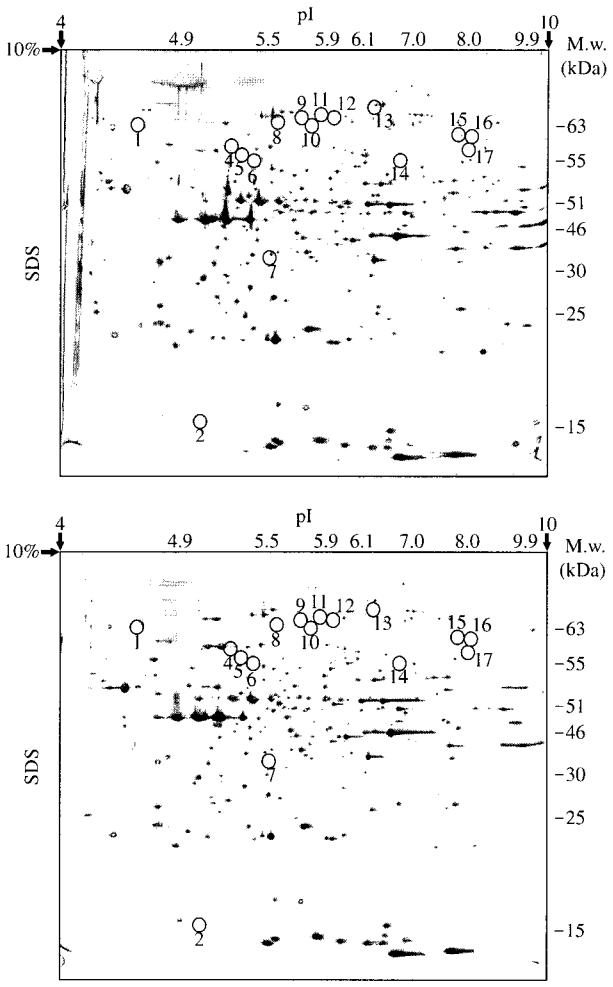

Fig. 1. 2D/E of whole body proteins from female adults of *C. riparius* exposed to BPA. (A) Control condition. (B) 10 μg L^{-1} BPA. Key 17 protein spots expressed increase or decrease at least 3-fold or were newly apparent in chemical treated animals.

RESULTS AND DISCUSSION

The polypeptide expression response to chemical treatments and reflecting I-18 C gene

In the preliminary study, *C. riparius* were placed into various concentrations (control, 0.3, 1 and 10 μg L^{-1}) of BPA. The mortality of control groups was little different from that of the low concentration. In the experimental process, the body volume of exposed female was thinner than that of the control female but those of male adults didn't show a difference. Kwak and Lee (2005) reported a similar result (the body volume of exposed female was thinner than that of the control female) and the difference of protein expression in *C. riparius* was induced after treatment of tebufenozide acting as an ecdysteroidal molting hormone using in two-dimensional gel electrophoresis analysis (2D/E).

In this study, 1108 protein spots were identified in the 2D/E gels in female using gel electrophoresis (Fig. 1). These visualized protein spots allowed for the extraction of 17 protein spots which differed by more than three-fold in BPA treated females, representing approximately 1.6% of the total protein spots (Table 1). Usually, protein expression sensitively changes with developmental stages and external environments. Accordingly, induction of protein is specific to the developmental process and exposure to EDCs. The fourth-instar larvae normally required 8~10 days to emerge as adults. During this period, many proteins are

expressed, corresponding to larvae-pupae-adult phases. Due to the emerged adults exhibited differing protein expression according to treatment conditions, the female adults in treatments have thinner body volume than that in control conditions. Accordingly, we considered that BPA interrupted protein expressions and could be induce a thin body in females.

Also, this study was focused that polypeptide expression of I-18 C gene, early-ecdysterone inducible gene, was observed adult of *C. riparius* exposure to BPA. Control group, the amount of polypeptide reflecting ORF II (14.4 kDa) of the I-18 C gene was larger than that of ORF I (21.5 kDa) of this gene (Table 2). After treatments, the amount of polypeptide expression of ORF I and ORF II of the I-18 C gene didn't show differences. Borowicz (2005) reported that ORF I was overexpressed as the polypeptide and ORF II of this gene expressed as the polypeptide. Also, exposure to insecticide, ORF I was overexpressed as the polypeptide but ORF II was clearly reduced as protein (Kwak 2005). In this study, BPA didn't induce polypeptide expressions reflecting early-ecdysterone inducible gene. However, BPA interrupted protein expressions (17 spots in 2D/E) using the other mode of action.

Table 1. The obvious different spots from female adults of *C. riparius* exposure to BPA. Total number of spots visible on 2D/E gels was 1108. The selected spots showed over 3 folds differences between treatment and control groups. SSP; Specific number of protein spots in Fig. 1. *: clearly increasing polypeptide after treatment, **: disappeared polypeptide after treatment group, ***: decreasing polypeptide after treatment

SSP (spot ID)	MW (molecular weight)	PI (isoelectric point)	
1	62.4	4.4	*
2	16.6	4.9	**
4	56.1	5.1	***
5	54.6	5.2	**
6	51.8	5.2	**
7	30.1	5.4	*
8	64.1	5.4	**
9	64.2	5.6	*
10	62.2	5.6	*
11	64.3	5.7	*
12	64.1	5.9	*
13	73.0	6.6	**
14	52.1	7.0	*
15	58.7	7.8	*
16	58.7	8.0	*
17	56.4	8.0	**

Table 2. The amount of expressed polypeptide of DNA fragment reflecting open reading frame I (ORF I; 21.5 kD) and open reading frame II (ORF II; 14.4 kD) of the I-18 C gene of *C. riparius*

kD	Isoelectric point	Control		Treatments	
		Male	Female	Male	Female
14.4	6.1	692.3	496.8	352.7	378.8
21.5	6.0	0	224.2	0	247.1

Difference of mitochondrial cytochrome oxidase I after treatments

Cytochrome oxidase I is highly conserved that it may be difficult to detect any amino acid change. However, amino acid evolution is faster in less conserved genes, and for reconstructions of data should be the most information. In this study, the difference of sequence for cytochrome oxidase I between control group and BPA treated group (0.3, 1, 10 μg L^{-1}) was less than 1~2% (Table 3). Now, BPA was affected a little in conserved mtDNA cytochrome oxidase I of *C. riparius*. The damage or change in mtgenome level (especially 1 μg L^{-1} BPA concentration) was suggested exposure to BPA in this preliminary research. We have to confirm damage of DNA level after chemical treatments (BPA etc.) in further study. And we make the design of universal primers and extract the stress gene reflecting expression protein response to BPA among 17 spots in 2D/E (Table 1).

Screening of gene expression at the RNA or protein level has been employed, in order to determine specific indicators for stresses (endocrine disruption chemicals, contaminated soil, chemical etc.). The entire protein complement expressed

Table 3. Homogeneity between control group and groups of exposure to bisphenol A, and difference DNA sequence region of the mitochondrial COI gene of *Chironomus riparius*

Bisphenol A treated groups (μg L^{-1})	Homogeneity (%)	Difference DNA sequence region of the mitochondrial COI gene					
		55~57	61~63	252~254	274~276	283~285	293~295
Control	100	TCG	TCA	CAC	CTA	AAC	AAG
0.3	99	.T.	.T.	...	...	...	...
1	98	.T.	.T.	.T.	T.C	.C.	.C.
10	99	.T.	.T.	...	...	...	...

at a given time has been concerned with the isolation of individual proteins for use as markers of the targets of drug action. Now, it is being employed in the isolation of protein markers associated with endocrine disruption. Techniques have been developed to analyze large numbers of proteins simultaneously, in order to discern subtle changes in protein expression (Herbert *et al.* 1997). In future studies, the sets of proteins induced and repressed by the environment will be assessed to detect indicators of endocrine disrupting chemicals.

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