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Changes in Proteome Following Exposure to Di (2-ethylhexyl) Phthalate in *Chironomus riparius* (Diptera: Chironomidae)

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Abstract - Due to the fourth-instar larvae of C. *riparius* have a sensitive to ecdysteroidal molting hormones for the life cycle developments, accordingly the emerged adult affected corresponding to larval phase's environments. The emerged female from larval phase exposure to DEHP observed a fat body and clumsy fling behavior in females. The body volume of treated female groups was clearly larger than that of control females. In the 2D/E gel 1108 protein spots were identified. The visualized protein spots allowed extraction of 27 protein spots differed more than 3 fold in DEHP treated animals, which was approximately 2.4% of the total protein spots. In this view, the body volume (or morphological characters) was well observed and detected faster than physiological detection for various EDCs. In this study, the body volume as a detecting point for EDCs suggested a bio-marker in individual levels.

Key words : proteome, *Chironomus riparius*, DEHP, endocrine disruption

INTRODUCTION

The most well known are the "xenoestrogens", man-made estrogen-mimicking chemicals, which interfere with functions of the female steroid hormone via interaction with the cellular receptor (Sumpter 1995; Jobling *et al.* 1996). In recent years, endocrine disruption (ED) has become common (Ankley *et al.* 1998), and it should be required to detect specific responses to EDCs. Therefore, endocrine specific endpoints have been proposed as the 'gold-standard' for risk assessment (Ingersoll *et al.* 1999). These tests can be designed to incorporate sensitive periods in the developmental process, including embryogenesis, gonadal development, molting or metamorphosis, growth and reproduction, all of which are regulated by the endocrine system and potentially susceptible to disruption. *Chironomus riparius* (Chironomidae), which has been extensively used in environmental assessment schemes and standardized chronic assays (USEPA 1994) and has a well studied endocrine system.

By far the most frequently reported phthalate, and that found at highest concentrations in the environment, is Di (2-ethylhexyl) phthalate (DEHP). This is to be expected, considering its high usage and greater persistence relative to the shorter chain phthalates. DEHP is widely used in the production of various plastics, polyvinyl chloride (PVC), inks and industrial oils. Especially, flexible PVC is employed for the production of floor tiles, furnishing, food packaging materials, and a variety of medical devices. The tolerable daily intake (TDI) for human is presumed as 40-140 $\mu g\ kg^{-1}\ day^{-1}$ (Inoue 2000). DEHP produced dose-related delays on surface righting in male offspring (Tanaka 2002) and opposite effects on the sex ratio of offspring of male and female mice (James 2003). DEHP should give rise to awareness about the animal and human exposure to these pollutants suspected to be carcinogenic and estrogenic (Harris *et al.* 1997).

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While chirinomid exposure to DEHP did not shows a sexual differentiation act as oestrogenic mode (Brown *et al.* 1996). In this point, we try to find effects of DEHP in chinomids. Accordingly, we suggested the effect of protein levels as detecting points for EDCs.

MATERIALS AND METHODS

Conditions were according to the suggestions for a standard procedure by Streloke and Kopp (1995). Egg masses of C. *riparius* 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 constant at 20±1°C in incubator chamber (Sanyo MIR-553, Japan). Twenty of fourth-instar larvae were introduced into each test vessel. For 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 sediment layer of 1 cm of fine sand (< 63 μm particle size). The test vessels were aerated continuously after midge larvae were introduced. Water loss due to evaporation was low, and if necessary, vessels were refilled with M4. Each vessel was provided 10 mg of ground fish food (Tetra-Werke, Melle, Germany). To achieve an exposure to constant substance concentrations through the midges' pupal phase and to avoid water quality changes from excess food, M4 was removed daily and replaced by new M4. To prevent escape of adults during test periods, each vessel was covered with 0.5 mm mesh net. F-test was employed to observe whether differences of body shape characters exist between control group and treated group (Zar 1984).

Solutions of DEHP (99%, Junsei Chemical Co. Ltd., Japan) had been dissolved in analytical grade acetone to provide stock concentration of 20 mg L^{-1} active ingredient. The nominal concentrations of DEHP were as follows: control, 0.3, 1, 10 and 30 μg L^{-1}. The half-time of DEHP is reported to be about 14-21 days.

Female adults of C. *riparius* were homogenated directly by mortor-driven homogenizer (PowerGen125, Fisher Scientific), and then the protein pellet was solublized in sample buffer composed with 7M urea , 2M Thiourea containing 4% (w/v) 3-[(3-cholamidopropy) dimethy-ammonio]-1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT) and 2% (v/v) pharmalyte and 1mM 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 soluble fraction was used for two-dimensional gel electrophoresis. Protein loading was normalized by Bradford assay. IPG dry strips were equilibrated for 12-16 hours with 7M urea, 2M thiourea containing 2% 3-[(3-cholamidopropy) dimethyammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), 1% pharmalyte and respectively loaded with 200 μg 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 3,500 V during 3 hours for sample entry followed by 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 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 (20-24 cm, 10-16%). SDS-PAGE was performed using Hoefer DALT 2D system (Amersham Biosciences) following manufacturer's instruction. 2D gels were run at 20°C for 1.7 kVh. And then 2D gels were silver stained as described by Oakley *et al.* (Anal. Biochem. 1980, 105:361-363) but 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. Quantity of each spot was normalized by total valid spot intensity. Protein spots were selected for the significant expression variation deviated over two fold in its expression level compared with control or normal sample. Each protein was compiled by Pi and molecular weight (MW).

RESULTS AND DISCUSSION

Due to the fourth-instar larvae of C. *riparius* have a sensitive to ecdysteroidal molting hormones for the life

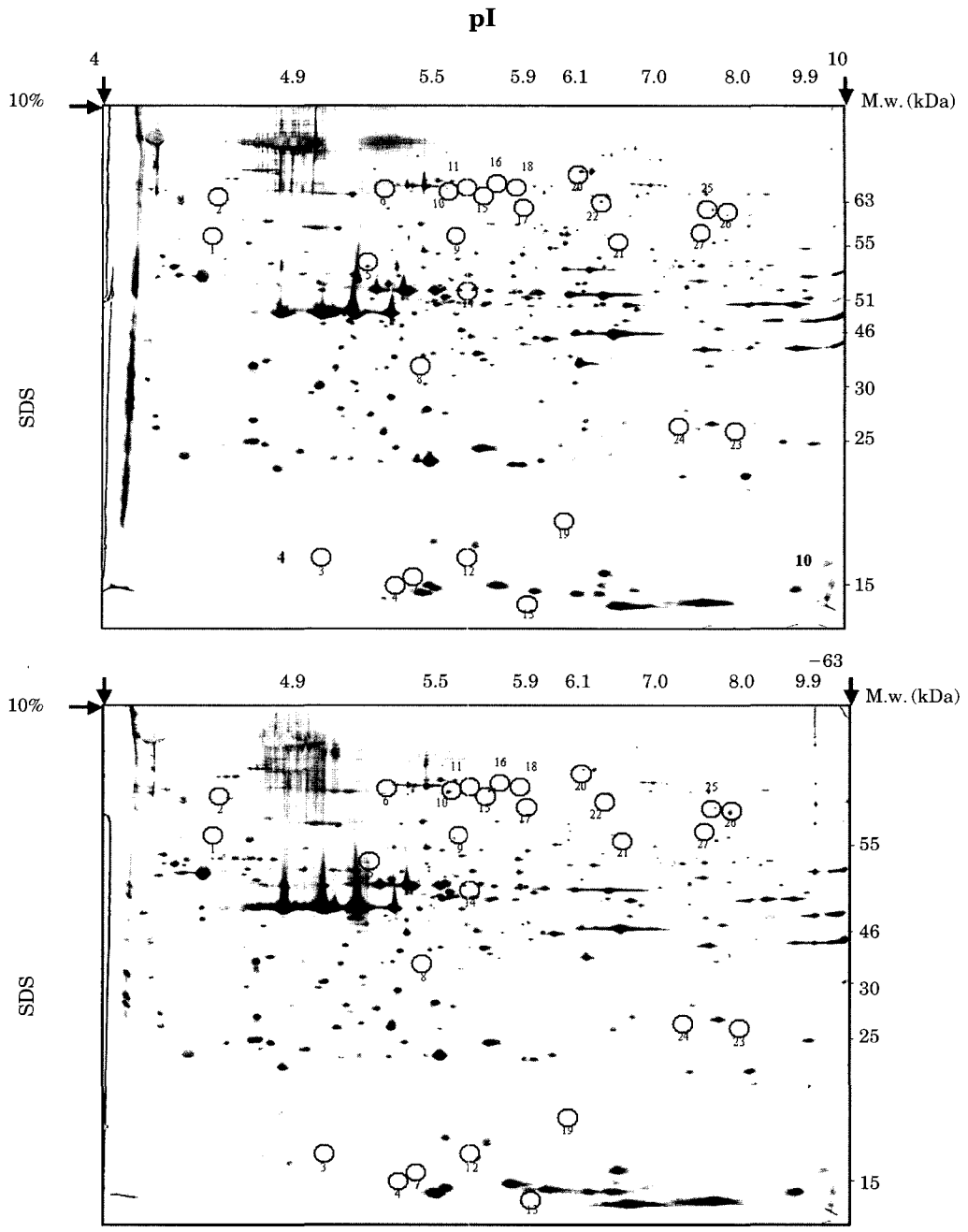

Fig. 1. 2D/E of proteins from female adults of C. *riparius* exposed to DEHP. The fourth-instar larval phase of C. *riparius* were given 10 μg L^{-1} DEHP developed pupae phase, and then emerged adults. After 8 days, whole body proteins were analyzed by 2D/E. (A) Control condition. (B) Contamination of DEHP. Key 27 protein spots expressed increasing/decreasing more than 3 fold or disappeared protain after DEHP treated animals.

cycle developments, accordingly the emerged adult affected corresponding to larval phase's environments. The emerged female from larval phase exposure to DEHP observed a fat body and clumsy fling behavior in females. The body volume of treated female groups was clearly larger than that of control females (Table 1).

In the 2D/E gel 1108 protein spots were identified (Fig. 1A and B). The visualized protein spots allowed extraction of 27 protein spots differed more than 3 fold in DEHP treated animals, which was approximately 2.4% of the total protein spots (Table 2). Usually protein expression sensitively changes with developmental stages and

Table 1. Body shapes of female adults of C. *riparius* such as head capsule length, head capsule width, body length, body width and body volume. Asterisks (*) denote a significant difference ($P < 0.05$). SD: standard deviation.

	Control		10 μg L^{-1}	
	Mean	SD	Mean	SD
Head capsule length (mm)	1.31	0.12	1.28	0.34
Head capsule width (mm)	0.70	0.12	0.75	0.14
Body length (mm)	9.07	0.68	9.63	1.36
Body width (mm)	1.22	0.20	1.46	0.27
Body volume (μL)	65.48	18.19	93.08*	22.20

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

SSP (spot ID)	MR (kDa)	PI (isoelectric point)	
1	53.0	4.4	*
2	62.4	4.4	*
3	16.6	4.9	**
4	15.1	5.2	*
5	47.4	5.1	*
6	63.6	5.2	*
7	15.4	5.3	*
8	30.1	5.4	*
9	53.2	5.5	**
10	64.2	5.5	*
11	64.2	5.6	*
12	16.7	5.6	*
13	14.3	6.1	*
14	41.9	5.6	**
15	62.2	5.6	*
16	64.3	5.7	*
17	59.8	6.1	*
18	64.1	5.9	*
19	18.7	6.5	**
20	73.0	6.6	**
21	52.1	7.0	*
22	61.9	6.9	*
23	25.0	8.2	*
24	25.3	7.6	**
25	58.7	7.8	*
26	58.7	8.0	*
27	55.1	7.8	**

external environments. Accordingly, induction of protein is specific to developmental process and EDCs. The exposed time in this study relatively was long days: the larvae required 8-10 days for em-erged adults. During this period, many proteins induce and express corresponding to larvae-pupae-adult phases. Finally, the emerged adults showed different protein expressions. In addition, due to the competition effect with a potential endocrine disruption chemical, DEHP, protein inductions of treated groups compared with those of control groups were generally increased and therefore, considered endocrine disruptions. In conclusion the disruption effect of DEHP interrupted protein expressions and induced a thin body in females. Nowadays many researches for detection of EDCs considered physiology and a toxic-chemical analysis, however, assessments for an ecological healthy were required ED of individual levels. In this view, the body volume (or morphological characters) was well observed and detected faster than physiological detection for various EDCs. In this study, the body volume as a detecting point for EDCs suggested a biomarker in individual levels.

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