Bull. Environ. Contam. Toxicol. (2005) 74:1159–1165

DOI: 10.1007/s00128-005-0702-3

Bulletin of Environmental Contamination and Toxicology

Two-Dimensional Gel Electrophoresis Analysis of Proteins Following Tebufenozide Treatment of *Chironomus riparius*

I.-S. Kwak, W. Lee

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

Received: 5 July 2004/Accepted: 11 March 2005

Compounds mimicking the action of juvenile hormones (JHs) may be used as safe insecticides. Numerous JH analogs have thus far been discovered, and some have already been used as commercial insecticides (Retnakaran et al. 1985; Staal 1975). Due, however, to the limited spectrum of their insect control and their slow toxic action, the agricultural use of these earlier JH analogs has remained limited. Recently, persistent efforts by the agrochemical industry have led to the discovery of several new and much more chemically diverse insecticidal agents, employing JH or ecdysteroid modes of action. The insecticide tebufenozide (N-tert-butyl-N'-[4-ethyl-benzoyl]-3,5-dimenthyl-benzohydrazide, formerly RH-5992), belongs to the group known as the insect growth regulators, and the benzoylhydrazines have been extensively studied. The benzoylhydrazines have been reported to function at the molecular level as agonists of ecdysteroidal molting hormones, and to exert a variety of hormonal effects in insects and crustacean arthropods (Wing 1988; Clare et al. 1992; Retnakaran et al. 1995; Dhadialla et al. 1998). In addition, tebufenozide is a non-steroidal agonist of 20E (20-hydroxyecdysone; molting hormone) and exhibits their insecticidal activity via interactions with the ecdysteroid receptor proteins. The molting process is initiated by an increase in the titer of 20E, and is completed subsequent to the decline of 20E titers and the release of eclosion hormone. The epidermal cells reorganize for massive protein synthesis, and secrete new epicuticle and cuticle. The morphological and structural changes occurring in the epidermis during insect growth and development are dependent upon the regulation of gene expression with different titers of 20E, in the absence or presence of JH (Riddiford 1996). *Chironomus 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 the insecticide tebufenozide.

In the preliminary study, C. *riparius* were placed into various concentrations (control, 10, 30, 60, 100 μg/L) of tebufenozide (LC_{50} 81.94 μg/L and NOEC 30 μg/L; Hahn et al. 2001). The mortality of control groups was little different from that of the low concentration (10 μg/L). 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. This study focused on tebufenozide acting

Correspondence to: W. Lee

as an ecdysteroidal molting hormone and evaluated changing protein expression through hormonal effects on the target insect, C. *riparius*. This study aims to detect changes in protein expression and to characterize changes in morphological characteristics.

MATERIALS AND METHODS

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 ℃ 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.

Tebufenozide (Sigma-Aldrich Laborchemikalien GmbH, 99.9%) was dissolved in analytical-grade acetone in order to create a stock concentration of 20 mg/L active ingredient. The nominal concentrations were the control, and 10 µg/L. 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 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 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 7M urea, 2M 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 96kVh. Prior to the second dimension, strips were incubated for 10 minutes in equilibration buffer (50 mM Tris-Cl, pH6.8 containing 6M 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 1.7kVh. 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 2-fold or more than the control value were selected. Each protein was then compiled according to pI and molecular weight (MW).

RESULTS AND DISCUSSION

Fourth-instar larvae of C. *riparius* exhibit marked sensitivity to ecdysteroidal molting hormones with regard to life cycle development. Accordingly, the emerged adults were affected, corresponding to the environment experienced in the larval phase. The 10 μg/L tebufenozide was lower than the NOEC (30 μg/L, Hahn et al. 2001). Generally, the bodies of females were larger than males. The head capsule, body length and body width were not significantly different in each sex. The female emerging from the larval phase after exposure to tebufenozide displayed a significantly thinner body than controls, but males had smaller differences in the body volume (Table 1).

Table 1. Morphological characteristics of C. *riparius* adults. Asterisks (*) denote a significant difference ($P < 0.05$). SD: standard deviation. 50 adult individuals were measured at each concentration.

	Female				Male			
	Control		10 μg/L		Control		10 μg/L	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Head capsule length (m	1.261	0.152	1.282	0.113	1.160	0.165	1.127	0.116
Head capsule width (mr	0.773	0.078	0.748	0.087	0.630	0.059	0.585	0.061
Body length (mm)	9.214	0.780	9.116	0.905	9.818	0.533	9.784	0.612
Body width (mm)	1.708	0.197	1.554	0.274	0.953	0.219	0.979	0.338
Body volume (μl)	79.521	18.361	70.298*	27.098	31.281	12.322	35.127	14.181

Upon gel electrophoresis, 1108 protein spots were identified in the 2D/E gels (Figure 1 and 2). The amount of protein induction for the male was relatively lower than for the female. These visualized protein spots allowed for the extraction of 19 protein spots which differed by more than three-fold in

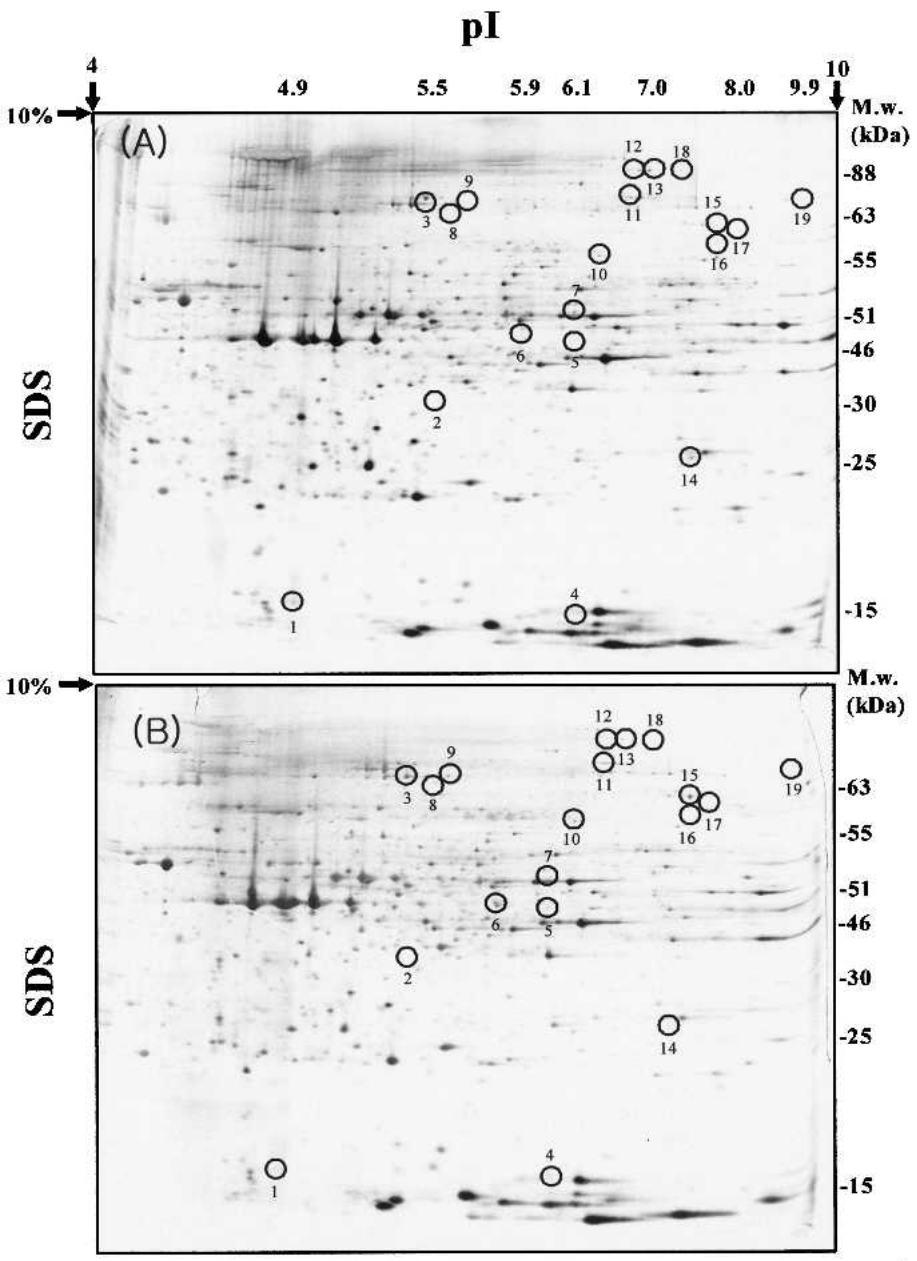

Figure 1. 2D/E of whole body proteins from female adults of C. *riparius* exposed to tebufenozide. (A) Control condition. (B) 10 µg/L tebufenozide. Key 19 protein spots expressed increase or decrease at least 3-fold or were newly apparent in tebufenozide-treated animals.

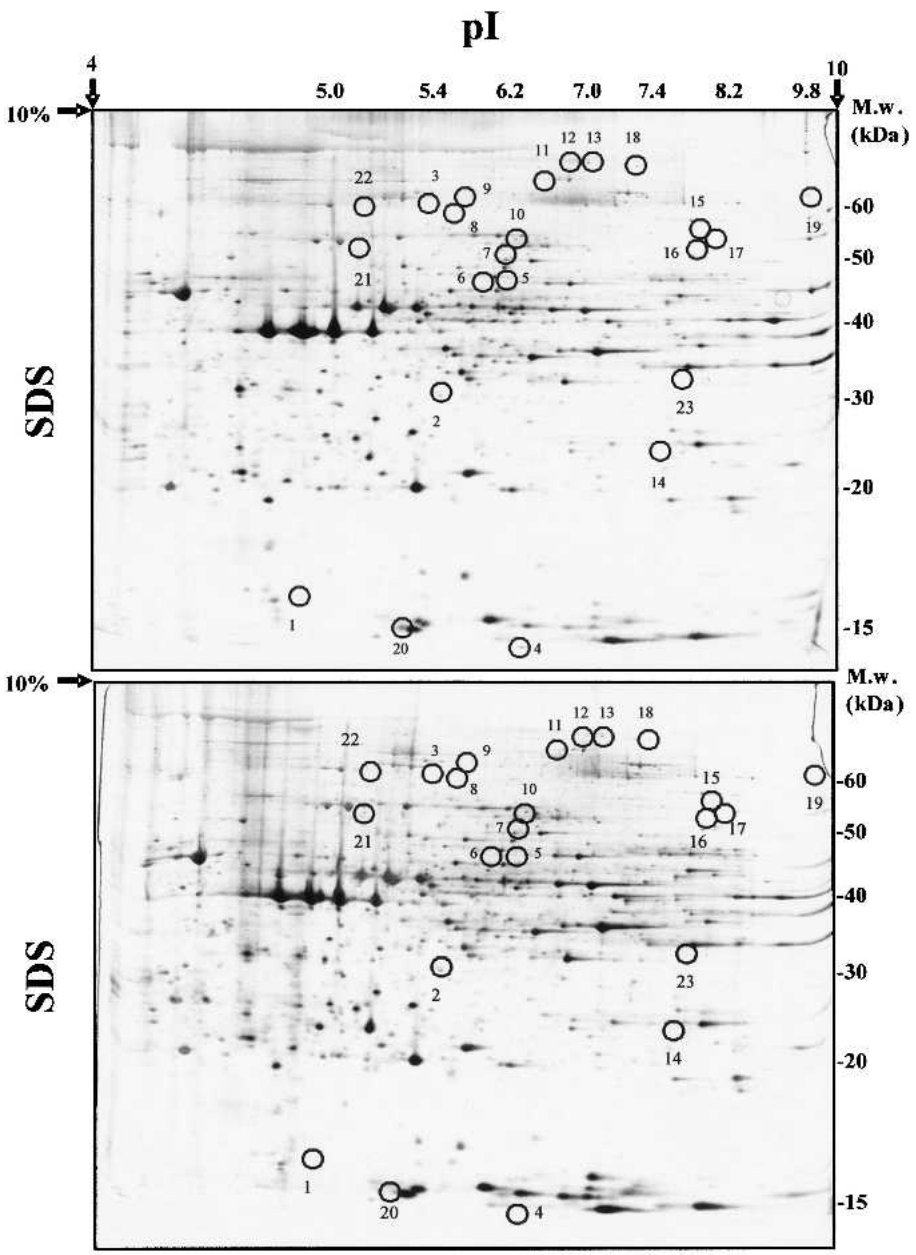

Figure 2. 2D/E of whole body proteins from male adults of C. *riparius* exposed to tebufenozide. (A) Control condition. (B) 10 µg/L tebufenozide.

tebufenozide treated females, representing approximately 1.7 % of the total protein spots (Table 2).

Table 2. The obviously different spots from C. *riparius* adults exposed to tebufenozide.

	Protein induction after treatment	Spot number (Figure 1 and 2)				
Both sex	Decreased	4	7	18	19	
	Increased	10				
Female	Decreased	1	2	12	13	
	Increased	3	5	6	8	9
Male	Decreased	13				
	Increased	20	21	22	23	

Total number of spots visible on 2D/E gels was 1108. The selected spots showed > 3 fold differences between treatment and control groups.

Usually, protein expression changes sensitively with developmental stages and external environments. Accordingly, induction of protein is specific to the developmental process and 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. Finally, the emerged adults exhibited differing protein expression according to treatment conditions. In conclusion, tebufenozide interrupted protein expressions and induced a thin body in females.

Recently, 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 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.

Acknowledgement. This study was supported by the Korea Research Foundation Grant (KRF-2002 -005-C00022).

REFERENCES

Bradford MM (1976) A rapid and sensitive method for microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-54

Clare AS, Rittschof D, Costlow JD (1992) Effects of nonsteroidal ecdysone

mimic RH 5849 on larval crustaceans. J Exp Zool 262: 436-440
Dhadialla TS, Carlson GS, LeDat P (1998) New insecticides with ecdysteroidal and juvenile hormone activity. Annu Rev Entomol 43: 545-569
Elendt BP, Bias WR (1990) Trace nutrient deficiency in *Daphnia magna* cultured in standard medium for toxicity testing; effects of the optimization of culture conditions on life history parameters of *Daphnia magna*. Water Res 24:1157-1167
Hahn T, Matthias L, Ralf S (2001) Effects of the hormone mimetid insecticide tebufenozide on *Chironomus riparius* larvae in two different exposure setups. Ecotox Environ Saf 49: 171-178
Herbert BR, Sanchez JC, Bini L (1997) Two dimensional electrophoresis: the state of the art and future directions. In: Wilkins MR, Williams KL, Appel RD, Hochstrasser DF (eds) Proteome Research: New Frontiers in Functional Genomics. Springer-Verlag, Berlin, pp 31-56
Oakley BR, Kirsch DR, Morris NR (1980) A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal Biochem 105: 361-363
Retnakaran A, Granett G, Ennis T (1985) Insect growth regulators. In: Kerkut GA, Gilbert LI (ed) Comprehensive Insect Physiology, Biochemistry and Pharmacology, Pergamon, Oxford, p 529-601
Retnakaran A, Hiruma K, Palli SR, Riddiford LM (1995) Molecular analysis of the mode of action of RH-5992, a lepidopteran-specific, non-steroid agonist. Insect Hem Mol Biol 25: 109-117
Riddiford LM (1996) Molecular aspects of juvenile hormone action in insect metamorphosis. In: Gilbert LI (ed) Metamorphosis, Academic, London, p 223-251
Staal GB (1975) Insect growth regulators with juvenile hormone activity. Annu Rev Entomol 20:417-60
Streloke M, Kopp H (1995) Long-term toxicity test with *Chironomus riparius*: development and validation of a new test system. Blackwell, Berlin/Vienna
USEPA (1994) Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates, United States Environmental Protection Agency EPA/600/R-94/024, Technical report, Washington DC pp133
Wing KD (1988) RH-5849, a nonsteroidal ecdysone agonist: Effects on a Drosophila cell line. Science 241: 467-469