1994

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Subcellular Distribution and Characterization of Esterase Isozymes from Insecticide-Resistant and -Susceptible Strains of German Cockroach (Dictyoptera: Blattellidae)

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J. Econ. Entomol. 87(3): 541–545 (1994)

ABSTRACT Esterases from insecticide-resistant and -susceptible strains of German cockroach, *Blattella germanica* (L.), were isolated and analyzed for differences in activity. The isozymes E5, E6, E7, and E8 of resistant strains indicated about two- to fivefold increases in activity compared with those of the susceptible strain. These isozymes constituted ≈60% of the total activity in the resistant strains, whereas they contributed only about 40% activity in the susceptible strain. Subcellular distribution studies showed that most esterase activity is present in the 100,000 × *g* fraction of the homogenate (cytosolic). Only a small portion of activity was membrane bound. Molecular weights of the esterase isozymes calculated based on their retardation coefficients ranged between 48 and 81 kd.

KEY WORDS *Blattella germanica*, esterase, isozyme

THE GERMAN COCKROACH, *Blattella germanica* (L.), is one of the most important pests of households, health institutions, and commercial food-processing establishments. This species conventionally is controlled using insecticides. However, several researchers have reported complete control failures caused by development of resistance to organophosphate (OP), carbamate, and pyrethroid insecticides (Schal 1988, Cochran 1989, Rust & Reierson 1991, Zhai & Robinson 1992). Resistance to OP and carbamate insecticides by German cockroaches has been correlated with enhanced oxidative and hydrolytic enzyme activity (Siegfried et al. 1990, Siegfried & Scott 1991).

Esterases, a group of hydrolytic enzymes displaying high levels of activity toward naphthyl esters and nitrophenyl esters, have been detected in insecticide-resistant German cockroaches (Siegfried & Scott 1992, Prabhakaran & Kamble 1993). These enzymes may be responsible for insecticide resistance through increased detoxification, or sequesteration, or both (Devonshire & Moore 1982).

Recent electrophoretic studies of German cockroach homogenates revealed 10 esterase isozymes that differ in isozyme composition between insecticide-resistant and -susceptible strains (Prabhakaran & Kamble 1993). However, no information is available on the biochemical comparisons of individual isozymes from resistant and susceptible strains of German cockroaches. Therefore, our study was undertaken to isolate and characterize esterase isozymes from insecticide-resistant and -susceptible strains of German cockroaches and to determine the subcellular distribution and molecular weight of these isozymes.

Materials and Methods

Cockroach Strains. The CSMA (susceptible strain reared in the laboratory without selection pressure), Baygon-R, and Dursban-R (multiresistant to several OP and carbamate insecticides [Siegfried et al. 1990, Prabhakaran & Kamble 1993]) strains were used in this study. The cockroaches were reared on Purina Dog Chow (Ralston Purina, St. Louis, MO) and water. The colonies were maintained in plexiglass containers (30 by 30 by 30 cm) at 27 ± 2°C, 60 ± 10% RH, and a photoperiod of 12:12 (L:D) h.

Chemicals. The esterase inhibitor paraoxon (90% purity) and the substrate α-naphthyl acetate were purchased from Sigma Chemical (St. Louis, MO). Fast garnet blue BB salt and the nonionic detergent Triton X-100 were obtained from Aldrich Chemical (Milwaukee, WI). All other chemicals and buffers were of reagent grade or better.

Enzyme Preparation. For isozyme analysis, 20 male German cockroaches of each strain were frozen at −20°C for 30 min and homogenized in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0) using a Tissue tearer homogenizer (Fisher Sci-

0022-0493/94/0541–0545$02.00/0

entific, St. Louis, MO). The homogenate was centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was used for isozyme separation by nondenaturing electrophoresis. For subcellular distribution studies, the homogenate was prepared with 25 male German cockroaches of each strain in 8 ml of 0.1 M phosphate buffer (pH 7.0), in both the presence and absence of 1% Triton X-100. The supernatant from 10,000 × *g* homogenate was further centrifuged at 100,000 × *g* for 60 min in a Beckman L7-65 ultracentrifuge equipped with a 70.1 titanium (Beckman Instruments, Ontario, CA) rotor to obtain microsomal and cytosolic fractions. The 10,000 × *g* pellet (mitochondrial and nuclear materials) and the 100,000 × *g* pellet (microsomal protein) were resuspended in 0.1 M phosphate buffer (pH 7.0).

Isozyme Separation. For isozyme studies, nondenaturing polyacrylamide gel electrophoresis (PAGE) was done in a vertical electrophoresis unit (Bio-Rad Protean II, Richmond, CA) by using 8.0% polyacrylamide gel and tris-glycine buffer. Each sample (1.5 ml) containing 10% glycerol was loaded into the wells formed by a preparative comb (Hoefer Scientific Instruments, San Francisco, CA). Electrophoresis was conducted at a constant 200 V at 0°C. After the run, a piece of the gel was cut out and stained for esterase activity in 100 ml of 0.2 M phosphate buffer (pH 6.5) containing 2% α-naphthyl acetate and 0.04 g of fast garnet blue BB salt for 5 min. By using this stained piece of gel, we marked esterase isozymes and separated them individually from the unstained gel. The cut gels were homogenized individually immediately in 2 ml of 0.1 M phosphate buffer (pH 7.0) and centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant was used as a source for individual isozyme activity. The separated isozymes were subjected to electrophoresis to ensure that only individual isozymes were included in the enzyme assay. The difference in isozyme activity between strains was analyzed by Fisher's least significant difference (LSD) at $P > 0.01$ (SAS Institute 1989).

The interaction of isozymes with an insecticidal substrate was studied by incubating the polyacrylamide gels in 10^{-6} M paraoxon for 20 min before the addition of α-naphthyl acetate and fast garnet blue BB salt. Nondenaturing PAGE was also done to determine the subcellular distribution of isozymes. The different fractions of the homogenate were resuspended in appropriate buffer and subjected to electrophoresis. The gels were stained using α-naphthyl acetate.

Protein and Esterase Assay. The protein concentration of the homogenates and isozymes was determined by the method of Bradford (1976). The esterase activities were assayed according to the technique of van Asperen (1962). The assay was done by measuring the production of α-naphthol from α-naphthyl acetate substrate. Each sample was added to a total volume of 1 ml reaction mixture containing 0.02 mM substrate and 0.02 M sodium phosphate buffer (pH 7.0). The reaction was initiated by addition of 20 μl of 10 mM α-naphthyl acetate substrate, and the solution was incubated at 26°C. The reaction was stopped after 15 min, and color was developed by addition of 166 μl diazo blue B (0.3% in 3.5% sodium lauryl sulfate). A_{605} was read 10 min later against an enzyme blank, and the concentration of hydrolyzed substrate was determined from a standard curve of α-naphthol.

Molecular Weight Determinations. The molecular weights of the esterase isozymes were determined based on the methods of Bryan (1977) and Davis (1964). Nondenatured-protein molecular-weight markers (Sigma Chemical, St. Louis, MO) were separated on a set of gels of various polyacrylamide concentrations in a Tube gel electrophoresis unit (Bio-Rad, Richmond, CA). The 100 log (R_f × 100) was plotted against the percentage gel concentration for each protein, and the slopes were determined. The logarithm of negative slope was plotted against the logarithm of the molecular weight of each protein to obtain a linear curve. Using this standard plot, the molecular weight of each isozyme was calculated.

Results

Subcellular Distribution. The subcellular esterase activities determined by comparing the activities of the supernatants and pellets obtained after 10,000 × *g* and 100,000 × *g* centrifugation of resistant and susceptible German cockroach strains are reported in Table 1. Maximum esterase activity was observed in the 100,000 × *g* supernatant (cytosolic fraction) of all three strains, which indicates that the largest part of the activity is caused by soluble enzymes. The 10,000 × *g* pellets (mitochondrial and nuclear fraction) displayed about one-fourth of the total activity in the nonsolubilized samples. However, a decrease in total activity was observed in the solubilized samples of 10,000 × *g* pellets for each strain. In addition, increased total activity was observed in the 100,000 × *g* supernatant with Triton X-100 for all three strains. This result suggests that some esterases are membrane bound and are released into the soluble fraction in the presence of a detergent. The 100,000 × *g* pellet (microsomal fraction) constituted <10% of the total esterase activity.

Nondenaturing electrophoresis of fractions obtained by differential centrifugation revealed different forms of esterases (Fig. 1). The 100,000 × *g* fraction displayed the highest diversity of esterases. The 10 esterase bands detected in this fraction were designated as E1 through E10. E1 was the slowest migrating esterase, whereas E10

Table 1. Subcellular distribution of esterase activity from German cockroaches

Sample[b]	Solubilization	% of total activity[a]		
		CSMA	Baygon-R	Dursban-R
Homogenate	None	100	100	100
10,000 × *g* pellet	None	22.8 ± 0.6	24.3 ± 1.2	25.1 ± 1.3
100,000 × *g* supernatant	None	71.5 ± 0.5	73.2 ± 3.3	72.8 ± 1.8
100,000 × *g* pellet	None	8.2 ± 0.9	5.7 ± 0.5	4.5 ± 0.6
% recovery		102.5 ± 0.3	103.2 ± 2.6	102.4 ± 1.2
Homogenate	1% Triton X-100	100	100	100
10,000 × *g* pellet	1% Triton X-100	13.5 ± 0.1	11.2 ± 0.9	14.0 ± 0.8
100,000 × *g* supernatant	1% Triton X-100	78.4 ± 0.3	84.2 ± 0.6	78.8 ± 5.4
100,000 × *g* pellet	1% Triton X-100	8.5 ± 2.6	6.0 ± 0.9	10.3 ± 3.1
% recovery		100.4 ± 2.4	101.4 ± 0.7	103.1 ± 1.5

[a] Results are the mean ± SEM of three preparations with three determinations each.
[b] Homogenates prepared from German cockroaches and subjected to 10,000 × *g* for 20 min and resulting supernatant centrifuged for 1 h at 100,000 × *g*.

was the fastest. The 10,000 × *g* pellet displayed a strongly stained E5 band and a faintly stained E1 band. Similar bands also were detected in the 100,000 × *g* pellet resuspended in the buffer. The esterase compositions of the gels obtained with the Triton X-100 solubilized fractions did not show any deviation from those of the gels with nonsolubilized fraction.

Individual Isozyme Activity. Data on the specific activities of total esterases and individual isozymes are presented in Table 2. Generally, the esterase activities in the insecticide-resistant German cockroach strains were two- to fourfold higher than those of the susceptible strain. The variation in specific activities of isozymes E1 through E4 was <2-fold, except E3 of Baygon-R, which was two times higher than that of the other two strains. In the same strain, significant increases in isozyme activities were observed in E5, E6, E7, E8, and E9 isozymes when compared with those of the susceptible strain. The Dursban-R strain also showed increased activities in E5, E6, E7, and E8 isozymes. However, when polyacrylamide gels were incubated in paraoxon, these isozymes were completely inhibited. Such inhibition indicates that these isozymes are also active against the insecticidal substrate.

The percentage of total activity of individual isozymes from the three strains of German cockroaches (Fig. 2) indicated that isozymes E5 to E8 were responsible for ≈60% of the total activity of esterases in the resistant strains. The same isozymes contributed only 40% of the total activity in the susceptible strain. However, the reduction in activity was compensated by the E3 and E4 isozymes in that strain.

Molecular Weight Determination. The molecular weight of each isozyme was determined indirectly by using the retardation coefficients (K_R) obtained from protein standards. The calculated molecular weights of different esterase isozymes (Table 3) generally ranged from 48 to 81 kd. The esterase isozymes with molecular weights of 55 to 65 kd were prominent in all three strains.

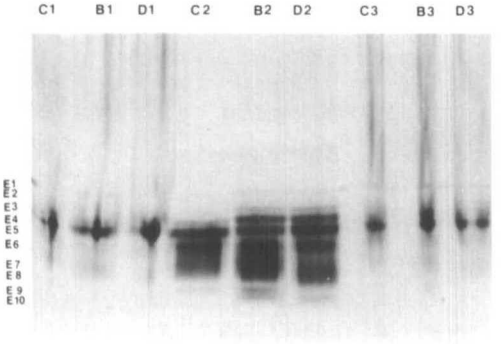

Fig. 1. Results of polyacrylamide gel electrophoresis of esterase isozymes from German cockroaches. C1, B1, D1, 10,000 × *g* pellet; C2, B2, D2, 100,000 × *g* supernatant; C3, B3, D3, 100,000 × *g* pellet. The letters C, B, and D designate CSMA, Baygon-R, and Dursban-R strains, respectively.

Discussion

Generally, the subcellular distributions of esterases in German cockroaches observed in this

Table 2. Individual esterase isozyme activity from German cockroaches

Isozyme	Specific activity (μMol/min/mg protein)[a]		
	CSMA	Baygon-R	Dursban-R
Homogenate	87.7 ± 1.8a	349.0 ± 3.0b	212.4 ± 7.3c
E1	112.0 ± 9.1a	125.8 ± 6.2a	121.4 ± 5.4a
E2	114.5 ± 7.4a	208.5 ± 4.9b	174.5 ± 2.7b
E3	152.6 ± 6.3a	335.3 ± 4.9b	144.0 ± 8.1a
E4	187.6 ± 4.1a	251.4 ± 12.4b	122.1 ± 2.6c
E5	257.0 ± 13.2a	443.1 ± 9.2b	503.2 ± 32.8b
E6	216.8 ± 6.4a	749.5 ± 6.0b	819.7 ± 25.5b
E7	122.1 ± 3.5a	387.6 ± 7.0b	393.3 ± 16.5b
E8	171.6 ± 5.4a	445.8 ± 10.7b	246.5 ± 3.1c
E9	184.1 ± 4.9a	407.2 ± 4.1b	173.8 ± 6.4a
E10	118.5 ± 4.9a	125.7 ± 7.1a	106.6 ± 4.6a

[a] Results are the mean ± SEM of three preparations with three determinations each. Means within the same row followed by the same letter are not significantly different ($P > 0.01$; least significant differences [SAS Institute 1989]).

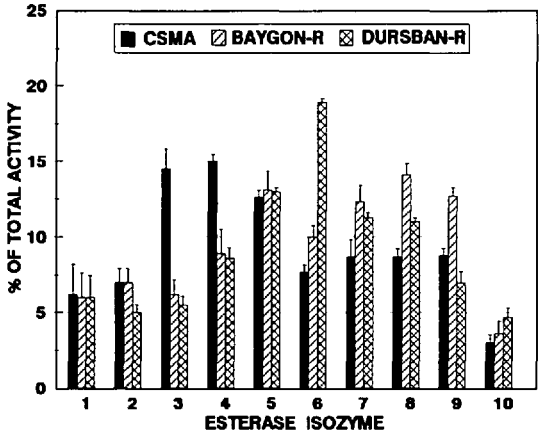

Fig. 2. Percentage of total activity of individual esterase isozymes from insecticide-resistant and -susceptible strains of German cockroach.

study parallel those reported for the mosquito *Culex pipiens* L. (Callaghan et al. 1991). Soluble enzymes (cytosolic fraction) are responsible for most of the total esterase activities in German cockroaches. However, an increase in percentage total activity in the cytosolic fraction caused by the solubilization of the homogenate in a detergent indicated that some forms of esterases are membrane bound or hydrophobic in nature. Nondenaturing electrophoresis confirmed the presence of all esterase isozymes in the soluble enzyme fraction. Similar esterase-activity distribution studies have been reported for green peach aphid, *Myzus persicae* (Sulzer) (Devonshire & Moore 1982). Insecticide-resistant strains of green peach aphid showed an increase in the activities of the detoxifying esterases. However, the esterases were mostly membrane bound.

As reported earlier by Siegfried & Scott (1992) and Prabhakaran & Kamble (1993), esterase activity was much higher in the Dursban-R and Baygon-R resistant strains. However, the insensitivity of acetylcholinesterase to inhibition by insecticidal substrates is not an important resistant mechanism in these strains (Siegfried et al. 1990). The suggested resistance mechanisms in these strains involve combined effects of oxidative and hydrolytic enzymes (Siegfried & Scott 1992). The comparison of individual esterase isozyme activities allowed us to identify which esterases are mainly responsible for the activities observed in crude homogenates. Based on comparisons, we concluded that isozymes E5, E6, E7, and E8 are accountable for the increased hydrolytic activity toward α-naphthyl acetate in resistant German cockroaches. The inhibition of these isozymes by paraoxon indicates that they can be associated in the insecticide resistance mechanism. However, at this point, it is not clear whether these isozymes are involved in the actual insecticide detoxification process or in sequestration of insecticides. The molecular-weight determination indicated that the E5 to E9 isozymes had molecular weights between 50 and 65 kd. Esterases with similar molecular weights were reported to be involved in insecticide resistance in mosquitoes (Callaghan et al. 1991).

Table 3. Estimated molecular weight of esterase isozymes from German cockroach

Isozyme	Molecular wt ($\times 10^3$ Daltons)
E1	81
E2	77
E3	73
E4	69
E5	65
E6	63
E7	60
E8	55
E9	50
E10	48

Acknowledgments

We thank Blair D. Siegfried, John E. Foster, and Z B Mayo (University of Nebraska, Lincoln) for their critical review of the manuscript. This research was funded in part by the Pesticide Impact Assessment Program. This is published as Paper 10420, Journal series, Nebraska Agriculture Research Division, and Contribution 835, Department of Entomology and Water Center/Environmental programs, University of Nebraska, Lincoln.

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Received for publication 8 September 1993; accepted 29 November 1993.