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Biochemical and Genetic Analysis of Epoxide-Metabolizing Enzymes in Susceptible and Resistant House Flies, *Musca domestica* L.

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Microsomal and cytosolic activities of glutathione *S*-transferase (GST) and epoxide hydrolase (EH) toward *trans*- and *cis*-stilbene oxides (TSO and CSO, respectively) were measured in homogenates of the house fly (*Musca domestica* L.) from strains that are either susceptible or metabolically resistant to insecticides. GST activity was detectable with CSO but not with TSO. Thus, the substrate selectivity of house fly GSTs differs from that in mammals and some other insects where the conjugation of both TSO and CSO has been measured previously. High levels of microsomal and cytosolic GST activities were present in the resistant, but not in the susceptible, strain. The genes controlling these high GST activities from both subcellular fractions were mapped to chromosome II. In contrast, no differences in levels, substrate selectivity, or subcellular locale of EH activity were detected between susceptible and metabolically resistant flies. These data suggest that EH activity is not coordinately regulated with GST or other enzymes relating to metabolic resistance in the house fly. If multiple resistance results from the coordinate regulation of detoxifying enzymes, the fact that EH is controlled differently may allow insecticides that contain epoxides and/or are activated by epoxidation to be used against insect populations where multiple resistance has arisen. © 1987 Academic Press, Inc.

INTRODUCTION

The resistance of insects to insecticides has rapidly become a dominating problem for contemporary agriculture. The number of insecticide-resistant species of insects has increased from 7 in 1938 to 447 in 1980 (1). A major reason that the number of insecticide-resistant populations of insects has risen so dramatically is cross-resistance. In general, the major classes of chemicals in use are directed at a small number of target sites and are detoxified by a limited number of enzymes. Thus, the evolution of a resistance mechanism, as a result of application of one class of compounds, may confer resistance to many members of that class and to different classes of chemicals as well. For this reason, emphasis is now being placed on the development of chemicals that have unique targets and/or routes of metabolism.

In addition to the need for the development of new and different classes of chemicals, a more thorough basic knowledge of resistance mechanisms is essential for the management of resistant populations (2). An avenue of research likely to be fruitful in this regard is the study of the genetic regulation of enzymes involved in metabolic resistance. In addition to being interesting from a basic standpoint, knowledge obtained by studying selection for resistance by insecticide application may enhance our understanding of multiple resistance. Multiple resistance arises in insect populations when two classes of insecticides with distinct pharmacodynamic properties are no longer efficacious.

Multiple or cross-resistance may result from a pleiotropic effect arising through selection for a mutant regulatory gene(s) that controls the expression of high levels of a number of enzyme activities involved in the detoxication of insecticides (3, 4). In resistant house flies, *Musca domestica* L.,

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high levels of the mixed-function oxidases (MFOs),[1] carboxylesterases, DDT dehydrochlorinase, and glutathione (GSH) *S*-transferases (GSTs) are all associated with gene(s) on chromosome II (5). GSTs (E.C. 2.5.1.18) are of interest because they metabolize, among other things, a wide array of organophosphorus (OP) insecticides (6–9). High levels of these enzyme activities have been found in the cytosol of the house fly and have been shown to be a factor contributing to resistance to insecticides (10–14).

Delaying the evolution of multiple/cross-resistance may be possible through the use of insecticides that are not detoxified by enzymes that are regulated coordinately in insects. The epoxide hydrolases (EHs, E.C. 3.3.2.30) are a group of detoxifying enzymes that are essential in mammals because they facilitate the elimination of a variety of epoxide-containing xenobiotics including potent carcinogens and mutagens (15). In insects, the importance of these enzymes for the metabolism of dietary epoxides and insecticides is unknown. However, in tests with a house fly strain resistant to dimethoate, the toxicity of an arylterpenoid with a terminal epoxide group was shown to be greater than the toxicity of the corresponding methoxyl or ethoxyl derivatives in both susceptible and OP-resistant house flies. In addition, cross-resistance to the epoxide was fourfold lower than that to the methoxyl compound (16), suggesting that, at least in this case, the metabolism of the epoxide and alkoxyl functionalities is regulated differentially. Therefore, the use of epoxides as insecticides (and the disruption of insect epoxide metabolism) as a strategy for insect control is an attractive alternative to contemporary targets but remains to be fully evaluated.

EH and GST activities with two epoxide substrates were genetically mapped to test the hypothesis that, like a number of other xenobiotic metabolizing enzymes, EH and GST activities were regulated coordinately by a gene (or genes) on chromosome II in the house fly. The regulation of a microsomal GST activity and its possible role in insecticide resistance were also examined. No differences were observed for EH activities between susceptible and resistant flies, while GST activity was 6–10 times higher in the resistant strain. Differences between patterns of epoxide hydration by epoxide hydrolase in the house fly and other insects are discussed.

MATERIALS AND METHODS

House flies. S-stw;bwb;ocra is a susceptible strain with recessive mutants for stubby wing, brown body, and ocra eyes controlled by chromosomes II, III, and V, respectively. R-Rutgers is resistant to OP and carbamate insecticides. The strain was initially selected with diazinon and periodically pressured. This R-Rutgers strain has high basal levels of MFO (17) and glutathione *S*-transferase activities (12), although this study concentrates on enzyme involved in epoxide metabolism. The resistant strain shows an IC_{50} of 4.6 μg diazinon per vial compared to 0.17 μg/vial for the susceptible sbo strain.

Genetic procedures. Females of the R-Rutgers strain were crossed with males of the S-stw;bwb;ocra strain as described by Motoyama *et al.* (18). F_1 males were backcrossed to S-stw;bwb;ocra females. This procedure eliminated recombination and allowed for the isolation of intact individual chromosomes as heterozygotes with chromosomes from the susceptible (marked) chromosomes. Thus, the eight B_1 phenotypes represented all possible combinations of the three susceptible chromosomes with the corresponding chromosomes from the resistant parent.

Preparation of enzymes. Twenty-five to fifty, 1- to 2-day-old female flies were

[1] Abbreviations used: MFO, mixed-function oxidase; DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane; GSH, glutathione; GST, glutathione S-transferase; OP, organophosphorus; EH, epoxide hydroxylase; LDH, lactate dehydrogenase; TSC, *trans*-stilbene oxides; CSO, *cis*-stilbene oxides.

frozen on dry ice. The abdomens were removed, pooled, and homogenized in cold 0.25 *M* sucrose. The resulting homogenate was filtered with glass wool and centrifuged at 10,000*g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 100,000*g* for 1 hr. The supernatant (cytosol) was removed and used directly while the resulting microsomal pellet was resuspended in 0.25 *M* sucrose. For all assays reported in this study, homogenates from 3–6 different generations of house flies were prepared and used immediately as enzyme source. Homogenates of flies from each generation were treated as an independent replicate.

Enzyme and protein assays. Glutathione *S*-transferase and epoxide hydrolase activities were measured according to Gill *et al.* (19) with slight modifications (20). Enzyme preparations (100 or 50 μl for microsomes or cytosol, respectively) were diluted in 1 ml cold potassium phosphate buffer (pH 8.9, $I = 0.2$). A typical reaction mixture contained 180 μl enzyme (0.2–1.0 fly equivalents) and 20 μl GSH (5 m*M* final concentration, freshly prepared in buffer). Reactions were started with the addition of 2 μl tritium-labeled *trans*- or *cis*-stilbene oxides (70 mCi/mmol, 5×10^{-5} *M* final) and terminated at 10 min by adding 200 μl *iso*-octane or *n*-hexanol for EH or GST, respectively, followed by vigorous vortexing and centrifugation (ca. 1500 rpm for 3 min). This partition step removes the lipophilic substrates from the aqueous phase. A 150-μl aliquot of the reaction products (aqueous phase) was measured into 3 ml of aqueous counting scintillant and counted in an LKB 1217 Rack Beta scintillation counter.

Lactate dehydrogenase activity (LDH, E.C. 1.1.1.27) was assayed according to the procedure of Bergmeyer and Bernj (21) in an effort to determine the levels of cytosolic contamination of microsomal preparations. Protein concentrations were estimated by the method of Bradford (22) with bovine serum albumin as a standard.

Statistical analyses. Pairwise and multiple comparisons were made using Student's *t* test and the method of Scheffe (23), respectively.

RESULTS

Epoxide hydrolase activities toward TSO and CSO were measured in both microsomal and cytosolic preparations of homogenates from susceptible and resistant house flies (Table 1). EH activities with CSO as substrate were higher than those when TSO was used, but with the exception of the microsomal activity in the resistant R-Rutgers strain, differences between the substrates were not statistically significant ($p > 0.05$). In tests with GST, clear substrate selectivity was observed: there was no detectable activity (limit of detection = 10 pmol/min · mg protein) with TSO as substrate while CSO hydrolysis was readily apparent.

With respect to the subcellular fraction assayed, statistically significant differences in activity ($p > 0.05$) were detected for GST, but not EH activities (Table 1). GST activity in the cytosol of the susceptible S-stw;bwb;ocra strain was 16 times greater than activity measured in the microsomes. Similarly, a 10-fold difference in activity was seen between microsomal and cytosolic fractions of the resistant Rutgers strain.

LDH activity was measured in these homogenates to determine whether the microsomal GST activity observed was a result of cytosolic contamination. The results of these tests revealed a 6.8% level of apparent cytosolic contamination in the microsomes (data not shown). This contamination does not account for all the microsomal GST activity measured. Furthermore, when the microsomes were resuspended in sucrose and centrifuged a second time at 100,000*g* for 60 min, LDH activity was reduced to undetectable levels, yet over 80% of the GST activity in the microsomes toward CSO remained. Therefore, a distinct microsomal GST activity was present in the strains tested.

TABLE 1

EH and GST Activities in Susceptible (S-sbo) and Resistant (R-Rutgers) House Flies[a]

	EH		GST	
	TSO	CSO	TSO	CSO
S-sbo				
Microsomes	65.5 ± 13.1	87.9 ± 26.4	ND[b]	35.0 ± 6.40
Cytosol	55.3 ± 14.3	92.6 ± 35.6	ND	573 ± 209
R-Rutgers				
Microsomes	88.0 ± 11.2	118 ± 20.5	ND	354 ± 127[c]
Cytosol	50.8 ± 10.3	166 ± 125	ND	3780 ± 970[c]

[a] Activities are expressed as picomoles of substrate metabolized per minute per milligram protein ±95% CI based on triplicate determinations of four to six different homogenates of flies from different generations.

[b] Not detectable under assay conditions where 10 pmol conjugated/min is detected as above background.

[c] Denotes activities that are significantly different ($p > 0.05$) from the corresponding susceptible activities.

EH activities in the susceptible and resistant strains were not statistically different ($p > 0.05$). However, EH activities with CSO tended to be higher in the resistant strain than those in the susceptible strain (Table 1). In contrast, GST activity in the resistant strain was, on average, 10-fold greater than that in the susceptible flies for the microsomal activity, and over 6.5-fold greater in the cytosol.

Microsomal and cytosolic EH activities in the parental, F_1, and eight backcross phenotypes of the R-Rutgers × S-stw;bwb;ocra) × S-stw;bwb;ocra cross are shown in Figs. 1 and 2, respectively. These were tested in an attempt to map EH activities to one or more of the three chromosomes containing visible markers (II-stw, III-bwb, or V-ocra). No statistical differences were measured in EH activities between the parental S-stw;bwb;ocra and R-Rutgers flies, or their F_1 progeny. In addition, EH activities in the backcross phenotypes were not significantly different from those of either parental strain, the F_1, or each other.

In contrast, major differences in GST activities were evident between strains and among the phenotypes tested (Fig. 3). Activity in the F_1 was not significantly different from that in the resistant, R-Rutgers strain, suggesting that the high level of activity was inherited in a dominant fashion. Comparable activity levels were also measured in the backcross phenotypes containing the resistant chromosome II, providing further evidence that factor(s) responsible for the elevated activity levels measured reside on this chromosome.

An analysis of the contributions of chromosomes II, III, and V to EH and GST activity appears in Table 2. No statistical differences were seen for EH activity with respect to the genetic composition of the flies tested. For GST, however, activities were dependent on the phenotype of the flies assayed. Increases in activity of 3.2-fold in the microsomes and 5.7-fold in the cytosol

FIG. 1. *Genetic analysis of the contributions of chromosomes II (stw), III (bwb), and V (ocra) on levels of microsomal epoxide hydrolase in the R-diazinon strain of the house fly. Bars represent mean activity ± 95% confidence interval (CI) at 30°C based on triplicate assays of five homogenates (25–50 female abdomens per homogenate) with each assay run on different days. Chromosomes from the wild-type (resistant) parent are designated by +.*

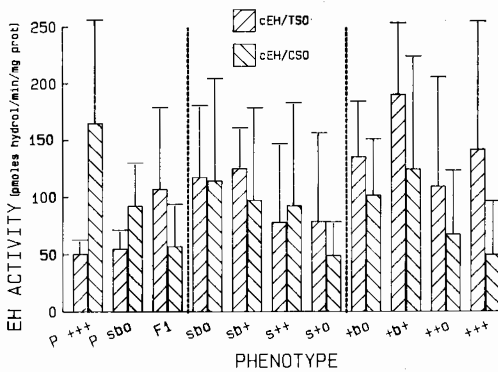

FIG. 2. *Genetic analysis of the contributions of chromosomes II (stw), III (bwb), and V (ocra) on levels of cytosolic epoxide hydrolase in the R-diazinon strain in the house fly. Bars represent mean activity ± 95% confidence interval (CI) at 30°C based on triplicate assays of five homogenates (25–50 female abdomens per homogenate) with each assay run on different days. Chromosomes from the wild-type (resistant) parent are designated by +.*

were measured when chromosome II from the resistant parent was expressed. In contrast, levels of activity were statistically lower when chromosome III from the resistant parent was expressed, providing additional evidence for a negative secondary effect of this chromosome in regulating activity levels in resistant house flies (14).

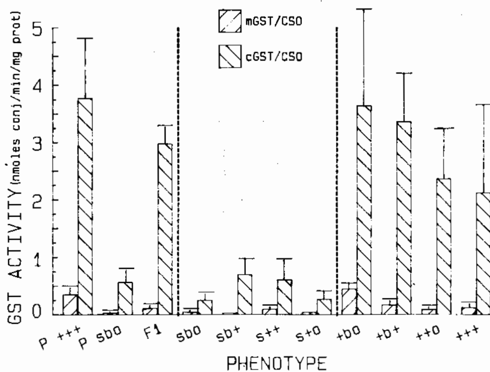

FIG. 3. *Genetic analysis of the contributions of chromosomes II (stw), III (bwb), and V (ocra) on levels of glutathione S-transferase in the R-diazinon strain of house fly. Bars represent mean activity ± 95% confidence interval (CI) at 30°C based on triplicate assays of five homogenates (25–50 female abdomens per homogenate) with each assay run on different days. Chromosomes from the wild-type (resistant) parent are designated by +.*

DISCUSSION

In this study, no differences in levels of EH activities were found between the susceptible and resistant house fly strains. Thus, the gene(s) on chromosome II that has been postulated to be instrumental in the regulation of metabolic resistance in the house fly (4) does not overtly influence levels of EH activity. This finding also suggets that high levels of EH activity are not a controlling factor in the resistance of this house fly strain to diazinon, DDT, or an array of carbamates (24). These results agree with those in an earlier report where no differences were found in EH activity toward styrene oxide between a susceptible and an OP-resistant strain of *Drosophila melanogaster* (25). In contrast, Mullin *et al.* (26) found large differences in EH activity with another epoxide substrate, *trans*-β-ethyl-styrene oxide, between susceptible and OP-resistant spider mites (*Amblyseius fallacis*). Therefore, the involvement of EH in OP resistance may vary and depend on the arthropod studied and/or the substrate used to monitor activity.

GST activity with CSO was higher in the resistant than in the susceptible strain and mapped to chromosome II. These results agree with earlier studies on the genetic location of GST activities with other substrates (12, 14, 17). A microsomal GST activity was also detected with CSO. This activity was also higher in the resistant strain and appeared to be regulated by gene(s) on chromosome II.

Levels of activity measured in house fly homogenates were similar to those in other insects studied previously using these substrates. However, qualitative differences exist in TSO and CSO hydrolysis among the house fly, *Trichoplusia ni* (27), and *D. melanogaster* (28). Apparently, patterns of epoxide metabolism (e.g., substrate selectivity and subcellular distribution) are variable in insects, and may differ between two closely related species (i.e., *M. domestica* and *D. melanogaster*). This variability may

TABLE 2
Analysis of EH and GST Activities in B_1 [F_1(S-sbo × R-Rutgers) × S-sbo] Phenotypes[a]

	II-stw			III-bwb			V-ocra		
	S[b]	R	R/S	S	R	R/S	S	R	R/S
Epoxide hydrolase/TSO									
Microsomes	102	113	1.1	97.7	116	1.2	120	95.1	0.8
Cytosol	107	144	1.3	144	112	0.8	120	131	1.1
Epoxide hydrolase/CSO									
Microsomes	116	123	1.0	108	129	1.2	135	105	0.8
Cytosol	87.1	85.0	1.0	110	66.6	0.6	79.7	91.3	1.1
GSH transferase/CSO									
Microsomes	57.6	186	3.2*	178	94.3	0.5*	146	115	0.8
Cytosol	480	2720	5.7*	1890	1480	0.8	1690	1670	1.0

[a] Values represent mean activity levels (pmol metabolized/min · mg protein) based on triplicate assays of three to five homogenates, each from a different generation of flies.

[b] The designations S and R indicate whether the chromosome studied was contributed by the susceptible or resistant parent, respectively. Asterisks signify that the reported ratio is statistically different ($p > 0.05$) from unity.

be a result of biological differences between these insects. A negative correlation has been established between herbivory and the activities of "*cis*" epoxide hydrolase and glutathione *S*-transferase (reviewed in (29)). In addition, differences in levels of MFO activities have been associated with polyphagy (30).

Like the results with EH, differences in glutathione *S*-transferase activity were also observed between the house fly and other insects studied earlier. In the present study, no GST activity was measurable with TSO as substrate. In contrast, GST activity was readily detectable with this substrate in *T. ni* (27) and *D. melanogaster* (28). Further, the absence of GST/TSO activity in house flies illustrates that the substrate specificity of the enzymes that conjugate dichloronitrobenzene, chlorodinitrobenzene, CSO, and other widely used substrates may be different from that metabolizing TSO. Alternatively, these substrates may each be metabolized by a different form of GST.

We have found that EH activity in a multiresistant strain of the house fly is not correlated with high levels of other enzymes involved in the detoxication of insecticides. Based on this finding, we posit that the efficacy of epoxide-containing insecticides may be similar in susceptible and multiresistant insects, and insecticides that are metabolized by EH may be successfully used against insect populations that possess metabolic resistance to other classes of insecticides.

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