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Biochemical characterization of insecticide resistance in the German cockroach, *Blattella germanica*, from Malaysia

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Abstract. The possible insecticide resistance mechanisms of four Malaysian field-collected strains of the German cockroach, *Blattella germanica* (Linnaeus) (Dictyoptera: Blattellidae), were characterized with biochemical assays and native polyacrylamide gel electrophoresis (PAGE). Elevated esterase activity (at low to moderate frequency) and altered acetylcholinesterase (low frequency) were detected in all field strains, while elevated glutathione S-transferase levels were present in only two strains. Seven esterase bands were separated by native PAGE; a greater intensity occurred in three bands in the resistant strains compared to the susceptible strain. Inhibition studies using specific inhibitors on polyacrylamide gels suggested that the slowest of these three esterases is a cholinesterase, while the other two are carboxylesterases with a preference for β- over α-naphthyl acetate.

Key words. *Blattella germanica*, carboxylesterase, cholinesterase, elevated esterase, elevated glutatione S-transferase, multi-resistance, Malaysia.

Introduction

The development of insecticide resistance in the German cockroach, *Blattella germanica*, is a common problem for the pest control industry. German cockroaches are resistant to all major groups of insecticides (Cornwell, 1976; Cochran, 1995). Most reports of *B. germanica* resistance involved dose–response bioassays (Bennett & Spink, 1968; Batth, 1977; Rust & Reierson, 1991; Zhai & Robinson, 1991), but do not detail underlying resistance mechanisms.

Resistance mechanisms involving both increased metabolism [elevated monooxygenase, esterase and glutathione S-transferase (GST) activities] and target site insensitivity [altered acetylcholinesterase (AChE) and altered sodium channel (*kdr*-type) resistance] have been detected in *B. germanica*. Scott & Matsumura (1981, 1983) found *kdr*-type resistance in a DDT-selected strain of *B. germanica*. Increased monooxygenase and esterase activity in organophosphate- and carbamate-resistant strains were detected through biochemical assays and *in vivo* and *in vitro* metabolism studies (Siegfried *et al*., 1990; Siegfried & Scott, 1991, 1992; Hemingway *et al*., 1993a). Hemingway *et al*. (1993b) reported elevated cytochrome P^{450} monooxygenase content of > 2-fold in seven of 15 pyrethroid-resistant strains studied. Recently, Valles & Yu (1996) reported increased activities of monooxygenases, aldrin epoxidase, phorate sulfoxidase, methoxyresorufin *O*-deethylase, GSTs, esterases, cytochrome P^{450} and cytochrome b_5, and cytochrome *c* reductase in a resistant strain of *B. germanica* from Georgia, U.S.A.

Earlier, we found that propoxur resistance levels in 10 Malaysian *B. germanica* strains reverted following pre-application of piperonyl butoxide and *S,S,S*-tributyl-phosphorotrithioate (DEF) (Lee *et al*., 1996). Biochemical assays using a microtitre plate reader were used to obtain endpoint and kinetic readings for non-specific esterase assays in four of these strains. Esterases were further separated and characterized by native polyacrylamide gel electrophoresis (PAGE). The possible involvement of AChE and GST resistance mechanisms were also studied. This is the first report on resistance mechanisms in *B. germanica* from South East Asia.

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Materials and methods

Cockroach strains

Four field strains of the German cockroach (Melia II, ChilliPadi I, TsimShaShui and HangTuah) were collected from

hotel and restaurants in Kuala Lumpur, Malaysia (Lee *et al*., 1996). Their resistance profiles are shown in Table 1. A susceptible strain (ICI) from Zeneca Agrochemicals, Jealotts Hill, U.K., was used for comparison.

Biochemical assays

Adult male *B. germanica* aged 2–4 weeks were frozen at –70°C prior to biochemical assays. For AChE assays, the head of each insect was homogenized in 200 µl of 1% Triton X-100 phosphate buffer, 0.1 M, pH 7.6 (Triton buffer). For the other biochemical assays, the remainder of the insect was homogenized in 500 µl of distilled water. Each homogenate was diluted 5×, spun at 10 000 **g** for 2 min and the resulting supernatant used as the enzyme source.

AChE, non-specific esterases and GST assays were performed as described by Hemingway *et al*. (1993a). For the AChE assay, 2 × 25 µl of individual homogenate were added to microtitre plate wells and the volumes were made up to 160 µl with Triton buffer, followed by 10 µl of 0.01 M 5,5-dithiobis-2-(nitrobenzoic acid). To the first replicate, 25 µl 0.01 M acetylthiocholine iodide (ATCI)/propoxur (9.88 ml ATCI + 0.12 ml 0.1 M propoxur) was added, while to the second replicate, only 25 µl 0.01 M ATCI was added. The kinetic rate of colour formation in each well was measured at 405 nm for 5 min using a UVmax Kinetic Microplate Reader (Molecular Devices Corp., Wokingham, U.K.).

Non-specific esterase activity was measured with the substrates α-, β-naphthyl acetate (NA) and *p*-nitrophenyl acetate (PNPA). For NA assays, 2 × 20 µl replicates of microfuged supernatant of each insect were used. To the first replicate, 200 µl of 0.3 mM α-NA in phosphate buffer 0.02 M (pH 7.2) was added, and to the second replicate, 200 µl of 0.3 nM β-NA. After 5 min incubation, 50 µl Fast Blue B solution (6 mM Fast Blue B in 3.5% sodium lauryl sulphate) was added and the mixture incubated for 5 min. The concentration of products were determined at 570 nm as an endpoint, calculated from a standard curve of α- or β-naphthol.

For the PNPA assay, 10 µl of microfuged *B. germanica* supernatant was added to the microtitre plate and 200 µl of PNPA working solution (300 µl 0.1 M PNPA in acetonitrile + 29.7 ml 0.05 M phosphate buffer pH 7.4). The plate was read at 405 nm for 2 min. The rate of formation of *p*-nitrophenol was determined kinetically and converted into absolute units based on protein concentration and the product extinction coefficient of 6.53 μM^{-1} (correction for path length).

GST activity was measured by adding 200 µl of 1-chloro-2,4-dinitrobenzene (CDNB)/reduced glutathione (GSH) working solution (23.75 ml 0.01 M GSH phosphate buffer 0.1 M, pH 6.5 + 1.25 ml 0.063 M CDNB in methanol) into 2 µl of 5× diluted supernatant. The reaction was then read at 340 nm for 5 min, and the activity of GST was calculated into absolute units based on the extinction coefficient 5.76 mM^{-1} (Hemingway *et al*., 1993a).

Protein assays were based on the method of Bradford (1976). Microfuged homogenate (10 µl) from each insect was added to 300 µl Bio-Rad Protein assay reagent (diluted 5× from stock), incubated for 5 min, and endpoint absorbance measured at 570 nm. Protein concentration was determined by converting the absorbance into concentration based on a bovine serum albumin standard curve.

Table 1. Resistance profiles (from Lee *et al*., 1996) of four resistant strains of German cockroaches used in this study.

Insecticide + synergist	Resistance ratio at LD_{50} (RR_{50})			
	Melia II	Chillipadi I	TsimShaShei	HangTuah
Propoxur	53.1	36.3	7.5	39.7
+ PBO	3.2	2.3	1.2	6.9
+ DEF	9.3	5.7	4.1	5.5
Bendiocarb	>61.4	48.8	11.3	>62.8
Chlorpyrifos	4.2	4.7	2.0	4.2
Cypermethrin	13.1	8.3	5.9	22.0
+PBO	21.3	24.2	8.3	–
+DEF	23.1	13.1	7.0	–
Permethrin	14.2	14.5	7.4	–
+PBO	17.0	17.1	15.3	–
+DEF	21.3	21.3	4.1	–
Phenothrin	–	52.3	15.5	–
Deltamethrin	19.8	23.8	5.6	–
DDT	>6.2	>6.5	>6.3	

Native PAGE

Groups of 10 adult males were homogenized in 3 ml phosphate buffer (pH 7.6) and spun at 11 000 **g** for 10 min at 4°C. The supernatant was used as the enzyme source.

Gel electrophoresis was performed on a 7.5% vertical polyacrylamide running gel. The samples were prepared by adding 20 µl of 5% xylene cyanol marker to 50 µl supernatant. Tris/EDTA/boric acid solution, 0.1 M (pH 8.0) was used as the electrode buffer. Electrophoresis was performed with a Bio-Rad Mini Protean II electrophoresis system. Ten microlitres of sample was loaded into each well and the gels were run at 150 V until the marker was within 1 cm of the gel base. Gels were incubated in 100 ml of 600 mM of α- and β-NA in phosphate buffer for 5 min and stained with the addition of 1 ml 0.05 M Fast Blue B.

Inhibition studies were done by incubating the electrophoresed gel in 96 ml of 10^{-4} M propoxur in phosphate buffer (pH 7.6) for 10 min. After incubation, 2 ml each of 0.03 M α- and β-NA were added and further incubated for 5 min before 1 ml of 0.05 M Fast Blue B was added. Experiments were repeated for other insecticides (paraoxon, malaoxon, permethrin and cypermethrin) at the same concentration.

Gene frequencies (GF) were estimated for altered AChE, elevated esterase and GST by assuming the populations were in Hardy–Weinburg equilibrium (Falconer, 1981) using the measured frequency of homozygous susceptibles in each strain.

Results

AChE assay

Distribution patterns of percentage propoxur inhibition of AChE activity in four resistant strains ($F_{\text{melia II}} = 3.3719$, $F_{\text{ChilliPadi I}} = 13.1932$, $F_{\text{TsimShaShui}} = 8.2398$ and $F_{\text{HangTuah}} = 3.1535$) were significantly different ($P < 0.01$) from those of the susceptible strain (Fig. 1). Their percentage activity also ranged widely, indicating heterogenous populations. The mean percentage AChE activity remaining in the propoxur-inhibited fractions were highest in ChilliPadi I and HangTuah strains, followed by the Melia II and TsimShaShui strains. Some individuals in the resistant strains showed percentage AChE activities of >100% in the propoxur-inhibited fraction. The results suggest a low frequency of altered AChE in all resistant strains based on the expected 66.5–80% propoxur inhibition of AChE activity in susceptible individuals.

Non-specific esterase assays

All strains demonstrated significantly ($F_{\text{Melia II}} = 13.4558$, $F_{\text{ChilliPadi I}} = 7.5106$, $F_{\text{TsimShaShui}} = 2.8800$, $F_{\text{HangTuah}} = 1.73476$; $P < 0.01$) elevated non-specific esterase activities with α-NA compared to the susceptible strain (Fig. 2). Esterase activity ranged from 0.004 to 0.158 nmol α-naphthol produced min^{-1} mg^{-1} protein in the susceptible strain. In the resistant strains, esterase activity was in the range 0.016–0.812, 0.056–0.547, 0.018–0.425 and 0.093–0.362 nmol α-naphthol produced min^{-1} mg^{-1} protein in Melia II, ChilliPadi I, TsimShaShui and HangTuah, respectively. These wider ranges reflect the heterogeneity of the resistant strains.

With β-NA, significantly higher ($F_{\text{melia II}} = 9.1829$, $F_{\text{ChilliPadi I}} = 7.5390$, $F_{\text{TsimShaShui}} = 1.5563$, $F_{\text{HangTuah}} = 2.1905$; $P < 0.01$) esterase activity was also detected in all resistant strains over the susceptible strain (Fig. 3). Distribution patterns of all resistant strains also differed significantly ($F_{\text{Melia II}} = 15.0951$, $F_{\text{ChilliPadi I}} = 8.0726$, $F_{\text{TsimShaShui}} = 2.8478$ and $F_{\text{HangTuah}} = 5.2185$; $P < 0.01$) from the susceptible strain when assayed against PNPA (Fig. 4).

Because homogenate from the same individuals were used in the assay with both α- and β-NA, comparisons between hydrolytic activity are possible. Non-specific esterase of the susceptible and three resistant strains demonstrated significantly greater ($t_{\text{ICI}} = 20.8554$, $t_{\text{ChilliPadi I}} = 5.4121$, $t_{\text{TsimShaShui}} = 2.7054$ and $t_{\text{HangTuah}} = 7.2750$; $P < 0.01$) hydrolytic activity against β-NA than α-NA. However, no significant difference ($t = 1.2893$; $P = 0.1983$) between hydrolytic activities with the two substrates was detected for the Melia II strain.

Mean esterase activity and propoxur resistance levels were well correlated when subjected to regression analyses. With α-NA, the relationship between esterase activity and propoxur RR_{50}s was $y = (0.090 \pm 0.017) + (0.004 \pm 0.001)x$, where y = nmol α-naphthol produced min^{-1} mg^{-1} protein and x = propoxur RR_{50}; $R^2 = 0.8276$; $P = 0.0321$. A good correlation

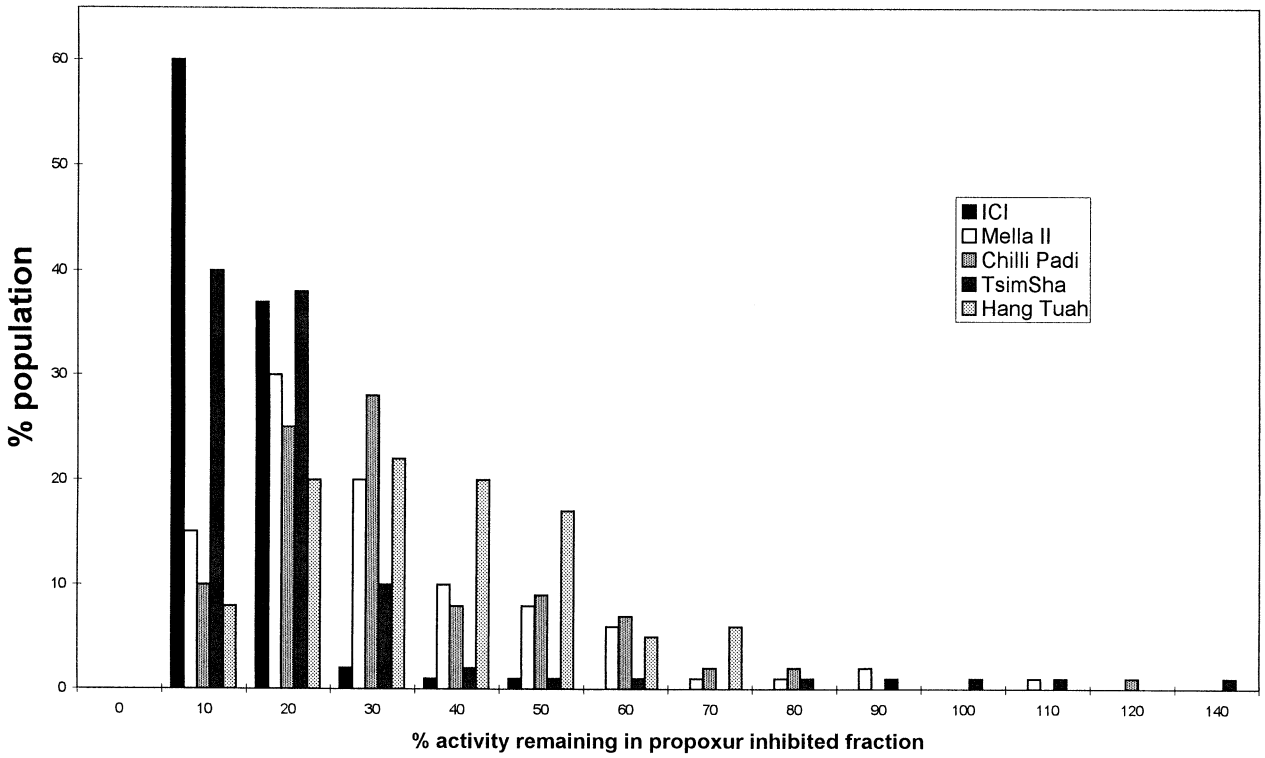

Fig. 1. Acetylcholinesterase inhibition profiles for susceptible (ICI) and resistant strains of the German cockroach (expressed as percentage acetylcholinesterase activity in the propoxur-inhibited fraction).

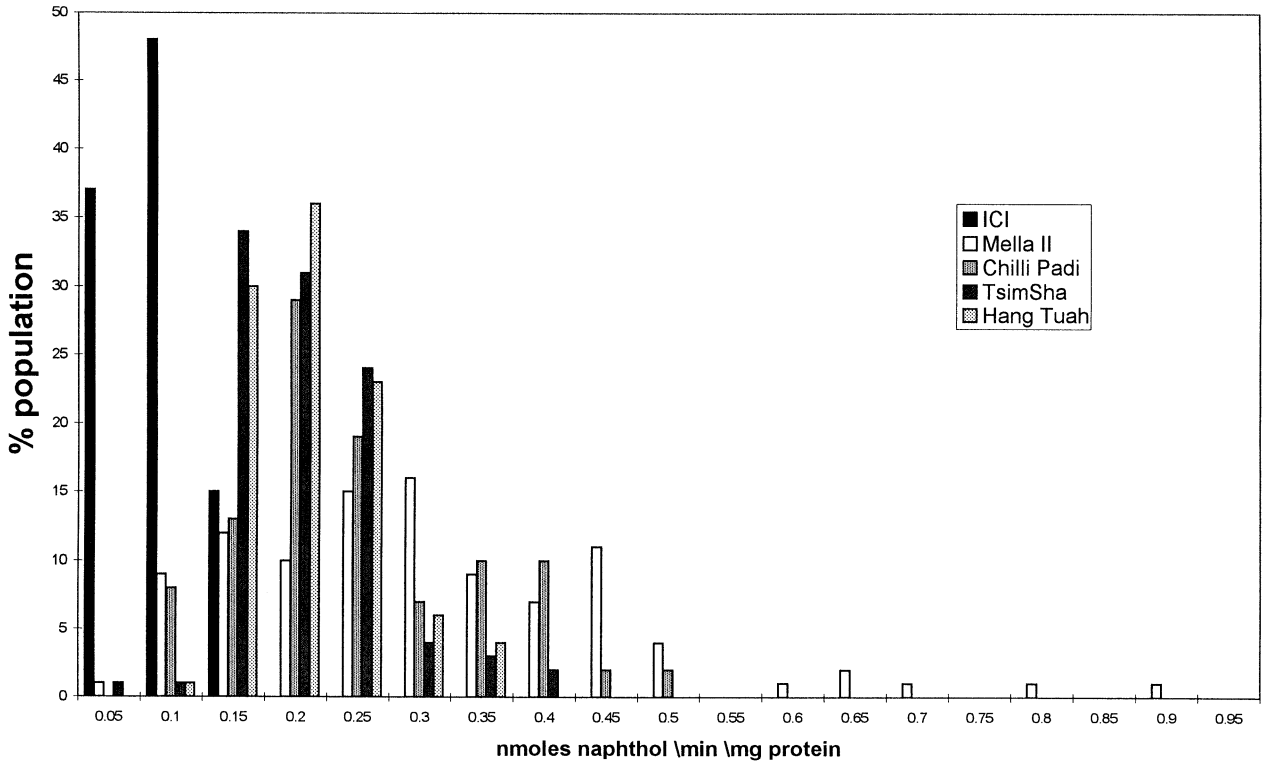

Fig. 2. Esterase activity profiles in populations of susceptible (ICI) and resistant strains of the German cockroach with the model substrate α-naphthyl acetate.

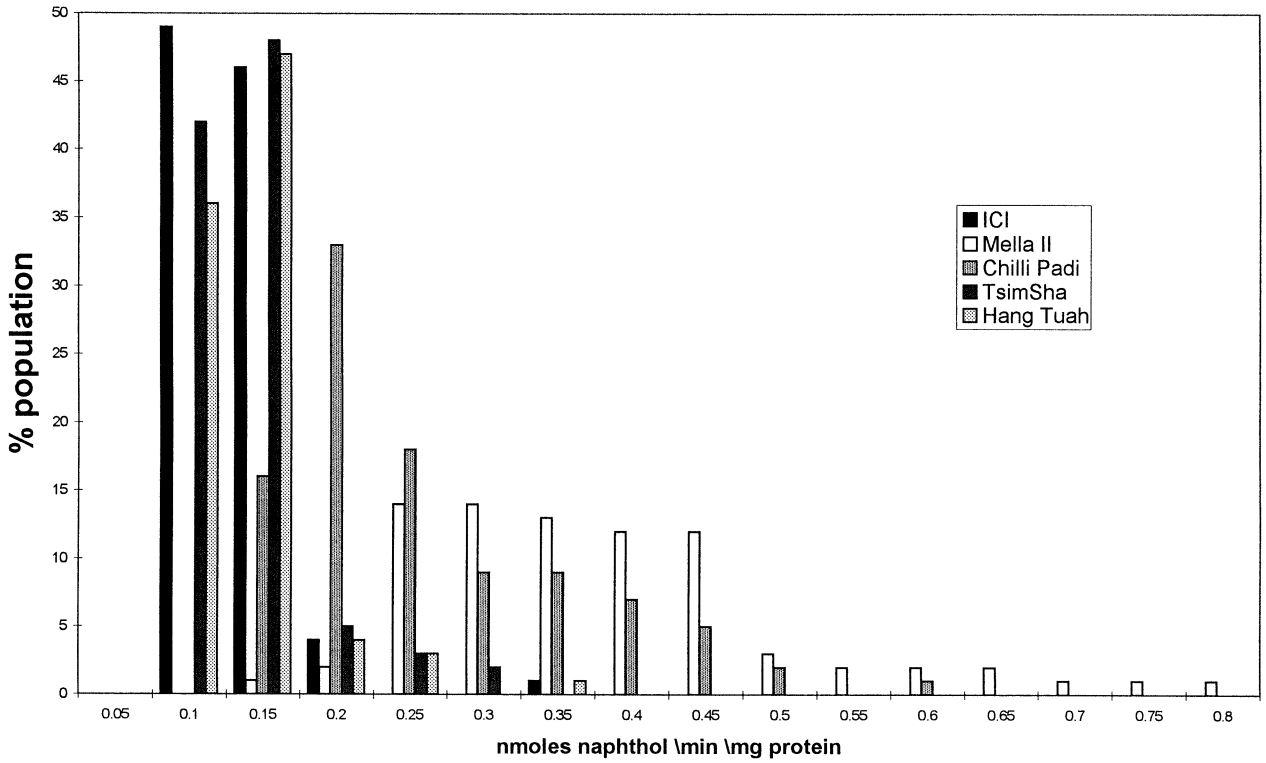

Fig. 3. Esterase activity profiles in populations of susceptible (ICI) and resistant strains of the German cockroach with the model substrate β-naphthyl acetate.

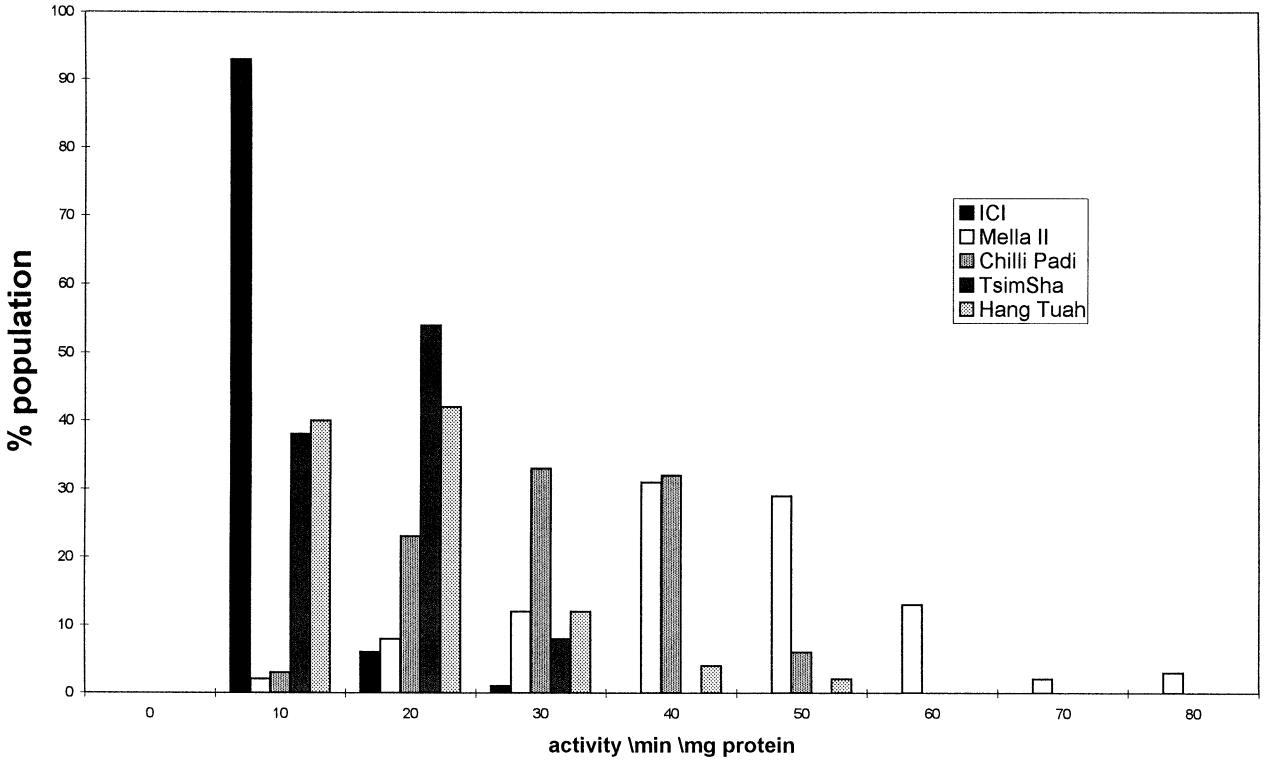

Fig. 4. Esterase activity profiles in populations of susceptible (ICI) and resistant strains of the German cockroach with the model substrate *p*-nitrophenyl acetate.

between esterase activity and propoxur RR_{50} also occurred with β-NA $[y = (0.161 \pm 0.005) + (0.0036 \pm 0.0002)x$, where y = nmol β-naphthol produced $min^{-1}\,mg^{-1}$ protein and x = propoxur RR_{50}; $R^2 = 0.9735$; $P = 0.0019$. With PNPA the relationship was $y = (14.83 \pm 1.38) + (0.45 \pm 0.04)x$, where y = µmol *p*-nitrophenol produced $min^{-1}\,mg^{-1}$ protein and x = propoxur RR_{50}; $R^2 = 0.8899$; $P = 0.0160$.

GST assay

Distribution patterns of GST activity in the susceptible strains were comparable to that in the TsimShaShui strain ($F = 1.4987$; $P > 0.05$), but significantly different from those of the Melia II ($F = 16.6743$; $P < 0.01$) and HangTuah ($F = 13.1119$; $P < 0.01$), which had greater activities (Fig. 5). The distribution pattern of the susceptible strain was also significantly different ($F = 1.8130$; $P < 0.01$) from that of the ChilliPadi I strain, which had lower GST activity. The GST activity distributions of the ICI, TsimShaShui and ChilliPadi I strains fall within a narrow range, implying that the population are homogenous. Of all strains, the HangTuah strain had the highest mean GST activity, followed by the Melia II strain.

Esterase banding patterns and insecticide inhibition studies

Seven esterase bands were detected on native PAGE in all strains. Bands E_1, E_5 and E_7 of Melia II, ChilliPadi I and HangTuah were more intense when compared with those of the susceptible strain (Fig. 6). Bands E_5 and E_7 of Melia II and ChilliPadi I were heavily stained. The staining intensity of esterase bands of TsimShaShui were quite similar to those of the susceptible strain; only the E_7 band was more heavily stained.

When subjected to propoxur (10^{-4} M) inhibition before staining, the E_1 and E_2 bands of all strains disappeared, while band E_7 had reduced staining (Fig. 6). Bands E_3–E_6 in all strains were not inhibited by propoxur. Malaoxon at 10^{-4} M inhibited bands E_1–E_4, and partially inhibited bands E_5–E_7 in all resistant strains. Band E_7 of the susceptible strain disappeared after malaoxon inhibition. Paraoxon at 10^{-4} M totally inhibited all esterase bands. None of the bands were inhibited by 10^{-4} M permethrin or cypermethrin.

Gene frequencies of altered AChE, elevated esterase and GST in all resistant strains were estimated based on the frequency of susceptible homozygotes. Altered AChE allele frequencies in the four resistant strains were low, ranging from 0.03 to 0.08 (Table 2). Melia II had the highest frequency of elevated esterase. The highest elevated GST frequency was in the HangTuah strain (0.68), followed by that of the Melia II strain (0.17), while the other two strains (ChilliPadi I and TsimShaShui) only contained susceptible levels of GST activity.

Discussion

The results from this study, and Lee *et al*. (1996), suggest that a range of resistance mechanisms occur in four resistant *B.*

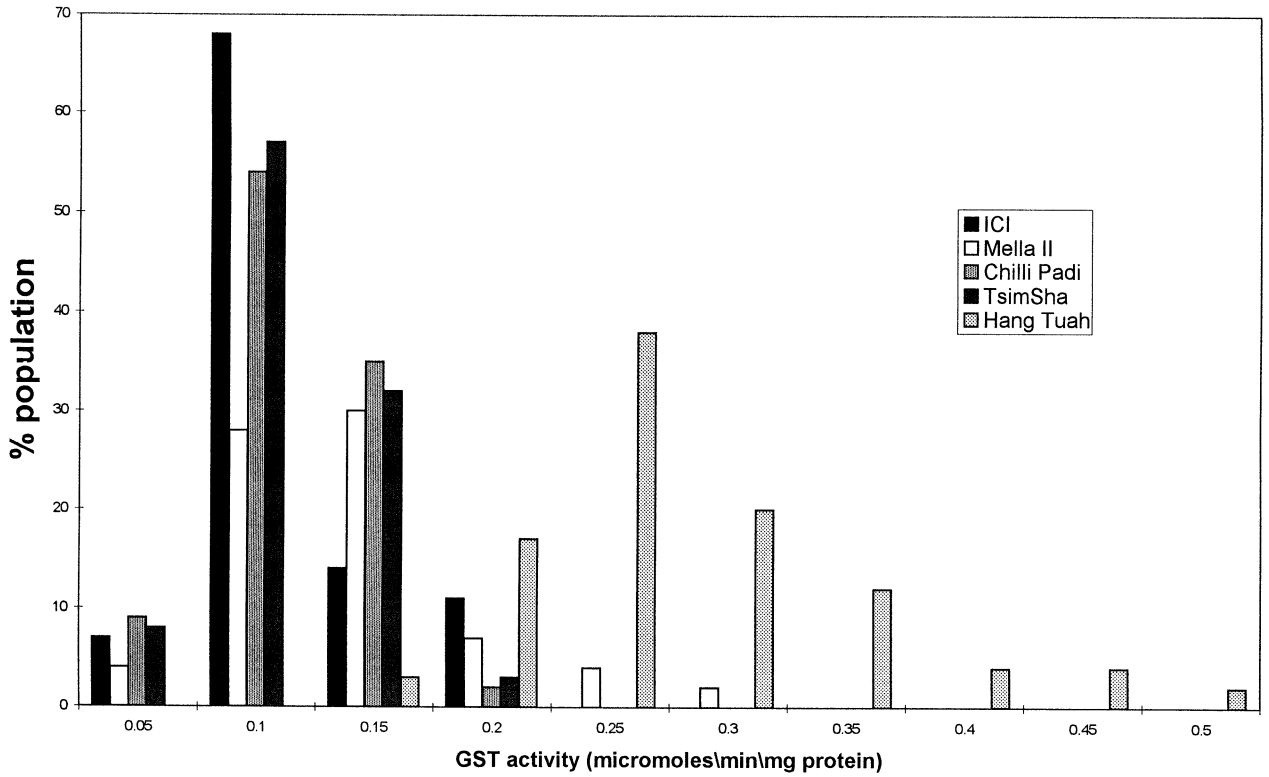

Fig. 5. Glutathione S-transferase activity profiles in populations of susceptible (ICI) and resistant strains of the German cockroach.

germanica strains from Malaysia. The Melia II, HangTuah, ChilliPadi I and TsimShaShui strains demonstrated multiple resistance mechanisms, i.e. altered AChE, elevated esterase and monooxygenase, and two of the strains had elevated GST activity. A further non-metabolic resistance mechanism (e.g. *kdr*-type) may be present in all four strains, suggested by the high levels of DDT and pyrethroid cross-resistance, which is not affected by synergists (Lee *et al*., 1996).

In all resistant strains, an altered AChE occurred at a very low frequency (< 10%). This suggests that this resistance mechanism is more common in Malaysian field populations than those from other continents. Hemingway *et al*. (1993a) detected one strain which possessed an altered AChE in 15 strains that were examined. The low organophosphate and high carbamate resistance characteristics of this strain were very similar to those of the resistant strains used in this study. Long-term propoxur use in Malaysia (> 10 years with bi-weekly or monthly application) probably accounts for the higher frequency of altered AChE found in the strains used in this study. Earlier, Siegfried & Scott (1990) did not find this mechanism in three multiple organophosphate- and carbamate-resistant U.S.A. strains. By contrast, altered AChE is common in other insect pests such as mosquitoes, leafhoppers and houseflies (Hama & Iwata, 1971, 1978; Tripathi & O'Brien, 1973; Hemingway *et al*., 1986; Raymond *et al*., 1986).

Elevated esterase activity has been much reported as a resistance mechanism in *B. germanica* (Siegfried & Scott, 1992; Hemingway *et al*., 1993a; Anspaugh *et al*., 1994; Lee *et al*., 1994; Schart *et al*., 1996). In this study, total non-specific esterase activity was studied using adult male German cockroaches. Elevated esterase activity was detected in resistant strains when compared to that of the susceptible strain. This may be due to increased catalytic efficiency or overproduction of esterases through gene amplification or over-expression (Hemingway & Karunaratne, 1998). Results in absolute units for the susceptible strain with α- and β-NA were similar to those of Hemingway *et al*. (1993a), who used first-instar nymphs, suggesting that there is no stage difference in esterase activity in the susceptible strain. Valles *et al*. (1994) also found no significant difference in esterase and GST activity and cytochrome P_{450} content between last instars and adult males.

Non-specific esterase in the susceptible strain and three resistant strains demonstrated greater hydrolytic activity towards β-NA, as compared to α-NA. This finding concurs with Hemingway *et al*. (1993a), where five of 15 strains of German cockroach preferred to hydrolyse β-NA rather than α-NA, while 10 further strains showed equal esterase activity between the two substrates. This phenomenon has also been observed in OP-resistant *Simulium damnosum* (Hemingway *et al*., 1989b). Total non-specific esterase activity correlated well with propoxur resistance levels. This has also been observed in some strains of chlorpyrifos-resistant *B. germanica* (Hemingway *et al*., 1993a).

Elevated GST causes DDT resistance in houseflies (Clark & Shamaan, 1984) and is associated with DDT and organophosphate resistance in mosquitoes (Hemingway *et al*., 1991; Ranson *et al*., 1997). Elevated GST activity was not correlated with chlorpyrifos resistance in Malaysian *B. germanica*, as we

Table 2. Estimated gene frequencies of some possible resistance mechanisms in four strains of German cockroach from Peninsular Malaysia.

Resistance mechanism	R allele frequency (± SEM)			
	Melia II	ChilliPadi I	TsimShaShui	HangTuah
Altered AChE	0.05	0.08	0.03	0.08
Elevated esterases	0.65	0.47	0.20	0.50
Glutathione S-transferase	0.17	0	0	0.68

previously reported for chlorpyrifos-resistant *B. germanica* from the U.S.A. (Hemingway *et al*., 1993a). Increase GST activity detected in Melia II and HangTuah may be attributed to DDT resistance.

Seven esterase bands were detected by non-denaturing polyacrylamide electrophoresis in all strains. No additional isozymes were detected in any of the strains. Prabhakaran & Kamble (1993) earlier reported 10 esterase bands. The additional three bands found by these authors may be due to different gel staining or the presence of Triton-X100 in their homogenization buffer.

Esterase band characterization by Prabhakaran & Kamble (1993) suggested that the 10 bands (E_1–E_{10}) separated belong to cholinesterase (bands E_1–E_5), phosphatase (E_6–E_7) and carboxylesterase (E_8–E_{10}) groups. The three bands, which were not detected in our study, probably belong to the cholinesterase group as expected, given the lack of a detergent in our homogenization buffer.

In the current study, there is no direct evidence to show which esterase isozymes are involved in insecticide resistance, although bands E_1, E_5 and E_7 showed more intense banding patterns in the resistant strains. Prabhakaran & Kamble (1994) suggested that the esterase band (E_6) is involved with insecticide resistance. This was the most active isozyme and was increased at least three-fold in specific activity when compared to the isozyme in the susceptible strain.

Mean total non-specific esterase activity with the substrate PNPA on the susceptible strain was much higher than that reported by Anspaugh *et al*. (1994) and Prabhakaran & Kamble (1993). There are two possible reasons: pH of the assay and the assay method used. In this study, the pH used in the esterase assay was 7.6, compared to 7.0 or 7.4 in other studies (Prabhakaran & Kamble, 1993; Anspaugh *et al*., 1994). Differences in pH can affect esterase activity in *Culex quinquefasciatus* (Hemingway & Karunaratne, 1998) and in *B. germanica* (Prabhakaran & Kamble, 1993). In addition, the single insect microplate technique used in this study is probably more sensitive than the spectrophotometric method used by Prabhakaran & Kamble (1993), which required groups of at least 10 insects per assay.

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