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PRELIMINARY CHARACTERIZATION OF INSECTICIDE DETOXIFYING ESTERASES IN SOME AGRICULTURALLY IMPORTANT INSECT PESTS

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ABSTRACT

Presence and the prevalence of elevated carboxylesterases, an important mechanism of insecticide resistance, and their interaction with different insecticide groups were investigated in ten species of agriculturally important insect pests *ie.* the rice brown planthopper (*Nilaparvata lugens*), four aphid species (*Toxoptera citricidus, Aphis gossypii, Myzus persicae, Aphis craccivora)*, diamond-backmoth larvae (*Plutella xylostella*) and four ant species (*Oecophylla* sp., *Crematogaster* sp., *Atta* sp. and *Camponotus* sp.).

Insect homogenates were run on native Polyacrylamide (7.5%) Gel Electrophoresis (PAGE) and the gels were stained for esterase activity using α- and β-napthyl acetate (NA) as substratres. Elevated carboxylesterase bands were present in all the species except *T.citricidus* and *Oecophylla* sp. These esterases were classified according to their preference for each substrate and R_f values. To examine the involvement of these isoenzymes with insecticides, gels were pre-incubated with insecticides of different groups for 10 mins. after the electrophoresis and then exposed to the substrates in the presence of insecticides. All the elevated bands except an 'α' type band (R_f = 0.32) of *Myzus persicae* were completely inhibited by paraoxon. None of the bands were completely inhibited by propoxur and permethrin, although some showed partial inhibition. Prevalence of elevated esterase activity in these populations was monitored by microtitre plate esterase assays using the same substrates. Assays were read at 490 nm. using a spectrophotometer. *Myzus persicae* had the highest esterase activity for both substrates (23.26±49.51 OD/mg/ml for α-NA and 39.07±75.70 OD/mg/ml for β-NA). Lowest activity was shown by *Oecophylla* sp. (0.23±0.26 OD/mg/ml for α-NA and 0.20±0.20 OD/mg/ml for β-NA).

INTRODUCTION

Despite the intensive use of insecticides, the pest populations are becoming a serious threat to the agriculture in Sri Lanka. Failure of the control is mainly due to the resistance shown by these insects to insecticides (Peiris, 1985). Usage of synthetic insecticides for more than 30 years has allowed the development and the establishment of insecticide resistance genes in these pest populations. Therefore the identification of the underlying molecular basis of insecticide resistance is of great importance for effective pest management.

Quantitative and/or qualitative differences of the enzymes which can metabolize insecticides is one of the major mechanisms of insecticide resistance (Karunaratne, 1996). Elevation of carboxylesterases has been reported from several agriculturally important insect pests outside Sri Lanka. However, no work has been conducted to find out the presence of these mechanisms in Sri Lankan insect pests of agricultural importance. Aphids are an important group of insect pests which feed on plant sap and transmit viral diseases. Presence

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of elevated-esterase based mechanism is well recorded in the aphid *Myzus persicae* (Devonshire, 1977; Devonshire and Moores, 1982). This mechanism has also been reported from the brown planthopper (*Nilaparvata lugens*) which causes hopper burn in rice, from Japan (Chen and Sun, 1994). Reports are not available in literature for the diamondback moth larvae (*Plutella xylostella*), which is a worldwide pest of cruciferous crops, and for ant species, which damage seeds and seedlings of crops. Present work was designed to identify the presence and the prevalence of elevated carboxylesterase-based mechanism in these insects in Sri Lanka. Reactivities of the elevated isoenzymes towards different types of insecticides were also tested.

MATERIALS AND METHODS

Insects

The rice brown planthoppers *Nilaparvata lugens* were collected from Batalagoda. Four species of aphid pests were collected from Gannoruwa vegetable farm from different vegetable crops ie. *Toxoptera citricidus* from *Citrus* sp., *Aphis gossypii* from brinjal, *Myzus persicae* from cabbage and *Aphis craccivora* from cowpea. Diamond-backmoth larvae (*Plutella xylostella*) from cabbage were also from Gannoruwa. Four ant species (*Oecophylla* sp., *Crematogaster* sp., *Atta* sp. and *Camponotus* sp.) were from the grasslands of University of Peradeniya campus. Collected insects were snap frozen at -20 ^{0}C until further use.

Chemicals

Biochemicals were purchased from Sigma Chemical Co. (UK). Permethrin [60:40 trans: cisratio] (3 – phenoxybenzyl (1RS,3RS; 1RS, 3SR)-3 -(2,2-dichlorovinyl)-2,2-dimethyl cyclopropane carboxylate) (97.2% pure), paraoxon (diethyl-4-nitrophenyl phosphate) (98% pure) and Propoxur (2-isopropoxyphenyl methylcarbamate) (97.5% pure) were a gift from Professor Janet Hemingway, University of Wales, Cardiff, UK.

Native polyacrylamide gel electrophoresis (PAGE)

Crude homogenate samples were performed in 7.5% PAGE in tris/borate buffer, *p*H 8.0 by the method of Davis (1964) using a Bio-Rad mini gel electrophoresis system. From each species, insects (100 mg) were homogenised in 200 µl of 50 mM phosphate buffer *p*H 7.4 and centrifuged at 13,000 g for 2 mins. 10 µl of the supernatant was loaded into each well with 4 µl of xylene cyanol marker and the gels were run at 150 V. After the electrophoresis gels were stained for esterase activity with 0.4% (w/v) α- and β-naphthyl acetate and 0.1% (w/v) Fast blue B salt in 20 mM phosphate buffer *p*H 7.4. For each species electrophoresis was repeated with five different samples (each 100 mg). Insecticide interaction experiments were carried out with three technical grade insecticides, representing three major synthetic insecticide groups (paraoxon - an organophosphate, propoxur - a carbamate, permethrin - a pyrethroid). After electrophoresis, gels were pre-incubated in 0.1 mM insecticide solutions in the phosphate buffer for 15 mins. and then exposed to the substrates as above in the presence of the insecticide. All the inhibition experiments were repeated at least for three times.

Biochemical assay

All the esterase and protein assays were carried out at 22°C. Individual insect was homogenized in 60 - 100 µl distilled water and the microfuged homogenate (2 X 20 µl) was used. To one replicate of homogenate, 200 µl of 0.3 mM α- naphthyl acetate (1 ml of 30 mM solution in acetone in 99 ml of 20 mM phosphate buffer *p*H 7.2) was added. To the other replicate, 200 µl of 0.3 mM β- naphthyl acetate (prepared as for α- naphthyl acetate) was

mg Fast Blue B salts in 15 ml distilled water + 35 ml of 5% sodium lauryl sulphate). Enzyme activity was measured at 490 nm as an endpoint assay in a UV_{max} microtitre plate reader (BIO-TEK Instruments, USA). Protein concentrations of the homogenates were determined by the method of Bradford (1976) using bovine serum albumin as the standard protein. 10 µl of the homogenate was mixed with 300 µl of working reagent (BIO-RAD, USA) and the absorbance was measured at 630 nm after 5 mins.

RESULTS

Native PAGE revealed that elevated carboxylesterase bands are present in eight of the ten pest species tested. No bands were detected in *T. citricidus* and *Oecophylla* sp. populations. In each of diamondback moth larvae, *A. gossypii* and *A. craccivora* two bands were elevated and in *M. persicae* three bands were elevated (Figure 1). Elevation was much higher in aphid species as measured by the colour intensities of the bands. Rate of flow (R_f) values were recorded for each band in relation to the mobility of the xylene cyanol marker and the bands were identified according to their substrate preference *ie.* α- naphthyl acetate preferred esterases stain in purple colour (α type) and β- naphthyl esterase preferred esterases stain in pink (β type) (Raymond *et al.*, 1987). Some bands were intermediate in colour indicating an equal preference for both substrates (Table 1). It is interesting to note that all the elevated isoenzymes of ant species preferred α- naphthyl acetate substrate.

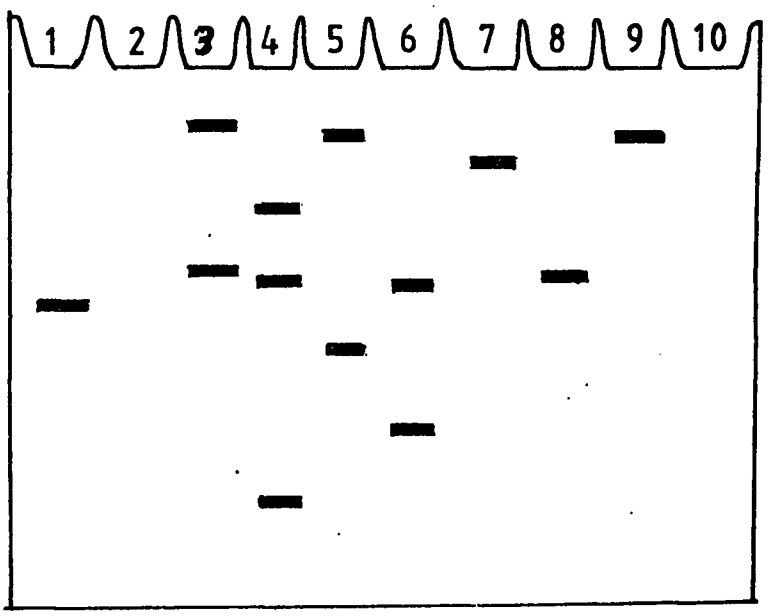

Figure 1. Native polyacrylamide gel electrophoresis (PAGE) of insect crude homogenates, stained for esterases activity with the substrate *α*- and *β*- napthyl acetate. 1. *Nilaparvata lugens*, 2. *Toxoptera citricidus*, 3. *Aphis gossypii*, 4. *Myzus persicae*, 5. *Aphis craccivora*, 6. *Plutella xylostella*, 7. *Camponotus* sp., 8. *Atta* sp., 9. *Crematogaster* sp. and 10. *Oecophylla* sp.

Results of gel inhibition studies are also given in Table 1. The organophosphate paraoxon completely inhibited all the elevated bands except one band of *M. persicae*. Partial inhibition was seen in six bands with the carbamate propoxur and in two bands with the pyrethroid permethrin. None of the elevated bands of ant species were inhibited by either propoxur or permethrin.

Table 1
Characterization of different carboxylesterase isoenzyme bands

Species	R_f value	Substrate Preference	Intensity	Inhibition		
				Paraxon	Propoxur	Permethrin
Nilaparvata lugens	0.6	α	++++	I	NI	NI
*Toxoptera citricidus**	-	-	-	-	-	-
Aphis gossypii	0.12	α	++++	I	P	NI
	0.48	β	++++	I	P	NI
Myzus persicae	0.32	α	++++	NI	NI	NI
	0.50	β	++	I	P	P
	1.06	α/β	++++	I	NI	NI
Aphis craccivora	0.12	α	++++	I	P	NI
	0.68	β	++++	I	P	P
Plutella xylostella larvae	0.55	α/β	++	I	P	NI
	0.95	α	++	I	NI	NI
Camponotus sp.	0.22	α	++	I	NI	NI
Atta sp.	0.53	α	+	I	NI	NI
Crematogaster sp.	0.16	α	+++	I	NI	NI
Oecophylla sp.*	-	-	-	-	-	-

*- no bands were detected. I- complete inhibition. P- partial inhibition. NI- not inhibited

Biochemical assays done with the same substrates show very high variations of the total esterase activity among the insects within each population (Table 2). As expected from the gel electrophoresis data, *M. persicae* shows the highest mean esterase activity for both substrates. Very high standard deviations show that *M. persicae* population is highly heterogeneous for their elevated esterase genes. Diamondback moth larvae also show a high

level of activity whereas all the ant species have very little total esterase activity. Lowest activities were shown by *Oecophylla sp.*, which did not show any elevation of esterases in native PAGE. However, *T. citricidus*, which also had no bands in PAGE showed a considerable amount of total esterase activity in microtitre plate assays.

Table 2
Mean esterase activities of insect crude homogenates with the substrates α- and β-napthyl acetate (n= 50)

Species	Mean enzyme activity $OD.mg^{-1}ml^{-1}$ (n = 50)	
	α	β
Nilaparvata lugens	15.5 ± 9.1	18.9 ± 10.5
Toxoptera citricidus	22.5 ± 60.5	23.4 ± 58.4
Aphis gossypii	19.7 ± 20.1	38.2 ± 32.7
Myzus persicae	23.3 ± 49.5	39.1 ± 75.8
Aphis craccivora	8.8 ± 16.3	12.3 ± 24.5
Plutella xylostella larvae	23.0 ± 33.7	35.2 ± 44.9
Camponotus sp.	2.6 ± 2.3	0.7 ± 0.7
Atta sp.	3.6 ± 3.6	5.4 ± 6.3
Crematogaster sp.	15.1 ± 31.4	12.5 ± 20.3
Oecophylla sp.	0.23 ± 0.26	0.2 ± 0.2

DISCUSSION

Elevation of carboxylesterases is an important mechanism in insecticide resistance. Carboxylesterases can hydrolyse insecticides which have ester bonds. Organophosphates are basically esters of phosphoric acid and are often metabolysed by carboxylesterases. Therefore the presence of these enzymes in great quantities causes very high tolerance especially to organophosphates. This mechanism is commonly found among insecticide resistant insect pest populations and has been well characterized in the aphid *M. persicae* and in the mosquito *C. quinquefasciatus* (Devonshire and Moores, 1982; Karunaratne *et al.*, 1993; 1995). It has been shown that the elevation or the increased amounts are due to the amplification of the esterase genes (Field *et al.*, 1993; Vaughan and Hemingway, 1995). In a resistant strain of *C. quinquefasciatus*, there were about 250 copies of the esterase gene in the genome (Mouches *et al.*, 1986). Using highly resistant strains of aphids and mosquitoes,

it has been estimated that these carboxylesterases comprise about 0.4 - 3% of the total protein of an insect. These enzymes have a very high binding capacity rather than a high capacity of breaking down the insecticide (Devonshire and Moores, 1982; Karunaratne *et al.*, 1993).

Native PAGE with the substrates α- and β- naphthyl acetate is commonly used to detect elevated isoenzymes of carboxylesterases (Raymond *et al.*, 1987). Bands can be seen at a standard range of homogenate concentration, only if the mechanism is present *ie.* only the resistant but not the susceptible strains of *C. quinquefasciatus* show bands (Karunaratne *et al.*, 1995); only the malathion resistant *C. tritaeniorhynchus* but not the malathion susceptible *C. gelidus* from Sri Lanka show bands (Karunaratne and Hemingway, 1997).

Data of the present work shows that this mechanism is common among Sri Lankan agriculturally important insect pests as well. Populations which have the mechanism were highly heterogeneous for the resistance. Mechanism was absent in *T. citricidus* and *Oecophyla* sp. according to the PAGE results. However, the aphid *T. citricidus* showed higher total esterase activity with the same substrates in biochemical assays. This may indicate the presence of a large number of non-elevated isoenzymes of carboxylesterases. Insecticide interaction experiments showed that all the elevated isoenzymes are highly reactive with organophosphates except for one isoenzyme from *M. persicae*. This enzyme band was not reactive with any of the three insecticides tested. Although this enzyme is not important with respect to the insecticide resistance, it is worth to be studied further in biochemical point of view.

Results basically indicate that the pest populations studied can give resistance especially to organophosphates except for two species. Mechanism is highly developed in the aphid *M. persicae*. The reactivity of these enzymes with propoxur and permethrin is only partial if there is any. Early work on british population of *M. persicae* have shown that the carboxylesterase isoenzyme E4 interact well with pyrethroids also (Devonshire and Moores, 1982). Isoenzymes of carboxylesterases which are involved in insecticide resistance in some of the Sri Lankan insect pests of agriculture were identified in this study. Further biochemical and molecular biological characterization of these esterases will answer at least some of the basic questions related to the evolution and the spread of insecticide resistance in these pest populations allowing us a better management of pests and pesticides.

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