CHARACTERIZATION OF MULTIPLE VARIANTS OF CARBOXYLESTERASES WHICH ARE INVOLVED IN INSECTICIDE RESISTANCE IN THE MOSQUITO *CULEX QUINQUEFASCIATUS*

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ABSTRACT

Overproduced carboxylesterases A_2 and B_2 (EC 3.1.1.1.), involved in insecticide resistance in the mosquito *Culex quinquefasciatus* were purified to homogeneity using 4th instar larvae from a Sri Lankan resistant strain, PelRR. Esterase B_2 ($M_r = 62,000$) was characterized and compared with the esterase A_2 characterized previously. The kinetic constants for interaction with several insecticides indicate, as for the esterase A_2, that the role of B_2 in insecticide resistance is mainly sequestration. The bimolecular rate constant, k_a, is the most important constant which correlates directly with the insecticide resistance ratios of the strain. A concentration of approximately 7.67 pmol of both A_2 and B_2 esterases accounting for about 0.4% of the total protein could be estimated per 4th instar larva. Using several different methods an $A_2 : B_2$ ratio of 1 : 3 could be obtained for this strain.

A_2, B_2, B_1 and susceptible non-amplified 'A' and 'B' type esterases were purified from one susceptible and six more resistant strains of *Cx quinquefasciatus*, which originated from different geographical areas. Significant differences in the k_a's for insecticide kinetics were obtained for the enzymes from the different strains. The susceptible enzymes were markedly less reactive with insecticides than the resistant enzymes and this was shown even at the crude homogenate level. The qualitative differences observed among the resistant populations indicate the

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presence of a greater number of amplified allelic forms for the esterase loci A_2, B_2 and B_1 than previously has been suggested.

The A_2 and B_2 esterases were partially purified from three sub-colonies selected with three different insecticides from a single parental colony. Significant intra-colony differences were observed in enzyme-insecticide interactions demonstrating the existence of different alleles of A_2 and B_2 within a single population.

Antiserum raised against PelRR A_2 esterase cross-reacted with both enzymes from the other strains although the reactivity of the B_2 enzyme was about 50-fold less than that of the A_2 esterase. Also the A_2 antiserum strongly cross-reacted with insect acetylcholinesterase, the target site of the insecticides. Esterase A_2 did not show any significant immunological relationship with the other resistance associated esterases tested from other insects. Some of the commercially available vertebrate carboxylesterases and cholinesterases also cross-reacted with the A_2 antiserum.

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Chapter 1

GENERAL INTRODUCTION

1.1. HISTORICAL BACKGROUND

Melander's observations of San Jose scale resistance to lime-sulphur in the Clarkston Valley of Washington in 1908, was the first documented arthropod resistance to pesticides (Melander, 1914). The number of reported cases increased gradually until the introduction of DDT in 1946, which resulted in the sudden appearance of a large number of new cases of resistance over a five year period. The subsequent wide scale use of new insecticides further increased the number of reported cases and by 1980 resistance had been detected in populations of at least 428 species representing 14 orders and 83 families of insects and acarines (Forgash, 1984). This rapid development of insecticide resistance was largely supported by the enormous reproductive capacity and genetic flexibility of insects.

Mosquitoes are vectors of many human diseases such as malaria, filariasis, dengue and encephalitis, as well as being a biting nuisance to man. The development of resistance by mosquitoes to pesticides was first observed in 1947, when the salt-marsh mosquitoes *Aedes taeniorhynchus* and *Ae. sollicitans* began to show resistance to DDT in Florida (Brown, 1986). By 1992, 56 species of

Anophiline and 46 species of Culicine mosquitoes worldwide, had been reported to be resistant to pesticides (WHO, 1992). An understanding of the mechanisms which underlie these resistances is of great importance for the development of rational strategies for the management of resistant populations. This understanding would also be helpful in designing new insecticides to use against resistant strains and to develop strategies to revert resistant strains back to their initial sensitivity levels.

1.2. INSECTICIDES AND THEIR TARGET SITES

Commonly used insecticides can be divided into four major groups; organochlorines, organophosphates (OPs), carbamates and pyrethroids (FIGURE 1.1). Today the use of organochlorines has been discontinued in many areas of the world because of resistance and concerns for the environment although DDT still remains as the most widely used insecticide for malaria control. All these insecticides attack the nervous system of the insect. For OPs and carbamates the target site is acetylcholinesterase (AChE), the enzyme which hydrolyses the neuro-transmitter acetylcholine. Cyclodienes, a sub-group of organochlorines, bind to the γ-aminobutyric acid (GABA) receptors in the Cl^- channels of the neurons and modulate the Cl^- conductance across the nerve membrane. The rest of the organochlorines (DDT + its analogues) and pyrethroids bind to Na^+ channel proteins of the neuron and inactivate its shut-down.

A) ORGANOCHLORINES

DDT

γ-HCH (a cyclodiene)

B) ORGANOPHOSPHATES

Chlorpyrifos

Malathion

Fenitrothion

Parathion

C) CARBAMATES

Propoxur

D) PYRETHROIDS

Lambda cyhalothrin
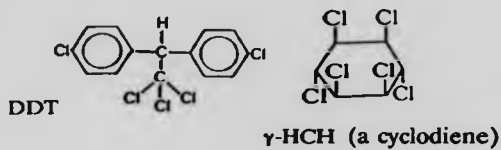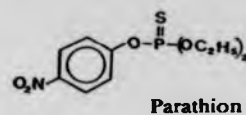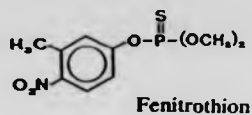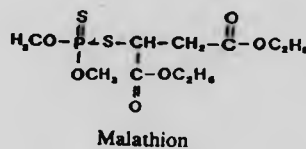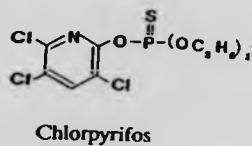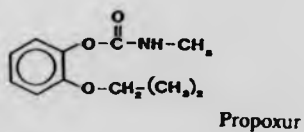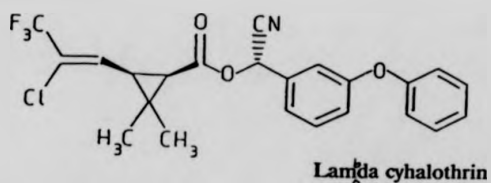
FIGURE 1.1. DIFFERENT TYPES OF INSECTICIDES

1.3. OTHER METHODS OF CONTROL

Chemical compounds targeting other areas of insect physiology have also been introduced although they are not in extensive use due to their expense and specificity of action. Juvenile hormone analogues (JHAs) and chitin synthesis inhibitors (CSIs) have specific actions against insects with very little effect on vertebrates. Sex and aggregation pheromone baited traps are used to attract the insects or to disrupt their mating behaviour. The effectiveness of these methods can be increased if they are used with insecticides (Edwards, 1993; Jones, 1993). Polystyrene beads, spread over the breeding sites of mosquitoes are also used in *Culex* and *Anopheles* control programmes (Curtis, 1993). Biological control of mosquitoes has also been tested for decades. The most promising results have been obtained from the bacteria *Bacillus thuringiensis* and *B. sphaericus* which produce proteins toxic to mosquito larvae (Becker and Rettich, 1993). Predatory fish species, dragon fly larvae, Copepods Mesocyclopes, Mermi thid nematodes and some species of Protozoa and fungi are also considered as possible control agents (Becker and Rettich, 1993; Curtis, 1993). Difficulties concerning the storage, transport and complex life cycles of these organisms remain as major drawbacks to their use. Among all these control methods the use of insecticides (this term will refer to organochlorines, OPs, carbamates and pyrethroids throughout this thesis unless otherwise stated) is the most effective and the most common method in insect control.

1.4. MECHANISMS OF INSECTICIDE RESISTANCE

Mechanisms developed by insects to bring about the resistance to insecticides can broadly be classified as follows:

1.4.1. BEHAVIORAL CHANGES

Developed to avoid or minimise the contact with and thereby the uptake of the insecticides. Insects may thus change their habits accordingly *eg.* indoor spray of DDT in Thailand changed the preferred habitat of *Anopheles minimus* from indoor to outdoor resting (Bang, 1985).

1.4.2. CUTICULAR RESISTANCE (REDUCED PENETRATION)

The uptake of the toxicant is reduced due to a thickening or a change in chemical composition of the insect cuticle. This has been observed in OP resistant strains of *Culex quinquefasciatus* (Stone and Brown, 1969) and *Cx tarsalis* (Apperson and Georghiou, 1975). Differences in insecticide transport across the cuticle was demonstrated in house flies by Golenda and Forgash (1989). It has been reported that cyclodiene resistance in red flour beetle *Tribolium castaneum* (Lin *et al.*, 1993), fenvalerate resistance in diamondback moth *Plutella xylostella* (Noppun *et al.*, 1989) and OP resistance in colorado potato beetle *Lepinotarsa decemlineata* (Argentine *et al.*, 1994) were partly due

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to reduced penetration.

1.4.3. TARGET SITE INSENSITIVITY

Changes in the insecticide target sites have developed, so that the toxicant molecules can not interact with them.

A) Knock-down Resistance (kdr)

Individuals with this mechanism are resistant to the rapid knock-down effect caused by the pyrethroid and DDT insecticides and are called 'kdr' type or super 'kdr' (highly resistant) type. Resistance is caused by reductions in the number and/or the affinity of the target site, Na^+ channel protein (Jackson *et al.*, 1984; Kasbekar and Hall, 1988; Pauron *et al.*, 1989). This mechanism has been observed in house flies (*Musca domestica*) (Sawicki, 1987; Rossignol, 1988; Grubs *et al.*, 1988), *Drosophila* (Jackson *et al.*, 1984; Ramaswami and Tanouye, 1989) *Ae. aegypti* (Hemingway *et al.*, 1989a) and German cockroaches (*Blattella germanica*) (Hemingway *et al*, 1993a).

B) Changes in GABA Receptors

Cyclodienes which constitute a large group of organochlorines, exert their action by binding to GABA receptors. Alteration of the structure of the target site

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results in decreased affinity to the insecticides. This has been demonstrated in several insect species including *Drosophila* (ffrench-Constant *et al.*, 1992; Steichen and ffrench-Constant, 1994), *Ae. aegypti* (Thompson *et al.*, 1993), *Blattella germanica* (Kadous *et al.*, 1983), *Tribolium castaneum* (Lin *et al.*, 1993) and *Musca domestica* (Anthony *et al.*, 1991).

C) Altered Acetylcholinesterases

This form of resistance is highly effective against carbamates and OPs and has been found among several strains of mosquito species; *Anopheles albimanus* from Central America (Georghiou and Pasteur, 1978; Hemingway *et al.*, 1984), *An. sacharovi* in Turkey (Hemingway *et al.*, 1992), *Cx quinquefasciatus* from Cuba (Bisset *et al.*, 1990; 1991; Rodriguez *et al.*, 1993) and Tanzania (Khayrandish and Wood, 1993a; 1993b), *Cx pipiens* from Italy (Villani and Hemingway, 1987; Bonning *et al.*, 1991) and *Cx tritaeniorhynchus* from Japan (Takahashi and Yasutomi, 1987). Selection of this mechanism in the field populations of *Culex* mosquitoes has been in combination with and preceded by the carboxylesterase mechanism (Villani and Hemingway, 1987; Rodriguez *et al.*, 1993). This is also common in other insects such as the tobacco bud worm *Heliothis virescens* (Brown and Bryson, 1992), the pear bug *Cacopsylla pyri* (Berrada *et al.*, 1994), *Lygus hesperus* (Hemiptera: Miridae) (Zhu and Brindly, 1992a; 1992b), citrus thrip *Scirtothrip citri* (Ferrai *et al.*, 1993), *Lepinotarsa decemlineata* (Argentine *et al.*, 1994) and *Blattella germanica* (Hemingway *et al.*, 1993b).

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1.4.4. METABOLIC RESISTANCE

This includes qualitative or quantitative changes in the enzymes, which metabolise or sequester the insecticides before they reach their target sites. There are three groups of such enzymes; glutathione-S-transferases, monooxygenases and carboxylesterases.

1.4.4.1. Glutathione-S-transferases (GSTs)

Resistance can occur due to an increased activity of GSTs. These enzymes principally catalyse O-dealkylation of OPs (phosphorothionates and their oxon analogues) (FIGURE 1.2) and the dehydrochlorination of DDT to DDE (Hayes and Wolf, 1988; Lamoureux and Rusness, 1989; Hassall, 1990).

This mechanism has been reported to be responsible for the OP and DDT resistance in house flies (Motoyama and Dauterman, 1978; Clark and Shamaan, 1984). GSTs have been shown as a possible mechanism for the insecticide resistance in german cockroaches (Hemingway *et al.*, 1993a; 1993b) and the mosquitoes *An. gambiae* (Hemingway *et al.*, 1985), *An. sacharovi* (Hemingway *et al.*, 1992) and *An. subpictus* (Hemingway *et al.*, 1991). In a DDT resistant *An. gambiae* strain, resistance has been shown to be conferred by qualitatively different GSTs that are present in higher concentrations in the resistant strain (Prapanthadara *et al.*, 1993).

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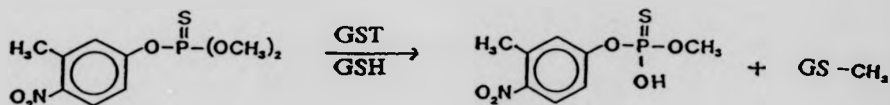
FIGURE 1.2 O-dealkylation of fenitrothion by GST

1.4.4.2. Oxidases

Detoxication of insecticides by increased activity of mixed function oxidases (mfo) or monooxygenases, due to changes in cytochrome P-450, the terminal oxidase of the microsomal electron transport system, is also found in insects. The most common type of reaction catalysed by this enzyme system is again O-dealkylation (Hassall, 1990). This is also a common mechanism for drug resistance in mammals (Wislocki *et al.*, 1980).

An mfo mediated mechanism has been shown to be responsible for organophosphate resistance in *An. subpictus* in Sri Lanka (Hemingway *et al.*, 1987) and resistance to both permethrin and pirimiphos-methyl in *Cx quinquefasciatus* from Saudi Arabia (Hemingway *et al.*,1990), to deltamethrin in the same species in West Africa (Magnin *et al.*,1988) and to permethrin in a

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Tanzanian strain (Khayrandish and Wood, 1993b). In houseflies oxidative degradation has been shown to be a major mechanism of insecticide resistance (Welling *et al.*, 1974; Scott and Lee, 1993). Chlorpyrifos resistance in several strains of *Blattella germanica* was also partly due to an oxidase mechanism (Hemingway *et al.*,1993a ;1993b).

Another important reaction catalysed by oxidases is the conversion of phosphorothionates to their oxon analogues. OPs are usually applied as thionates because of their low toxicity to humans and their relatively high solubility in lipids which enables them to penetrate the insect integument rapidly. Once inside the body, the formation of oxon analogues, which are highly toxic, is catalysed by 'mfo's (FIGURE 1.3). Malaoxon has shown approximately 2000 times greater anticholinesterase activity than malathion in *Cx tarsalis* (Matsumura and Brown, 1961). Antiserum raised against purified P-450 of housefly has inhibited the activation of chlorpyrifos to its oxon analogue in housefly microsomes (Hatano and Scott, 1993). The use of oxidase synergists such as piperonyl butoxide in bio-assays has increased the level of resistance to organophosphates due to the inhibition of the formation of highly toxic oxon analogues. An increase in resistance to OPs has been observed in *An. stephensi* (Hemingway, 1982) and in *Cx quinquefasciatus* (Magnin *et al.*, 1988; Khayrandish and Wood, 1993b) after pretreatment of the mosquito larvae with piperonyl butoxide.

$$H_3CO-\underset{\underset{OCH_3}{|}}{\overset{\overset{S}{\|}}{P}}-S-\underset{\underset{\underset{O}{\|}}{C-OC_2H_5}}{CH}-CH_2-\overset{\overset{O}{\|}}{C}-OC_2H_5 \longrightarrow H_3CO-\underset{\underset{OCH_3}{|}}{\overset{\overset{O}{\|}}{P}}-S-\underset{\underset{\underset{O}{\|}}{C-OC_2H_5}}{CH}-CH_2-\overset{\overset{O}{\|}}{C}-OC_2H_5$$

Malathion   **Malaoxon**
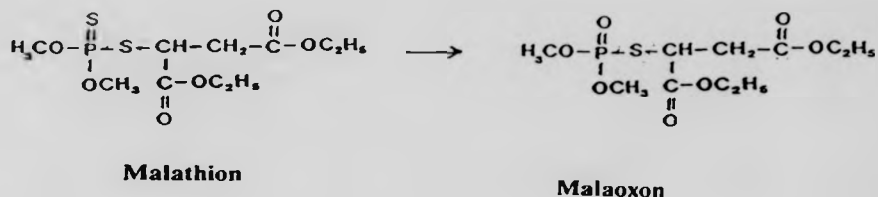
FIGURE 1.3. Formation of the oxon-analogue of malathion by oxidases

1.4.4.3. Carboxylesterases

Qualitative and/or quantitative changes in carboxylesterases can lead to increased levels of insecticide resistance. This mechanism is usually developed against OP and carbamates and common in mosquitoes, aphids and many other insect species (FIGURE 1.4).

A) *Qualitatively Different Carboxylesterases*

The existence of highly active variants can increase the resistance by increasing the rate of interaction with and/or hydrolysis of insecticides. This mechanism is present in some Anophiline mosquitoes, where the resistant individuals can hydrolyse the insecticides rapidly without any detectable increase in

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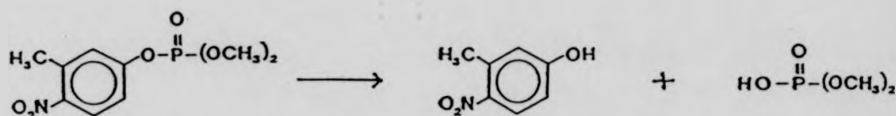
FIGURE 1.4. Hydrolysis of Fenitrooxon by carboxylesterases

carboxylesterase activity to general substrates when compared with susceptibles. *eg. An. arabiensis* from Sudan (Hemingway, 1983), *An. culicifacies* from India (Malcolm and Boddington, 1989) and *An. stephensi* from Pakistan (Hemingway, 1982).

B) *Quantitative Differences (Elevation) of Carboxylesterases*

A correlation between insect resistance to insecticides and increased esterase activity detected by enzyme assays or by native gel electrophoresis using general esterase substrates such as α- and β-naphthyl acetates, has been reported in the mosquito species *Cx quinquefasciatus* (Georghiou and Pasteur, 1978; Georghiou *et al.*, 1980; Hemingway and Georghiou, 1984; Raymond *et al.*, 1987; Magnin *et al.*, 1988; Bisset *et al.*, 1990; 1991; Hemingway *et al.*, 1990; Peiris and Hemingway, 1990b; Wirth *et al.*, 1990), *Cx pipiens* (de Stordeur, 1976; Pasteur *et*

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al., 1981a; 1981b; Villani *et al.*, 1983; Maruyama *et al.*, 1984; Fournier *et al.*,1987; Villani and Hemingway, 1987), *Cx tarsalis* (Matsumura and Brown, 1961; Apperson and Georghiou, 1975; Prabhakar *et al.*, 1987), *Cx tritaeniorhynchus* (Takahashi and Yasutomi, 1987), *Ae. aegypti* (Field *et al.*, 1984; Mourya *et al.*, 1993); the peach-potato aphid *Myzus persicae* (Devonshire, 1977); the black fly *Simulium damnosum* (Diptera: Simuliidae) (Hemingway *et al.*, 1989b), the housefly *Musca domestica* (Kao *et al.*, 1985a; 1985b), the two-spotted spider-mite *Tetranychus urticae* (Matsumura and Voss, 1964), *Lygus hesperus* (Zhu and Brindley, 1992a), the brown plant-hopper *Nilaparvata lugens* (Chen and Sun, 1994), the tobacco white fly *Bemsia tabaci* (Byrne and Devonshire, 1991; 1993), *Scirtothrips citri* (Ferrari *et al.*, 1993) and *Blattella germanica* (Prabhakaran and Kamble, 1993; Hemingway *et al.*, 1993a; 1993b). Involvement of the esterases in the resistance can be verified by synergistic studies using carboxylesterase inhibitors such as DEF (*S,S,S*- tributyl phosphorothioate), TPP (triphenyl phosphate) and IBP (*S*-benzyl *O,O*-diisopropyl phosphorothionate) (Apperson and Georghiou, 1975; Georghiou and Pasteur, 1978; Hemingway, 1982; 1983; Hemingway and Georghiou, 1984; Magnin *et al.*, 1988; Hemingway *et al.*, 1989b; Bisset *et al.*, 1990; Wirth *et al.*, 1990).

1.5. CROSS-RESISTANCE AND MULTIPLE RESISTANCE

Resistance is usually high to the insecticide which induced it, with some cross

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resistance to other insecticides. The range of resistance can be very narrow (*eg*. to a few insecticides within a pesticide group) or broad, encompassing several pesticide groups. This can result in resistance to a range of insecticides to which the insect has not been previously exposed. Where more than one mechanism of insecticide resistance operates in a single insect, it is termed multiple resistance. The contribution of each mechanism to the resistance varies according to the type of insecticide.

1.6. STAGE SPECIFICITY OF RESISTANCE

Generally the selection of larvae with an insecticide selects the resistance in the adult as well and the converse is also true. But stage specificity has been reported in some cases. Exposure of adult *An. arabiensis* to malathion for three years in Sudan resulted in adult, but not larval, malathion resistance (Hemingway, 1983). A decrease in the adult resistance to malathion with age has been observed in *An. stephensi* (Rowland and Hemingway, 1987). Adults of *Cx quinquefasciatus* were less susceptible than larvae after a larval selection with OPs (Amin and White, 1985). Differences in the esterase bands in native gel electrophoresis between larvae and adults have been observed in a field population of *An. subpictus* (Hemingway *et al.*, 1987). Maruyama *et al.* (1984) reported that there is only one esterase band in the eggs of *Cx pipiens* with the gradual appearance of many bands during larval development followed by the loss of most anodal bands on pupation. However, the correlation of these stage

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specific esterases with the insecticide resistance has not been investigated.

1.7. FITNESS OF THE RESISTANT INSECTS

Generally the diurnal activities of resistant individuals are the same as the susceptible individuals. This has been shown with *Cx quinquefasciatus* strains (Amin, 1983). However, depending on the type and the extent of the resistance mechanism, differences in the fitness may be observed. In *An. gambiae*, the selection of resistance to γHCH/dieldrin resulted in a reduction in fecundity, activity and competitiveness at mating (Rowland, 1988). A resistant strain of *Drosophila melanogaster* also has been shown to be more sensitive to higher temperatures than susceptibles (ffrench-Constant *et al.*, 1993a). Increased fitness has been shown for malathion resistant *Tribolium castaneum* where the resistance mechanism is thought to be carboxylesterase-based (Beeman and Nanis, 1986).

1.8. ESTERASES

1.8.1. CLASSIFICATION

"Carboxylesterase" or "esterase" is usually a collective term for the molecules which have a hydrolytic action on carboxylic esters. The term carboxylesterase covers a wide variety of enzymes and most of them are non-specific esterases. Phosphoric acid esters such as OPs can also be hydrolysed by some of these

enzymes. The generally accepted nomenclature of the International Union of Biochemistry (I.U.B.) is not well suited for a carboxylesterase unless its physiological role is known (Heymann, 1980). Moreover, overlapping substrate specificities make individual classification difficult. An esterase classification introduced by Aldridge (1953a, 1953b) remains valuable. According to this, esterases can be divided into two major groups; 'A' esterases and 'B' esterases depending on their interactions with paraoxon (later extended to other OPs as well). Those inhibited by OP compounds in a progressive and temperature dependent reaction are called 'B' esterases and those which are not inhibited (hydrolysis of OPs is not an obligatory requirement) are called 'A' esterases (Aldridge, 1993). 'A' esterases hydrolyse OPs possibly through an acylated cysteine in the active site. Their activity requires metal ions and is sensitive to inhibition by metallic salts which have affinity to S-H groups (Aldridge, 1993). The 'A' esterase classification has recently been revised. They are now grouped under phosphoric triester hydrolases (EC 3.1.8) (Reiner, 1993; Walker, 1993). The term 'carboxylesterase' (EC 3.1.1.1) is now mainly attributed to B-esterases (Reiner, 1993; Walker, 1993). The active site of all B-esterases has a serine residue which reacts with the OP. Therefore, the terms 'B esterases' and 'serine hydrolases' are synonyms. The physiological functions of most of these esterases are unknown and therefore they are called non-specific-esterases, although their role is thought mainly to be the detoxication of xenobiotics.

Apart from these major categories of classification, different nomenclatures have

been developed to describe the different types of esterases present in a particular species or a group of closely related species. Mentlein *et al.* (1984; 1985a) worked on rat liver microsome esterases and proposed that individual esterase should be listed according to their most prominent natural substrate. *eg.* monoacylglycerol lipase, palmitoyl-CoA hydrolase, palmitoyl-carnitine hydrolase *etc.* However, mobility differences shown in the native electrophoresis is the most commonly used characteristic in this type of classification. *eg.* mouse and rat esterases ES-1, ES-2 *etc.* (Peters, 1982; Simon *et al.*, 1985), *Drosophila* esterases Est.2, Est.5 *etc.* (Zorus *et al.*, 1982), German cockroach esterases E-1, E-2 *etc.* (Prabhakaran and Kamble, 1993). In mosquitoes the esterase nomenclature has been developed on the basis of their electrophoretic mobility and their preference for hydrolysing the synthetic esters α- and β-naphthyl acetate (Georghiou and Pasteur, 1978; Raymond *et al.*, 1987). An esterase which preferentially hydrolyses α-naphthyl esters is classified as an "esterase A" and that with a preference for β-naphthyl esters as an "esterase B". Subscripts 1,2 *etc.* were used after "A" or "B" to indicate the order, starting from the slowest running esterase. Unless stated otherwise, this classification will be used in this report for mosquito carboxylesterases. However, to date, all insect esterases, found to be involved in OP resistance are B-type esterases according to Aldridge's classification and belong to carboxylesterases (EC 3.1.1.1). Four major esterase types are very common and have been observed in natural populations of the *Cx. pipiens* complex; A_1 in Southern France and Italy (Pasteur *et al.*, 1981b; Severini *et al.*, 1993), B_1 mainly in North America, Cuba and China (Georghiou and

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Pasteur, 1978; Bisset *et al.*, 1990; Qiao and Raymond, submitted) and the most common A_2 and B_2, always associated together, in Africa, North America, Europe, the Middle East and Asia (Villani *et al.*, 1983; Raymond *et al.*, 1987; Hemingway *et al.*, 1990). However B_4 and B_5 (associated with A_4 and A_5 respectively) recently described by Poirie *et al.* (1992) from Southern France and Mediterranean countries are not compatible with this nomenclature.

1.8.2. LOCALIZATION

In cell fractionation experiments, carboxylesterases have been predominantly found in the microsomal and mitochondrial fractions, showing their intracellular location (Matsumura and Brown, 1961; Heymann, 1980; Chen and Sun, 1994). In rat liver, the presence of xenobiotic metabolising carboxylesterases has been shown in the cytosolic fraction (McCracken *et al.*, 1993). Parenchymal cells have shown the highest carboxylesterase activity in a study of the differential distribution of carboxylesterases among the cell types of rat liver (Gaustand *et al.*, 1992). In *Drosophila*, Est-5 has appeared at high concentration in the haemolymph and fat body (Zorus *et al.*, 1982) while Est-6 of adult males was concentrated in the anterior ejaculatory duct (Sheehan *et al.*, 1979; Karotam and Oakshott, 1993). Increased esterase activity has also been found in the larval body wall and digestive tract of *Drosophila* (Healy *et al.*, 1991). Gut tissues of *Cx tarsalis* have shown an increased carboxylesterase activity when compared with head, thorax and the rest of abdomen (Matsumura and Brown, 1963). However,

currently no comprehensive study has been performed to show the exact distribution of carboxylesterases in the insect body.

1.8.3. GENETICS, IMMUNOLOGY AND MOLECULAR BIOLOGY OF ESTERASES

1.8.3.1. Genetics

Insect resistance to insecticides is an inherited characteristic. Formal genetic studies of mosquitoes have been based on the polymorphism of adult or larval esterases shown by the native gel electrophoresis. Highly active esterase forms are co-dominant and there have been no hybrid forms in heterozygotes (de Stordeur, 1976). Many investigators have considered that different elevated esterases are governed by different genes. Inheritance and linkage relationships have been analyzed from the esterase banding patterns or esterase activity of the progeny which resulted from mass crosses between resistant and susceptible strains followed by backcrosses to the susceptible parents (de Stordeur, 1976; Georghiou *et al.*, 1980; Pasteur *et al.*, 1981a; 1981b; Villani *et al.*, 1983; Prabhaker *et al.*, 1987; Takahashi and Yasutomi, 1987; Peiris and Hemingway, 1993). Resistance is not sex-linked as detected by the reciprocal crossings (de Stordeur, 1976; Georghiou *et al.*, 1980, Peiris and Hemingway, 1993). In *Cx quinquefasciatus* a minor maternal effect on resistance has been shown with back-crosses involving resistant (F_1) females, giving consistantly and significantly lower

mortalities than those involving resistant (F_1) males (Peiris and Hemingway, 1993). Mosquitoes have three pairs of chromosomes (Besansky *et al.*, 1992). The pattern of inheritance with known recessive morphological mutations, belonging to each linkage group, has indicated that the resistant gene(s) are on linkage group (chromosome) II for *Cx tritaeniorhynchus* (Takahashi and Yasutomi, 1987) and chromosome III for the *Cx pipiens* complex (Pasteur *et al.*, 1981a). Pasteur *et al.* (1981a; 1981b) attributed the 'A' and 'B' type esterases to two closely linked gene loci Est-3 and Est-2 respectively. Wirth *et al.* (1990) showed that genes for the esterases B_2, A_2 and B_1 in *Cx quinquefasciatus* are located 2.9 and 3.9 centimorgans apart respectively in a separate linkage group from A_1. Esterases A_4 and B_4, recently described for a *Cx. quinquefasciatus* strain from France, have been shown to be under the control of two distinct loci on the same chromosome at approximately 0.8 centimorgans apart. Using restriction maps of esterase 'B' regions, an unrooted consensus tree has been constructed describing the genetic divergence of six esterase 'B' haplotypes (Poirie *et al.*, 1992) (FIGURE 1.5).

In vertebrates, inheritance and the linkage relationships of esterase genes have been well established. Polymorphism is a common phenomenon and most of the gene loci have more than two alleles (TABLE 1.1). In mouse, *Mus musculus*, most of the esterase genes are found on chromosome 8 and arranged in two clusters 6.9 centimorgans apart. Gene loci within a cluster are very closely arranged and code for esterases which are more similar to each other than to

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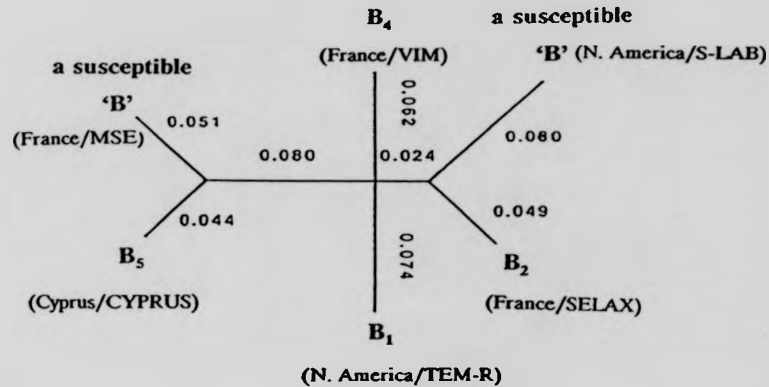
FIGURE 1.5. Proposed unrooted evolutionary tree describing the genetic divergence of six esterase 'B' haplotypes (numbers are estimates of nucleotide substitutions along each segment). Country and the strain are shown in parentheses. Adopted from Poirie et al. (1992).

those coded by the loci of the other cluster. Orthologous chromosome regions have been identified in other mammals including rat (Linkage group V) and rabbit (linkage group VI) (FIGURE 1.6) (Peters, 1982; Hedrich and Deimling, 1987; Zutphen *et al.*, 1987). Recently the mouse Es-29 was shown to be under the control of two independent genes, a structural gene (linked to cluster 2 on chromosome 8) and a modifying/regulatory gene (on chromosome 12) (Deimling and Gaa, 1992).

ESTERASE GENE	CHROMOSOME	NO. OF ALLELES
Es-1	8	5
Es-2	8	7
Es-3	11	6
Es-5	8	2
Es-6	8	3
Es-7	8	4?
Es-8	7	2
Es-9	8	4?
Es-10	14	3
Es-11	8	2
Es-12	?	2
Es-13	9	2
Es-14	9	2
Es-15	?	3
Es-16	3	3
Es-17	9	2
Es-20	8	4
Es-22	8	7
Es-23	8	5
Es-24	8	4
Es-25	12	2
Es-29	8	3

TABLE 1.1. Location and observed number of alleles for mouse esterases.
Compiled from Peters *et al.*, 1982; Deimling *et al.*, 1983; Eisenhardt and
Deimling, 1983; Deimling, 1984; Berning *et al.*, 1985; Medda *et al.*, 1986;
Deimling and Taylor, 1987; Deimling and Gaa, 1992.
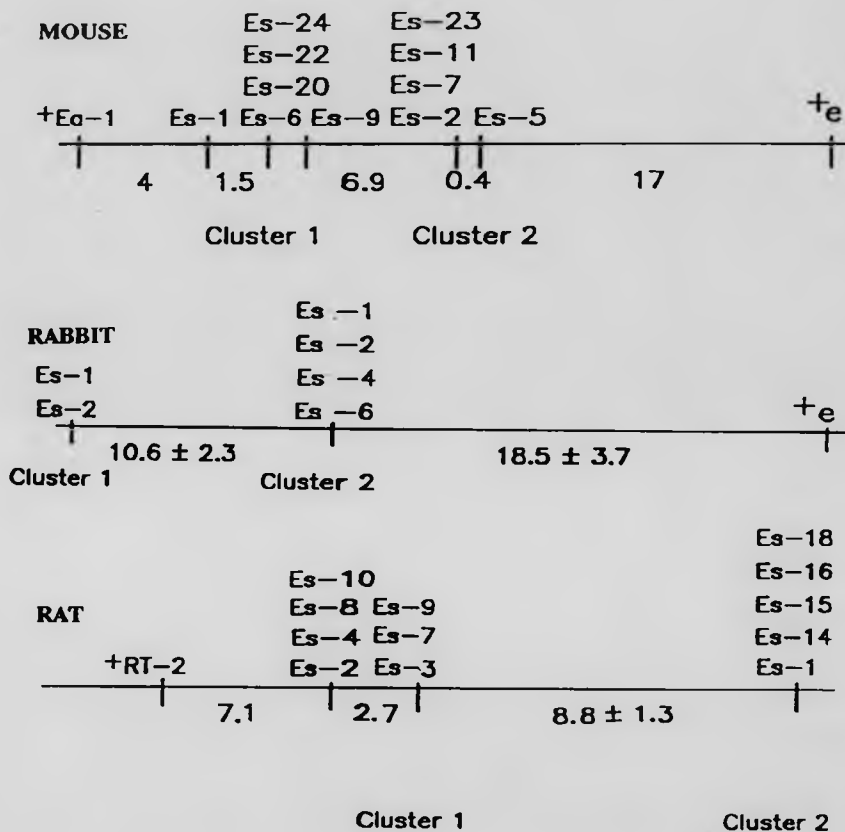
FIGURE 1.6. Orthologous chromosome regions of the mouse, the rat and the rabbit. *RT, *Ea-1: two blood group genes, *e: a coat colour gene

Compiled from Peters, 1982; Ronai *et al.*, 1985; Hedrich and Deimling, 1987; Zutphen *et al.*, 1987 and Deimling and Gaa, 1992.

1.8.3.2. Immunology

Recent immunological and molecular biological studies clearly show that the amplification of esterase genes, which allows an increased production of different detoxifying esterases, is a common mechanism for organophosphate resistance in insects. Reactions of antisera, raised against esterase B_1 of OP resistant *Cx quinquefasciatus* (TEM-R) and A_1 of OP resistant *Cx pipiens* (S54), with immunoblots of various strains have shown that esterase B_1 and A_1 are overproduced in these strains by factors of at least 500-fold and 70-fold respectively as compared with the corresponding susceptible strains. B_1 antiserum was found to cross-react with other types of B esterases but not with type A esterases (Mouches *et al.*, 1987). Similarly, A_1 antiserum reacted with other types of A esterases but not with type B esterases. Furthermore, proteins immunologically related to type B esterases could be detected in *Cx tarsalis, An. albimanus, An. stephensi, Ae. aegypti, Myzus persicae* and *Musca domestica* strains (Mouches *et al.*, 1987; Beyssat-Arnaouty *et al.*, 1989). Antiserum, raised against E4 esterase of peach potato aphid *Myzus persicae*, was used to show the presence of increased amounts of these esterases in crude homogenates of resistant strains compared to those of susceptibles. This antiserum cross-reacted with the closely related variant of E4, FE4, and esterases of *Phorodon humuli* (Hemiptera: Aphidae), showing the homology among these enzymes (Devonshire *et al.*, 1986a). In the house mouse, antisera raised against cluster 1 esterases cross-react with all the esterases of cluster 1 but not with those of cluster 2 and vice versa.

Using the immunological and biochemical characteristics, a phylogenic tree has been constructed to show the evolution of these two clusters each forming a multigene family (FIGURE 1.7) (Ronai *et al.*, 1985). It was proposed that the multiplicity of the carboxylesterase isozymes of the house mouse is the result of repeated duplication of an ancestral gene.
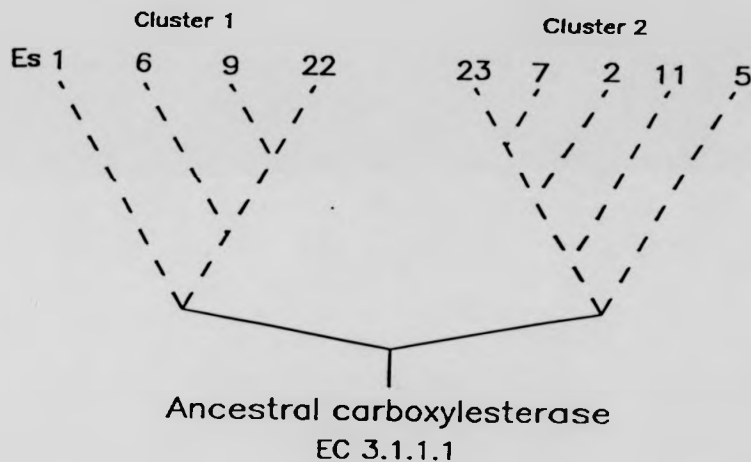
FIGURE 1.7. Evolutionary tree representing possible divergence of mouse carboxylesterase isozymes. Figure from Ronai et al. (1985).

1.8.3.3. Molecular Biology

Many studies on cultured mammalian cells have shown the development of resistance to toxins by amplification of genes which are responsible for

detoxication mechanisms (Devonshire and Field, 1991). This has also been observed in the insects, including mosquitoes. A partial cDNA, which could be used to synthesise a protein that reacts with B_1 antiserum, was isolated from the TEM-R strain. This was used as a probe to detect gene copy number of B_1 in different strains. Adults of the resistant strains with esterase B_1 were found to possess up to 250 times more copies of the gene than the adults of the susceptible strain (Mouches *et al.*, 1986). Utilizing a partial cDNA of the same gene Raymond *et al.* (1989) showed that gene amplification is also common in natural populations of *Cx quinquefasciatus*. A probe prepared from the partial B_1 DNA hybridized with other 'B' type esterase genes of field collected mosquito larvae and adults, demonstrating different levels of amplifications of 'B' type genes in field populations (Raymond *et al.*, 1989). In contrast 'A' type genes did not hybridized with this B_1 probe confirming the immunological findings that there is a high level of homology among type B esterases but not between B and A esterases (Mouches *et al.*, 1987).

Later, the gene coding for esterase B_1 of the TEM-R strain was sequenced and the structure of the amplified unit in the mosquito genome, (the amplicon) which includes the structural gene, was partially characterised (Mouches *et al.*, 1990). Each amplicon was found to contain a highly conserved 25 kilobase "core" sequence within a sequence of at least 30 kilobases and was present in a large number of copies in the genome of resistant mosquitoes but not in susceptible mosquitoes. The esterase B_1 gene was 2773 base pairs long with three introns

and the deduced amino acid sequence of the enzyme contained 540 residues. The active site polypeptide sequence was similar to those of eukaryotic serine esterases. In addition, esterase B_1 contained regions strongly similar to human butyrylcholinesterase, *Torpedo* AChE and Est-6 and AChE from *Drosophila melanogaster* (Mouches *et al.*, 1990).

Raymond *et al.* (1991) cloned and sequenced a *HindIII/BamHI* fragment of the esterase B_2 gene and compared this with the homologous B_1 fragment. The two fragments showed 96% nucleotide sequence homology, and 97% homology in the predicted amino acid sequence. Using restriction fragment length polymorphism (RFLP) and partial sequencing, they have also shown that structural genes of electrophoretically dissimilar type B esterases were similar whereas their flanking regions varied considerably. However, the flanking sequences of esterase B_2 from different geographical locations (Africa, Asia and North America) were identical. Therefore it was suggested that amplified esterase B_2 genes originated from an initial event, and by migration this type of OP insecticide resistance has subsequently spread throughout the world (Raymond *et al.*, 1991). Later Poirie *et al.* (1992) found two 'B' type esterases (B_4 and B_5) with RFLP patterns of the flanking regions different from each other and from those of B_1 and B_2 and thus argued that amplification of esterase 'B' locus has occurred at least 4 times. Using the restriction maps built with three restriction enzymes and with male genitalia as morphological markers to discriminate tropical, temperate and hybrid forms of the *Cx pipiens* complex of mosquitoes, Rivert *et al.* (1993) have

shown the recent migration of A_2 and B_2 esterases into France. However, the possibility of co-evolution can not be overlooked when the patchy distributions and different insecticide cross-resistance spectra shown by the resistant populations are considered.

Chromosomal organization of the B_1 amplified gene has been studied by *in situ* hybridization using a labelled esterase B_1 gene probe. Esterase B_1 gene copies were found to be clustered in the intermediate region (between the centromere and the apex) of the chromosome II, in a tandem arrangement making it a little longer in resistant types (Nance *et al.*, 1990). Wirth *et al.* (1990), after observing the pattern of inheritance and linkage relationships of these esterases, stated that these amplified genes may be inherited as clusters showing a monofactorial inheritance. Tandem gene duplication of *Drosophila* Est-6 (on chromosome 3) has also been demonstrated by *in situ* hybridization experiments using a 1.8 kb cDNA probe of the Est-6 gene (Procunier *et al.*, 1991).

Some progeny of a cross between OP resistant TEM-R and a susceptible strain showed significantly higher esterase activity than the mean esterase activity of their parents suggesting an enhanced expression of the amplified B_1 genes when they are present in only one of the two homologous chromosome pair (Ferrari and Georghiou, 1990). The same phenomenon has also been shown with Sri Lankan *Cx quinquefasciatus* by Peiris and Hemingway (1993).

Apart from the above studies on mosquito esterases intensive molecular biological studies have been carried out on the esterase E4 and its closely related variant FE4 in *Myzus persicae*. Again gene amplification is responsible for the increased production of these esterases and the degree of amplification is well correlated with the level of resistance and the amount of esterase activity in different strains (Field *et al.*, 1988). Increased amounts of mRNA coding for these esterases has been shown for resistant strains using the immunologically precipitated products of their *in vitro* translation. No such protein has been detected in the experiments done with susceptible aphids (Devonshire *et al.*, 1986b). The amplified E4 related sequences were highly methylated at restriction enzyme *MSP1* sites in all resistant strains but not in those where resistance had reverted. This suggested that the subsequent loss of methylation had resulted in the loss of transcriptional expression of these genes, giving an apparently susceptible phenotype (Field *et al.*, 1989). Recently, Field *et al.* (1993) have cloned and fully characterised the genes of E4 and FE4 esterases. The open reading frame of E4 cDNA was 1656 nucleotides long coding 552 amino acids. The gene was 4.3 kb long and the coding region was interrupted by six introns. Its closely related variant FE4 gene had a 99% identity over this region with a single nucleotide substitution which resulted in the FE4 mRNA having an extra 36 nucleotides at the 3' end. The deduced amino acid sequence shows the possibility that the activity of the native protein involves a charge-relay system with a catalytic triad (serine, histidine and glutamic acid) in the active site as in AChE. Strong similarities have been shown between the amino acid sequence

surrounding the active site of E4 and that of the other serine hydrolases, including mammalian AChEs and carboxylesterases, mosquito esterase B_1, *Drosophila* Est-6 and the juvenile hormone esterase from *Heliothis virescens* (Field *et al.*, 1993).

1.8.4. PHYSICOCHEMICAL PROPERTIES

1.8.4.1. Physical Properties

Liver, kidney and intestinal carboxylesterases of mammals generally consist of units of molecular weight of about 60 kDa. The majority of these are monomers which tend to associate at higher concentrations, while a few form stable trimers (Heymann, 1980). Native molecular weights of several esterases found on mouse chromosome 8 were 45-55 kDa (Peters, 1982) although that of 130 kDa has been reported recently for ES-29 (Deimling and Gaa, 1992). Recently Alexon *et al.* (1993) also have shown a monomeric molecular weight of 59 kDa for a rat liver microsomal esterase.

Molecular weights of purified mosquito esterases A_2 and B_2 were 67 and 60 kDa respectively and both were monomers in their native forms (Ketterman *et al.*, 1992; Jayawardana, 1992). Merryweather *et al.* (1990) reported a mosquito carboxylesterase of 62 kDa. A monomeric structure of 67 kDa and a homodimeric structure of 118-134 kDa have been reported for partially purified

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B_1 and A_1 (Fournier *et al.*, 1987). Although the dimeric structure for A_1 here contrasts with those of other reported esterases, it may be due to temporary associations between monomers. However, a molecular weight of 16 kDa, shown for a partially purified mosquito carboxylesterase by Matsumura and Brown (1963), is an exception. Estimated molecular weights for the esterases of other insects also fall into the above general range; 65 and 66 kDa for esterase E4 and FE4 respectively in *Myzus persicae* (Devonshire *et al.*, 1986b), 65 kDa for esterase-C in *Drosophila melanogaster* (Holwerda and Morton, 1983) and 62-64 kDa for esterases E_1, E_2 and E_3 in *Nilaparvata lugens* (Chen and Sun, 1994). Again an exceptionally higher molecular weight range of about 220 kDa has been reported for partially purified housefly esterases (Kao *et al.*, 1985a).

The iso-electric point (pI) of B-type carboxylesterases (according to Alridge's classification) is usually in the range of pH 4.7-6.5 (Heymann, 1980). Two purified human liver carboxylesterases have shown pI values of 5.2-5.8 and 4.2-4.8 (Ketterman *et al.*, 1989) while a pI range of 4-7 has been observed in mouse, rat and rabbit liver carboxylesterases (Kao *et al.*, 1985b, Simon *et al.*, 1985). Mentlein *et al.* (1984; 1985a; 1985b) reported a pI range of 5.2 - 6.4 for several rat liver microsomal carboxylesterases. In insects, 5.1 and 5.3 pI values have been reported for two active esterases in insecticide resistant houseflies (Kao *et al.*, 1985a). Carboxylesterases E_1, E_2 and E_3 in *Nilaparvata lugens* have shown pI values of 4.7 - 4.9 (Chen and Sun, 1994). Twenty two soluble esterases of *Drosophila melanogaster* have shown a pI range of 3.8 - 6.2 (Healy *et al.*, 1991).

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Reported pI values of mosquito carboxylesterases are similar to those in other organisms, being 5.2 for A_2 (Ketterman *et al.*, 1992), 5.1 for B_2 (Jayawardena, 1992) and between 5-6 for A_1 and B_1 (Fournier *et al.*, 1987).

The glycosylated nature of esterase E4 of *Myzus persicae* has been shown by its high affinity for lectin (con-A sepharose) and by non-denaturing electrophoresis gels, stained for sugars. Differences in glycosylation may be responsible for about 6 - 8 kDa difference between the native esterases, E4 and FE4, and their respective nascent forms, shown by both *in-vitro* translation of mRNA experiments (Devonshire *et al.*, 1986b) and deduced amino acid sequences (Field *et al.*, 1993). Five and three possible glycosylation sites have been shown for esterases E4/FE4 and B_1 amino acid sequences respectively (Field *et al.*, 1993, Mouches *et al.*, 1990). The predicted molecular weight of esterase B_1 was 59,000 kDa which is 8 kDa less than its native form (Fournier *et al.*, 1987; Mouches *et al.*, 1990).

1.8.4.2. Chemical Properties

The complex involvement of carboxylesterases in detoxication in the mammalian liver has been well studied. In insects, most of the earlier work was reviewed by Ahmad and Forgash (1976).

The majority of serine hydrolases contain a serine-histidine-glutamic acid

catalytic triad, but some (*eg.* cholesterol esterase) have aspartic acid as the acidic member of the triad (Cygler *et al.*, 1993). Carbamate and many OP insecticides inhibit B-type carboxylesterases (according to Aldridge's classification) by rapid esterification of the serine residue in the active site. This reaction is often followed by a slow hydrolysis of the new ester bond. Therefore considering the overall reaction, these insecticides are only inhibitors because they are such poor substrates for these esterases.

The generally accepted reaction mechanism is;

$$E \; + \; I \; \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \; EI \; \overset{k_2}{\rightarrow} \; EI' \; + \; P_1 \; \underset{H_2O}{\overset{k_3}{\rightarrow}} \; E \; + \; P_2$$

or;

$$E \; + \; I \; \overset{k_a}{\rightarrow} \; EI' \; + \; P_1 \; \underset{H_2O}{\overset{k_3}{\rightarrow}} \; E \; + \; P_2$$
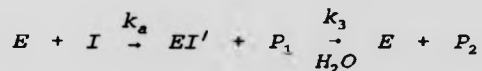
where, E is the enzyme, I the inhibitor, EI Michaelis complex, EI' acylated enzyme, P_1 first product (alcohol) and P_2 second product (acid).

It is believed that the formation of the Michaelis complex (k_1) is probably diffusion controlled and the fastest of all reactions making it impossible to

measure (Aldridge and Reiner 1972). For substrates, the whole reaction is very fast so that free enzyme is regenerated and the substrate is rapidly hydrolysed. However it is not conclusive that substrates go through an acylated esterase intermediate step because the intermediate has not been isolated (Aldridge 1993). For inhibitors, the acylated enzyme is formed very quickly (k_a is very high) but it is either stable or its rate of hydrolysis (k_3) is very slow becoming a rate limiting step. Hence carboxylesterases have a very high affinity for the OPs and carbamates with a very low capacity because of the irreversible 1 : 1 stoichiometry of the reaction in the active site. In the presence of large amounts of these enzymes the insecticides are rapidly sequestered before they reach their targets. This is the basis for the resistance mechanism with increased amounts of carboxylesterases.

Kinetic constants, showing the rates of acylation and the affinities of binding of the purified mosquito carboxylesterase A_2, have been reported for four insecticides (three OPs and one carbamate), supporting its role in sequestration (Ketterman *et al.*, 1992). Slow turnover of the insecticides, determined by the kinetic constant k_3, has been shown for both purified esterases A_2 (Ketterman *et al.*, 1992) and E4 of *Myzus persicae* (Devonshire, 1977; Devonshire and Moores, 1982).

Kinetic constants for hydrolysis of various xenobiotic substrates by esterases, have been investigated by many workers using crude homogenates of insects.

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Cuany *et al.* (1993) have reported I_{50} values of several insecticides for TEM-R crude homogenate of *Cx quinquefasciatus*. Studies with purified esterases have been reported for mosquito esterase A_2 (Ketterman *et al.*, 1992), peach-potato aphid esterase E4 (Devonshire, 1977) and housefly esterases (Kao *et al.*, 1985a). The purified esterases E4, from both resistant and susceptible strains, have shown similar kinetic constants towards paraoxon and α-naphthyl acetate, indicating, that the enzyme from both strains are biochemically similar and the difference between the strains must be caused by different amounts of the same enzyme resulting from amplification rather than a change in the structural gene (Devonshire, 1977). In contrast, qualitative differences for the susceptible and resistant esterases of pI 5.1 have been shown in house fly strains (Kao *et al.*, 1985a). I_{50} values for several insecticides have been reported recently for purified E_1, E_2 and E_3 carboxylesterases of *Nilaparvata lugens* (Chen and Sun, 1994).

Extensive biochemical work has been performed to determine the possible physiological functions of different rat liver microsomal esterases (Mentlein *et al.*, 1984; 1985a; 1985b). Ketterman *et al.* (1992) also attempted to investigate the role of A_2 on possible physiological substrates. The mosquito A_2 carboxylesterase showed acylglycerol lipase activity, which has also been reported for vertebrate carboxylesterases. It was suggested that this A_2 enzyme, like the mammalian carboxylesterases, may be involved in lipid-fatty acid metabolism in addition to its role in detoxication.

1.9. BACKGROUND AND AIMS OF THE PRESENT STUDY

1.9.1. BACKGROUND OF THE STUDY

The use of pesticides, both directly and indirectly, against the mosquito Cx *quinquefasciatus* has resulted in the selection of the elevated esterase-based mechanism in populations throughout the world. The majority of these involve the co-elevation of two esterases, A_2 and B_2. The overproduction of 'B' esterases has been shown to be due to gene amplification (Mouches *et al.*, 1986; 1990; Raymond *et al.*, 1989). On the basis of identical restriction digest patterns of the B_2 amplicon from the resistant strains of Cx *quinquefasciatus* from major ports, Raymond *et al.* (1991) have put forward the hypothesis that this resistance mechanism has occurred only once, and spread worldwide by a recent migration. According to this hypothesis all the amplified A_2 and B_2 esterases would be the same. To date, no work has been performed to test this hypothesis using the purified enzymes.

1.7.2 AIMS OF THE STUDY

The ultimate goals of this study are;

1) Purification and characterization of the elevated mosquito esterases A_2 and B_2 to reveal their properties.

2) To establish the role of these esterases in the insecticide resistance.

3) To investigate whether or not the amplified enzymes in different strains (which originate from different geographical regions of the world) are functionally identical, using the purified enzymes. If there are multiple variants, the possibility of the presence of more than one form within a single population will also be examined.

4) To establish the identity of the susceptible non-amplified esterases.

CHAPTER 2

PURIFICATION AND CHARACTERIZATION OF THE CARBOXYLESTERASES A_2 AND B_2 FROM PELRR STRAIN.

2.1. INTRODUCTION

To determine the role of the carboxylesterases which are responsible for the insecticide resistance in the mosquito *Cx quinquefasciatus*, the PelRR strain was used as a model enzyme source. The 'Pel' original colony was collected in 1984 from Peliyagoda, Sri Lanka and was heterogenous for OP resistance. The major resistance mechanism was the increased activity of carboxylesterases A_2 and B_2. The PelRR strain was selected from the 'Pel' colony with temephos for 13 consecutive generations and was 29-fold more resistant to the selecting insecticide than the susceptible PelSS strain which was also derived from the 'Pel' colony by single family selection for low esterase activity. The basic biochemistry and the genetics of OP resistance of the PelRR strain had already been studied and the increased activity of esterases A_2 and B_2 was the only resistance mechanism detected (Peiris and Hemingway, 1990a; 1990b). Later, the methodologies were developed to purify both A_2 and B_2 enzymes and esterase A_2 was characterized (Ketterman *et al.*, 1992; Jayawardena, 1992).

At the start of this project, purified enzymes could no longer be obtained using

the published purification methods. To characterize the properties and to define the role in insecticide resistance it was important to obtain the enzymes pure. This chapter details the re-development of the purification methodologies for both enzymes and the characterization of the B_2 enzyme from the PelRR strain. Most of the characterization of esterase A_2 had already been done previously (Ketterman *et al.*, 1992) and this was completed by the other members of the research group from enzyme purified as part of this project.

2.2. MATERIALS AND METHODS

2.2.1. MOSQUITO COLONY MAINTENANCE

Adult mosquitoes were kept in cages of dimensions 45 x 45 x 45 cm with 20% (w/v) glucose solution for food. Females were blood fed once a week on young chicks confined by wire-mesh cages and placed inside the mosquito cages overnight. Small plastic tubs containing water were placed inside the cages for egg laying. Adult cages were maintained in a 12 hour light/dark regime at 25°C and 80% relative humidity. Egg rafts or hatched first instar larvae were transferred to rearing bowls containing tap water. The bowls were covered with nets and maintained at 25°C. A mixture of liver powder + yeast was added as required as the larval food. Pupae or late 4th instar larvae were transferred to the plastic emergence tubs which were placed inside the adult cages. In the PelRR

strain, larval selection for temephos resistance was maintained by exposing the fourth instar larvae of every third generation to the calculated LD_{50} concentration of temephos. Larval bioassays were undertaken according to standard World Health Organization procedures (WHO, 1981).

2.2.2. CHEMICALS AND EQUIPMENTS

Q-Sepharose Fast Flow, Phenyl Sepharose Fast Flow, PD-10 columns, Nap-5 columns and Nick spin columns were purchased from Pharmacia, UK. Hydroxylapatite, Prep-Cell model 491 and the protein assay kit were purchased from Bio-Rad, UK. The *p*-chloromercuribenzoate was from Pierce (Chester, UK). Chemicals were purchased from Sigma (UK) except when stated otherwise. O,O,O'O'-tetramethylO,O'-thiodi-*p*-phenylenebis(phosphorothioate)(temephos, 99.7% pure), O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate (chlorpyrifos, 99.5% pure), O,O-dimethyl O'-4-nitro-m-tolyl phosphorothioate (fenitrothion, 97% pure) , diethyl (dimethoxythiophosphorylthio) succinate (malathion, 97% pure) and its oxon analogue (malaoxon, 87.5% pure), diethyl-4-nitrophenyl phosphate (paraoxon, 97.4% pure) and 2-isopropoxyphenyl methylcarbamate (propoxur, 97% pure) were purchased from British Greyhound (Birkenhead, Merseyside, U.K.). The oxon analogues of chlorpyrifos (chlorpyrifos oxon, analytical grade)and fenitrothion (fenitrooxon, 98.3% pure) were gifts from DowElanco (Midland, U.S.A.) and Sumitomo (Osaka, Japan) respectively. α-cyano-3-phenoxybenzyl 3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-

dimethylcyclopropanecarboxylate (lambda cyhalothrin, 81.2% pure) was a gift from Zeneca Public Health (Bracknell, Berks., U.K.).

2.2.3. ENZYME ASSAYS

Enzyme activities were assayed with the substrate 1mM p-nitrophenyl acetate in 50 mM phosphate buffer (pH 7.4) at 22°C, unless otherwise stated. A stock solution of 100 mM p-nitrophenyl acetate was prepared in acetonitrile and stored at 4°C. 200 μl of a 1 mM working solution, prepared in 50 mM sodium phosphate buffer (pH 7.4), was mixed with 10 μl of enzyme solution in a microtitre plate well and the increase in absorbance at 405 nm was continuously monitored for 0.5-1.0 minute in a UVmax microtitre plate reader (Molecular Devices, USA). An extinction co-efficient of 6.53 mM^{-1} (corrected for a path length of 0.6 cm for 200 μl) was used to convert the absorbance to millimoles.

2.2.4. PROTEIN DETERMINATION

The protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin as the standard protein. In a microtitre plate well, 10 μl of protein sample was mixed with 300 μl of working solution (prepared according to the instructions of the manufacturer) and the absorbance was read at 570 nm after a five minute incubation at 22°C.

2.2.5. PURIFICATION OF CARBOXYLESTERASES

2.2.5.1. Purification of esterase A_2

Unless stated otherwise, the purification steps were performed at 4°C. Fourth instar larvae were snap-frozen in liquid nitrogen and stored at -70°C until used. The larvae (15g) were homogenised in 100 ml of bis-tris propane buffer (pH 6.5 with the conductivity adjusted to 2.0 mS/cm) containing 25 mM DL-dithiothreitol (DTT). The homogenate was centrifuged at 10,000g for ten minutes and the supernatant (pH and conductivity adjusted) was applied to a Q-Sepharose Fast Flow column (4.4 x 4 cm) equilibrated with homogenisation buffer. The enzyme was eluted with a 10 bed volume salt gradient (0-0.5 M NaCl in bis-tris propane buffer) and elution profiles were determined for esterase activity, protein and conductivity. The esterase activity eluted in one peak and the salt concentration of the pooled fractions was adjusted to 3M NaCl. This sample was then applied to a Phenyl-Sepharose Fast Flow column (2.2 x 8 cm) equilibrated with 25 mM bis-tris propane buffer (pH 6.5) containing 10 mM DTT and 3M NaCl. The absorbed esterase activity was eluted with 5 bed volumes of a decreasing salt gradient (3-0 M NaCl in the equilibration buffer). Elution was continued isocratically once the gradient ended. The esterase A_2 peak was eluted first and the fractions were combined and dialysed against dry sucrose. Further de-salting and buffer exchange into the hydroxylapatite buffer was performed on PD-10 columns. The sample was then applied to an hydroxylapatite column (2.2 x 5.4

cm) equilibrated with 10 mM phosphate buffer, pH 6.8, containing 10 mM DTT. The esterase activity was eluted with a 5 bed volume gradient of phosphate buffer (10-200 mM, pH 6.8). To avoid the crystallization of the phosphate buffer, which occur at lower temperatures, the hydroxylapatite chromatography was performed at 22°C. The esterase activity was eluted as a single peak and the fractions were combined and concentrated to 500 μl in Amicon centriprep 10 concentrator units. Buffer exchange into tris-borate native PAGE electrode buffer (pH 8.0) was performed using a Nap-5 column. The sample (with xylene cyanol marker and 10% glycerol) was applied onto a 9% acrylamide gel (17 ml) with a 4% acrylamide stacking gel (9 ml) in a Prep-Cell model 491. The gel was run at 15 W constant power and the enzyme was eluted with the same buffer containing 10 mM DTT. Pooled fractions of activity were concentrated and the buffer exchanged into 50 mM phosphate buffer (pH 7.4) on Nap-5 columns. Purified enzyme was stored in 50 mM phosphate buffer (pH 7.4) containing 50% (v/v) glycerol and 25 mM DTT at -20°C.

2.2.5.2. Purification of esterase B_2

The esterase B_2 peak was the second esterase peak eluted from the Phenyl-Sepharose column as described above. Fractions were pooled separately from those of A_2 and dialysed against dry sucrose. De-salting and buffer exchange into the hydroxylapatite buffer was performed on PD-10 columns. The sample was then applied to a hydroxylapatite column (2.2 x 5.4 cm) equilibrated with 10 mM

phosphate buffer (pH 6.8) containing 50 mM NaCl and 10 mM DTT. The esterase activity was eluted with a 5 bed volume gradient of the phosphate buffer (10-200 mM, pH 6.8) containing no NaCl. The hydroxylapatite chromatography was performed at 22°C. The esterase activity was eluted as a single peak and the fractions were combined and concentrated in Amicon centriprep 10 concentrator units. Buffer exchange into 0.1 M phosphate buffer containing 10 mM EDTA (pH 7.8) was performed on Nap-5 columns. This sample was applied to a *p*-chloromercuribenzoate column (1.5 x 5 cm) equilibrated with the same buffer. The esterase activity was eluted with a 5 bed volume gradient of the equilibrating buffer and 20 mM phosphate buffer, pH 6.8, containing 30 mM β-mercaptoethanol. Fractions with esterase activity were combined and concentrated. Buffer exchange was performed on Nap-5 columns and the purified enzyme was stored at -20°C in 50 mM phosphate buffer (pH 7.4) containing 50% (v/v) glycerol and 25 mM DTT.

2.2.6. CHARACTERIZATION OF ESTERASES A_2 AND B_2

2.2.6.1. Kinetic constants

All specific activities are given in units/mg of protein. A unit corresponds to the hydrolysis of 1 μmol of substrate in 1 min under the assay conditions used. Kinetic constants were determined from at least three experiments for each substrate or insecticide using enzymes from several different purifications.

2.2.6.1.1. DETERMINATION OF THE MICHAELIS CONSTANT (K_m) AND THE MAXIMUM VELOCITY (V_{max}) FOR THE SUBSTRATES

The hydrolytic production of p-nitrophenol at different substrate concentrations was measured to determine the constants for p-nitrophenyl acetate and p-nitrophenyl hexanoate (p-nitrophenyl caproate) using the assay described previously (see section 2.2.3.). For α- and β-naphthyl acetate the production of naphthol was measured at 235 nm in a Beckman Du-70 spectrophotometer (Buckinghamshire, UK). An extinction co-efficient of 24 mM^{-1} (corrected for the path length) was used to convert the absorbance to moles. The Michaelis constant (K_m) and V_{max} for these substrates were calculated by non-linear regression using the Enzfitter programme (by R.J.Leatherbarrow, Biosoft)

2.2.6.1.2. DETERMINATION OF KINETIC CONSTANTS FOR SEVERAL INSECTICIDES

For the inhibition kinetics, stopped time inhibition assays were performed using p-nitrophenyl acetate or p-nitrophenyl hexanoate as the substrate. An enzyme concentration which gives a reading of about 300 mOD in the control was used. Insecticide stock solutions (100 mM) were always prepared in acetonitrile and diluted in phosphate buffer (pH 7.4) prior to each experiment. The purified B_2 enzyme was incubated with a series of concentrations of the test insecticide (acetonitrile concentration of the medium never exceeded 1% [v/v]) and at 0.5-

5.0 min time intervals aliquots were withdrawn and diluted with the substrate immediately to stop further inhibition. Residual activity was determined by measuring the rate of substrate hydrolysis. The activities were divided by those measured in the absence of insecticide (control). The inhibitor concentrations were in large excess so that linear pseudo-first-order kinetics were obtained (Aldridge and Reiner, 1972). For each insecticide concentration log percentage remaining activity at each time point was plotted against time and the slope of each graph gave the observed rate of inhibition (k_{obs}). The following equation was used to determine the constants K_a and k_2 (Aldridge and Reiner, 1972).

$$[I]/k_{obs} = K_a/k_2 + [I]/k_2$$

where; $[I]$ = insecticide concentration, K_a = Michaelis constant for the inhibitor and k_2 = first order rate constant for the formation of acylated enzyme from the Michaelis complex.
by dividing with $[I]$ the following equation is derived;

$$1/k_{obs} = K_a/k_2 \cdot 1/[I] + 1/k_2$$

Therefore, when $1/k_{obs}$ values are plotted against $1/[I]$, the k_2 and K_a constants are given by the (1/Y-axis intercept) and (slope x k_2) respectively.

The bimolecular rate constants for the formation of acylated enzyme (k_a) were

derived from the following equation (Aldridge and Reiner, 1972).

$$k_a = 1/[I]t \ \ln \ 100/X$$

where; t = time of preincubation of the enzyme and X = percentage remaining activity.

Whenever the $[I]$ could not be maintained in large excess and thereby pseudo-first-order kinetics were not obtained, the k_a values were determined in the presence of the substrate using the following equation (Main and Dauterman, 1963).

$$k_a = 2.303 \ /(t_2-t_1)(1-\alpha)[I] \ \ \log \ V_1/V_2$$

and, $(1-\alpha) = K_m/(K_m+[S])$

where; K_m = Michaelis constant for the substrate, $[S]$ = substrate concentration, V_1 is the velocity after inhibition for time t_1 and V_2 is the velocity after inhibition for time t_2.

To minimise the effect of the reversible enzyme-substrate complex on the rate of acylation, the substrate concentration was maintained at a very low concentration so that the [substrate concentration]/K_m ratio was always less than

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0.5 (Aldridge and Reiner, 1972).

The re-activation experiments were performed by incubating the enzyme with the respective insecticide for 10-15 min so that the enzyme was more than 90% inhibited. Concentration of the enzyme was determined as earlier. The unbound insecticide and enzyme-insecticide complex were separated on a Nick spin column. 10 μl Aliquots were removed to measure the esterase activity of the re-activating enzyme, as well as a control, over time. The slope of the curve, obtained by plotting the percentage remaining activity against the time, gave the reactivation constant k_3.

2.2.6.2. Influence of effectors

Several compounds which may effect the enzyme activity and are commonly used to characterize different esterase types were used. Solutions of bis-(p-nitrophenyl) phosphate (0.1 mM), paraoxon (0.1 μM and 0.1 mM), EDTA (1 mM), eserine (10 μM) and several metal ions were prepared either in 50 mM phosphate buffer (pH 7.4) or in 25 mM bis-tris propane buffer (pH 7.4) depending on their solubility. Each effector was pre-incubated with the purified enzyme for 30 min at 22°C. Esterase activity was then measured in the presence of each effector. Enzyme concentration was determined as described in the previous section.

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2.2.6.3. Polyacrylamide gel electrophoresis

Bio-Rad Miniprotean II vertical gel electrophoresis units were used. Electrophoresis of native protein samples was performed in 7.5% acrylamide gels in tris/borate buffer, pH 8.0, by the method of Davis (1964). The gels were stained for esterase activity with 0.04% (w/v) α- and β-naphthyl acetate and 0.1% (w/v) Fast Blue B in 100 mM phosphate buffer, pH 7.4. SDS-polyacrylamide gel electrophoresis was performed with standard proteins using 10% acrylamide gels in tris/glycine/SDS buffer, pH 8.3, as described by Laemmli (1970). Coomassie Blue R250 was used to stain for protein.

2.2.6.4. Determination of Molecular Weight

For each SDS-polyacrylamide gel, log molecular weight versus the relative mobility for each standard were plotted to obtain a regression line. The molecular weight of esterase B_2 was determined using its relative mobility compared to the standards. This was repeated on several gels.

2.2.6.5. N-terminal analysis

N-terminal analysis of purified B_2 (for each trial a sample of about 10 μg in 100 μl of distilled water was sent) was done by automated Edman degradation on an Applied Bio Instruments 477a protein sequencer by the protein sequencing unit

at Royal Holloway and Bedford New College, Egham Hill, Egham, Surrey TW20 0EX, England.

2.3. RESULTS AND DISCUSSION

2.3.1. PURIFICATION OF ESTERASES

Purification procedures for the esterases A_2 and B_2 were developed by modifying the methods of Ketterman *et al.* (1992) and Jayawardena (1992). In the previous methodologies pure esterases were obtained by sequential chromatography on Q-Sepharose, phenyl-Sepharose and hydroxylapatite. Both esterases eluted from the ion-exchange column in a single peak as both had similar isoelectric points (between 5.0-5.1). The A_2 and B_2 esterase activity peaks were separated on the hydrophobic phenyl-Sepharose column. Each esterase was then purified to homogeneity by hydroxylapatite columns. However, probably due to batch to batch variation of the chromatography media, an inefficiency in binding of both enzymes to Q-Sepharose and of esterase B_2 to hydroxylapatite was later observed under the established conditions. Experiments carried out under different conditions revealed that the conductivity should not exceed 2.5 mS/cm in order to bind these esterases firmly to Q-Sepharose material at pH 6.5. Esterase B_2 could be bound to hydroxylapatite material by reducing phosphate ion concentration (by changing the buffer system) or by introducing NaCl. $MgCl_2$ or

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$CaCl_2$ could not be used because of their inhibitory effect on B_2 in high concentrations. As a result of extensive experiments it was found that the maximum purity with a sharp esterase peak in the elution could be attained only by the method given in section 2.2.5.2. The necessity of a fourth step in the purification procedures arose as the samples still had several other protein contaminants. For the fourth step various materials were tested with both enzymes under different conditions.

Esterase A_2 could be bound to Cibacron Blue, a dye ligand immobilised on agarose, but was difficult to elute from it. When the same dye was used to elute the esterase, it could not be dissociated from the enzyme afterwards. Esterase A_2 would also bind to immobilised p-chloromercuribenzoate but was not eluted by various standard methods. Finally using the differences in the mobility in native PAGE, esterase A_2 could be separated from the other contaminating proteins with the Prep Cell model 491.

Esterase B_2 did not bind to any of the several dye ligands tested (Cibacron Blue, Reactive Blue 4-Agarose [R 8754], Reactive Brown 10-Agarose [R 8629], Reactive Green 19-Agarose [R 4004], Reactive Red 120-Agarose [R 9129] and Reactive Yellow 86-Agarose [R 8504]). It also did not bind to chelating Sepharose charged with Zn^{++} binding sites. The glycosylated nature of this enzyme is questionable as it did not bind to Con-A Sepharose (with binding sites for glucose and mannose residues). Since most of the contaminating proteins

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gave a single band together with esterase B_2 in native PAGE, the Prep Cell could not be used. Finally, pure esterase B_2 could be isolated using immobilised p-chloromercuribenzoate.

The disulphide bonds must be very important in maintaining the active forms of A_2 and B_2 since it was essential to maintain a high concentration of DTT, which serves as an reducing agent and protects the S-S groups, during the purification procedures and storage. However, DTT was removed before the samples were introduced to p-chloromercuribenzoate material to facilitate the binding.

Chromatography profiles are shown in FIGURES 2.1-2.4. Esterase B_2 was eluted from p-chloromercuribenzoate column in a broad peak of activity and the protein concentrations of these fractions were not detectable (profile not shown). Data for a single purification procedure is given in TABLE 2.1. Multiple preparations routinely gave approximately 40% final recovery of enzyme activity (as a percentage of the total esterase activity [$ie.$ A_2 + B_2] observed in the beginning) and 350 -fold purification for esterase A_2 and 3% final recovery and 50-60 -fold purification for esterase B_2. Using the outlined procedures approximately 800 μg of purified esterase A_2 and 200-300 μg of purified esterase B_2 were obtained from 15 g wet weight of larvae. The final esterase preparations appeared to be homogeneous as determined by SDS-PAGE (FIGURES 2.5 and 2.6).

FIGURE 2.1

Elution profile of Q-Sepharose chromatography for esterases A$_2$ and B$_2$ purification

5 ml fractions of the elution of 0-0.5 M NaCl gradient in 25 mM bis-tris propane buffer (pH 6.5) were collected.
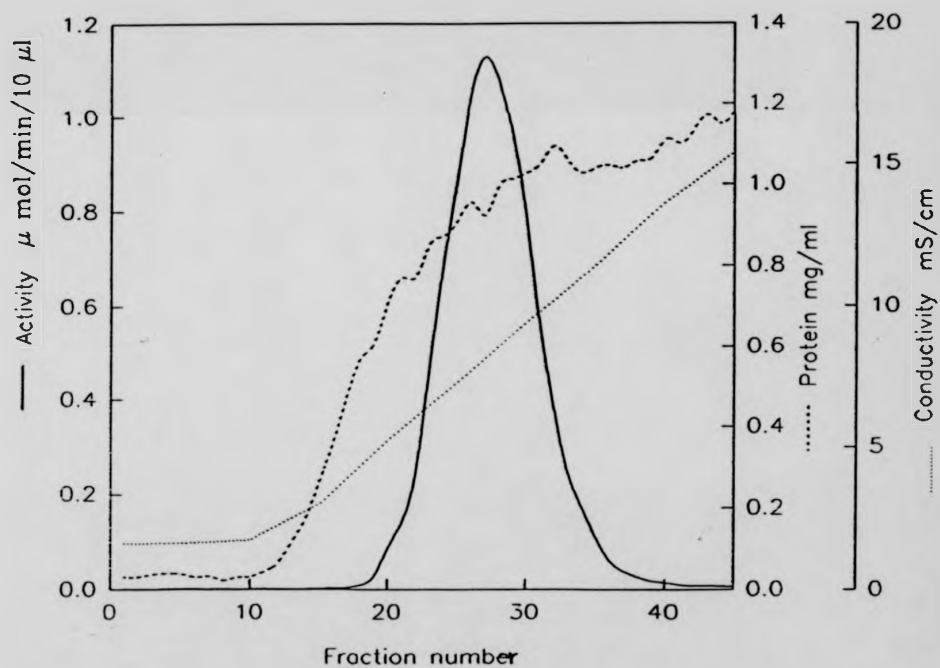
FIGURE 2.2

Elution profile of phenyl-Sepharose chromatography for esterases A_2 and B_2
5 ml fractions of the elution of 3-0 M NaCl gradient in 25 mM bis-tris propane
buffer (pH 6.5) were collected. The two esterases were eluted in two separate
peaks (see the text for details).
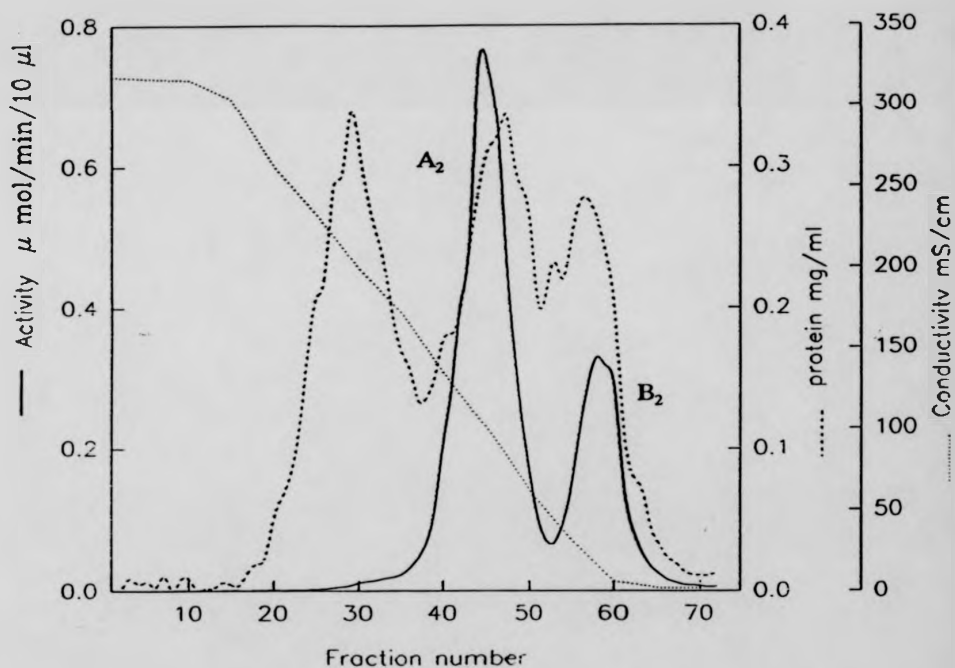
FIGURE 2.3

Elution profile of hydroxylapatite chromatography for esterase A$_2$
1 ml fractions of the elution were collected. Enzyme was applied to the column
in 10 mM phosphate buffer (pH 6.8) and eluted with a 10-200 mM phosphate
gradient.
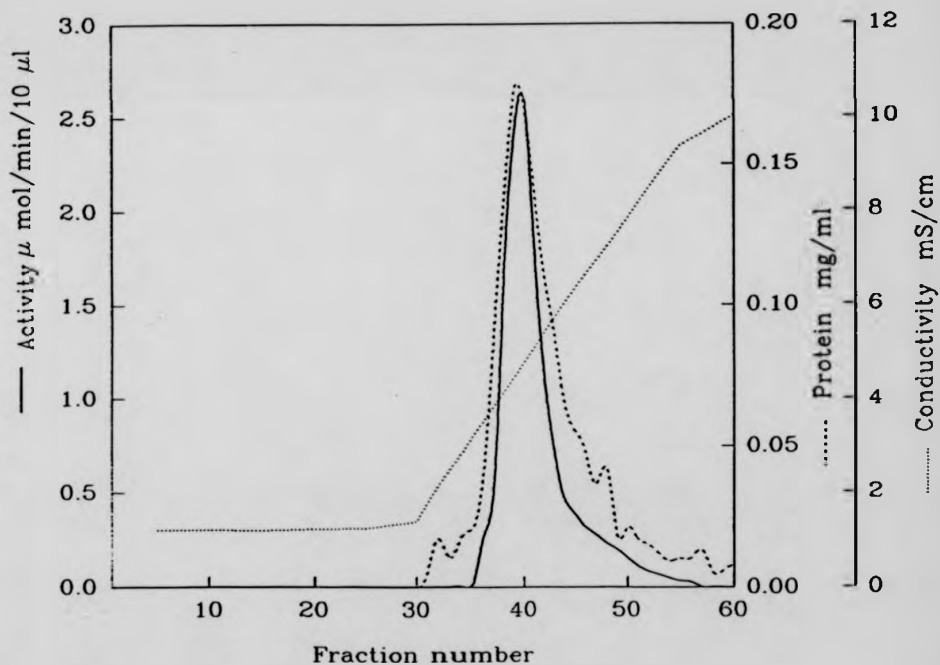
FIGURE 2.4

Elution profile of hydroxylapatite chromatography for esterase B$_2$
1 ml fractions of the elution was collected. Enzyme was applied to the column
in 10 mM phosphate buffer (pH 6.8) with 50 mM NaCl and eluted with a 10-200
mM phosphate gradient without NaCl.
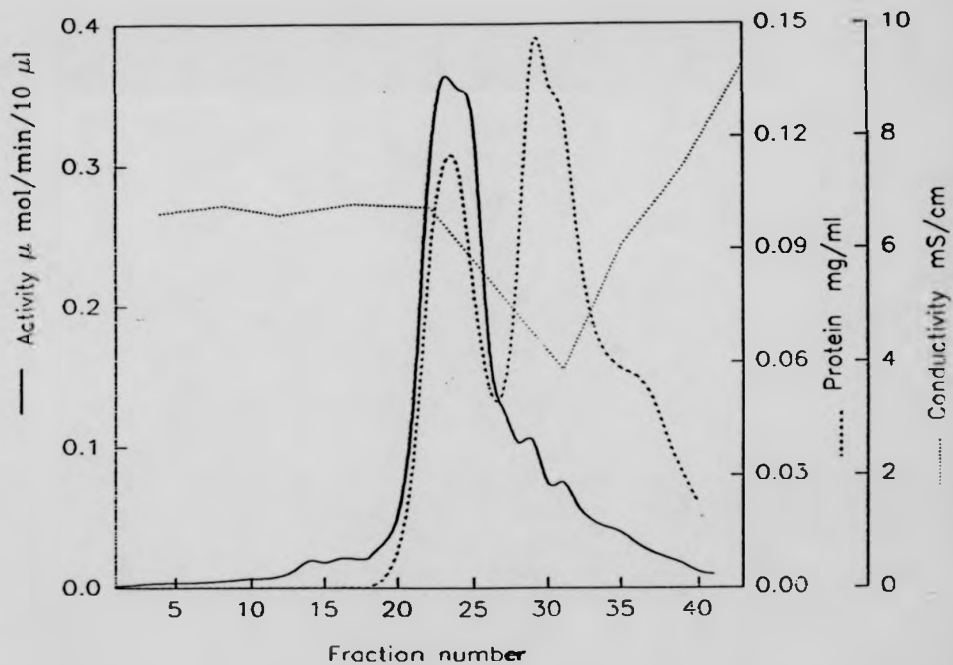
TABLE 2.1

Purification of the esterases A_2 and B_2

The purification was monitored by the assay of 1 mM *p*-nitrophenyl acetate hydrolysis at 22°C. The two esterases were separated after the phenyl-Sepharose column.

Step		Specific Activity (Units/mg)	Protein (mg)	Purification (fold)	Recovery (%)
10,000 g supernatant		1.03	692.5	-	-
Q-Sepharose		13.15	63.93	12.74	117.5
Phenyl-Sepharose	A_2	33.22	12.67	32.20	58.90
	B_2	21.49	3.98	20.83	11.96
Hydroxylapatite	A_2	204.78	1.63	198.8	46.84
	B_2	47.27	1.45	45.81	9.60
Prep-Cell	A_2	363.15	0.80	352.6	40.70
p-chloro-mercuribenzoate	B_2	51.82	0.395	50.22	2.86

FIGURE 2.5

SDS-PAGE of purified esterase A_2

Molecular weight markers on 10% gel are; α_2-macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactate dehydrogenase (36.5 kDa) and triosephosphate isomerase (26.6 kDa).

A_2

180.0 KDa

116.0 KDa

84.0 KDa

58.0 KDa
48.5 KDa

36.5 KDa

26.6 KDa
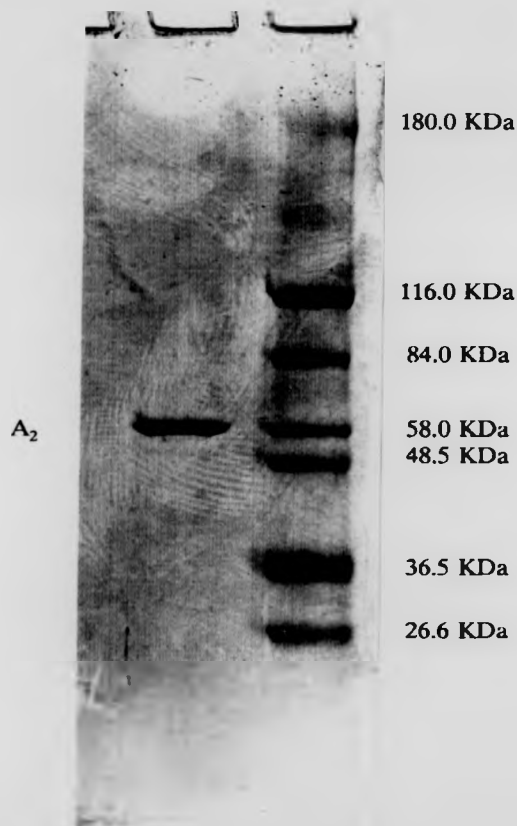
FIGURE 2.6

SDS-PAGE of purified esterase B$_2$

Molecular weight markers on 10% gel are; β-galactosidase (119 kDa), fructose-6-phosphate kinase (98 kDa), pyruvate kinase (80.6 kDa), fumarase (64.4 kDa), lactic dehydrogenase (44.6 kDa) and triosephosphate isomerase (38.9 kDa) (Markers are pre-stained and therefore slightly heavier than the similar markers in FIGURE 2.5 which are not pre-stained).
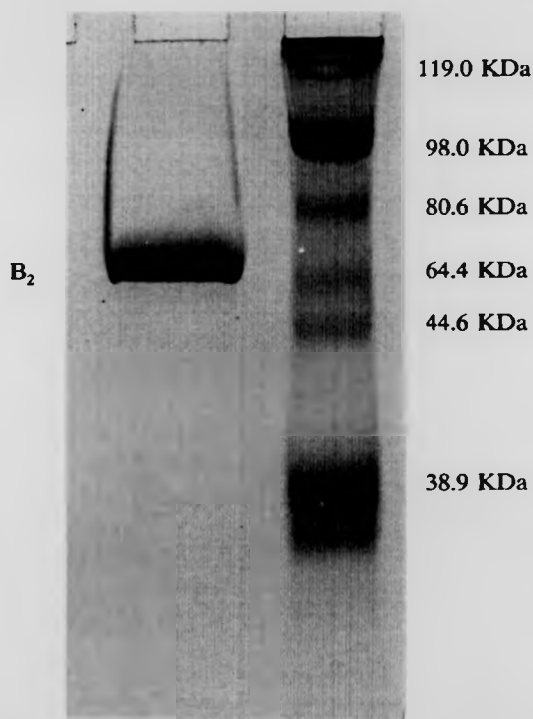
2.3.2 CHARACTERIZATION OF ESTERASES A_2 AND B_2

2.3.2.1. Physical Characterization

The monomeric M_r of the purified esterase B_2, estimated from SDS/PAGE was 62.8 ± 2.4 kDa (FIGURE 2.7). This is in close agreement with the previous observations for the same enzyme (Jayawardena, 1992) and for esterase A_2 (Ketterman *et al.*, 1992) (see section 1.5.4.1.). The pI of the esterase B_2 was previously determined to be 5.0 (Jayawardena, 1992).

N-terminal sequencing of both esterases A_2 and B_2 was not possible, probably due to a blocked N-terminus of the enzyme molecule. One purified sample of esterase B_2 and two purified samples of esterase A_2 (obtained from two different purifications) were analysed. In each attempt only about an 8% total recovery, spread over several amino acid signal peaks, was obtained.

2.3.2.2. Chemical Characterization

2.3.2.2.1. Substrate specificity

Michaelis constants (K_m) and V_{max} values were determined for four general esterase substrates (TABLE 2.2). Both esterases showed a higher affinity towards *p*-nitrophenyl hexanoate (C-6) than *p*-nitrophenyl acetate (C-2). A preference for

FIGURE 2.7

Subunit molecular weight estimation of purified esterase B_2

R_f values of the SDS-PAGE standard proteins were plotted against their log molecular weights. Molecular weight of the B_2 was determined graphically for each gel.
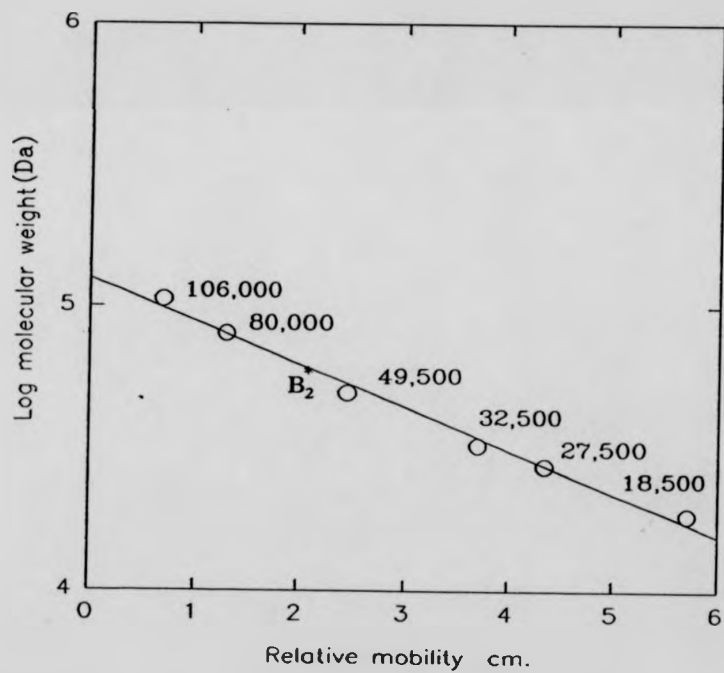
TABLE 2.2

Substrate interactions of esterases A_2 and B_2

The rate of substrate hydrolysis was measured at 405 nm for p-nitrophenyl acetate and p-nitrophenyl hexanoate, and at 235 nm for α-naphthyl acetate at 22°C. A unit corresponds to the hydrolysis of 1 μmol of substrate in 1 min under the assay conditions used.

	A_2		B_2	
	V_{max} (units/mg)	K_m (μM)	V_{max} (units/mg)	K_m (μM)
p-nitrophenyl acetate	472.0 ± 51.4	145.8 ± 45.0	63.4 ± 0.8	140.0 ± 50.0
p-nitrophenyl caproate	788.2 ± 74.8	35.4 ± 9.7	83.3 ± 9.8	17.0 ± 6.0
α-naphthyl acetate	717.9 ± 48.4	30.5 ± 6.1	200.9 ± 15.0	172.5 ± 40.6

medium chain length mono- and di-acylglycerols had been observed earlier for both enzymes (Ketterman *et al.*,1992; Jayawardena, 1992). When the data for the two enzymes are compared, the rate of hydrolysis is considerably slower with esterase B_2 for both substrates. Pig and rat liver carboxylesterase isoenzymes have shown K_m and V_{max} values of 30-220 μM and 66-110 units/mg respectively for *p*-nitrophenyl acetate (Heymann,1980). For the same substrate, two purified human liver carboxylesterases have shown K_m values of 190 and 130-870 μM and V_{max} values of 13.3 and 67.3-142.7 units/mg (Ketterman *et al.*, 1989) and Mentlein *et al.* (1984) reported K_m values and V_{max} values of 87-740 μM and 30-176 units/mg respectively for five esterases purified from rat liver microsomes. The range of K_m and V_{max} values of the esterases E_1, E_2 and E_3 purified from *Nilaparvata lugens* were 150-250 μM and 20-100 units/mg for the substrate *p*-nitrophenyl acetate (Chen and Sun, 1994). These observations are very close to the observations reported here for purified mosquito carboxylesterases although the V_{max} of the esterase A_2 for *p*-nitrophenyl acetate is slightly higher. The two substrates used to define esterases A_2 and B_2 originally, α- and β-naphthyl acetate were also examined. By definition, esterase A_2 was more reactive towards α-naphthyl acetate and esterase B_2 was more reactive towards β-naphthyl acetate when given a choice between the two substrates. This could be shown with α-naphthyl acetate (TABLE 2.2) but with β-naphthyl acetate the K_m and V_{max} values could not be determined because of the insolubility of the substrate at the concentrations necessary. Slightly lower V_{max} values (15-70 units/mg) have been reported for the E_1, E_2 and E_3 esterases of *Nilaparvata lugens* for α- and β-

naphthyl acetate (Chen and Sun, 1994).

2.3.2.2.2. Influence of effectors

The influence of several metal ions and known carboxylesterase effectors are presented in TABLE 2.3. Enzyme activity of B_2 was inhibited only by $CuCl_2$, $FeCl_3$ and $HgCl_2$ among several metal ions tested. Inhibition by $HgCl_2$ was also observed for esterase A_2 (Ketterman *et al.*, 1992) and this again implicates the involvement of a thiol group in catalysis, or more likely, in conformational stability of these enzymes as discussed earlier (Section 2.3.1.). 'A' esterases (according to Aldridge's classification) and arylesterases are characteristically inhibited by mercuric chloride because of the presence of -SH groups of the cystein residue at the active site (Aldridge 1993; McCracken *et al.*, 1993). A phosphorotriester hydrolase, purified from the insect *Heliothis virescens*, a pest of cotton, showed complete inhibition with Hg^{2+} and slight inhibition with Cu^{2+}, but not with Fe^{3+} at 1 mM concentrations (Konno *et al.*, 1990). Rat liver microsomal esterases have been inhibited to various degrees with $MgCl_2$, $ZnSO_4$, $CuSO_4$ and $HgCl_2$ (Mentlein *et al.*, 1984).

Esterase B_2 was completely inhibited by 0.1 mM paraoxon but only to about 50% by 0.1 μM. Esterase A_2 was completely inhibited by 0.1 μM paraoxon. These results confirm that both esterases are 'B' type serine hydrolases according to Aldridge's classification (Aldridge 1953a; 1953b; 1993). Bis-(p-nitrophenyl)

TABLE 2.3

Influence of the effectors on the activity of purified esterase B_2

The individual effectors were pre-incubated with the purified enzyme for 30 min at 22°C. Esterase activity was then measured with 1 mM p-nitrophenyl acetate in the presence of each effector.

Effector	Concentration	% Activity
$CaCl_2$	1.0 mM	105.9
$CuCl_2$	1.0 mM	4.8
$FeCl_3$	1.0 mM	3.5
$HgCl_2$	1.0 mM	11.7
$MgCl_2$	1.0 mM	113.1
$MnCl_2$	1.0 mM	96.7
$ZnCl_2$	1.0 mM	102.3
EDTA	1.0 mM	104.1
Eserine	10.0 μM	103.4
Bis-(p-nitrophenyl)phosphate	0.1 mM	5.9
Paraoxon	0.1 μM	51.3
	0.1 mM	5.9

phosphate, which has been shown to be a specific carboxylesterase inhibitor in rats (Brandt *et al.*, 1980) also inhibited the purified mosquito esterase B_2. EDTA did not inhibit the enzyme indicating that there is no metal ion requirement for catalysis. In contrast, 'A' esterases (according to Aldridge's classification) are inhibited with EDTA (Reiner, 1993). Esterase A_2 also behaved in the same manner as B_2 with the latter two effectors. Purified esterase B_2 was not inhibited by the acetylcholinesterase inhibitor eserine. In contrast esterase A_2 was completely inhibited by 10 μM eserine although it showed no significant activity with commonly used cholinesterase substrates (Ketterman *et al.*, 1992). However, any structural similarity of esterase A_2 to the target site enzyme AChE does not account for any greater contribution to resistance, since both A_2 and B_2 are almost equally effective at binding various insecticides (see section 2.3.2.2.3.). Complete inhibition with paraoxon and inhibition to various degrees with bis-(p-nitrophenyl) phosphate and eserine have also been demonstrated for rat liver esterases (Mentlein, *et al.*, 1984; McCracken *et al.*, 1993).

2.3.2.2.3. *Interaction of esterase B_2 with insecticides*

The interaction of esterase B_2 was determined for four OPs (chlorpyrifos, fenitrothion, malathion, parathion and their oxon analogues), one carbamate (propoxur) and one pyrethroid (lambda cyhalothrin). No significant interaction of purified mosquito esterase B_2 with phosphorothionates could be detected. Slight inhibitions caused by some of the thionates at their maximum solubility

limits may be attributed to a possible contamination by the highly reactive oxon analogues. There was no interaction with the pyrethroid lambda cyhalothrin. Therefore the inhibition constants could be determined only for chlorpyrifos-oxon, fenitrooxon, malaoxon, paraoxon and propoxur and are presented in TABLE 2.4 together with k_3 values obtained from reactivation kinetic experiments. The k_a values for the interaction of esterase A_2 with the insecticides are also presented for comparison.

Linear pseudo-first order kinetics for the inhibition of the esterase B_2 were obtained for the insecticides fenitrooxon, malaoxon and propoxur (FIGURES 2.8 and 2.9). Therefore all three inhibition constants (K_a, k_2 and k_a) could be determined for these insecticides. Chlorpyrifos-oxon and paraoxon were very potent inhibitors, such that the inhibition rates could be measured only at very low inhibitor concentrations. With these inhibitors, the concentrations of insecticides approached or were lower than that of the B_2 enzyme and pseudo-first order kinetics could no longer be obtained. Therefore, K_a and k_2 values of esterase B_2 could not be determined for these two insecticides and only the k_as were determined in the presence of substrate (TABLE 2.4). Esterase A_2 was always used in much lower concentrations than esterase B_2 in enzyme assays because of its greater reactivity towards the assaying substrates. Also the reactivity of A_2 with the chlorpyrifos-oxon was much lower than that of B_2 and a greater amount of insecticide was needed to inhibit the enzyme. Therefore in the insecticide interaction experiments done with esterase A_2, the inhibitor

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TABLE 2.4

Kinetic constants for the interaction of esterase B_2 with the insecticides

Enzyme activity was measured by the assay of 1 mM p-nitrophenyl acetate hydrolysis at 22°C. The value in parentheses is the resistance ratio of LD_{50} concentrations from resistant-versus-susceptible larval bioassay experiments (for organophosphates the value represents the resistance ratio for the thionate analogue). The k_a's for the esterase A_2 are presented for comparison.

Insecticide	$10^2 \times k_2$ (min^{-1})	$10^4 \times k_3$ (min^{-1})	$10^5 \times K_a$ (M)	$10^{-5} \times k_a$ (M^{-1} min^{-1})	$10^{-5} \times k_a$ (M^{-1} min^{-1})
			B_2		A_2
Malaoxon[a](7)	16.3 ± 3.8	18.1 ± 3.7	0.43 ± 0.11	0.50 ± 0.17	0.17 ± 0.07
Fenitrooxon[a](4)	37.4 ± 15.4	13.5 ± 3.9	0.56 ± 0.21	1.73 ± 0.60	0.91 ± 0.37
Chlorpyrifos-oxon[a](37)	-	2.4 ± 1.7	-	1550 ± 140	145.3 ± 59.7
paraoxon[a](20)	-	5.1 ± 2.0	-	170 ± 53	178.0 ± 47.7
propoxur[b] (2)	40.2 ± 10.2	10.9 ± 4.4	135.7 ± 64.8	0.0052 ± 0.0017	0.012 ± 0.003

a -Organophosphate.

b -Carbamate.

FIGURE 2.8

Time dependent inhibition of esterase B_2 by malaoxon

For each inhibitor concentration, log % remaining activity was plotted against time. k_{obs} for each concentration was obtained by the slope of the graph.

Malaoxon concentrations: ○ 2.5 μM, ● 4 μM, ▽ 5 μM, ▼ 6 μM, □ 10 μM, ■ 20 μM.
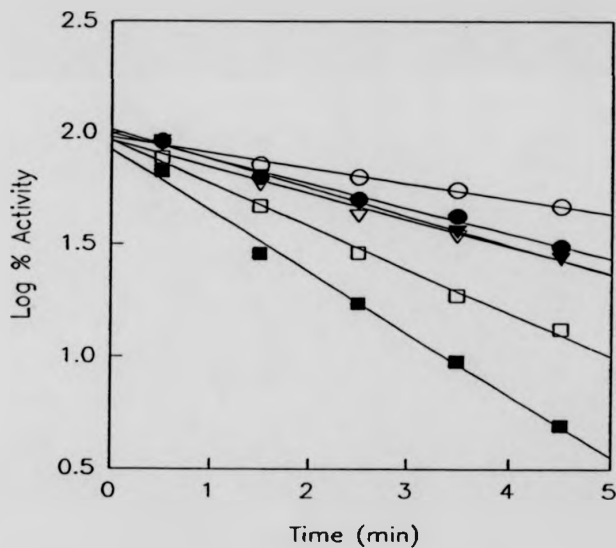
FIGURE 2.9

Relationship between the inhibitor concentrations and their k_{obs} values for malaoxon

k_{obs} values obtained from the FIGURE 2.8 were plotted against their relative inhibitor concentration to determine k_2 and K_a constants (see the text for details).
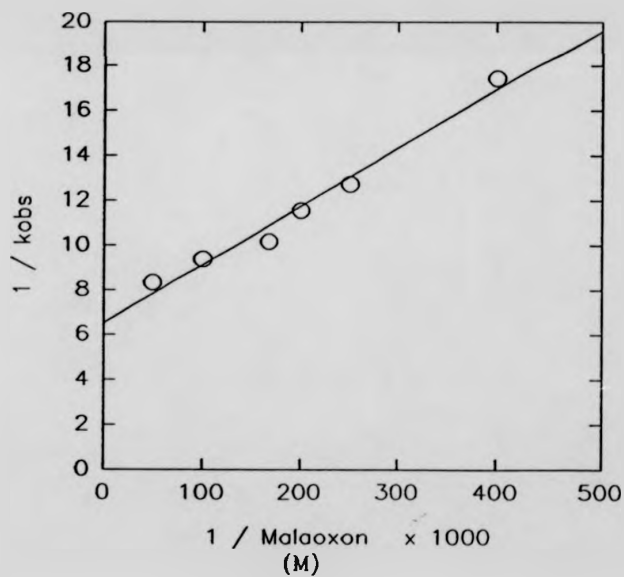
concentration could always be maintained in excess. The k_a values for the esterase A_2 were determined in both the presence and absence of substrate in order to compare the data obtained by the two methods and were shown to agree irrespective of the manner of determination. Data presented here agree with the fact that oxons are far more potent inhibitors than the thionate analogues. Both esterases were also tested with temephos, with which the mosquito strain PelRR was selected. Again no interaction was observed with the thionate form and the oxon analogue could not be tested as it was not available. Apart from OPs, the carbamate propoxur interacted with the purified esterase B_2, but at a very slow rate. The reactivation rates (k_3) were very low even with the highly active oxon forms indicating a slow turn over of the insecticides (FIGURE 2.10). Therefore the sequestration role of this enzyme is confirmed.

The inhibition rates of esterases A_2 and B_2 can be compared with the insecticide cross-resistance spectrum showed by the PelRR strain (Peiris and Hemingway, 1990a). If the toxicity is mainly brought about by the oxons, formed *in vivo*, the resistant ratios shown for the thionates are due to the interaction of their oxon analogues with these esterases. A causal relationship therefore appears to exist between the bimolecular rate constant (k_a) and the insecticide cross-resistance spectrum. The greatest resistance ratios shown for chlorpyrifos (37) and parathion (20) may be due to the greater interaction (as shown by higher k_a values) of these esterases with their oxon analogues. This is clearly shown by the respective k_a values; $1550 \pm 140 \times 10^5$ and $170 \pm 53 \times 10^5$ M^{-1} min^{-1} for esterase B_2

FIGURE 2.10

Reactivations of the B_2 esterases inhibited by fenitrooxon, malaoxon and propoxur

Acylated enzyme was separated and the reactivation of the enzyme was measured and plotted against time. The reactivation constant (k_3) was obtained from the slope of each graph.
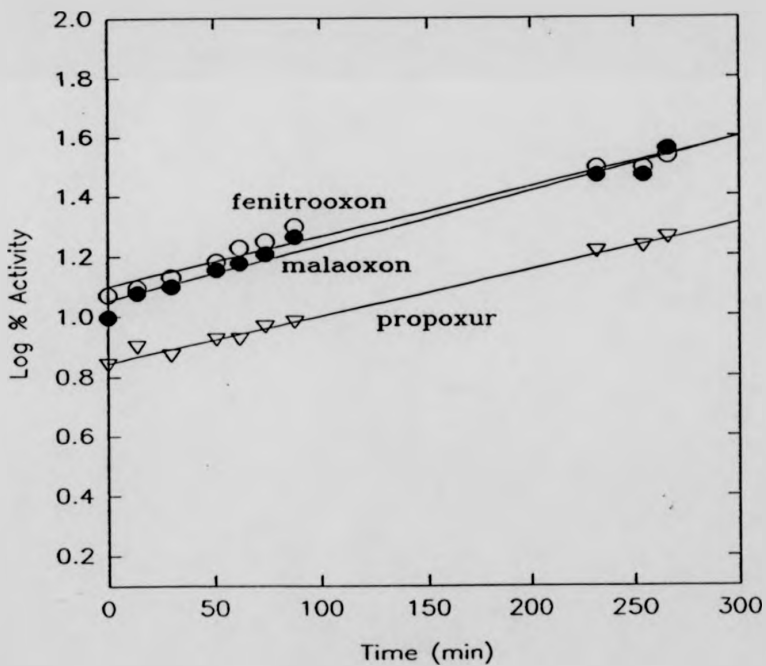
and $145.3 \pm 59.7 \times 10^5$ and $178.0 \pm 47.7 \times 10^5$ M^{-1} min^{-1} for esterase A_2. The k_a values obtained for fenitrooxon and malaoxon are much lower than these and correlate well with the lower resistance ratios shown for fenitrothion (4) and malathion (7) respectively. The slightly higher resistance ratio of malathion compared with fenitrothion can not be explained in a similar manner. The contribution of other minor mechanisms may slightly alter the direct relationship between the k_a values and the insecticide cross-resistance spectrum. For the carbamate propoxur, which is not converted into any other active form *in vivo*, k_a values of both enzymes are very low and can directly be compared with the low resistance ratio shown for it. The mosquito strain PelRR has shown a negative cross-resistance (0.75 x) for the pyrethroid permethrin (Peiris and Hemingway, 1990a), which suggests that the resistant enzyme may be less reactive with pyrethroids than the susceptible enzyme, although further work is required to confirm this.

In general, no major difference was observed between the two esterases with their insecticide interactions indicating that both A_2 and B_2 probably have a similar role in resistance. Differences in k_a's of A_2 and B_2 for the same insecticide may reflect the degree of their relative contributions for the resistance to that particular insecticide. Purified carboxylesterase E4, which is responsible for the insecticide resistance in peach-potato aphid, had a k_a of $1330.0 \pm 80.0 \times 10^5$ M^{-1} min^{-1} for paraoxon (Devonshire, 1977). A k_a value of $46.0 \pm 7.0 \times 10^5$ M^{-1} min^{-1} has been reported for rat plasma carboxylesterases (Maxwell, 1992). In rats,

it has been shown *in vivo* that carboxylesterases are more sensitive to organophosphates and carbamates than AChE and protect AChE from these compounds (Gupta and Dettbarn, 1993). In sheep erythrocytes, the bimolecular rate constant (k_a) of the target site, AChE, for paraoxon has been shown to be 11×10^5 M^{-1} min^{-1} (Aldridge & Davison, 1952). A rat brain AChE has shown a k_a value of $9.0 \pm 1.1 \times 10^5$ M^{-1} min^{-1} for the same insecticide (Maxwell, 1992). For an insect AChE, purified from *Lygus hesperus* (Hemiptera: Miridae) this value was 9.44×10^5 M^{-1} min^{-1}. This insect enzyme has also been shown to have k_a values of $10^4 - 10^6$ (M^{-1} min^{-1}) for five other oxons tested (Zhu and Brindley, 1992b). Susceptible AChE from the tobacco budworm, *Heliothis virescens*, was determined to have a k_a of approximately 0.3×10^5 M^{-1} min^{-1} for paraoxon (Brown and Bryson, 1992). These data indicate that the insect carboxylesterases which have the putative role of sequestration in protecting the target site, AChE, interact with these organophosphates more readily than the target site itself, thereby demonstrating the effectiveness of this resistance mechanism.

The correlation between the k_a and the insecticide resistance is also reflected by the respective K_a value (Michaelis constant for the insecticides). The constants k_2 and k_3 do not have any apparent relationship with the insecticide cross-resistance spectrum of the mosquito strain (see TABLE 2.4). Slow deacylation rates (k_3) have been observed for the interactions of aphid esterase E4 with insecticides showing the major role of this enzyme in detoxication is also sequestration (Devonshire and Moores, 1982). TABLE 2.5 compares the

TABLE 2.5

Comparison of the deacylation rates (k_3) of the mosquito esterases A_2, B_2 and the aphid esterase E4 in their interactions with insecticides.

Insecticide	k_3 hr^{-1}		
	A_2	B_2	E4
Malaoxon	0.038	0.109	3.120
Paraoxon	0.031	0.031	0.330
Propoxur	0.374	0.065	0.093

deacylation rates for esterases B_2, A_2 and E4. The aphid esterase E4 hydrolysed paraoxon at 10× the rate of the mosquito esterases A_2 and B_2. For malaoxon, esterase E4 hydrolysis was 30× the rate of the mosquito esterase B_2 and 100× the A_2 rate. Therefore in addition to the sequestration, hydrolysis was also shown to be important in organophosphate detoxication by aphid esterase E4. In contrast, for the carbamate propoxur, E4 had a similar rate to esterase B_2, but this was about 4× less than the rate for A_2.

2.3.3. DETERMINATION OF THE PROPORTIONS OF ESTERASES A_2 AND B_2 PER PELRR LARVA

The relative amounts of esterases A_2 and B_2 present in Pel RR crude homogenate was investigated using the acetylcholinesterase inhibitor eserine. Previous experiments showed that 10 μM eserine completely inhibits the activity of A_2 but not the activity of B_2 (see section 2.3.2.2.2.). When the crude homogenate of Pel RR was inhibited with 10 μM eserine a remaining activity of about 30%, which probably represents the activity of B_2, was obtained. Since the V_{max} of B_2 is about 7.5 fold less than that of A_2 for the assaying substrate p-nitrophenyl acetate (see TABLE 2.2), a rough estimation of about three times more B_2 than A_2 (30x7.5 B_2 : 70 A_2) can be made. However, when the inhibited crude homogenate was run on a native PAGE gel a faint A_2 band also appeared in addition to the B_2 band. The deacylation rate (k_3) of esterase A_2 for the carbamate eserine was then investigated to explain this observation. The k_3 for

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eserine was 0.237 hr^{-1}. This was similar to the k_3 of the other carbamate tested, propoxur (see TABLE 2.5). Upon entering into the gel the acylated enzyme is separated from the excess inhibitor and about 20-25% of the inhibited A_2 is reactivated during the electrophoresis run which takes about an hour. This amount is enough to produce a small band of esterase A_2 in the gel.

After the phenyl Sepharose column the A_2 and B_2 enzymes were separated and the recovered enzymatic protein ratio was about 60% A_2 and 12% B_2 (see TABLE 2.1). After the difference in V_{max} for the substrate for the two enzymes was normalised a proportion of 60% B_2 and 40% A_2 (12x7.5 B_2 : 60 A_2) can be determined for the recovered enzymes. Since the esterase B_2 is more labile than A_2, a higher proportion of B_2 than this can be expected in the crude homogenate. Higher availability of esterase B_2 in the crude homogenate was also demonstrated using native PAGE gels stained for the esterase activity. To obtain an A_2 band and a B_2 band with similar intensities to the respective crude homogenate bands, about 3-4 times more purified B_2 than purified A_2 had to be applied (FIGURE 2.11).

2.3.4. SUMMARY OF THE ROLE OF A_2 AND B_2 ESTERASES IN MOSQUITO LARVAE

In a purification procedure begun with 600-800 mg of total protein, about 1-1.5

FIGURE 2.11

Native PAGE of different amounts of purified A_2 and B_2 proteins (Stained for the activity with both α- and β-naphthyl acetate).

Lane a) PelRR crude homogenate, b) $0.1\mu g$ of B_2, c) $0.02\mu g$ of A_2, d) $0.02\mu g$ of B_2, e) $0.002\mu g$ of A_2.

mg of pure esterases A_2 and B_2 could be obtained with a final recovery of about 40-45% esterase activity (TABLE 2.1). Assuming that the esterases A_2 and B_2 are mainly responsible for the high p-nitrophenyl acetate activity of the crude homogenate, it can be estimated that they comprise about 3 mg [(1.25x100)/42.5] or 0.4% of the total protein. According to the relative proportions determined previously about one fourth of this amount is esterase A_2 and the rest is esterase B_2. A mean protein estimation of 0.115 ± 0.038 mg per 4th instar larva could be obtained from the crude homogenates prepared from individual larvae (20 independent determinations). Therefore each larva has about 460 ng of A_2 and B_2 (0.115 mg x 0.4%). Since each esterase has a molecular weight of approximately 60,000, a concentration of approximately 7.67 pmol total of both A_2 and B_2 esterases per mosquito larva of PelRR strain can be estimated (460 ng/60,000). Thus the amount per mg of the larval protein is 66.7 pmol (7.67/0.115). The wet weight of a 4th instar mosquito larva is about 2.4 mg (determined by weighing 100 larvae). Therefore the concentration of both these esterases per mg of mosquito larva is 3.2 pmol (7.67/2.4). The calculated amount of paraoxon (molecular weight = 275.2), for example, that can be sequestered by esterases A_2 and B_2 in a 4th instar mosquito larva is about 2.1 ng (275.2 x 7.67 pmol). The deacylation rate (k_3) of the mosquito crude homogenate for paraoxon was determined to be 0.056 hr^{-1} and was similar to that of the pure enzymes. Therefore a single mosquito larva can hydrolyse about 0.118 ng of paraoxon per hour (2.1 x 0.056), which is 5.59% of the sequestered amount. Concentrations of the esterase E4 in resistant aphids have been shown to be in the range of 0.85-

24.7 pmol/mg aphid (Devonshire & Sawicki, 1979). For the most resistant aphid variant G6, E4 per aphid (10 pmol) could sequester 2.5 ng paraoxon and hydrolyse 0.83 ng per hour which was 33.2% of the sequestered amount. For the susceptible aphid variant these figures were 0.04 ng, 0.01 ng hr^{-1} and 25% respectively (Devonshire & Moores, 1982). Differences in the percentage hydrolysis of the sequestered amount between the resistant and susceptible enzymes may indicate a qualitative difference although the authors have not discussed it. It is clear that unlike the aphid E4, which can sequester and significantly hydrolyse the insecticide, the main role of the mosquito esterases A_2 and B_2 in insecticide resistance is sequestration. The rates of interaction of the mosquito esterases with organophosphates are also apparently slower than those of E4 as shown by their respective k_a values for paraoxon. It has been shown that unlike A_2 and B_2 esterases, aphid E4 is capable of interacting with pyrethroid insecticides. The (1S)trans-enantiomer of the pyrethroid permethrin is hydrolysed rapidly by E4 (Devonshire and Moores, 1982). In the most resistant variant of aphid, the E4 enzyme accounted for approximately 3% of the total protein of an aphid (Devonshire & Moores, 1982) whereas both esterases A_2 and B_2 comprise only 0.4% of the total protein in 4th instar PelRR larvae. The lower percentage content of these esterases in PelRR homogenate was also evident since a significant band at the level of A_2 and B_2 could not be observed when the crude homogenate was run on SDS-PAGE gels. In contrast, E4 protein could be identified as a prominent band in SDS electrophoresis of resistant aphid homogenates (Devonshire and Moores, 1982). However, our results are not

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compatible with the percentages estimated for the esterases A_1 and B_1 by Fournier *et al.* (1987). Using partial purification procedures they showed that esterase A_1 constitutes 1-3% of the proteins of OP resistant *Cx pipiens* (S54 strain) whereas esterase B_1 constitutes 6-12% of the proteins of TEM-R *Cx quinquefasciatus* (Fournier *et al.*, 1987).

Greater efficiencies of the carboxylesterase based resistance mechanism in aphids may have an additional advantage of detoxifying the poisonous compounds found in the plant materials on which they feed. It is now known that OPs are not always man-made *eg.* a naturally occurring organophosphorous compound which inhibits AChE in a progressive manner has been found in cyanobacterium *Anabeana flos-aquae* (Edward and Carmichael, 1991). Most of the distribution studies show that increased activity of carboxylesterases is mainly found in association with the digestive tract of the resistant insects (see section 1.8.2.). These factors probably indicate that the evolution of the esterase-based resistance mechanisms in insects may have primarily occurred as an adaptation to the toxic materials found in their environment.

2.4. SUMMARY

Characterization of the purified mosquito carboxylesterases A_2 and B_2 revealed that their major role in insecticide resistance is sequestration (rapid binding followed by slow turn over of the insecticide) and the bimolecular rate constant,

k_a is the most important constant which correlates directly with the insecticide resistance ratios of the strain. A concentration of approximately 7.67 pmol of both A_2 and B_2 esterases accounting for about 0.4% of the total protein could be estimated per 4th instar larva of PelRR strain. It was also estimated that the $A_2 : B_2$ ratio present in this strain is about 1 : 3.

CHAPTER 3

Chapter 3

CHARACTERIZATION OF 'A' AND 'B' TYPE ESTERASES FROM DIFFERENT POPULATIONS OF *CULEX QUINQUEFASCIATUS*

3.1. INTRODUCTION

From the studies performed with the esterases A_2 and B_2 of the PelRR strain it was clear that both enzymes are equally important in insecticide resistance and the role of these elevated carboxylesterases in resistance is sequestration. According to the hypothesis put forward by Raymond *et al.* (1991), amplification of A_2 and B_2 esterases has arisen only once and through a recent migration spread worldwide (see section 1.6.3.3.). If this is true then all amplified A_2 and B_2 esterases would be the same. This hypothesis does not however account for the different resistance patterns observed in the *Culex* strains which contain only this resistance mechanism (Georghiou and Pasteur, 1978; Amin and Peiris, 1990; Hemingway *et al.*, 1990; Peiris and Hemingway, 1990a; 1990b). The present study was carried out to investigate whether the qualitative differences occur between the amplified A_2 esterases and B_2 esterases. Identity of the susceptible non-amplified forms were also investigated. Both esterases A_2 and B_2 were purified and characterized from one susceptible and a further four resistant (*ie.* in addition to the PelRR) strains of *Culex quinquefasciatus*, which originated from

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different geographical areas. Since the purified susceptible 'B' esterase appeared to be similar to the esterase type B_1 in native electrophoretic mobility, two B_1 esterases were also purified from two different strains and characterized for comparison. The strains used in this study were chosen to represent different samples of enzymes in space and time, as well as representing different pesticide regimes. Two of the locations were also identical to those used in Raymond's study.

3.2. MATERIALS AND METHODS

3.2.1. MOSQUITO STRAINS

The strains **Dar91, Tanga85** and **Muheza** were collected respectively from Dar es Salaam, Tanga and Muheza in Tanzania. The **Dar91** strain originated from a resistant population which had been selected in the field by chlorpyrifos for a number of years and then by fenitrothion, since 1988. It was colonized in 1991. Since colonization this strain was maintained without any selection pressure. The **Tanga85** strain originated from a chlorpyrifos resistant field population and was colonized in 1985. This strain has been maintained in the laboratory under intermittent chlorpyrifos selection pressure. The field collection sites for **Dar91** and **Tanga85** are approximately 200 km apart. **Muheza** was collected in 1987 from an area where pesticides were not routinely used for *Culex* control. It has been maintained under intermittent chlorpyrifos selection since colonization.

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Muheza is approximately 60 km from Tanga and 140 km from Dar es Salaam. **SPerm** was collected from Jeddah, Saudi Arabia in 1989. Field selection of this population had been with temephos and then with a range of different pyrethroids. Immediately after colonization, it was selected for 20 generations with permethrin and subsequently selected intermittently with malathion and temephos in the laboratory. The strains **Col** and **Trinidad** were collected from Nuqui-Choco, Colombia and Port of Spain, Trinidad respectively in 1993. The area where **Col** originated was not under the pressure of insecticides used in mosquito control. The field population of **Trinidad** had been selected with malathion. Both **Col** and **Trinidad** strains were maintained in the laboratory without any selection pressure. The susceptible **PelSS** strain was obtained by single family selection for low esterase activity from the Sri Lankan **Pel** strain and lacks all the resistance mechanisms (see section 2.1).

All the strains except **PelSS** were OP resistant and showed elevated carboxylesterases as the major resistance mechanism.

3.2.2. *PURIFICATION AND CHARACTERIZATION OF CARBOXYLESTERASES*

Chemicals used were as detailed in section 2.2.2.. All the 'A' type and 'B' type esterases were purified using the methods previously adopted for PelRR A_2 and B_2 respectively (see section 2.2.5.1. and 2.2.5.2.). Characterizations of the purified

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esterases were also carried out according to the procedures described previously in chapter 2. Crude homogenates for the insecticide interaction experiments were prepared in ice-cold 50 mM phosphate buffer (pH 7.4) with 5% (v/v) glycerol and 10 mM DTT. Fresh or frozen 4th instar larvae were homogenized thoroughly and centrifuged at 15,000g for 5 minutes and the supernatant was used for the experiments.

3.3. RESULTS AND DISCUSSIONS

3.3.1. PURIFICATION OF CARBOXYLESTERASES

Initially esterases A_2 and B_2 were purified from Dar91, Tanga85, Muheza and SPerm strains. All these strains had increased activity of A_2 and B_2 as the major resistance mechanism (FIGURE 3.1). Using the previous methodologies both esterases A_2 and B_2 could be purified to homogeneity from these strains without any difficulty. Final enzyme preparations appeared as a single band on SDS-PAGE after Coomassie staining.

None of the carboxylesterases were elevated in the susceptible PelSS crude homogenate (see FIGURE 3.1). During the purification of PelSS, esterase peaks were difficult to detect with the substrate p-nitrophenyl acetate (especially after the phenyl Sepharose column). The specific activity of PelSS crude homogenate

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FIGURE 3.1

Equal amounts of crude homogenates from different strains on a native PAGE gel stained for esterase activity.
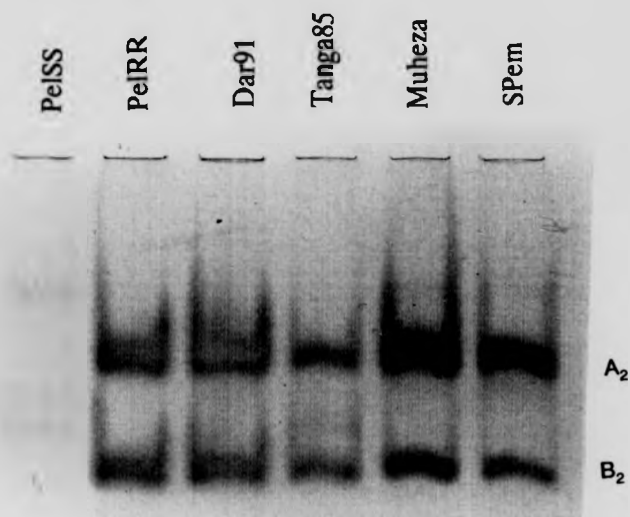
for the substrate p-nitrophenyl acetate was 0.02 units/mg. This is approximately 50x less than the 1.03 units/mg observed routinely for PelRR crude homogenate. It was shown in the previous chapter that the reactivity of both A_2 and B_2 esterases of PelRR was much greater towards p-nitrophenyl hexanoate than p-nitrophenyl acetate. For PelSS crude homogenate also a higher specific activity of 0.14 units/mg could be observed for p-nitrophenyl hexanoate. Therefore this was used as the assaying substrate during the PelSS purification. However when assaying several fractions at a time with this substrate, extra care had to be taken because of the nature of the p-nitrophenyl hexanoate hydrolysis which was linear only for about 30 seconds. Thus, in all purifications other than PelSS, p-nitrophenyl acetate hydrolysis was used, as this reaction was linear for at least 2 minutes. During the purification of PelSS also two esterase peaks corresponding to A_2 and B_2 were obtained after the phenyl Sepharose column. These were pooled separately and confirmed to be an 'A' type and a 'B' type esterase on a native PAGE gel. These two esterases were further purified as if they were A_2 and B_2 esterases and at the end of the purification about 5-10 μg of purified protein from each enzyme, which was barely enough to check the purity, could be obtained. Purifications started with greater amounts of materials did not result in any better recoveries. Therefore subsequent PelSS purifications were discontinued at the end of the hydroxylapatite step to obtain enough enzyme for characterization.

The B_1 esterases were also purified to homogeneity using the method developed

for esterase B_2. In both the Col and Trinidad strains, B_1 was the only 'B' type esterase elevated in the crude homogenates.

3.3.2 PHYSICAL CHARACTERIZATION

SDS-PAGE with purified esterases demonstrated that the molecular weights of the different A_2 enzymes were similar to that of PelRR A_2 (about 67 kDa) and the different B_2 and B_1 enzymes were similar to that of PelRR B_2 (about 62 kDa) (see section 2.3.2.1). A native PAGE gel with the purified 'A' type esterases is shown in FIGURE 3.2. It should be noted that the electrophoretic mobility of the susceptible 'A' was reproducibly faster than that of the resistant A_2s. In contrast FIGURE 3.3 of the 'B' esterases of the same strains showed that the susceptible 'B' esterase had a slower mobility than that of the resistant B_2s. Slight consistent variations between the A_2 bands and between the B_2 bands could also be observed. However the results clearly showed that the 'A' and 'B' type esterases purified from PelSS are markedly different from those esterases of the resistant strains in their electrophoretic mobility. According to the classification of Raymond *et al.* (1987) these susceptible 'A' and 'B' type esterases should be classified as A_n (where n is a number greater than 2) and B_1 respectively because of their electrophoretic mobilities (see section 1.6.1.). This similarity of the susceptible 'B' esterase to the B_1 esterases could be shown by PAGE with the purified PelSS 'B' esterase and the B_1 esterases purified from Col and Trinidad strains (FIGURE 3.4). PelRR B_2 esterase was also run on the

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FIGURE 3.2

A native PAGE gel of purified 'A' type carboxylesterases stained for esterase activity
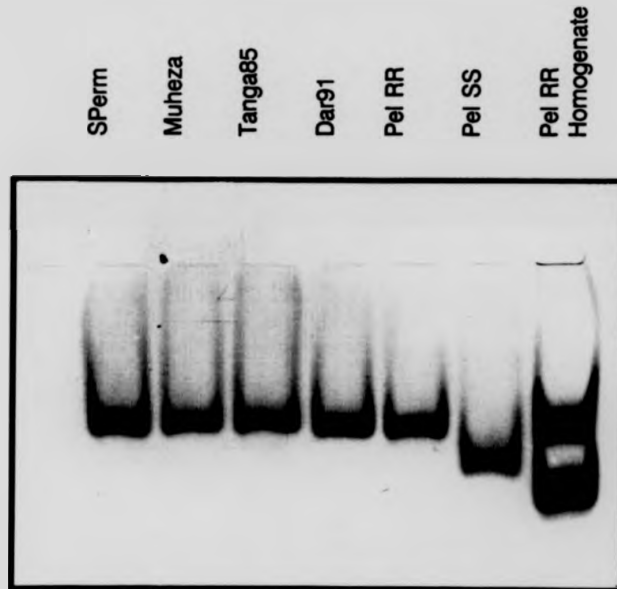
FIGURE 3.3

A native PAGE gel of purified 'B' type carboxylesterases stained for esterase activity
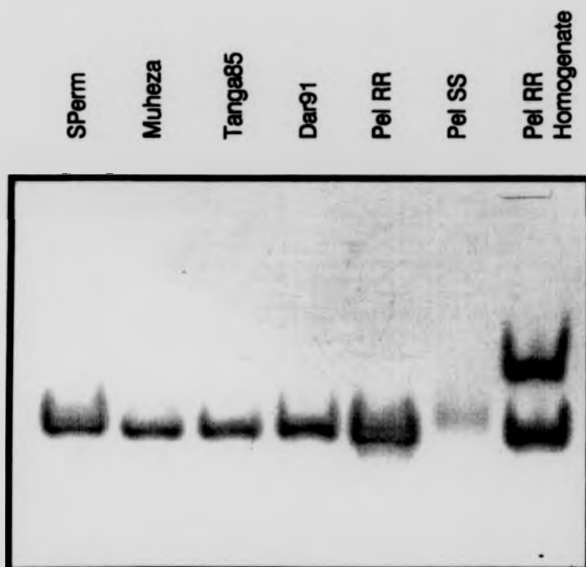
FIGURE 3.4

A native PAGE gel of purified B_1, B_2 and susceptible 'B' carboxylesterases stained for esterase activity

same gel for comparison.

3.3.3. CHEMICAL CHARACTERIZATION

Chemical characterization of 'A' type esterases was performed by the other members of the group. To examine possible kinetic differences between the purified esterases, they were all tested with the two substrates and five insecticides used previously. Kinetic data presented here are the means from at least three experiments for each substrate or insecticide using the enzymes from several different purifications for each strain.

3.3.3.1. Interactions with substrates

TABLE 3.1 shows the V_{max} and K_m values of two A_2 esterases and all four B_2 esterases for the substrates p-nitrophenyl acetate and p-nitrophenyl hexanoate. Values for PelRR enzymes are also presented for comparison. For the partially purified PelSS 'B' esterase V_{max} values could not be obtained because of the presence of other proteins and only the K_m values are presented. It should be noted that the K_m of the susceptible 'B' esterase for p-nitrophenyl acetate is about 2-3 times higher than that of the resistant B_2s. Several significant differences were observed among the K_m and V_{max} values of different A_2 esterases and B_2 esterases. The V_{max} of Tanga85 A_2 is twice than that of PelRR A_2 for both substrates. For p-nitrophenyl hexanoate the V_{max} of Muheza B_2 is

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TABLE 3.1

The substrate interactions of A_2 and B_2 carboxylesterases and the susceptible 'B' type carboxylesterase purified from different strains (Data for PelRR strain are also presented for comparison).

	p-nitrophenyl acetate		p-nitrophenyl hexanoate	
	V_{max} (units/mg)	K_m (μM)	V_{max} (units/mg)	K_m (μM)
A_2				
Dar91	609.4 ± 21.0^a	182.2 ± 18.3^a	954.6 ± 80.3^a	28.0 ± 6.9^a
Tanga85	893.1 ± 25.9^b	209.5 ± 17.0^a	1594 ± 171.0^b	29.0 ± 8.9^a
Pel RR	472.0 ± 51.4^a	145.8 ± 45.0^a	788.2 ± 74.8^a	35.4 ± 9.7^a
B_2				
Dar91	98.81 ± 1.33^a	85.41 ± 3.94^a	159.8 ± 15.4^a	13.7 ± 4.2^a
Tanga85	70.09 ± 1.49^b	90.11 ± 6.49^a	111.8 ± 7.21^b	7.45 ± 2.09^a
Muheza	77.56 ± 0.88^c	95.28 ± 3.72^a	83.43 ± 3.41^c	6.85 ± 1.51^a
SPerm	92.74 ± 1.62^d	86.76 ± 5.20^a	117.5 ± 5.98^b	7.05 ± 1.61^a
Pel RR	63.16 ± 0.75^e	140.8 ± 5.24^b	183.3 ± 17.2^a	15.3 ± 4.34^a
Sus. 'B'				
PelSS	-	247.2 ± 23.3^c	-	12.2 ± 2.21^a

The data are means ± standard deviations. In the same column different superscript letters indicate a significant difference ($P < 0.05$) among 'A' esterases or among 'B' esterases.

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same gel for comparison.

3.3.3. CHEMICAL CHARACTERIZATION

Chemical characterization of 'A' type esterases was performed by the other members of the group. To examine possible kinetic differences between the purified esterases, they were all tested with the two substrates and five insecticides used previously. Kinetic data presented here are the means from at least three experiments for each substrate or insecticide using the enzymes from several different purifications for each strain.

3.3.3.1. Interactions with substrates

TABLE 3.1 shows the V_{max} and K_m values of two A_2 esterases and all four B_2 esterases for the substrates p-nitrophenyl acetate and p-nitrophenyl hexanoate. Values for PelRR enzymes are also presented for comparison. For the partially purified PelSS 'B' esterase V_{max} values could not be obtained because of the presence of other proteins and only the K_m values are presented. It should be noted that the K_m of the susceptible 'B' esterase for p-nitrophenyl acetate is about 2-3 times higher than that of the resistant B_2s. Several significant differences were observed among the K_m and V_{max} values of different A_2 esterases and B_2 esterases. The V_{max} of Tanga85 A_2 is twice than that of PelRR A_2 for both substrates. For p-nitrophenyl hexanoate the V_{max} of Muheza B_2 is

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TABLE 3.1

The substrate interactions of A_2 and B_2 carboxylesterases and the susceptible 'B' type carboxylesterase purified from different strains (Data for PelRR strain are also presented for comparison).

	p-nitrophenyl acetate		p-nitrophenyl hexanoate	
	V_{max} (units/mg)	K_m (μM)	V_{max} (units/mg)	K_m (μM)
A_2				
Dar91	609.4 ± 21.0^a	182.2 ± 18.3^a	954.6 ± 80.3^a	28.0 ± 6.9^a
Tanga85	893.1 ± 25.9^b	209.5 ± 17.0^a	1594 ± 171.0^b	29.0 ± 8.9^a
Pel RR	472.0 ± 51.4^a	145.8 ± 45.0^a	788.2 ± 74.8^a	35.4 ± 9.7^a
B_2				
Dar91	98.81 ± 1.33^a	85.41 ± 3.94^a	159.8 ± 15.4^a	13.7 ± 4.2^a
Tanga85	70.09 ± 1.49^b	90.11 ± 6.49^a	111.8 ± 7.21^b	7.45 ± 2.09^a
Muheza	77.56 ± 0.88^c	95.28 ± 3.72^a	83.43 ± 3.41^c	6.85 ± 1.51^a
SPerm	92.74 ± 1.62^d	86.76 ± 5.20^a	117.5 ± 5.98^b	7.05 ± 1.61^a
Pel RR	63.16 ± 0.75^e	140.8 ± 5.24^b	183.3 ± 17.2^a	15.3 ± 4.34^a
Sus. 'B'				
PelSS	-	247.2 ± 23.3^c	-	12.2 ± 2.21^a

The data are means ± standard deviations. In the same column different superscript letters indicate a significant difference ($P < 0.05$) among 'A' esterases or among 'B' esterases.

half of the V_{max} values of Dar91 B_2 and PelRR B_2. In general, the V_{max} differences are much greater for p-nitrophenyl acetate than for p-nitrophenyl hexanoate. These results demonstrate that qualitative differences exist between both the A_2 esterases and the B_2 esterases purified from the different strains. It is also shown that the susceptible 'B' enzyme is markedly different from the B_2 esterases both in electrophoretic mobility and the kinetics of its interaction with these two substrates.

3.3.3.2. Interactions with insecticides

Since it was shown in the previous chapter on the PelRR resistant strain that the k_a is the most important constant to determine the rate of interaction between these enzymes and the insecticides, only the k_a values were determined for the esterases from the four other resistant strains and the susceptible PelSS strain. TABLE 3.2 shows the k_a values for the interaction of all the purified 'A' type esterases with chlorpyrifos-oxon, fenitrooxon, malaoxon, paraoxon and propoxur. Data for PelRR A_2 are also presented for comparison. Several significant differences were observed between the k_a values for the different enzymes for the same insecticide. Differences are about 10x between Dar91 and PelSS for chlorpyrifos-oxon and also between PelRR and Tanga85 for fenitrooxon. PelSS 'A' enzyme reacted more slowly than all the resistant A_2 enzymes with chlorpyrifos-oxon and paraoxon showing that it is not only amplified but also less able to bind the insecticides than its respective 'resistant' counterparts. The

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TABLE 3.2

The kinetic constant k_a (M^{-1} min^{-1}) for insecticide interactions with elevated A_2 and susceptible (PelSS) 'A' carboxylesterases (Data for PelRR A_2 are also presented for comparison).

	$10^{-6} \times k_a$					
	Pel SS	Pel RR	Dar91	Tanga85	Muheza	SPerm
Chlorpyrifos-oxon	53.3 ± 10.7[a]	144 ± 32.9[b]	521 ± 91.9[c]	374 ± 74.3[c]	709 ± 122[c]	726 ± 158[c]
Fenitrooxon	3.43 ± 1.19[ab]	0.958 ± 0.201[a]	4.53 ± 0.80[b]	9.35 ± 1.54[c]	2.89 ± 0.57[b]	3.16 ± 0.649[b]
Malaoxon	0.113 ± 0.022[ab]	0.219 ± 0.033[a]	0.130 ± 0.031[ab]	0.086 ± 0.010[b]	0.229 ± 0.032[a]	0.204 ± 0.034[a]
Paraoxon	131 ± 16.4[a]	190 ± 20.7[ab]	143 ± 28.6[a]	119 ± 24.3[a]	387 ± 72.4[c]	351 ± 67.0[bc]
Propoxur	0.0018 ± 0.0004[a]	0.0124 ± 0.0027[bc]	0.0151 ± 0.0023[b]	0.0063 ± 0.0011[c]	0.0203 ± 0.0040[b]	0.0211 ± 0.0047[b]

The data are means ± standard deviations. In the same row different superscript letters indicate a significant difference ($P < 0.05$).

respective k_a values for the 'B' type esterases are presented in TABLE 3.3. Although the differences among the 'B' esterases were less than those for the 'A' enzymes, between the susceptible 'B' and B_2 enzymes the differences were significant. Efficiencies in detoxifying chlorpyrifos-oxon and paraoxon of PelSS 'B' enzyme is respectively 1000x and 100x less than those of the resistant strains. In addition, the organophosphates malaoxon and fenitrooxon were of use in distinguishing between some of the B_2 enzymes from the resistant strains.

The kinetic differences presented here further elaborate the idea that functional variants exist among the A_2 and the B_2 esterases and more than one allele from each of the A_2 and B_2 esterase genes are amplified. Two of the resistant strains used in the present study (PelRR and Dar91) were collected from the same locations as in Raymond's study. It should be noted that the esterases from Dar91 and Tanga85, which were collected at 6 year interval from the same country, are also kinetically different from each other. This suggests the possibility that more than one amplified allelic form of the A_2 gene and the B_2 gene may occur in the same geographical location. The susceptible 'A' and 'B' enzymes are less reactive with a range of insecticides and electrophoretically different from the respective resistant enzymes. Since both the PelSS and PelRR strains were derived from the same Pel parental colony it is clear that different non-amplified susceptible alleles can co-exist with amplified resistant alleles in the same population. Differences between the resistant enzymes and the susceptible enzymes in insecticide binding suggest that there has been a positive

TABLE 3.3

The kinetic constant k_a (M^{-1} min^{-1}) for insecticide interactions with elevated B_2 and susceptible (PelSS) 'B' carboxylesterases (Data for PelRR B_2 are also presented for comparison).

	$10^{-5} \times k_a$					
	Pel SS	Pel RR	Dar91	Tanga85	Muheza	SPerm
Chlorpyrifos-oxon	2.29 ± 0.811[a]	1550 ± 140[b]	1670 ± 269[b]	2100 ± 397[b]	1750 ± 324[b]	2090 ± 524[b]
Fenitrooxon	0.328 ± 0.008[a]	1.60 ± 0.300[b]	5.67 ± 0.957[c]	3.08 ± 0.183[d]	4.06 ± 0.630[cd]	5.24 ± 0.503[c]
Malaoxon	0.400 ± 0.050[a]	0.496 ± 0.168[ab]	0.553 ± 0.195[ab]	0.513 ± 0.097[ab]	0.383 ± 0.057[a]	0.615 ± 0.015[b]
paraoxon	1.94 ± 0.054[a]	170 ± 53.1[b]	181 ± 24.3[b]	139 ± 42.0[b]	170 ± 24.3[b]	154 ± 29.2[b]
propoxur	<0.0001[a]	0.0052 ± 0.0017[b]	0.0074 ± 0.0014[b]	0.0048 ± 0.0004[b]	0.0065 ± 0.0007[b]	0.0061 ± 0.0018[b]

The data are means ± standard deviations. In the same row different superscript letters indicate a significant difference ($P<0.05$).

insecticide selection pressure for the selection and amplification of the resistant enzymes.

As the 'B' esterase in the PelSS susceptible strain had identical electrophoretic mobility to the amplified B_1 esterase, the hypothesis that these are identical isoenzymes was tested. The PelSS 'B' esterase was compared with the two elevated B_1 esterases purified from Col and Trinidad strains with respect to their interaction with the insecticides (TABLE 3.4). Data for PelRR B_2 are also presented for comparison. It should be noted that the reactivity of PelSS 'B' is significantly different from that of B_1 esterases for all the insecticides tested. B_1 esterases are 1000x and 100x more reactive than PelSS 'B' esterase for chlorpyrifos-oxon and paraoxon respectively. The two elevated B_1s are, therefore more similar to the elevated B_2 esterases than to the isoenzyme from the susceptible strain. For malaoxon and fenitrooxon, the Col B_1 and Trinidad B_1 are not only significantly different from that of PelSS, but also show a 20- and 10-fold difference from each other. In summary all four enzymes presented in TABLE 3.4 are kinetically different from each other, and the differences are much greater between the susceptible 'B' type esterase and either of the elevated resistant B_1 or B_2 esterases. This illustrates that in their insecticide interactions, the elevated B_1 esterases are more similar to the B_2 esterases (despite the different electrophoretic mobilities) than the susceptible 'B' esterases with identical electrophoretic mobilities (see FIGURE 3.4). Hence, the electrophoretic mobility of the esterases is an extremely poor indicator of the

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TABLE 3.4

The kinetic constant k_a (M^{-1} min^{-1}) for insecticide interactions with B_1 carboxylesterases purified from Columbian and Trinidad strains. Data for PelRR B_2 and PelSS 'B' are also presented for comparison.

Insecticide	k_a x 10^{-5}	(M^{-1} min^{-1})		
	Colombian B_1	Trinidad B_1	PelSS 'B'	PelRR B_2
Chlorpyrifos-oxon	$1230.0^a \pm 203.0$	$1210.0^a \pm 248.0$	$2.290^b \pm 0.811$	$1550.0^a \pm 140.0$
Fenitrooxon	$7.092^a \pm 1.279$	$0.223^b \pm 0.034$	$0.328^c \pm 0.008$	$1.600^d \pm 0.300$
Malaoxon	$1.300^a \pm 0.130$	$0.074^b \pm 0.014$	$0.400^c \pm 0.050$	$0.496^c \pm 0.168$
paraoxon	$100.0^a \pm 6.901$	$73.80^a \pm 9.330$	$1.942^b \pm 0.054$	$170.2^a \pm 53.10$
propoxur	$0.0033^a \pm 0.0005$	$0.0041^a \pm 0.0007$	$<0.001^b$	$0.0052^a \pm 0.0017$

The data are means ± standard deviations. In the same row different superscript letters indicate a significant difference ($P < 0.05$).

actual allelic variant or variants present in a given strain and the classification introduced by Raymond *et al.* (1987) (see section 1.8.1.) needs to be revised.

3.3.3.3. Interaction of crude homogenates with insecticides

To examine whether the kinetic differences shown by the purified enzymes of the resistant and susceptible strains could be seen at the crude homogenate level, the k_a values for the insecticide interactions were determined for PelRR and PelSS larval crude homogenates (TABLE 3.5). The data revealed that even at this level the rate of interaction can be seen to the same magnitude as for purified enzymes (see TABLE 3.2 and 3.3). As with the purified 'B' enzymes, in the crude homogenates also the greatest differences were for chlorpyrifos-oxon and paraoxon. However, for PelRR, any correlation between the crude homogenate data and the purified A_2 and B_2 data with the previously determined A_2 and B_2 proportions (see section 2.3.2.3.) could not be made. This may be due to the presence of other enzymes and interfering factors in the crude homogenate. It was shown earlier that the kinetic differences among the resistant enzymes are less prominent than they are between the resistant and susceptible enzymes. Also, the A_2/B_2 proportion may vary from strain to strain. Therefore the differences observed among the resistant enzymes may not be expected to be seen at crude homogenates. Still, this may be used as a crude method in detecting the level of interaction between these enzymes and insecticides. Insecticide interaction experiments with the TEM-R crude homogenate were

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TABLE 3.5

The kinetic constant k_a (M^{-1} min^{-1}) for insecticide interactions with the larval crude homogenates of PelSS and PelRR strains.

	$10^{-5} \times k_a$	
	PelSS	PelRR
Chlorpyrifos-oxon	1.25 ± 0.33	694 ± 98.0
Fenitrooxon	0.554 ± 0.070	2.05 ± 0.47
Malaoxon	0.040 ± 0.004	0.251 ± 0.028
Paraoxon	7.52 ± 0.78	168 ± 24.2
Propoxur	0.0003 ± 0.0000	0.0075 ± 0.0018

carried out by Cuany *et al.* (1993) and the I_{50} values have been reported for several insecticides. I_{50} values are not accurate measures for the time dependent rapid inhibitions of these esterases and can not be compared with the kinetic constants determined in the present study.

3.4. SUMMARY

Results presented in this chapter demonstrate the presence of qualitatively different A_2 and B_2 esterases in different populations of *Cx quinquefasciatus*. Kinetic differences among the A_2 esterases and the B_1 esterases were more profound than the differences between the B_2 esterases. Susceptible non-amplified enzymes were markedly less reactive than the resistant enzymes with some of the insecticides and these differences could be identified even in the crude homogenates of these mosquitoes. It was also shown that the esterases with similar electrophoretic mobilities can be significantly different from each other in their insecticide kinetics.

CHAPTER 4

CHARACTERIZATION OF DIFFERENT A_2 AND B_2 ESTERASES DERIVED FROM A SINGLE POPULATION OF *CULEX QUINQUEFASCIATUS*

4.1. INTRODUCTION

After observing the differences among both the A_2 esterases and the B_2 esterases purified from different populations of *Cx quinquefasciatus*, it was evident that each of the esterase loci A_2 and B_2 overproduces more than one amplified allelic form. The present study was designed to investigate whether different isoenzymes of elevated A_2 and B_2 can be found within a single population. Three sub-colonies were selected with different insecticides from a single parental colony. This population was a field collected strain which was heterogeneous with respect to resistance. The major resistance mechanism in the parental population was elevation of the A_2 and B_2 esterases. After selection the sub-colonies were examined for qualitative differences in their A_2 and B_2 esterases. Selection also resulted in larger than expected differences in the cross-resistance spectra of the different sub-colonies. Hence, the presence of resistance mechanisms other than the esterase based mechanism were also investigated.

4.2. MATERIALS AND METHODS

The chemicals used were as described in Chapter 2.

4.2.1. MOSQUITO STRAINS

The field collected Pel colony, described previously in section 2.1., was used as the parental colony. Since colonization it has been maintained without insecticide selection pressure. Three sub-colonies; Pel-Chl, Pel-Mal and Pel-Pro were selected for four generations with chlorpyrifos, malathion and propoxur respectively by exposing the 4th instar larvae of each generation to the calculated LC_{80} concentrations. The PelRR and PelSS strains, both of which were derived from the Pel parental colony, were also used for comparative purposes.

4.2.2. LARVAL BIOASSAYS

Larval bioassays were performed by exposing batches of 25 fourth instar larvae to known insecticide concentrations in 250 ml of distilled water (WHO, 1981). Insecticide solutions were made in ethanol and 1 ml of the alcohol solution added to the water. For each bioassay at least five concentrations giving mortality between 0-100% were tested, and four replicates were set for each concentration. Bioassays were always performed including a control of 1 ml of alcohol alone. There were no mortalities in the control. After 24 hr pesticide

exposure at $25 \pm 2^{\circ}C$ the number of dead larvae were counted. Mortality data were subjected to probit regression with an unpublished program written by C. J. Schofield (WHO, Geneva) based on the method of Finney (1971) and the lethal concentrations which gave 50% (LC_{50}) and 80%(LC_{80}) mortalities were calculated. The calculated LC_{80} for each insecticide for each generation was then taken as the dose with which to select the bulk of the larvae in that generation. Survivors of the pesticide treatment were transferred to clean water, fed as usual and reared to adulthood to establish the next generation.

4.2.3. BIOASSAYS WITH PIPERONYL BUTOXIDE

The possible involvement of monooxygenases in resistance was investigated by bioassays using the synergist piperonyl butoxide (PB) with the insecticides chlorpyrifos and propoxur. Bioassays were carried out by exposing the larvae to known insecticide concentrations with and without PB. 75 μl of the PB stock solution (in acetone) was added into 250 ml of distilled water with insecticide to give a final PB concentration of 3 mg/L. Control experiments confirmed that 24 hrs exposure to this concentration of PB alone was not lethal to mosquito larvae. Log dosage mortality regression lines were plotted for each insecticide with and without PB for each sub-colony.

4.2.4. GLUTATHIONE S-TRANSFERASE ASSAY

95 fourth instar larvae were placed in a microtitre plate (one larva per well) and each was homogenised thoroughly in 150 μl of distilled water using a multiple homogeniser (ffrench-Constant and Devonshire, 1987). 10 μl of the homogenate from each larva was placed in a microtitre plate and 200 μl of the substrate solution [95 parts of 10.5 mM reduced glutathione (GSH) in 100 mM phosphate buffer + 5 parts of 63 mM 1-chloro-2,4-dinitrobenzene (CDNB) in methanol] was added to each well. The reaction was measured at 340 nm for 5 minutes. An extinction co-efficient of 5.76 mM^{-1} (corrected for the path length) was used to convert the absorbances to moles. Protein concentration of each supernatant was measured according to the method described previously (see section 2.2.4.).

4.2.5. PURIFICATION OF CARBOXYLESTERASES

After four generations of insecticide selection, the unexposed fifth generation was used as the enzyme source for esterase purification work. Carboxylesterases A_2 and B_2 were partially purified from the frozen batches of 4th instar larvae from each sub-colony by sequential column chromatography on Q-Sepharose, phenyl Sepharose and hydroxylapatite as described in the section 2.2.5.1. and 2.2.5.2. respectively.

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4.2.6. *KINETIC CONSTANTS FOR CARBOXYLESTERASES AND ACETYLCHOLINESTERASES*

Inhibition kinetics of partially purified A_2 and B_2 esterases with the insecticides were carried out using the same methods detailed in section 2.2.6.1.2.

For the inhibition kinetics of AChE crude homogenates were prepared from frozen 4th instar larvae in ice-cold 50 mM phosphate buffer pH 7.4, with 25 mM dithiothreitol and 5% (v/v) glycerol. The supernatant of the centrifuged sample (15,000g for 5 minutes) was incubated with 5-100 μM of propoxur. 20 μl aliquots were withdrawn at 10 second intervals for two minutes and each aliquot was immediately added to an excess of substrate solution [25 μl of 10 mM acetylthiocholine iodide in distilled water + 10 μl of 10 mM 5,5' dithio-bis- (2 nitrobenzoic acid) (DTNB) in 100 mM phosphate buffer (pH 7.0) + 145 μl of 100 mM phosphate buffer (pH 7.8) with 1% Triton X-100] to stop the inhibition. Remaining AChE activity was read at 405 nm for 5 minutes. Uninhibited homogenate was assayed to obtain the 100% activity control.

Log % remaining activity was plotted against time for each experiment. The inhibition co-efficients (k_is) were calculated according to the following equation.

$$k_i = (\Delta \log V)2.303 \, / \, [I]t$$

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where; 'V' is the % remaining activity at the time 't' and [I] is the propoxur concentration.

The k_i values were calculated for each time point separately and averaged to obtain the final value.

4.3. RESULTS AND DISCUSSION

4.3.1. CROSS-RESISTANCE SPECTRA OF THE SUB-COLONIES

Increased activity of A_2 and B_2 was the major resistance mechanism for the observed organophosphate resistance of the field-collected Pel strain (Peiris and Hemingway, 1990a). In the present study, three sub-colonies were selected from the parental strain using two organophosphates and one carbamate. During the selection resistance to the selecting insecticide increased dramatically with associated cross-resistance increasing to the other two insecticides. TABLE 4.1 presents the resistance ratios for the parental colony prior to selection and for the three sub-colonies after four generations of selection at the 80% mortality level. Irrespective of the insecticide used for the selection, all three sub-colonies showed the greatest resistance ratio to chlorpyrifos. Also the colony selected with chlorpyrifos (Pel-Chl) had the highest resistance to all three insecticides. It was 1.7x more resistant to malathion and about 5x more resistant to chlorpyrifos and

TABLE 4.1

Resistance ratios (LD_{50} of larvae/ LD_{50} of PelSS larvae) for the parental colony and the three insecticide selected sub-colonies of *Cx quinquefasciatus* for the insecticides used for the selection.

Insecticide	Pel	Pel-Chl	Pel-Mal	Pel-Pro
Chlorpyrifos	32.0	1300	226.7	303.3
Malathion	4.82	175.0	100.6	100.6
Propoxur	2.30	948.3	161.9	154.2

propoxur than the other two sub-colonies. Cross-resistance spectra of Pel-Mal and Pel-Pro showed a similar pattern of resistance even though they were selected with insecticides belong to two different classes.

4.3.2. DIFFERENCES AMONG THE CARBOXYLESTERASES ELEVATED IN THE SUB-COLONIES

Esterases A_2 and B_2 were elevated in the crude homogenates of all three colonies (FIGURE 4.1). Larvae of the 5th generation, which had not been exposed to insecticides, were used for enzyme purifications. During the purification, esterases A_2 and B_2 were separated from each other after the phenyl Sepharose column and each enzyme was pooled separately and further purified by hydroxylapatite chromatography. A purification factor of about 200 for esterase A_2 and about 50 for esterase B_2 were obtained (see TABLE 2.1). Identification of each of the partially purified enzyme was confirmed by a native PAGE gel (FIGURE 4.2).

Previously it was shown that the bimolecular rate constant, k_a, is the most important constant which correlates directly with the insecticide resistance ratios of the strain (see section 2.3.2.2.3.). Interactions of all six partially purified enzymes with the three insecticides, used for selection, were investigated. TABLE 4.2 shows the k_a values of the sub-colony A_2 esterases together with those of PelRR A_2. The strain PelRR was also selected from the same Pel

FIGURE 4.1

A native PAGE gel of crude homogenates from the insecticide selected sub-colonies of Pel stained for esterase activity. Pel and PelRR crude homogenates were also run for comparison.
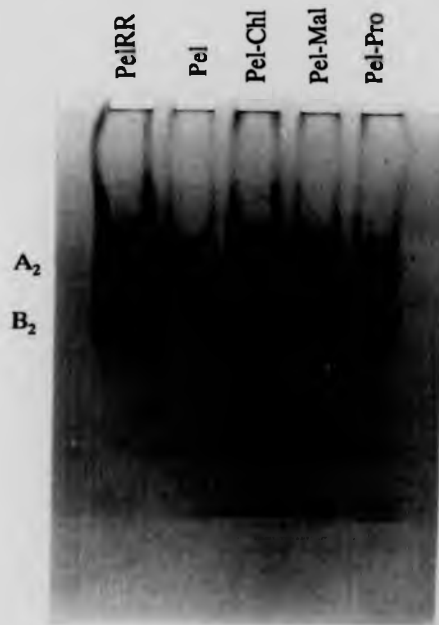
FIGURE 4.2

A native PAGE gel of A_2 and B_2 esterases partially purified from insecticide selected sub-colonies of Pel stained for esterase activity.
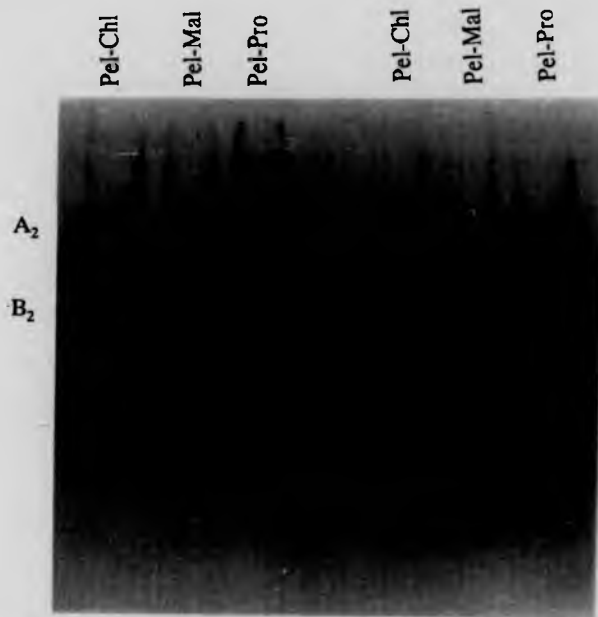
TABLE 4.2

The k_a values for the interaction of partially purified sub-colony A_2 esterases with the insecticides used for the selection. (data for PelRR A_2 are also presented for comparison).

Insecticide	$10^{-5} \times k_a$		$(M^{-1} min^{-1})$	
	Pel-Chl	Pel-Mal	Pel-Pro	PelRR
Chlorpyrifos-oxon	$289.1^a \pm 58.90$	$1220^b \pm 128.1$	$557.0^c \pm 60.32$	$144.2^a \pm 32.90$
Malaoxon	$0.266^a \pm 0.045$	$0.427^b \pm 0.032$	$0.415^{ab} \pm 0.059$	$0.219^c \pm 0.033$
Propoxur	$0.012^a \pm 0.0016$	$0.018^a \pm 0.0054$	$0.018^a \pm 0.0038$	$0.012^a \pm 0.0027$

The data are means ± standard deviations. In the same row different superscript letters indicate a significant difference ($P < 0.05$).

colony with the organophosphorus insecticide temephos (see section 2.1.). Therefore it is also a sub-colony of the Pel parental colony. However, to avoid confusion it will be referred to as PelRR throughout. The three sub-colony A_2 esterases were significantly different from each other in their interactions with chlorpyrifos-oxon and two of them, Pel-Mal A_2 and Pel-Pro A_2, were also different from PelRR A_2. For malaoxon, significant differences were observed among the A_2 enzymes from Pel-Chl, Pel-Mal and PelRR and also between Pel-Pro A_2 and PelRR A_2.

Enzyme-insecticide interaction data for B_2 esterases are presented in TABLE 4.3. Only malaoxon interacted differently with the different B_2 enzymes showing a significant difference between Pel-Pro and PelRR. It was shown in Chapter 3 that the B_2 esterases from different strains are also less different from each other kinetically than the A_2 enzymes (see section 3.3.3.2.). However these data for enzyme-insecticide interactions indicate that different alleles or allelic mixtures of esterase loci A_2 and B_2 exist in the different sub-colonies selected from the same population, therefore the parental 'Pel' colony must contain an allelic mixture. These colonies were selected only for four generations and a greater allelic segregation may have occurred if the colonies were selected further.

According to the reactivities of the sub-colony A_2 and B_2 esterases the resistance of Pel-Chl to all the insecticides tested should be less than that of the other two colonies which is not in agreement with the resistance ratio data in TABLE 4.1.

TABLE 4.3

The k_a values for the interaction of partially purified sub-colony B_2 esterases with the insecticides used for the selection. (data for PelRR B_2 are also presented for comparison).

Insecticide	$10^{-5} \times$ k$_a$ (M^{-1} min^{-1})			
	Pel-Chl	Pel-Mal	Pel-Pro	PelRR
Chlorpyrifos-oxon	1440^a ± 158.0	1560^a ± 208.2	1511^a ± 181.1	1550^a ± 140.1
Malaoxon	1.200^{ab} ± 0.213	1.293^{ab} ± 0.245	1.260^a ± 0.146	0.500^b ± 0.170
Propoxur	0.0058^a ± 0.001	0.0059^a ± 0.002	0.0104^a ± 0.020	0.0052^a ± .002

The data are means ± standard deviations. In the same row different superscript letters indicate a significant difference ($P < 0.05$).

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During the purifications slightly higher amounts of A_2 and B_2 esterases could be recovered from Pel-Chl sub-colony suggesting the presence of higher quantities of these enzymes. But this could not account for the very high resistance shown by Pel-Chl. Specific activities of the crude homogenates of the Pel-Chl, Pel-Mal and Pel-Pro for the substrate p-nitrophenyl acetate were 0.54, 0.52 and 0.50 μmol min^{-1} mg^{-1} respectively indicating the lack of quantitative differences. Also the relative differences of the affinities of the enzymes towards different insecticides do not correlate with the relative differences of the resistance ratios shown by each sub-colony. The presence of resistance mechanisms other than the esterase-based mechanism were investigated to explain the differences observed in the cross-resistance spectra .

4.3.3. THE PRESENCE OF OTHER RESISTANCE MECHANISMS

4.3.3.1. Cytochrome P-450 mechanism

The different classes of insecticides may select different isozymes of cytochrome P-450s. Therefore, the effect of the synergist PB with an phosphorothionate (chlorpyrifos) and a carbamate (propoxur) was determined. None of the sub-colonies showed significant differences at the 95% confidence level between their log probit mortality lines plotted for the bioassays with and without PB (FIGURES 4.3-4.8). This indicates that monooxygenases are not responsible for the observed resistance of the sub-colonies. Although some investigators have

FIGURE 4.3

Log-dosage probit mortality lines for the sub-colony Pel-Chl tested with chlorpyrifos with and without PB (95% confidence limits are indicated by dashed lines).
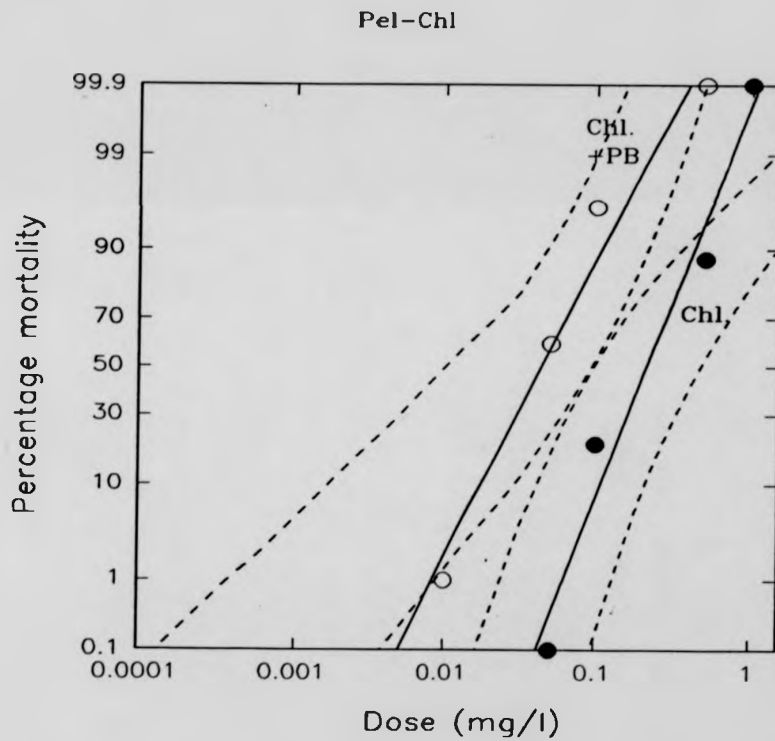
Pel-Chl

FIGURE 4.4

Log-dosage probit mortality lines for the sub-colony Pel-Chl tested with propoxur with and without PB (95% confidence limits are indicated by dashed lines).

Pel—Chl
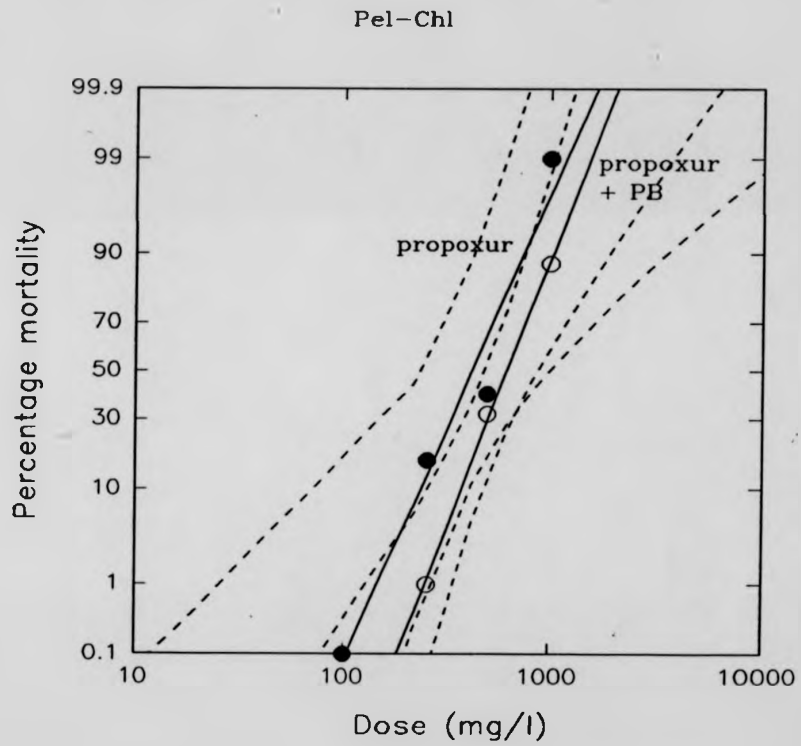
FIGURE 4.5

Log-dosage probit mortality lines for the sub-colony Pel-Mal tested with chlorpyrifos with and without PB (95% confidence limits are indicated by dashed lines).
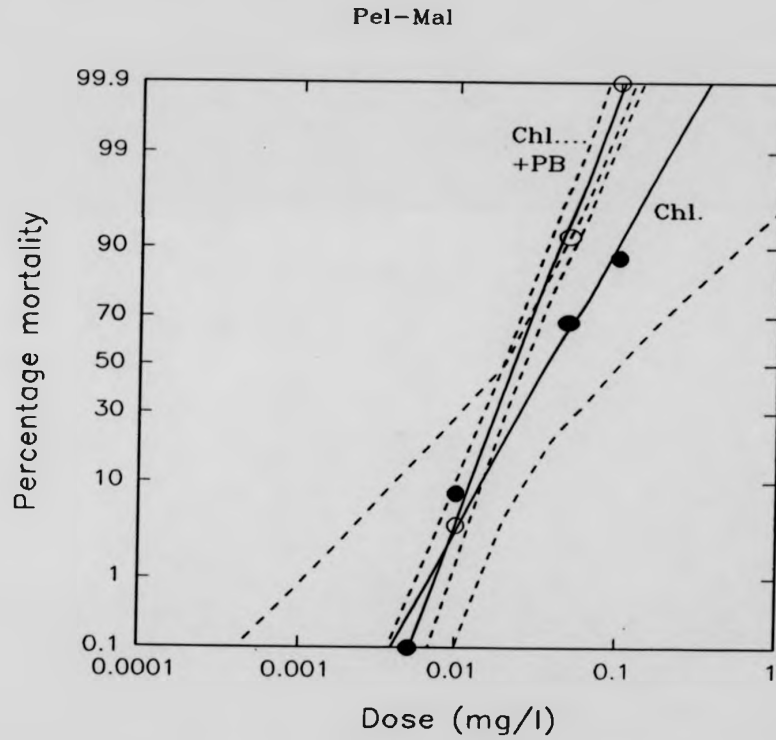
Pel—Mal

FIGURE 4.6

Log-dosage probit mortality lines for the sub-colony Pel-Mal tested with propoxur with and without PB (95% confidence limits are indicated by dashed lines).

Pel-Mal
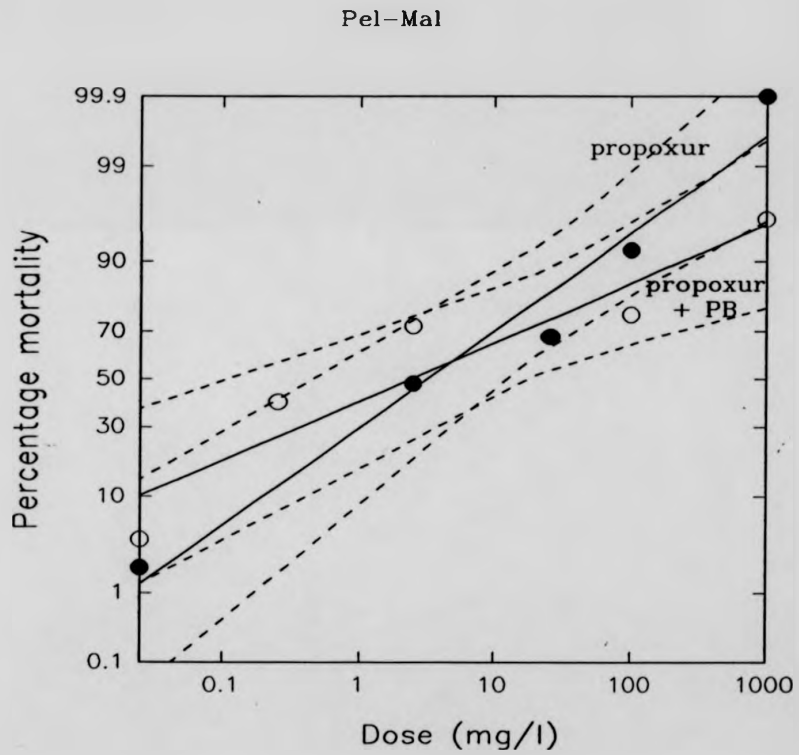
FIGURE 4.7

Log-dosage probit mortality lines for the sub-colony Pel-Pro tested with chlorpyrifos with and without PB (95% confidence limits are indicated by dashed lines).
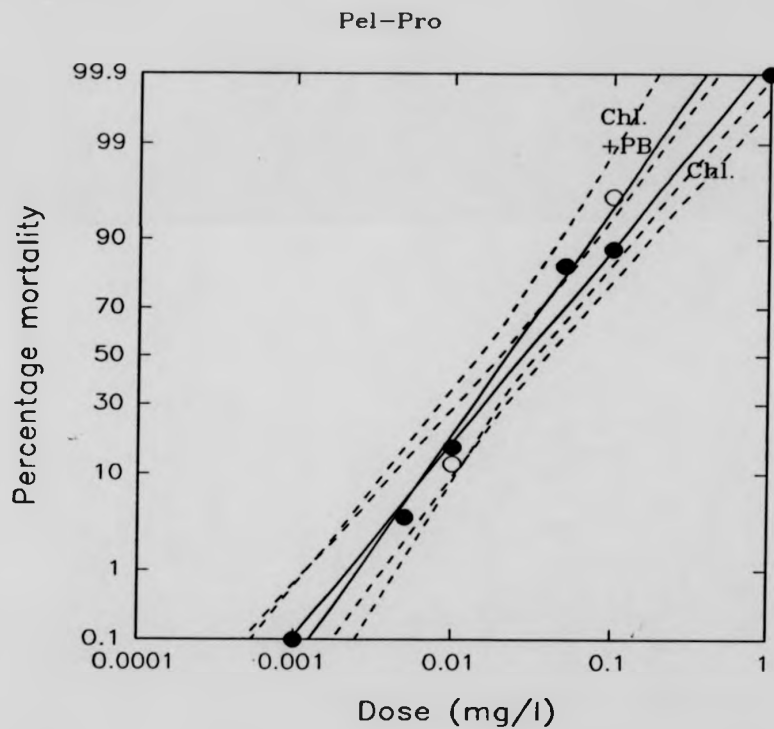
Pel-Pro

FIGURE 4.8

Log-dosage probit mortality lines for the sub-colony Pel-Pro tested with propoxur with and without PB (95% confidence limits are indicated by dashed lines).

Pel-Pro
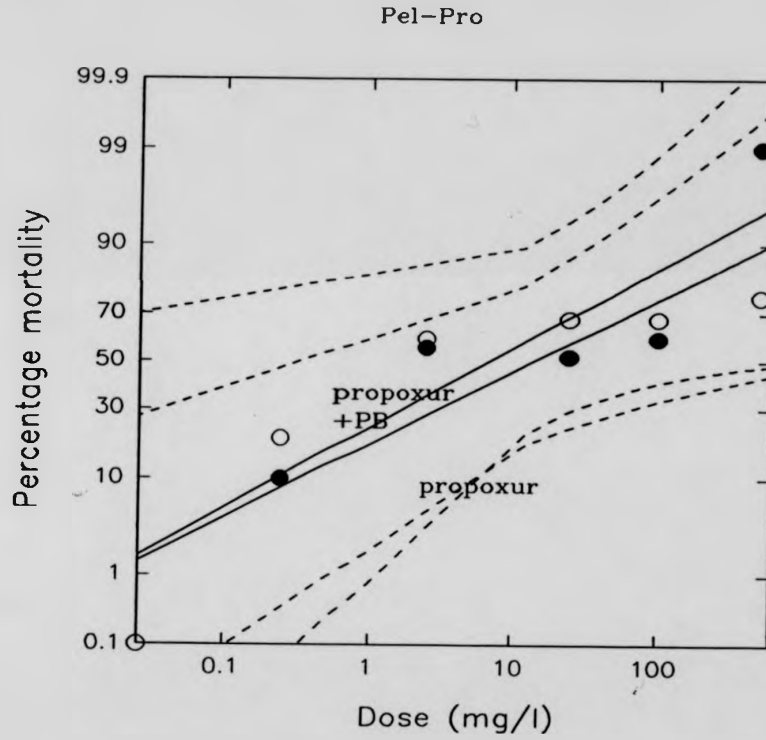
reported that the inhibition of oxidases increase the resistance to organophosphates (see section 1.2.4.2.), this was not observed. The current experiments differed from much of the earlier work in that PB was present throughout the bioassay, rather than being used as a 1 hr pre-exposure. However, there is no evidence that this variation in the exposure method affects insecticide toxicity significantly.

4.3.3.2. Glutathione S-transferase mechanisms

The specific activities of GST in the crude homogenates for the substrate CDNB were 0.386 ± 0.070, 0.396 ± 0.105 and 0.252 ± 0.080 for Pel-Chl, Pel-Mal and Pel-Pro respectively. These are not significantly different from each other or from the value of 0.340 ± 0.071 observed for PelSS. The distribution patterns of the populations are shown in FIGURE 4.9. The results suggested that the involvement of GSTs in the insecticide resistance of the sub-colonies is unlikely.

4.3.3.3. Altered acetylcholinesterase mechanism

Inhibition studies on the acetylcholinesterases showed that an altered AChE mechanism has been selected differently in the three sub-colonies. Selection of this mechanism along with the esterase based mechanism is very common among the field populations of *Cx quinquefasciatus* (Bisset *et al.*, 1990; 1991; Villani and Hemingway, 1987). AChE activity of Pel-Mal, Pel-Pro and the susceptible PelSS

FIGURE 4.9

Distribution pattern of GST specific activities of the sub–colony crude homogenates for the substrate CDNB. Data for PelSS are also presented for comparison.

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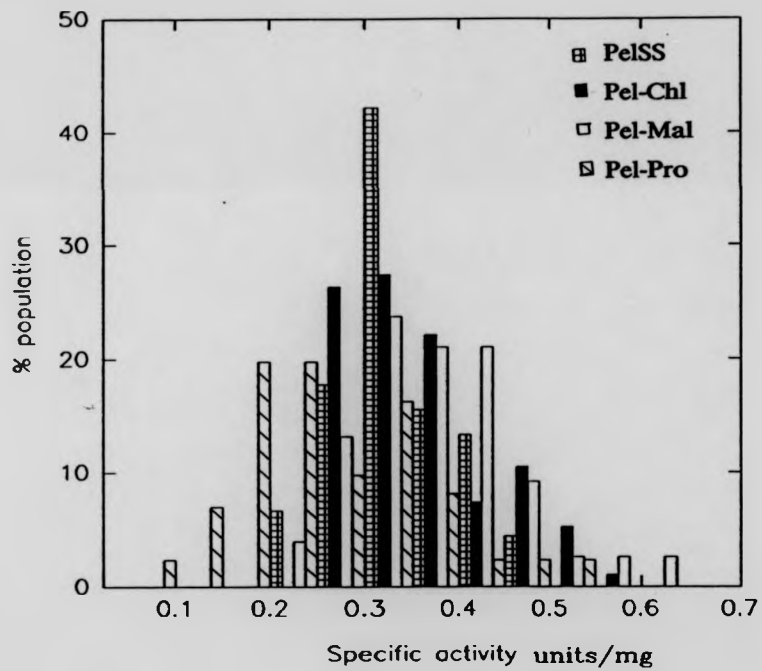
crude homogenates were inhibited by 60-80% with 5 μM propoxur over a period of 2 minutes. In contrast, Pel-Chl AChE activity was not inhibited at that concentration and 100 μM propoxur only inhibited it to 60% (FIGURE 4.10). The calculated inhibition co-efficient (k_i) values for AChEs from Pel-Chl, Pel-Mal , Pel-Pro and PelSS were $5.12 \pm 0.61 \times 10^3$, $1.39 \pm 0.15 \times 10^5$, $1.02 \pm 0.08 \times 10^5$ and $1.83 \pm 0.16 \times 10^5$ M^{-1} min^{-1} respectively and the k_i for Pel-Chl AChE was significantly less than the others ($P < 0.01$). The difference between the k_i values of Pel-Pro and PelSS was also significant ($P < 0.05$). Therefore the greater resistance ratios shown by Pel-Chl are due to the additional resistance conferred by the altered AChE resistance mechanism. Esterase and AChE kinetic data explain the differences in cross-resistance spectra shown by each sub-colony towards different insecticides. According to the reactivities of both the esterases A_2 and B_2 all sub-colonies should express the resistance in the order chlorpyrifos > malathion > propoxur (see TABLES 4.2 and 4.3). But the resistance of Pel-Chl sub-colony to propoxur is much higher than the expected level, while in the other two sub-colonies the resistance to propoxur is similar to that of malathion (TABLE 4.1). The altered AChE mechanism in mosquitoes has been reported to be invariably more efficient at giving carbamate than organophosphate resistance (Georghiou, 1972; Ayad and Georghiou, 1975). In the present study also resistance to propoxur has been increased relative to the organophosphate resistance due to the presence of this mechanism in the sub-colonies. The highest propoxur resistance in Pel-Chl is well correlated with its AChE resistance mechanism.

FIGURE 4.10

Time dependent inhibition of AChEs of the sub-colony homogenates. Data for PelSS are also presented for comparison.

Chapter 4
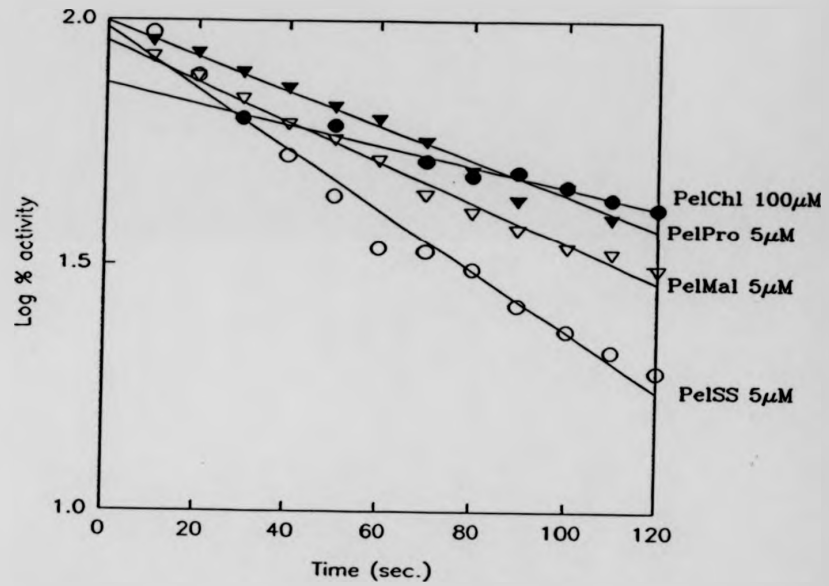
4.4. SUMMARY

During the present study three sub-colonies were selected with three different insecticides from a single parental colony of *Cx quinquefasciatus*. Elevated A_2 and B_2 esterases were partially purified from each sub-colony and interacted with different insecticides. Kinetic data revealed that there is more than one elevated isoenzyme for each of the esterases A_2 and B_2, in the parental population. However, the differences of the sub-colony cross-resistance spectra were mainly due to a variously selected altered AChE mechanism.

CHAPTER 5

IMMUNOLOGICAL CROSS-REACTIVITY OF THE ANTISERUM RAISED AGAINST PElRR A_2

5.1. INTRODUCTION

Polyclonal antiserum against the purified PelRR A_2 was raised previously and shown to have no cross-reactivity with the purified PelRR B_2 esterase (Jayawardena, 1992). Immunological cross-reactivity of this antiserum was further investigated in the present study. Carboxylesterases purified from several strains of *Cx quinquefasciatus* and mosquito crude homogenate samples on PAGE gels were examined for cross-reactivity using more sensitive detection methods. The antiserum was also interacted with an esterase purified from a malathion resistant strain of the saw-toothed grain beetle (*Oryzaephilus surinamensis*) and crude homogenate samples of *An. stephensi* and German cockroach (*Blattella germanica*) on PAGE gels to examine its cross-reactivity with other insect enzymes. The immunological relationships of esterase A_2 with the commercially available vertebrate carboxylesterases and cholinesterases were also investigated.

5.2. MATERIALS AND METHODS

5.2.1. *MATERIALS*

'A' and 'B' type carboxylesterases purified for the studies in Chapters 2 and 3 were used. Purified grain beetle esterase was a kind gift from Dr. Chris Walter, Central Science Laboratory, Berkshire. Mosquito crude homogenates were prepared from the PelSS, PelRR, Dar91 and Tanga85 strains of *Cx quinquefasciatus* and 'STMal' (malathion resistant) and 'Iraq' (susceptible) strains of *An. stephensi* (currently held at London School of Hygiene and Tropical Medicine). Mosquito larvae were homogenized in ice-cold 50 mM phosphate buffer (pH 7.4) with 10 mM DTT using fresh or frozen fourth instar larvae. The supernatants of the centrifuged homogenates (15,000g for 5 minutes) were used for the experiments. Cockroach crude homogenates were also prepared in a similar manner using the first instar nymphs from an OP resistant strain, 'Muncie' (colonized by Purdue University, USA and currently held at LSH&TM).

The polyclonal antiserum was previously produced against the purified PelRR A_2 esterase in white New Zealand rabbits (Jayawardena, 1992) and stored at -70°C in 20 μl aliquots.

Polyvinylidene difluoride (PVDF) transfer membranes (0.2 μ) were from

Millipore (Bedford, MA, USA). Anti-rabbit IgG, horseradish peroxidase linked white antibody (from donkey) and ECL (enhanced chemiluminescence) detection reagents were from Amersham International plc. (Buckinghamshire, England). The trans-Blot SD Semi-Dry Electrophoretic Transfer Cell was from Bio-Rad, UK. Purified vertebrate carboxylesterases, cholinesterases and all the chemicals were from Sigma Chemical Co., UK.

5.2.2. IMMUNOBLOTTING TECHNIQUES

All the immunoblotting experiments were carried out at room temperature (22°C).

5.2.2.1. Dot-Blot Assays

Purified esterases were diluted with distilled water to obtain the required protein concentrations. A 5 μl dot for each sample was placed directly on the PVDF membrane. After blocking non-specific sites with 5% (w/v) non-fat milk and 0.05% (v/v) Tween-20 in PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl, pH 7.5) the PVDF membrane was incubated with the A_2 antiserum (1/2500 dilution in the blocking solution) (1 hr each). After washing in 0.05% (v/v) Tween-20 in PBS, 0.05% (v/v) Tween-20 plus 0.5 M NaCl in PBS and in 0.05% (v/v) Tween-20 in PBS, for 5 min each, the membrane was incubated for 1 hr with the horse-radish-peroxidase labelled secondary antibody [1/1000 -

1/5000 dilution in 0.05% (v/v) Tween-20, 1% (w/v) non-fat milk in PBS]. The washing was repeated as described above. Membrane was then incubated for about 1 min in the ECL detection reagent and the bound antibody was detected by exposing to hyperfilm ECL for 10 seconds to 2 minutes.

5.2.2.2. Western-blotting

Electrophoresis of purified esterases or crude homogenates was performed on native or SDS PAGE and the gels were removed and equilibrated in transfer buffer (25 mM Tris, 0.2 M glycine, pH 8.3) for 15 minutes. While this was in progress a PVDF membrane (9 x 8 cm) and 10 pieces of Whatman 3 MM filter papers (each 9 x 8 cm) were also equilibrated in transfer buffer. The 'blotting sandwich' was assembled on the middle of the cathodic plate of the Transfer Cell in the following order; 5 pieces of filter paper were rolled out on each other and the PVDF membrane was placed on that followed by the gel and another 5 pieces of filter paper. It was essential to avoid trapping any air bubbles between the layers. The proteins were transferred at 20 V for 30 minutes. The sandwich was dissembled carefully once the process was completed and the PVDF membrane was directly placed in the blocking solution. The immunoblotting was then carried out as described in the previous section.

5.3. RESULTS AND DISCUSSION

5.3.1. CROSS-REACTIVITY WITH 'A' AND 'B' TYPE ESTERASES

To investigate whether the antiserum raised against PelRR A_2 cross-reacts with the other 'A' type esterases, both native and SDS PAGE gels were used with the 'A' esterases purified from Dar91, Tanga85, Muheza, SPerm and PelSS strains and were subjected to immunoblotting. Purified PelRR A_2 was also used as a control. The antiserum cross-reacted with all the A_2 esterases and the susceptible 'A' esterase both in their native and denatured forms (gels not shown). However a greater affinity could be observed for the native forms probably because the antiserum was raised against the native form of the PelRR A_2 (Jayawardena, 1992). It was also apparent that the reactivities of other 'A' esterases were similar to that of PelRR A_2. All the purified B_2 esterases and the purified PelSS 'B' esterase were also examined in a similar manner. The results revealed that the A_2 antiserum does cross-react with 'B' type esterases although the extent of reactivity was slightly lower than that with 'A' esterases (gels not shown). Cross-reaction between 'A' and 'B' types has not been reported previously and is in contrast to the previous observations. One possible explanation for this is that the highly sensitive ECL detection method was used in the present study. When using the much less sensitive 3,3'-diaminobenzidine tetrahydrochloride dye method for visualization on the immunoblots no cross-reaction with the PelRR B_2 protein was observed, as reported previously, with the same antiserum

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(Jayawardena, 1992). Mouches *et al.* (1987) have also reported that the antiserum raised against A_1 esterase cross-reacted with all the 'A' type esterases but not with 'B' type esterases. Similarly their B_1 antiserum cross-reacted with all the other types of 'B' esterases but not with type 'A' esterases (see section 1.8.3.2.). Therefore this is the first report to show the immunological cross-reactivity between the 'A' and 'B' type mosquito carboxylesterases. To determine the extent of cross-reactivity between the A_2 esterases and B_2 esterases, dot-blot immunoassays of the native proteins of PelRR A_2 and PelRR B_2 were performed. From serial diluted dot blots it was estimated that the A_2 antiserum is about 50× less reactive with esterase B_2 than esterase A_2 (FIGURE 5.1).

5.3.2. CROSS-REACTIVITY WITH OTHER INSECT PROTEINS

FIGURE 5.2 shows an immunoblot of a native PAGE gel run with equal amounts (12 μg from each) of crude homogenate proteins of PelRR, Dar91 and Tanga85 strains. Several protein bands are observed to have cross-reacted with the anti-esterase A_2 antiserum. Of these bands the low mobility and highly reactive band, found in all the samples, can be identified as acetylcholinesterase (AChE) according to the studies carried out on the PelRR strain previously (Peiris, 1989). The strong immunological cross-reactivity of the A_2 antiserum with AChE suggests that both enzymes contain similar motifs. Esterase A_2 and B_2 bands are prominent in all the resistant crude homogenates. In PelSS crude homogenate, the band between the resistant A_2 and B_2 esterases probably

FIGURE 5.1

Dot-blots of serial dilutions of the PelRR carboxylesterases A_2 and B_2 immunodetected with A_2 antiserum.
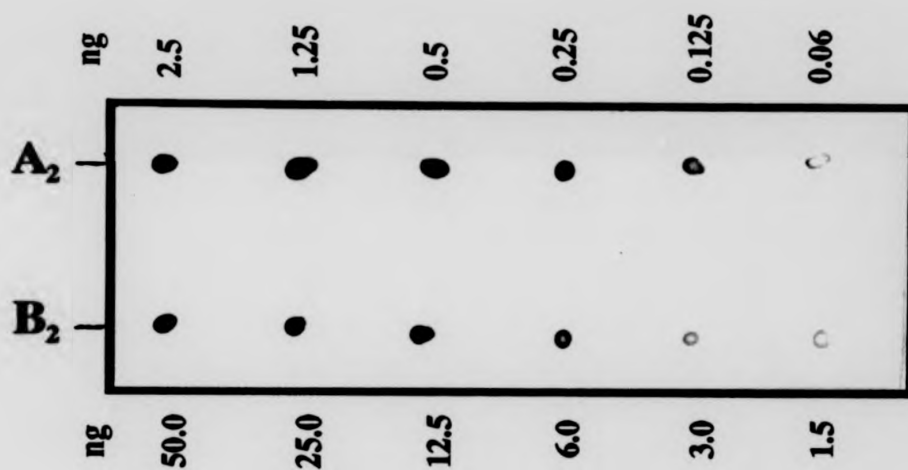
FIGURE 5.2

Equal amounts of crude homogenates on native PAGE gel immunodetected with A$_2$ antiserum.
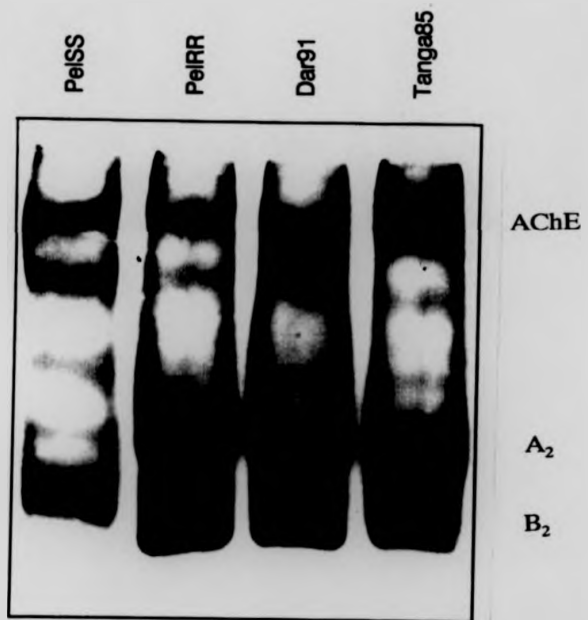
represents the co-migrating susceptible 'A' and 'B' enzymes, which are faster than A_2 and slower than B_2 respectively in their electrophoretic mobilities (see FIGURE 3.2 and 3.3). The weak PelSS band with an identical mobility to PelRR A_2 may represent the unamplified (or non-elevated) A_2 esterase in the PelSS strain. It is clear that several other bands present in the mosquito crude homogenate have also reacted with the A_2 antiserum and some of these bands may represent other forms of 'A' and 'B' esterases. The esterase B_2 band was always more diffuse than A_2 (see FIGURE 5.2) and after observing several films of the repeated experiments it was apparent that the broad B_2 band comprised more than one band in all the resistant crude homogenates. This indicates the possibility of having several isoenzymes with slightly different electrophoretic mobilities for each of the esterase bands elevated on native gels which are stained for esterase activity.

Cross-reactivity of A_1 and B_1 antisera with the proteins present in *Culex* crude homogenates was investigated by Mouches *et al.* (1987). After denatured electrophoresis the gels were electroblotted to nitrocellulose sheets. Both antisera identified their own esterase types. In addition, A_1 antiserum cross-reacted with an unidentified 40 kDa protein band also. However any cross-reactivity between these antisera and the AChE was not observed (Mouches *et al.*, 1987). Beyssat-Arnauty *et al.* (1989) extended these observations and introduced dot-blot immunoassays with this B_1 antiserum as a method of monitoring the resistance, caused by the overproduction of 'B' type esterases, in

the field. It is clear that the immunological cross-reactivity among 'A' and 'B' esterases or between these esterases and AChE is not detectable when dyes are used as the method of detection *ie.* Beyssat-Arnauty *et al.* (1989) used goat anti-rabbit alkaline phosphatase conjugate as the secondary antibody and 5 -bromo-4-chloroindoxyl phosphate with nitroblue tetrazolium salt to reveal its activity. Therefore it should be possible to monitor the amount of the 'B' esterases present in a given individual/strain, on comparative basis, using their method.

The purified esterase which is associated with the malathion resistance in saw-toothed grain beetle (*Oryzaephilus surinamensis*), was subjected to a dot-blot assay with A_2 antiserum. No cross-reactivity was observed even at a concentration of 0.75 μg per dot while the control (purified PelRR A_2- 0.001 μg per dot), on the same membrane, gave a strong cross-reactivity. Crude homogenate immunoblots with similar amount of proteins from a malathion resistant strain (STMal) and a susceptible strain (Iraq) of *An. stephensi* were also examined (FIGURE 5.3). STMal strain has a qualitatively different carboxylesterases as the major resistance mechanism (Hemingway, 1982) (see section 1.4.4.3.A). Only a single band, AChE, cross-reacted from both strains under the established conditions. On longer exposure of the film a few other bands also appeared. Out of these, an esterase band in STMal crude homogenate was very prominent and had the same electrophoretic mobility as that of the PelRR B_2. In the susceptible crude homogenate also a similar band, but with much less reactivity, was identified (labelled as 'Est' in FIGURE 5.3).

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FIGURE 5.3

Equal amounts of crude homogenates of *An. stephensi* (susceptible 'Iraq' and malathion resistant 'STMal' strains) and PelRR on native PAGE gel immunodetected with anti-A$_2$ antiserum. The film has been over exposed (see the text for details).

In the resistant homogenate, this band may represent a qualitatively different esterase involved in the malathion resistance. When a similar gel was stained for esterase activity, no such bands or any other elevated bands were observed in both strains. 'Muncie' strain of *Blattella germenica* has elevated esterases as an OP resistance mechanism. However, in the crude homogenates of these cockroaches none of the esterases except AChE cross-reacted with the antiserum even after long exposure. From these results it is clear that the A_2 antiserum is highly cross-reactive with the insect AChEs although the cross-reactivity with the resistance associated esterases from other insects is very poor.

5.3.3. CROSS-REACTIVITY WITH VERTEBRATE CARBOXYLESTERASES AND CHOLINESTERASES

Cross-reactivity of the PelRR A_2 antiserum with the commercially available vertebrate carboxylesterases and cholinesterases was examined using the dot-blot technique (FIGURE 5.4). Different concentrations of PelRR A_2 were used as controls. A strong immunological affinity was seen with rabbit liver esterase (1-10 fold less reactive than esterase A_2) although the antiserum affinity was insignificant for the porcine liver esterase. Cross-reactivity of electric eel AChE, bovine AChE, human AChE and horse butyrylcholinesterase were about 10^4, 10^2, 10^3 and 10^4 fold respectively, less than that of A_2 esterase. The results show that the mosquito carboxylesterase A_2 is immunologically related to several carboxylesterases and cholinesterases present in the vertebrates. Cross-reactivity

FIGURE 5.4

Dot-blots showing the cross-reactivity of A_2 antiserum with purified A_2 and vertebrate esterases. a-rabbit liver esterase, b- porcine liver esterase c- AChE from electric eel, d- AChE from bovine erythrocytes, e- AChE from human erythrocytes, f- butyrylcholinesterase from horse serum.
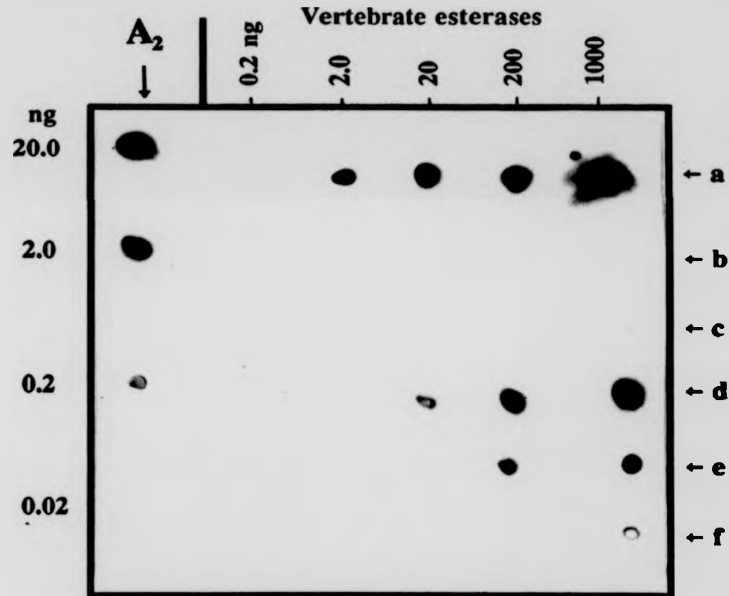
with the commercially available vertebrate esterases has also been shown recently for an antiserum raised against a partially purified rat liver microsomal carboxylesterase multigene family (Alexon *et al.*, 1993). However this is the first report to show the immunological relationships between mosquito and vertebrate esterases.

5.4. SUMMARY

Immunological studies presented in this chapter reveal that the 'A' and 'B' type mosquito carboxylesterases are immunologically related and the A_2 antiserum strongly cross-reacts with the mosquito and other insect AChEs. Esterase A_2 did not show any significant immunological relationship with the other resistance associated insect esterases tested. A_2 antiserum cross-reacted with some of the commercially available vertebrate esterases indicating its similarities with the enzymes found in completely different groups of animals.

CHAPTER 6

GENERAL DISCUSSION

6.1. ESTERASE-BASED RESISTANCE MECHANISMS

Increased activity of non-specific carboxylesterases is a major resistance mechanism against the organophosphorus and carbamate insecticides in a variety of insect groups. As discussed in Chapter 2, these esterases may have primarily evolved as an adaptation to the toxic compounds found in their diet and environment. However, mosquito esterases A_2 and B_2 have also shown a preference for intermediate length mono- and diacylglycerols suggesting their possible involvement in other physiological functions, such as fatty acid-lipid metabolism (Ketterman *et al.*, 1992; Jayawardena, 1992).

In the present study, characterization of the purified carboxylesterases A_2 and B_2 revealed that the esterase-based resistance mechanism in the mosquito *Cx quinquefasciatus* is due to the overproduction of carboxylesterases for the sequestration (rapid binding followed by slow turnover) of the insecticides. Hence the role of the mosquito esterases is very similar to that of the well characterized E4 esterase of aphids (Devonshire, 1977; Devonshire and Sawicki, 1979; Devonshire and Moores, 1982). To maintain the mechanism effectively resistant individuals have to produce a considerable amount of enzymes and for

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a mosquito larva and an aphid this amount is respectively up to 0.4% and 3% of their total proteins (see section 2.3.2.4). It is surprising that such a mechanism, which results in considerable energy loss to the insect, has been selected. However there is no evidence to show that the resistant individuals with elevated esterases are less fit in their diurnal activities than the susceptible individuals with no elevated esterases (Amin,1983). Still, it has been observed in the field that resistant esterase genes have a selective disadvantage over the susceptible genes when there is no insecticidal pressure (Severini *et al.*, 1993). The same observation was made with most of our laboratory colonies as well, although some strains, *eg.* Dar 91, maintained their increased esterase levels even in the absence of any selection pressure.

In contrast to the overproduced esterase-based mechanism, non-elevated esterases also can act in OP resistance. In humans and rabbits, OP resistance is mainly due to the presence of serum paraoxonases (which belong to the 'A' type esterases of the Alridge's classification) that can hydrolyse the insecticides rapidly (Du *et al.*, 1993; Furlong *et al.*, 1993; Li *et al.*, 1993). The overproduction of these enzymes is not necessary for their function as they do not act by sequestration. A similar type of mechanism has also been observed in a malathion resistant *An. stephensi* and *An. arabiensis* strains. Esterases involved in insecticide resistance, are not elevated as observed in native PAGE gels and qualitatively different from the susceptible enzymes (Hemingway, 1982; 1983). It has been reported that resistant *An. stephensi* carboxylesterases can hydrolyse malathion but are

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inhibited by paraoxon (Boddington, 1992). It is still to be investigated whether they belong to 'A' type or 'B' type esterases of the Alridge's classification. However unlike the resistant *Cx quinquefasciatus* mosquitoes with overproduced 'B' type (Alridge's classification) esterases, the malathion resistant *An. stephensi* mosquitoes have a much narrower cross-resistance spectrum (Hemingway *et al.*, 1982). Therefore, the most important factor which favours the selection of the overproduced esterase-based mechanism may be its ability to sequester a wide range of insecticides, giving the insect a broader cross-resistance spectrum. In the *Cx quinquefasciatus* strains studied, it is highly unlikely that any esterase-based mechanism, other than the one with overproduced esterases, is present because the insecticide interaction rates of the crude homogenate were very similar to those of the purified elevated esterases (see section 3.3.3.3.). However, in a malathion resistant strain of *Cx tarsalis*, both elevated and non-elevated types of carboxylesterase resistance mechanisms have been reported to co-exist (Ziegler *et al.*, 1987).

6.2. MOLECULAR HOMOLOGY OF THE MOSQUITO CARBOXYLESTERASES TO OTHER SERINE HYDROLASES

The relationship between the amino acid sequence and the three dimensional structure of a large family of esterases and related proteins (including TEM-R B_1 [see section 1.6.3.3.]) has recently been studied (Cygler *et al.*, 1993). It was shown that 25 amino acid residues were conserved in all the esterases studied.

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It is thought that these residues are vital for the structure (residues used for packing, salt bridges and disulphide bridges) and function (residues in the active site) of these proteins. TABLE 6.1. shows the amino acid residues surrounding the active site serine residue in some of the published serine hydrolases including the TEM-R B_1. A considerable similarity is seen between the mosquito esterase B_1 and the insecticide target site AChEs (8-10 out of 12 identical residues). Over the entire coding region of the gene, the number of identical residues is higher between the B_1 and the human AChE than between the B_1 and the AChE of the mosquito *An. stephensi*. Recently the amplified esterase genes of PelRR A_2, PelRR B_2, MRES (an OP resistant Cuban *Cx quinquefasciatus* strain) B_1 and a partial length of the susceptible non-amplified PelSS 'B' were sequenced. The percentage identities of A_2 esterase and 'B' type esterases with vertebrate AChEs are about 40% and 12% respectively at the amino acid level. The identities with the AChE of *An. stephensi* are much lower (less than 14% and 2% respectively) (Vaughan *et al.*, submitted[a]; [b]). The AChE gene of *Culex* has not been sequenced so far and that of *An. stephensi* may not represent a typical mosquito AChE as enzyme inhibition assays have shown that this AChE behaves differently from that of other mosquitoes (Dr. J. Hemingway, personal communication).

The antiserum raised against the esterase A_2 cross-reacted with purified esterase B_2 and several other vertebrate esterases and the differences were estimated (see Chapter 5). These immunological relationships of esterase A_2 were compared

Hydrolase	Sequence
Serine proteinases, subtilisin family	
Subtilisin BNP	AYNGTSMASPHV
Serine proteinases, trypsin family	
Bovine trypsin	SCQGDSGGPVVC
Drosophila trypsin-like	ACQGDSGGPLVS
Hornet trypsin-like	ACHGDSGGPLVS
Serine esterases	
Torpedo AChE	TIFGESAGGASV
Human AChE	TLFGESAGAASV
Drosophila AChE	TLFGESAGSSSV
An. stephensi AChE	TLFGESAGGSSV
Rat carboxylesterase	TIFGESAGGVSV
Mouse carboxylesterase	TIFGESSGGISV
Rabbit carboxylesterase	TIFGESAGGQSV
Drosophila Est-6	LLVGHSAGGASV
Heliothis JHE	TIAGQSAGASAA
Culex Esterase-B$_1$	TLAGHSAGAASV
Aphid E4/FE4	TTTGMSAGASSV

TABLE 6.1. Amino acid sequence surrounding the active-site serine residue (S) in some serine hydrolases including TEM-R B$_1$ (adopted from Field *et al.*, 1993).

with the homologies of their amino acid sequences (TABLE 6.2.). There is no apparent relationship between the homologies shown by the two methods. The immunological data show the presence of specific binding sites on the molecular surfaces which may be correlated with the conformations of the actual proteins.

The mosquito esterases A_2 and B_2 have been compared biochemically with other esterases during their characterization in Chapter 2. Unlike B_2, A_2 was completely inhibited by the specific AChE inhibitor eserine at 10 μM concentration, showing a very high structural similarity of the active sites of A_2 and AChEs. To assess the similarity of the conformation around the active site, the specific AChE inhibitors propidium iodide and BW284C51 were tested with purified esterase A_2. These inhibitors are thought to be bind to an anionic sub-sites situated around the rim of the active site gorge of the AChE molecule and modify activity through conformational change (Friboulet *et al.* 1990; Shafferman *et al.* 1992; Marchot *et al.* 1993). However esterase A_2 was not inhibited by either of these compounds (Dr. A. J. Ketterman, personal communication).

	Immunological reactivity[a]	Sequence identity[b]
PelRR B₂	2.0%	70%
Rabbit liver esterase	>10.0%	20%
Porcine liver esterase	0.01%	<14.0%
Electric eel AChE	0.01%	40.0%
Bovine AChE	1.0%	35.0%
Human AChE	0.1%	40.0%
Horse BChE[*]	0.01%	<14.0%

a- cross-reactivity of the A_2 antiserum is expressed as a percentage of its reactivity with purified esterase A_2

b- percentage identity is expressed at the amino acid level

[*]Butyrylcholinesterase

TABLE 6.2. Comparison of the immunological and sequence data obtained for molecular homologies of esterase A_2 with the mosquito esterase B_2 and several vertebrate esterases (see the text for details).

6.3. POLYMORPHISM OF MOSQUITO CARBOXYLESTERASES

During the present study, different A and B type carboxylesterases were purified and characterised from several different strains of *Cx quinquefasciatus*. The observed kinetic differences clearly indicated the existence of multiple variants showing the polymorphism in mosquito carboxylesterases. Recent molecular biological data show that 'B' type esterases are very similar to each other. Gene sequences of PelRR B_2, MRES B_1 and the partial PelSS 'B' have been compared with the sequence of TEM-R B_1 and a previously sequenced partial B_2 gene (Mouches *et al.*, 1990, Vaughan *et al.*, submitted[a]). The four esterase nucleotide sequences (excluding the partial B_2 sequence) are from 95.2% (TEM-R B_1 with MRES B_1 and PelSS 'B') to 98.8% (MRES B_1 with PelSS 'B') identical. Deduced amino acid identity between the esterases ranges from 95.2% (TEM-R B_1 with PelSS 'B') to 98.6% (partial sequenced B_2 with PelRR B_2) (Vaughan *et al.*, submitted[a]). The high identities at the molecular level suggest that the esterase 'B' genes are allelic. Esterase A_2 shows about 70% amino acid sequence homology with all the sequenced 'B' type esterases (Vaughan *et al.*, submitted[b]). The relationships among different A esterases are yet to be established.

In *Drosophila* there are at least 22 different soluble esterases which can be distinguished electrophoretically using naphthyl acetates as substrates and

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multiple alleles are present at some of the corresponding loci (Healy *et al.*, 1991). Human and rabbit paraoxonases are also present as highly polymorphic isoenzymes (Du *et al.*, 1993; Furlong *et al.*, 1993; Li *et al.*, 1993). In mouse, polymorphic esterase genes are arranged in two clusters and the orthologous chromosome regions have been identified in other mammals as well (see section 1.6.3.1.). The isoenzymes are very similar to each other and share a common origin (see section 1.6.3.2. and FIGURE 1.7.). It was shown that A and B type mosquito esterases are immunologically related and both have the same role in insecticide resistance. This suggests that both 'A' and 'B' gene loci may have evolved from a tandem duplication of a common ancestral locus and different 'A' and 'B' type genes have risen secondarily from each of these loci forming a multigene family, a situation somewhat similar to the organization of the mammalian esterase genes.

Specific glycosylation may be a possible explanation for the mobility differences of the different 'A' and 'B' electromorphs. There are five possible glycosylation sites amongst all the B esterases sequenced so far and only one of these is common to all four sequences (Mouches *et al.*, 1990; Vaughan *et al.*, submitted[a]). Since they have different sites of glycosylation, allele specific glycosylation leading to mobility differences on native PAGE is possible. The same phenomenon may cause the differences among the 'A' electromorphs as well. However during the development of the purification procedures it was

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noted that PelRR B_2 did not bind to Con-A Sepharose which contains binding sites for glucose and mannose residues (see section 2.3.1.). This implies that either the glycosylation of PelRR B_2 or the participation of glucose and mannose residues in glycosylation is insignificant.

6.4. THE THEORY OF MIGRATION

On the basis of identical restriction digest patterns of the flanking regions of the B_2 gene from different populations, Raymond *et al.* (1991) proposed the hypothesis that amplification of the B_2 allele of the esterase 'B' locus has occurred only once and spread worldwide (see section 1.6.3.3.). Esterase A_2 is always associated with esterase B_2 and shows almost complete linkage disequilibrium. It is now known that both esterases A_2 and B_2 sit on the same amplification unit (Vaughan *et al.*, submitted[b]). Therefore the event causing A_2 elevation must have occurred and spread concurrently with B_2. If OPs have been the main selecting agents for the A_2/B_2 esterase amplification worldwide, this selection has only been operative over the last forty years at most, and in the majority of countries the time scale would be much shorter than this. Thus, the amplification of the A_2/B_2 unit and its subsequent selection and migration have to be considered as a recent event and the time scale is not sufficient for the amplified genes to diversify significantly. Therefore if Raymond's migration theory is correct, all the amplified A_2 and B_2 genes would be almost identical (in

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actual terms Raymond *et al.* [1991] postulated the hypothesis assuming that all the B_2 genes are the same).

The A_2 and B_2 esterases purified in this study were from strains which originated in different geographical areas of the world (two of the strains were collected from identical sites to those used in Raymond's study) but they were kinetically different from each other. Slight differences among the electrophoretic mobilities of esterase B_2s were also observed (see Chapter 3). It was also shown that kinetically different A_2 and B_2 enzymes can be selected even within a single population of *Cx quinquefasciatus* (see Chapter 4). These data clearly demonstrate that different isoenzymes of esterases A_2 and B_2 occur in natural populations and suggest that Raymond's hypothesis is too simplistic.

Although it is possible that the post-transcriptional changes of the same gene product lead to the formation of different forms, existence of such a mechanism to generate kinetically different forms is highly unlikely. Sex-, tissue- and age-specific regulation of the genes of esterase 6 and esterase S in *Drosophila* have been studied but there is no data to show that they are enzymologically different (Karotam and Oakeshott, 1993; Ludwig *et al.*, 1993; Sergeev *et al.*, 1993). Extensive polymorphism of the gene product after mRNA processing and subsequent post-translational modifications has been reported for AChE (Chatonet and Lockridge, 1989; Taylor, 1991; Li *et al.*, 1993). Post-translational

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proteolytic processing and core-glycosylation have also been studied in rat liver esterases (Robbi and Beaufay, 1986; 1987; 1988). Two of the forms, of which one was formed as a result of proteolytic processing of the other, were similar to the pI 6.0 and 6.4 esterases which were shown to have different substrate preferences by Mentlein *et al.* (1985). However, both groups were uncertain whether such proteolytic processing can occur *in vivo* (Mentlein *et al.*, 1985; Robi and Beaufay, 1988). It is hard to believe that the kinetic differences observed among A_2 and B_2 esterases are due to post-transcriptional or post-translational modifications of the same gene product. A difference of three amino acid residues has been observed between the deduced amino acid sequences of the partial B_2 esterase sequence (Mouches *et al.*, 1990) and the corresponding region of the PelRR B_2 gene (Vaughan *et al.*, submitted[a]). In human serum paraoxonases, kinetic differences between two variants occur due to a single amino acid substitution at a position which is not near the active site (Humbert *et al.*, 1993). Therefore the observed differences in mosquito carboxylesterases must certainly be due to the differences in their gene sequences.

Two elevated B esterases found in *Cx pipiens* in France and Cyprus have been reported to possess restriction fragment length polymorphisms different from each other and from the previous patterns reported for B_2 esterases (Poirie *et al.*, 1992). Although both enzymes have almost identical mobilities to that of B_2 on starch gels, they have been classified as B_4 and B_5 because of their RFLP

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patterns and their respective association with A_4 and A_5 esterases which are different from esterase A_2 in electrophoretic mobility. It has now, therefore, been suggested that amplification of the esterase 'B' locus has occurred at least four times with two electrophoretically distinguishable allelic forms of the gene (B_1 and $B_2/B_4/B_5$), but it is maintained that B_2 is the most common form which was spread by migration throughout the majority of the world (Poirié *et al.* 1992). According to the results of the present study, B_2 esterases are also expected to vary in their actual gene sequences. Since B_4 and B_5 have different RFLP patterns and different 'A' types in association, these may be distinctly different from other B_2 esterases representing two early off-shoots (A_4/B_4 and A_5/B_5) of the main evolutionary stream of A_2/B_2 esterases.

RFLP analysis of the 'B' locus and its flanking region in susceptible *Culex* strains (which have non-amplified 'B' esterase genes) have shown a high degree of polymorphism (Raymond *et al.*, 1991). In the present study it was shown that the susceptible enzymes are markedly different from the resistant enzymes (see Chapter 3). However, gene sequencing data show a high identity (95.2% - 98.8%) between the PelSS 'B' and the other sequenced resistant 'B' types (Vaughan *et al.*, submitted[a]).

Insecticide-interaction data obtained for different resistant 'B' esterases showed that the two B_1 esterases differ greatly from each other whereas different B_2

types were more similar to each other (see TABLE 3.3 and 3.4). Deduced amino acid sequences have also shown that the percentage identity of TEM-R B_1 and MRES B_1 is only 96.1%. Interestingly both of these have shown greater identities (97.4% and 98.0% respectively) to PelRR B_2 (Vaughan *et al.*, submitted[a]). From both the kinetic and molecular biological data it is clear that B_1 esterases are more diversified. Although the enzymological data suggest that B_2 esterases are more conserved the situation is yet to be analysed with gene sequences. It is possible that the B_1 esterases have diverged at different times from the main B_2 stream during the process of evolution .

The enzymological data presented in this report and the molecular biological studies which are still in progress clearly show that a great number of alleles exist for the resistant esterase loci 'A' and 'B'. Such a variation in amplified alleles can not be expected to occur as a result of a single amplification event and a subsequent recent diversification. If they have been amplified only once, the original amplification has not occurred recently and can not be within the time span over which OPs have been used. Another possibility is that the amplification has occurred several times amplifying several different mutated alleles of the 'A' and 'B' loci. Amplification is a rare event and its frequency in cultured mammalian cells is reported to be 10^{-6}-10^{-4} (Schimke, 1984). Mutation is also a rare event and the sequenced data of 'B' esterases show that the changes that have occurred between alleles are due to single nucleotide substitutions rather

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than chromosomal rearrangements, as the nucleotide changes are scattered throughout the coding sequence (Vaughan *et al.*, submitted[a]). However, chromosomal rearrangement have also undoubtedly played an important role during the evolution of the 'A' and 'B' esterase loci, because it has been shown that all the 'A' and 'B' type genes are not situated on the same linkage group (Wirth *et al.*, 1990).

Transposable elements or long interspersed repetitive elements (LINEs) which are capable of accelerating the frequency of gene mutation and gene amplification have been found in association with the mosquito carboxylesterases. Mouches *et al.* (1990) identified LINEs, designated as Juan-C, closely associated with the amplified esterase B_1 gene in the TEM-R strain of *Cx quinquefasciatus*. Transposable elements (LINEs) are DNA sequences that are capable of movements (transposition), with or without a RNA intermediate, within or between genomes and considered as powerful mutagenic agents which cause increased genetic variability. Transposition can be influenced by environmental factors such as insecticidal pressure and therefore may play an important role in adaptation (Wilson, 1993). These elements can insert into genes or excise with parts of genes causing severe rearrangements. Truncated elements or the elements with deletions have also been observed commonly (Mouches *et al.*, 1992; Agarwal *et al.*, 1993). Many full length copies of Juan-C lines have been described recently from the genomes of different strains of *Cx quinquefasciatus*

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that originated from different continents. All these amplified elements had nearly identical copies suggesting a recent spread. They also display strong homologies with Juan-A elements of *Aedes* mosquitoes and similarities with some of the *Drosophila* LINE types indicating a common precursor. Juan-A elements have been identified in the genomes of various strains belonging to three non-sibling species of *Aedes*. It has been suggested that these elements have spread by horizontal transfer between the species (Mouches *et al.*, 1992; Agarwal *et al.*, 1993). Lack of the terminal repeats in the Juan-C sequence was observed to cause duplication of the host DNA at the site of their integrations (Agarwal *et al.*, 1993). Therefore the presence of LINEs in mosquito genomes, especially the close association of Juan-C with the esterase gene, indicates the possible involvement of these elements in accelerating the diversification of the mosquito esterase genes which are favoured by the environment. Although the different levels of amplification of the amplicon core and extremities make it unlikely that active transposition has been involved in amplification (Besansky *et al.*, 1992).

The frequent appearance of new esterase phenotypes has been observed near international ports suggesting the importance of migration in their spread (Rivert and Pasteur, 1993). Migration must have undoubtedly played an important role in the spread of the amplified resistant genes in the presence of the positive selection pressure of the insecticides. But the whole process can not be as simple as Raymond *et al.* (1991) overviewed. Mutations and amplifications of the gene

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loci 'A' and 'B' must have occurred on several occasions over a long period of time resulting in different forms of amplified 'A' and 'B' alleles.

6.5. RELATED EVENTS IN OTHER RESISTANCE MECHANISMS

A basic overview on the mechanisms which can cause the insecticide resistance in insects is given in the section 1.4. It is well known that different types of oxidases and GSTs belonging to different families can be involved in detoxication of xenobiotic substances such as insecticides. The recent advances in the understanding of qualitatively and quantitatively different insecticide target sites is of great interest and can be compared with the elevated esterase-based mechanism in the light of their physiological functions.

AChE is the target site of the OP and carbamate insecticides. Target site insensitivity is caused by the mutated forms of AChE with decreased sensitivity to insecticide inhibition. Four point mutations, associated with the resistance, have been identified in the *Drosophila* AChE gene and resistant wild populations are expected to carry a greater number of mutated alleles (Fournier *et al*, 1992; 1993). Resistance due to the altered AChEs in houseflies has been shown to be often accompanied by a modification in the kinetics of hydrolysis of its physiological substrate, acetylcholine, although there is no definitive correlation between its insensitivity to inhibition and the activity (V_{max}) or the affinity (K_m)

towards acetylcholine (Devonshire and Moores, 1984). The flexibility of the insect to tolerate slight changes in the kinetics of the AChE/acetylcholine interaction, means that a range of mutations in the AChE are possible leading to different pesticide insensitive variants. Unlike AChE, non-specific esterases appear to have no significant physiological function and therefore, there are fewer restrictions on their diversification.

The possibility of the involvement of increased quantities of AChE in OP resistance has also been investigated. Fournier *et al.* (1992) introduced additional copies of the AChE gene to *Drosophila melanogaster* individuals by P-mediated transformation and constructed different strains with various amounts of the enzyme. Toxicological analysis of these strains demonstrated that the resistance to OPs is correlated with the amount of AChE present in the central nervous system (Fournier *et al.*, 1992). By increasing the gene expression, the soluble form of AChE was secreted to the haemolymph of *D. melanogaster* in greater quantities. These flies showed high levels of OP resistance as a result of the excess AChE, present outside the nervous system (Fournier *et al.*, 1993). Therefore, as implied by the latter experiment, the presence of increased quantities of AChE can function in the sequestration of the OPs in a manner similar to the elevated mosquito esterases. It has been strongly suggested that in natural populations of insects increased production of AChE occurs as a mechanism for the insecticide resistance within the limitation that it must not

alter the normal physiology of the animal (Fournier *et al.*, 1992; 1993). It is hard to believe that such a mechanism would be selected in the presence of the elevated esterase-based mechanism because the esterases are more efficient than the AChE in binding some insecticides as shown in Chapter 2 (see section 2.3.2.2.3.) and also unlike AChE, esterases do not have any distinct physiological function which could be altered in the presence of the excess enzyme. Furthermore, the molecular weight of the AChE is higher than that of the esterases and since both enzymes interact with insecticides in 1:1 stoichiometry, production of AChE is more energy consuming than the production of esterases.

The number of mutated forms of a protein tend to be extremely limited when the protein of interest has a highly specific function in the physiology of the animal. Two such examples are exhibited by the GABA receptor proteins and the Na^+ channel proteins of the insect nervous system.

The γ- aminobutyric acid subtype A ($GABA_A$)-gated chloride channel of the insect nervous system is the target site for cyclodiene insecticides. The evidence suggests that the cyclodiene resistance is due to the insensitivity of these receptors (Deng *et al.*, 1991). The $GABA_A$ receptor/chloride ion-channel gene was cloned and sequenced from an insecticide susceptible *Drosophila melanogaster* strain (ffrench-Constant *et al.*, 1991). A single base pair mutation causing a single amino acid substitution (Ala^{302} to Ser) within the second

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membrane-spanning region of the channel was found to be the only consistent difference between resistant and susceptible strains (ffrench-Constant *et al.*, 1993b). Site-directed mutagenesis of this amino acid and functional expression of the resulting GABA gated chloride ion channels in *Xenopus* oocytes has confirmed the functionality of the resistance associated mutation (ffrench-Constant *et al.*, 1993c). In the resistant strains of *Drosophila simulans*, most insects showed the same mutation while a few displayed an alternative single base pair mutation in the same codon resulting in the substitution of a different amino acid. It has been shown that these were the only mutations present in both species using 122 resistant and 58 susceptible strains collected from two continents. Both mutations cause a loss of a restriction endonuclease *Hae*II site allowing the resistant gene to be detected by a polymerase chain reaction (PCR)/ restriction endonuclease (REN) treatment. The type of the resistant allele involved could be identified by the PCR amplification of specific alleles (PASA) treatment (ffrench-Constant *et al.*, 1993b; Steichen and ffrench-Constant, 1994). In *Aedes aegypti* the membrane spanning region of the gene has been shown to be almost identical to that of *D. melanogaster* and the same mutation, causing the same amino acid substitution, was shown to be involved with the resistance. This also could easily be detected by PCR/REN or PASA techniques (Thompson *et al.*, 1993; ffrench-Constant *et al.*, 1994). Most recently the same Ala^{302} to Ser mutation in $GABA_A$ protein has been shown to be involved with the cyclodine resistance in three species which belong to three different orders

of insecta; *Musca domestica* (Diptera), *Tribolium castaneum* (Coleoptera) and *Peripleneta americana* (Dictyoptera) (ffrench-Constant, 1994). It is interesting to note the conservation of the same mutation between widely separated insects groups. It has been suggested that the same mutation has occurred independently in different groups showing parallel evolution (Thompson *et al.*, 1993; ffrench-Constant, 1994). Unlike in the esterases and AChEs, the number of viable resistant mutations in this important functional region of the protein may therefore be extremely limited.

In *D. melanogaster* the presence of an *Eco*R1 site in the neighbouring intron was found to be associated with the resistance in all but 3 of 48 strains examined (ffrench-Constant *et al.*, 1993b). This shows a difference in the RFLP patterns of the resistant genes within only 770 base pairs of the resistance associated mutation whereas Raymond *et al.* (1991) claimed that the restriction maps of the amplified esterase B_2 genes were identical across several kilobases near the resistance gene in *Culex* strains from around the world. Perhaps the restriction enzymes used in the Raymond's study have not identified the mutations present among the resistant B_2 genes as they were not selected with the knowledge the DNA sequence.

The Na^+ channel protein of the insect nervous system is the target site for pyrethroids and DDT. Resistant insects have a 'kdr' type nerve insensitivity which

is caused by the reduction in the density of the target sites/ Na^+ channels in the neuronal membrane (Jackson *et al.*, 1984; Kasbekar and Hall, 1988; Rossignal, 1988) or as most of the studies have shown by the reduced affinity (alteration of the structure) of the target site (Jackson *et al.*, 1984; Grubs *et al.*, 1988; Pauron *et al.*, 1989; Amichot *et al.*, 1992). Deduced amino acid sequence of the Na^+ channel protein of *Drosophila* shows a high degree of homology with vertebrate channel proteins in all the areas presumed to be critical for channel function indicating a high level of evolutionary conservation (Salkof *et al.*, 1987). In *Drosophila* two putative Na^+ channel genes, 'DSCI' and 'para', have been identified to be responsible for producing channel proteins with decreased affinity (Salkoff *et al.*, 1987; Ramaswami and Tanouye, 1989). Cloning and sequencing of the 'DSCI' gene revealed a single non-silent nucleotide change between the susceptible and resistant genes that may be responsible for the resistance (Amichot *et al.*, 1992). Using a fragment of the *Drosophila* 'para' gene, the resistance associated 'Msc' gene has been isolated from house flies. The amino acid identity between 'para' and 'Msc' is 99%. Different RFLP patterns at the 'Msc' locus have been identified in susceptible, 'kdr' and 'super kdr' flies (Williamson *et al.*, 1993). The evidence shows that more than one gene is involved in the 'kdr' resistance mechanism in insects. Whether the resistance associated mutations, which are found in the resistant alleles of each gene type, are conserved among different insect groups is yet to be established. However, as was shown for GABA receptors, there may be severe functional constraints

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on the formation of functional Na^+ channels which are insensitive to insecticide binding.

6.6. PROBLEMS IN THE NOMENCLATURE OF MOSQUITO CARBOXYLESTERASES

In the A_2/B_2 classification, subscripts are based on the R_f values of different esterases on native PAGE gels (Raymond *et al.* 1987) and the time has come to assess the validity of this nomenclature. In the present study significant differences were shown among the enzymes with similar R_f values. In addition, two B_2- type enzymes which have the same mobilities as esterase B_2 on starch gels have been classified as B_4 and B_5 after observing the differences in their restriction fragment length polymorphism patterns (Poirié *et al.* 1992). Obviously the electrophoretic mobility of the esterases on either starch or acrylamide gels is an extremely poor indicator of the actual allelic variant or variants present in a given strain within the A_2 or B_2 classification. Furthermore, an esterase activity band observed on a native gel may not be a single enzyme but a mixture of isozymes representing an allelic mixture. The terms 'A' and 'B' are also misleading since both these types are referring to serine hydrolases which can be classified under the 'B' type esterases in Alridge's classification (Aldridge, 1953a; 1953b). In keeping with the rat and mouse nomenclature (Zutphen, 1983; Bender *et al.*, 1984; Zutphen & Bieman, 1984; Simon *et al.* 1985), we therefore propose

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the two enzymes be referred to as ESα and ESβ and the genes be called *Esα* and *Esβ*. Esterase B_1 and B_2 would thus become ESβ-1 and ESβ-2. Fully characterized allelic forms might then be superscripts following the digits for the forms, for example, for isoforms of ESβ-2 they might be ESβ-2^1 or ESβ-2^4.

6.7. CONCLUSIONS AND FUTURE STUDIES

In conclusion, characterization of the purified esterases A_2, B_2 and B_1 from several different strains of *Cx quinquefasciatus* revealed that the role of elevated carboxylesterases in the insecticide resistance is sequestration (rapid binding followed by slow turn over of the insecticide) and that more than one isoenzymes exists for each of these esterase isozymes. Non-elevated susceptible enzymes were markedly less reactive with some of the insecticides. Both esterase types 'A' and 'B' were immunologically related to each other and revealed a high molecular homology to the insecticide target site AChE. The presence of multiple variants of 'A' and 'B' type esterases show a polymorphism among the mosquito carboxylesterases, but the esterases are similar enough in their characteristics through purification to suggest a common origin. Diversification of the amplified resistant alleles of the esterases indicate that amplification of the gene loci have occurred several times and/or such events are not recent.

The knowledge of kinetic and catalytic properties and molecular structure of the elevated carboxylesterases should lead to their manipulation to facilitate insect control. The present work is an enzymological approach to this problem. A number of the A_2, B_2 and B_1 genes have already been sequenced (Vaughan *et al.*, submitted[a]; [b]). Polymorphism among the B_1 esterases has already been shown in both their DNA sequence and RFLP patterns (A.Vaughan, personal communication). Similar experiments for A_2/B_2 genes will show whether the multiple variants observed in the present study are due to differences in their gene sequences or whether there is any post-transcriptional or post-translational modifications in the expressed gene products. Different types of esterase genes will now be expressed in baculovirus systems and their subsequent purification and characterization will confirm their identity. Knowledge of the possible events/factors which can influence the mutation and amplification of these esterase genes would also be of great importance in developing new strategies in insect control. Subsequent construction of the 3-D structure of the carboxylesterase protein will allow the identification of the residues which are important in the catalytic activity of the enzyme. A thorough understanding of the molecular structure of these enzymes will aid in designing novel insecticides which are less reactive with these enzymes.

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APPENDIX

Refereed and non-refereed publications already arising from this study

Refereed:

1) KARUNARATNE, S.H.P.P., JAYAWARDENA, K.G.I., HEMINGWAY, J. AND KETTERMAN, A.J. (1993) Characterization of a B-type esterase involved in insecticide resistance from the mosquito *Cx quinquefasciatus*. *Biochem. J.* **294**: 575-579.

2) KETTERMAN, A.J., KARUNARATNE, S.H.P.P., JAYAWARDENA, K.G.I. AND HEMINGWAY, J. (1993) Qualitative differences between populations of *Cx quinquefasciatus* in both the esterases A_2 and B_2 which are involved in insecticide resistance. *Pestic. Biochem. Physiol.* **47**: 142-148.

3) JAYAWARDENA, K.G.I., KARUNARATNE, S.H.P.P., KETTERMAN, A.J. AND HEMINGWAY, J. (1994) Determination of the role of elevated B_2 esterase in insecticide resistance in *Cx quinquefasciatus* from studies on the purified enzyme. *Bull. Entomol. Res.* **84**: 39-43.

Non-refereed

1) KARUNARATNE, S.H.P.P., JAYAWARDENA, K.G.I., HEMINGWAY, J. AND KETTERMAN, A.J. (1993) Evidence for polymorphism in mosquito esterases involved in insecticide resistance. *Biochemical Society Transactions* **647**: 480.

2) KARUNARATNE, S.H.P.P., JAYAWARDENA, K.G.I., HEMINGWAY, J. AND KETTERMAN, A.J. (1993) The function of esterases in insecticide resistance in *Cx quinquefasciatus* mosquitoes from Sri Lanka. *Biochemical Society Transactions* **647**: 482.

3) HEMINGWAY, J., KETTERMAN, A.J., KARUNARATNE, S.H.P.P., JAYAWARDENA, K.G.I. AND VAUGHAN, A. (1993) Amplified esterases A_2 and B_2. Has resistance occurred once or several times in *Proc. 1st Int. Con. Insect Pest Urban Envir.* (eds K.B. Wildey and W.H. Robinson) BPCC Wheatons Ltd, Exeter, U.K., 319-328.

4) KARUNARATNE, S.H.P.P., JAYAWARDENA, K.G.I., HEMINGWAY, J. AND KETTERMAN, A.J. (1994) Immunological cross-reactivity of a mosquito carboxylesterase-A_2 antibody to other mosquito and vertebrate esterases and cholinesterases. *Biochemical Society Transactions* **649**: 127.

5) VAUGHAN, A., RODRIGUEZ, M., KARUNARATNE, S.H.P.P., SMALL, G.J., KETTERMAN, A.J., CHADEE, D. AND HEMINGWAY, J. (1994) Characterization of non-specific esterases in *Culex quinquefasciatus* insecticide resistance (Abstract) *8ᵗʰ IUPAC Cong. Pestic. Chem.*.