Kinetic and Molecular Differences in the Amplified and Non-amplified Esterases from Insecticide-resistant and Susceptible Culex quinquefasciatus Mosquitoes*

S. H. P. Parakrama Karunaratne, Janet Hemingway, Kamburapola G. I. J. ayawardena, Vasanthi Dassanayake, and Ashley Vaughan

From the Department of Pure and Applied Biology, University of Wales Cardiff, P. O. Box 915, Cardiff CF1 3TL, Wales, United Kingdom and the Department of Zoology, Open University, NawaI, Nugegoda, Sri Lanka

Two non-amplified esterases were purified from the insecticide-susceptible Pel SS strain of Culex quinquefasciatus. These were the two major esterase activity peaks in this strain. The two corresponding amplified carboxylesterases, Estα2 and Estβ2, involved in organophosphate sequestration were purified from two resistant C. quinquefasciatus strains. The Pel SS esterases were significantly less reactive with the organophosphates than those from the resistant strains. One of the Pel SS esterases was electrophoretically identical to amplified Culex Estβ1. However, it differed kinetically, and in its nucleotide and predicted amino acid sequences from the two characterized amplified Estβ1s, it is classified as Estβ3. Restriction fragment analysis suggested Pel SS has only one Estα and one Estβ gene, while the resistant Pel RR has both amplified and non-amplified forms of Estα and Estβ. The EcoRI fragments for both Pel SS esterases were distinct from those of the amplified Estα2, Estβ2, or Estβ1. An esterase with the same size EcoRI fragment as Estβ2 was also present in Pel RR. This restriction enzyme fragment analysis of C. quinquefasciatus field populations suggest that variability of the susceptible alleles may be lower than previously suggested. A non-amplified Estα with a unique EcoRI band was present in Pel RR. The previous esterase purification procedures may not have separated these amplified and non-amplified alleles. Hence, the small differences between the purified esterases from resistant strains may reflect mixtures of identical amplified alleles with different non-amplified alleles, which have significantly different kₐ values.

The use of pesticides, both directly and indirectly against the mosquito Culex quinquefasciatus, has resulted in the selection of broad spectrum organophosphate and carbamate resistance. The most commonly selected resistance mechanism is the increased activity of Estα and Estβ carboxylesterases (EC 3.1.1.1) (A₂ and B₂ esterases on an earlier classification) (1–6).

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+ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) Z11698, Z32696, Z32694.

† Supported by the Association of Commonwealth Universities.
‡ Supported by The Royal Society. To whom all correspondence should be addressed. Tel.: 44-1222-874000 (ext. 5050); Fax: 44-1222-874305; E-mail: sabjh@cardiff.ac.uk.
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Classification of these esterases is based on their preferences for α- or β-naphthyl acetate, their mobility on native polyacrylamide gel electrophoresis (PAGE), and their nucleotide sequence (1). The overproduction of the Estα2 and a series of Estβ esterases is due to gene amplification (1, 5, 7, 8). Identical EcoRI restriction fragment sizes of the amplified Estβ2 from resistant C. quinquefasciatus worldwide has been reported, in contrast to a high level of variability in Estβ from insecticide-susceptible mosquitoes (9). A cDNA with 97% identity to Estβ2 has been cloned from an insecticide susceptible strain (Pel SS) of C. quinquefasciatus from Sri Lanka (8); however, the protein has not been characterized. After native starch or PAGE of homogenates of individual resistant Culex larvae of the amplified Estα2/Estβ2 esterase phenotype, two electrophorons can be visualized. Under the same conditions, no esterase bands are visible in homogenates of susceptible insects. This has lead to the suggestion that the susceptible insects have null alleles for these loci (10).

Here, we report the purification and characterization of both an α- and a β-naphthyl acetate-specific esterase from the Pel SS strain of mosquito and compare these at a biochemical and basic molecular level to the elevated esterases Estα2, Estβ2, and Estβ1. The role of the amplified esterases in resistance is sequestration, which is rapid binding and slow turnover of insecticides (11–13). The current study elucidates the relative efficiencies of the purified esterases from a susceptible and two further resistant strains at binding the carbanes and biologically active oxon analogues of the organophosphorus insecticides. Characterization of the amplified and non-amplified esterases will facilitate future site-directed mutagenesis studies on the essential amino acid residues involved in the enzyme-insecticide interaction.

EXPERIMENTAL PROCEDURES

Four mosquito strains of C. quinquefasciatus were used. Pel was established from a large (>1,000) sample of larvae collected from Peliyagoda (Sri Lanka) in 1984. The population was heterogeneous for insecticide resistance, and both Pel SS and Pel RR were derived from this strain. The insecticide-susceptible Pel SS strain was obtained by selection and pooling of multiple single families for low esterase activity over three generations. The resistant Pel RR strain was selected from the Pel strain by mass larval selection with temephos (2, 3). The Muheza strain was collected from Tanzania in 1987 and maintained under intermittent chlorpyrifos selection. SPerm was collected from J eddah (Saudi Arabia) in 1980. It was selected for 20 generations with permethrin and then intermittently with malathion and temephos (15). The three resistant strains both have the
Elevated esterases Estα2 and Estβ2.

Field collections, each of >1000 larvae, were made in six suburbs of Colombo (Sri Lanka) in 1994. The areas included Peliyagoda, where the 1984 collection had been made. The frequency of the amplified esterases in these field populations was analyzed by nonspecific esterase assays of 100–200 larvae (3). Batches of 100 larvae of the remaining insects were used for DNA extraction.

Esterases were copurified from the resistant and susceptible strains to ensure that any differences in the enzymes seen between this and previous studies were not due to minor variations in our purification and kinetic determination procedures over time.

Q-Sepharose Fast Flow, phenyl-Sepharose Fast Flow, PD-10 columns, and Nick spin columns were purchased from Pharmacia (United Kingdom). Hydroxyapatite and the protein assay kit were purchased from Bio-Rad (UK). The p-chloromercuribenzoate was from Pierce (Chester, UK). Biochemicals were purchased from Sigma (UK) except as stated. The diethyl dimethoxythiophosphorylthio) succinate (malaaxon, 87.5% pure), diethyl-4-nitrophenyl phosphophate (paraoxon, 97.4% pure), and 2-isopropoxyphenyl methylcarbamate (propoxur, 97% pure) were purchased from British Greyhound (Birkenhead, Merseyside, UK). The O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate (chlordimeform, analytical grade) and O,O-dimethyl-O-4-nitro-m-tolyl phosphorothioate (fenitrooxon, 98.3% pure) were gifts from Dow Elanco (Midland) and Sumitomo Chemical Co. (Osaka, Japan), respectively.

Enzyme activities were assayed with the substrate p-nitrophenyl acetate (pNPA) (1 mM) in 50 mM phosphate buffer (pH 7.4) at 22°C. The protein concentration was estimated by the method of Bradford (16) using bovine serum albumin as the standard protein.

The purification of Carboxylesterases—Batches of 4th instar larvae were homogenized and centrifuged at 15,000 × g for 5 min, and the supernatant was taken. Carboxylesterases Estα and Estβ were purified several times from numerous batches of larvae of each strain by sequential column chromatography and preparative electrophoresis as described previously (12, 17). Enzyme preparations were homogeneous as determined by SDS-PAGE. 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 2-mercaptoethanol.

All specific activities are given in units/mg of protein. A unit corresponds to the hydrolysis of 1 mol of substrate in 1 min under the assay conditions used. Kinetic constants were determined from at least three experiments for each substrate or inhibitor using enzymes from several different purifications. For the inhibition kinetics, stopped time inhibition assays were performed using pNPA or p-nitrophenyl hexanoate (pNHP) as the substrate. Insecticide stock solutions were prepared in acetonitrile and diluted in phosphate buffer (pH 7.4). The purified enzyme was incubated with a series of concentrations of the test insecticide (acetomine concentration of the medium never exceeded 1% (v/v)) for fixed time intervals. Residual activity was determined from the rate of substrate hydrolysis.

Inhibitor concentrations were usually in large excess so that linear pseudo-first order kinetics were obtained. The bimolecular rate constants for the formation of acylated enzyme (k cat) were derived as described previously (18). If inhibitor concentration could not be maintained in large excess, k cat values were determined in the presence of substrate (19). To minimize 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 [S]/[K m] ratio was always less than 0.5 (18).

Electrophoresis of native protein samples was performed in 7.5% acrylamide gels in Tris borate buffer, pH 8.0 (20). 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-PAGE was performed with standard proteins using 4–20% acrylamide gels in Tris/glycine/SDS buffer, pH 8.3 (21).

Genomic DNA Studies—DNA was extracted (8), precipitated with ethanol, resuspended in a small volume of TE, and stored at 4°C until used for Southern blotting. Pel RR Estβ2 and Estα2 esterase cDNA fragments (8, 22) were used as probes to determine the haplotype of the esterases in each laboratory strain or field collection of mosquitoes. Genomic DNA (10 μg) was digested to completion with EcoRI, HindIII, or BamHI and separated on 0.8% (w/v) agarose gels. The DNA was transferred to charged nylon membranes (Amersham) and hybridized with 32P-labeled probe (specific activity > 2 × 108 cpm/μg) at 65°C for 16 h in hybridization buffer (5 × Denhardt’s solution, 6 × SSC, 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate, 5% (w/v) polyethylene glycol 8000, 100 μg/ml boiled sheared herring sperm DNA). The final membrane washes were at 65°C in 0.1 × SSC and 0.1% (w/v) SDS for 20 min. Membranes were probed first with the Estβ2 cDNA then stripped and probed with Estα2 cDNA.

RESULTS

On native PAGE gels, the susceptible Pel SS strain had no visible esterase bands, whereas all the resistant strains had the amplified esterase bands Estα2 and Estβ2 (Fig. 1). The specific activity of Pel SS crude homogenate for the substrate pNPA was 0.02 units/mg, approximately 50-fold less than that routinely observed for resistant crude homogenates. For pNPH, the specific activities were much higher (0.14 units/mg for Pel SS crude homogenate), and this was used as the assaying substrate in subsequent esterase purifications from Pel SS, as the higher specific activity made peak detection simpler. However, the first purification was followed with both pNPA and pNPH, and the major peaks detected were the same with both substrates. Since hydrolysis of pNPH is linear for less than 1 min, pNPA was still the substrate of choice for all purifications from the resistant strains. After purification, only 5–10 μg of each of the Estα and Estβ enzymes were obtained from 10–15 g, wet weight, of Pel SS larvae.

SDS-PAGE with purified esterases demonstrated that the SDS-PAGE pattern of the esterases from the susceptible Pel SS and five resistant strains of C. quinquefasciatus on native PAGE stained for esterase activity. The elevated esterases Estα2 and Estβ2 are marked.

FIG. 1. Equal amounts of crude homogenates of individual insects from the susceptible Pel SS and five resistant strains of C. quinquefasciatus on native PAGE stained for esterase activity. The elevated esterases Estα2 and Estβ2 are marked.

FIG. 2. A native PAGE of purified Estα2 type carboxylesterases from the susceptible Pel SS and five resistant strains of C. quinquefasciatus stained for esterase activity. Crude homogenate of Pel RR, which has elevated Estα2 and Estβ2, is shown for reference.
strains were similar to those previously reported for Estα21 and Estβ21 (11, 12). The electrophoretic mobility on native PAGE of the Pel SS-purified Estα was reproducibly faster than that of the resistant Estα21 (Fig. 2). In contrast, the Pel SS Estβ had a slower mobility than that of the resistant Estβ21 (Fig. 3) but exactly the same mobility as the elevated C. quinquefasciatus Estβ1 (14).

For the substrates NPA and pNPH, the K_m values of the Pel SS Estβ enzyme were 247.2 ± 23.3 and 12.2 ± 2.21 μM, respectively. K_m values of the Pel SS Estα enzyme for NPA and pNPH were 119.3 ± 5.7 and 40.0 ± 7.2, respectively.

Previous studies showed K_m to be the most important constant in the interaction between the Culex esterases and the insecticides (12). 10-fold differences in that are not significantly different from either single letter alone. pyrifos-oxon and paraoxon of the Pel SS Est b were 119.3 and 6.49 μM, respectively.

Fining binding chlorpyrifos-oxon and paraoxon of the Pel SS Estβ enzyme were, respectively, 1000- and 100-fold less than those of the resistant strains (Table II). Thus, the non-amplified enzymes from Pel SS are less able to bind the insecticides than their respective amplified counterparts from various resistant strains. The ratios of the reaction rates with the insecticides for the crude homogenates of Pel RR and Pel SS were similar to those observed for the purified enzymes (Table III). As with the purified enzymes, the greatest differences were for chlorpyrifos-oxon and paraoxon, which suggests that this method may be valid as a crude means of detecting the level of interaction between these enzymes and insecticides.

The genomic EcoRI digests of the Pel SS and Pel RR strains and the field-collected insects sequentially probed for the Estα and Estβ esterases are shown in Fig. 4. The amplified Estα21 and Estβ21 bands in Pel RR are clearly distinguishable. The elevated Estα21 esterase is seen as a 3.5-kb band in the Pel RR strain, along with a fainter (non-amplified) 4-kb band. In Pel SS, a single non-amplified 4.1-kb Estα band is present. In both the Pel RR and Pel SS strains, there is a fainter non-amplified 3.3-kb Estβ band. The six field collections of insects had elevated esterase frequencies ranging from 0.12 to 0.84 on the basis of nonspecific esterase assays. When DNA digests from field-collected insects were probed, the amplified Estα21 and Estβ21 bands had amplification levels broadly in agreement with the different frequencies of elevated esterase individuals in each of the populations. There were three distinct non-amplified bands of each of Estα and Estβ bands in all six field collections analyzed with each of the restriction enzymes (Fig. 4). These bands were identical in all field collections. Blots of DNA digests from individual Pel SS and field-caught insects showed that the amplified bands were easily visible in these samples, but the non-amplified bands were not visible under our experimental conditions. The non-amplified bands from Pel SS were, however, visible in the pooled DNA from 4 to 5 insects. Hence, the non-amplified restriction bands seen in the field material must be present in more than one insect from the pool of 100.

**DISCUSSION**

Both Estα and Estβ esterases are expressed in the insecticide-susceptible Pel SS strain of C. quinquefasciatus, although at a lower level than in resistant strains where these esterase genes are amplified (1, 8). The two esterases from Pel SS now need classifying in line with the system adopted for mosquito esterases (1). Recently, two Estβ1 esterases were found to be different from each other in their nucleotide sequence and inferred amino acid sequence, demonstrating that multiple alleles of the Estβ locus with identical electrophoretic mobility occur (8). The TEM-R strain of C. quinquefasciatus has an amplified Estβ1 EcoRI band of 2.1 kb (9), while the MRES Estβ1 has a double band of 3 and 3.2 kb (8). We have now shown that the Estβ esterase in Pel SS electrophoretically would be classified as Estβ1, but it has an EcoRI band of 3.3 kb, which is distinct and elevated in those from both the MRES and TEM-R strains, and has 98% identity at the amino acid level with the amplified Estβ (8), hence the Pel SS esterase should be classified as Estβ1. The high stringency washing conditions for the Southern blots show that the non-amplified and amplified Estα esterases must be closely related. The Estα esterase in Pel SS has a different electrophoretic mobility to Estα2 and Estα1 (formerly A1), hence it should be classified as Estα3.

The Pel SS Estβ1 K_m for pNPA was significantly higher than those of the resistant Estβ2 previously observed for Pel RR (140.8 ± 5.24 μM), Dar91 (85.41 ± 3.94 μM), and Tanga85 (90.11 ± 6.49 μM) (12, 17). In contrast, the Pel SS Estα3 and Estβ1 esterase pNPH K_m values are not significantly different from those previously reported for Estα21 and Estβ21 (17). The differences (up to 1000-fold) between the inhibition kinetic

**Table I**

| 10-5 × K_m | Pel SS | Pel RR | Dar91 | Tanga85 | Muheza | SPerm |
|---|---|---|---|---|---|---|
| Chlorpyrifos-oxon | 53.3 ± 10.7a | 144 ± 32.9b | 521 ± 91.9b | 374 ± 74.3b | 709 ± 122b | 726 ± 158b |
| Fenitrooxon | 3.43 ± 1.19a,b | 0.958 ± 0.201a | 4.53 ± 0.80b | 9.35 ± 1.54b | 2.89 ± 0.57ab | 3.16 ± 0.649b |
| Malaoxon | 0.113 ± 0.022a,b,c | 0.219 ± 0.033a | 0.130 ± 0.031a,b,c | 0.086 ± 0.010b,c | 0.229 ± 0.032c | 0.204 ± 0.034a,b,c |
| Paraoxon | 131 ± 16.4a | 190 ± 20.7b | 143 ± 28.6b | 119 ± 24.3b | 387 ± 72.4c | 351 ± 67.0b,c |
| Propoxur | 0.0018 ± 0.0004a,b | 0.0124 ± 0.0027c | 0.0151 ± 0.0023b,c | 0.0063 ± 0.0011b,c | 0.0203 ± 0.0040b,a,c | 0.0211 ± 0.0047b,c |

The kinetic constant k_m (μM⁻¹ min⁻¹) for insecticide interactions with the susceptible (Pel SS) Estα3 carboxylesterases and the elevated Estα21 esterase from five resistant strains of C. quinquefasciatus

The data are means ± S.D. In the same row, different superscript letters indicate a significant difference (p < 0.05). Two letters indicate values that are not significantly different from either single letter alone. k_m values for the enzymes purified from Pel RR, Dar91, and Tanga85 were reported previously (17) and given here for comparison.

**Fig. 3.** A native PAGE of purified Estβ type carboxylesterases from the susceptible Pel SS and five resistant strains of C. quinquefasciatus stained for esterase activity. Crude homogenate of Pel RR, which has elevated Estα21 and Estβ21, is shown for reference.
The kinetic constant $k_a$ (m$^{-1}$ min$^{-1}$) for insecticide interactions with the susceptible (Pel SS) Est$\beta 1$ carboxylesterases and the elevated Est$\beta 2$ esterases from five resistant strains of C. quinquefasciatus.

The data are means ± S.D. In the same row, different superscript letters indicate a significant difference ($p < 0.05$). $k_a$ values for the enzymes purified from Pel RR, Dar91, and Tanga85 were reported previously (17) and are given here for comparison.

| Insecticide          | 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$^b$ | 3.08 ± 0.183$^b$ | 4.06 ± 0.630$^a$ | 5.24 ± 0.503$^c$ |
| Malaoxon             | 0.400 ± 0.050$^a$ | 0.496 ± 0.168$^{a,b}$ | 0.553 ± 0.195$^{a,b}$ | 0.513 ± 0.097$^{a,b}$ | 0.383 ± 0.057$^b$ | 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$^a$ | 0.052 ± 0.001$^b$ | 0.0074 ± 0.0014$^b$ | 0.0048 ± 0.0004$^b$ | 0.0065 ± 0.0007$^b$ | 0.0061 ± 0.0018$^b$ |

| FIG. 4. Southern blots of EcoRI genomic digests probed with cDNA fragments of the Pel RR Est$\beta 2$ and Est$\beta 2$ esterases. Track 1, Pel RR; track 2, Pel SS probed with Est$\beta 2$; track 3, Pel RR; track 4, Pel SS; track 5, Kaduwela; and track 6, Peradeniya Culex field samples. 10 μg of DNA were digested per sample. Final washes were at 65 °C with 0.1% SDS and 0.1 × SSC (approximately 29 and 84% of the Peradeniya and Kaduwela C. quinquefasciatus populations, respectively, contained elevated esterases). |

The kinetic differences between the purified amplified Est$\beta 2$ and Est$\beta 2$ enzymes from different strains were previously suggested to be due to either allelic mixtures of the esterases or different single allele forms of both Est$\alpha 2$ and Est$\beta 2$ in each of the resistant strains (12, 13, 17). However, the Est$\beta 2$ EcoRI digest pattern, unlike that for Est$\beta 1$, does not appear to vary (9), hence the observed kinetic differences may not be reflected at the DNA level. Our current data show that Pel RR has the invariant Est$\beta 2$ EcoRI band and that it apparently contains an allelic mixture of both Est$\alpha$ and Est$\beta$, with a minor non-amplified band of each of the esterase types being present along with the prominent amplified band. Thus, in this and previous biochemical studies, all the purified Est$\alpha$ and Est$\beta$ esterases from the resistant strains may have been mixtures of amplified and non-amplified alleles, as the purification conditions used may not have separated these minor variants from each other. Such allelic mixtures would not have been detected, as both the Pel SS Est$\beta 1$ and the Pel RR Est$\beta 2$ esterase genes code for proteins of 540 amino acids, whose predicted molecular weights would not allow their separation by SDS-PAGE (8). This may explain the reported differences between purified esterases from different resistant strains, as variability could arise from different proportions of the amplified and non-amplified alleles, given the big differences in $k_a$ values between the amplified and non-amplified alleles of both the Est$\alpha$ and Est$\beta$ esterases. Alternatively, different non-amplified alleles could be mixed with an identical amplified allele to give a similar result. Our results also suggest that there is limited variability in the non-amplified Est$\alpha$ and Est$\beta$ alleles of the Sri Lankan field population, as only single non-amplified Est$\alpha$ and Est$\beta$ EcoRI bands were apparent from DNA obtained from mass homogenates of Pel SS, suggesting that this colony contains only a single Est$\alpha$ and a single Est$\beta$ esterase, even though the strain originated from numerous pooled single families selected from a large field collection of insects. Several years of laboratory colonization could have resulted in the loss of much of the variability. However, if the latter is true, it is surprising that the non-amplified Est$\beta$ allele of Pel RR has an identical EcoRI fragment to the Est$\beta 1$ allele of Pel SS, given that the two strains are maintained in separate insectaries. Similarly, restriction digest analysis of recent field collections of C. quinquefasciatus also suggest that the variability of the non-amplified Est$\alpha$ and Est$\beta$ alleles in the Sri Lankan field population is limited, with three common restriction fragments, as well as the amplified EcoRI fragment, occurring with each of three different restriction enzymes in six independent field collections from the Colombo area for each esterase.

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