Characterization of a B-type esterase involved in insecticide resistance from the mosquito *Culex quinquefasciatus*

S. H. P. Parakrama KARUNARATNE, K. G. Indrananda JAYAWARDENA, Janet HEMINGWAY and Albert J. KETTERMAN*

Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K.

The enzyme, esterase B₂, involved in insecticide resistance has been purified and characterized from the mosquito *Culex quinquefasciatus*. The monomeric enzyme has an *M_r* of 62000 and a pI of 5.0. This enzyme is compared with the esterase A₂ previously characterized [Ketterman, Jayawardena and Hemingway (1992) *Biochem. J.* **287**, 355–360]. The kinetic constants for interaction with several insecticides indicate, as for the esterase A₂, that the

B₂ enzyme has a role in resistance. The rates and affinities of binding observed support the hypothesis that the role mainly is sequestration followed by the slow turnover of the insecticide. Although the B₂ esterase appears to have a slightly higher rate of interaction with insecticides, the A₂ has a much greater *V_{max}*, with the xenobiotic substrates studied. The B₂ esterase also appears to be present in the larvae to a lesser extent than the esterase A₂.

INTRODUCTION

The mosquito *Culex quinquefasciatus*, Pel RR strain, from Sri Lanka has been shown to have organophosphorus insecticide resistance which is associated with increased activity of the esterases A₂ and B₂ (Peiris and Hemingway, 1990a,b). The putative mechanism of esterase-mediated resistance involves an overproduction of these insect enzymes for the sequestration of and cross-resistance to various organophosphorus and carbamate insecticides (Devonshire, 1977; Mouchès et al., 1986, 1987; Raymond et al., 1989). Two esterases responsible for the insecticide resistance of mosquito strain Pel RR are referred to as 'A₂' and 'B₂' because of their different preferences for the substrates α - and β -naphthyl acetate [and not based on the Aldridge (1953) classification] (Raymond et al., 1987). Immunological and molecular-biological evidence has shown a high level of sequence similarity among type 'B' esterases, but not between the type 'B' and 'A' esterases (Mouchès et al., 1987; Raymond et al., 1989; 1991). A limited enzymic characterization has been reported using crude homogenates and 100000 g supernatants as the enzyme source for esterases A₁ and B₁ (Pasteur et al., 1981; Fournier et al., 1987). It was reported, in the studied strains of *Culex*, that the analogous A and B esterases represented 1–3% and 6–12% of the total protein in the insect respectively (Fournier et al., 1987). Here we show that both the A and B esterases together comprise only 0.4% of the protein in the Pel RR strain.

Previously we have reported the purification and characterization of esterase A₂ (Ketterman et al., 1992). We now report the purification and characterization of the second enzyme for the strain Pel RR, esterase B₂. This is an essential first step in the elucidation of the biochemical mechanisms involved in resistance, because characterization of the purified esterases provides valuable information on the properties of the enzymes, highlighting differences in expression within and between strains and possibly even quantitative changes of these enzymes in different populations. Several possible physiological substrates were tested with purified esterase B₂ as was previously reported for esterase A₂ (Ketterman et al., 1992). Kinetic constants for rates of interaction and affinity of these esterases for various insecticides were investigated. The role of both esterases in

insecticide resistance is discussed and compared with that of the aphid esterase E4.

EXPERIMENTAL

Materials

Q-Sepharose Fast Flow, phenyl-Sepharose Fast Flow, PD-10 columns, Nap-5 columns, Nick spin columns and Ampholine PAGplates pH 3.5–9.5 were purchased from Pharmacia. Hydroxyapatite, Prep-Cell model 491 and the Protein Assay Kit were purchased from Bio-Rad. The *p*-chloromercuribenzoate-agarose was from Pierce.

Biochemicals were purchased from Sigma, except when stated otherwise. The NEFA C kit was purchased from Wako Pure Chemicals (Osaka, Japan). *OOO'O'*-tetramethyl-*OO'*-thiodi-*p*-phenylene bis(phosphorothioate) (temephos, 99.7% pure), *OO*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate (chlorpyrifos, 99.5% pure), *OO*-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 Dow Elano (Midland, MI, U.S.A.) and Sumitomo Chemical Co. (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 ICI (Bracknell, Berks., U.K.). The source of carboxylesterases reported here is a pesticide-selected resistant mosquito (*Culex quinquefasciatus*) from Sri Lanka. This strain, Pel RR, is 29-fold more resistant to temephos than a susceptible strain (Peiris and Hemingway, 1990a,b).

Assays

During purification, esterase activity was assayed at 405 nm using 1 mM *p*-nitrophenyl acetate in 50 mM phosphate buffer,

Abbreviation used: AChE, acetylcholinesterase.

* To whom all correspondence should be sent.

pH 7.4, at 22 °C. Unless stated otherwise, all enzyme assays were performed at 22 °C. Enzymic hydrolysis of diethyl succinate, malathion, palmitoyl-CoA, acetylthiocholine, butyrylthiocholine and the acylglycerol substrates were measured as reported previously (Ketterman et al., 1992). Protein concentration was estimated by the method of Bradford (1976), with BSA as the standard protein.

Kinetic constants

All specific activities are given in unit/mg of protein. A unit corresponds to the hydrolysis of 1 μ mol of substrate in 1 min under the assay conditions used. Michaelis constants (K_m) and maximum specific activities were calculated by non-linear regression by the ENZFITTER program (by R. J. Leatherbarrow; Biosoft). For the inhibition kinetics, stopped time inhibition assays were performed using *p*-nitrophenyl acetate or *p*-nitrophenyl hexanoate as the substrate. The purified enzyme was incubated with a series of concentrations of the respective insecticide, and at various times aliquots were withdrawn and 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 chlorpyrifos-oxon and paraoxon k_a values were determined in the presence of substrate by the method of Main and Dauterman (1963). The re-activation experiments were performed by incubating the purified enzyme or the crude homogenate with the respective insecticide for 15 min so that the enzyme was more than 90% inhibited. The unbound insecticide and enzyme-insecticide complex were separated on a Nick spin column, following the manufacturer's instructions. Aliquots were removed to measure the esterase activity of the re-activating enzyme, as well as a control, over time. The pseudo-first-order inhibition rate constants were calculated and used to determine the various inhibition constants (Aldridge and Reiner, 1972). Carbamate and organophosphate insecticides inhibit B-type carboxylesterases [according to Aldridge's (1953) classification] by rapid esterification of a serine residue in the active site. This reaction is followed by a rate-determining slow deacylation. The generally accepted reaction mechanism is:

where, EI is the Michaelis complex and EI' is the acylated enzyme. Linear pseudo-first-order kinetics were obtained for the inhibition of the esterases in the insecticide interaction experiments (Aldridge and Reiner, 1972).

Influence of effectors

Solutions of bis-(*p*-nitrophenyl) phosphate (0.1 mM), paraoxon (0.1 μ M and 0.1 mM), EDTA (1 mM), eserine (10 μ M) and several metal ions were prepared either in 50 mM phosphate buffer, pH 7.4, or in 25 mM Bistris propane buffer, pH 7.4. Each effector was 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.

Purification of carboxylesterases

Carboxylesterase B₂ was purified by a further development of the previous method for esterase A₂ (Ketterman et al., 1992). Using the same procedure as for esterase A₂, chromatography was performed on Q-Sepharose and phenyl-Sepharose. The esterase B₂ was eluted as a second activity peak in the abovementioned

phenyl-Sepharose column. These fractions were combined and dialysed against dry sucrose. Buffer exchange into the hydroxyapatite buffer was performed on PD-10 columns according to the manufacturer's instructions. The sample was applied to a hydroxyapatite column (2.2 cm $\times$ 5.4 cm) equilibrated with 10 mM phosphate buffer, pH 6.8, containing 50 mM NaCl and 10 mM dithiothreitol. The esterase activity was eluted with a 5-bed-vol. gradient of the phosphate buffer (10–200 mM, pH 6.8) containing no NaCl. The hydroxyapatite chromatography was performed at 22 °C. The esterase activity was eluted as a single peak, and the fractions were combined and concentrated in Amicon centrprep 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 cm $\times$ 5 cm) equilibrated with the same buffer. The esterase activity was eluted with a 5-bed-vol. gradient of the equilibrating buffer and 20 mM phosphate buffer, pH 6.8, containing 30 mM β -mercaptoethanol.

PAGE

Electrophoresis of native protein samples was performed in 7.5% acrylamide gels in 0.1 M 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 present simultaneously and 0.1% (w/v) Fast Blue B in 100 mM phosphate buffer, pH 7.4. SDS/PAGE was performed with Bio-Rad standard proteins (M_r 18 500–106 000) using a Bio-Rad Mini-Protean II electrophoresis unit and a 4–20% gradient acrylamide gel (Laemmli, 1970). Coomassie Blue R250 was used to stain for protein. Isoelectric focusing was performed on LKB Ampholine PAGplates pH 3.5–9.5 focused at 30 W constant power, at 10 °C, for 1.5 h.

N-terminal analysis

N-terminal analysis of purified enzymes was performed by automated Edman degradation on an Applied Bio Instruments 477a protein sequencer by the Protein Sequencing Unit at Royal Holloway and Bedford New College, University of London, Egham Hill, Egham, Surrey TW20 0EX, U.K.

RESULTS AND DISCUSSION

Purification

Both esterases were eluted from the ion-exchange column in a single peak, as both had similar isoelectric points. The B₂ enzyme was then separated from esterase A₂ on the phenyl-Sepharose column in a second, smaller, esterase activity peak. Multiple preparations routinely gave approx. 40% final recovery of enzyme activity and 350-fold purification for esterase A₂ and 3% final recovery and 50–60-fold purification for esterase B₂. With *p*-nitrophenyl acetate as the substrate, final specific activities for esterase A₂ were approx. 360 μ mol/min per mg and for esterase B₂ were approx. 52 μ mol/min per mg. By using the outlined procedures, approx. 800 μ g of purified esterase A₂ and 200–300 μ g of purified esterase B₂ can be obtained from 15 g wet weight of larvae. For esterase B₂ it was critical to maintain a minimum dithiothreitol concentration of 25 mM at the beginning of the purification. In the absence of this, pigments in the mosquito homogenate oxidized rapidly (within minutes) to produce a black-coloured solution in which the esterase quickly lost activity. After purification the enzymes were stable, with no decrease in specific activity after several months if stored in the presence of 25 mM dithiothreitol and 50% (v/v) glycerol

Table 1 Influence of the effectors on the activity of purified esterase B₂

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-(<i>p</i> -nitrophenyl) phosphate	0.1 mM	5.9
Paraoxon	0.1 µM	51.3
	0.1 mM	5.9

at -20 °C. The final esterase preparations appeared to be homogeneous as determined by SDS/PAGE, and any contaminating protein must be less than 0.6 % of the total protein (results not shown). In addition, the esterase classification of B₂ and A₂ was confirmed on non-denaturing PAGE gels stained for esterase activity.

Physical characterization

The relative monomeric mass of the purified esterase B₂, estimated from SDS/PAGE was 62 800 ± 2400. On gradient-gel PAGE native carboxylesterase had an estimated *M_r* of 55 700 ± 3700. The pI of 5.0 was determined by isoelectric focusing on Ampholine PAGEplates. This pI is typical for carboxylesterases, being in the general range of pH 4.7–6.5 (Heymann, 1982). The monomeric nature of the native form, *M_r* and the pI of this carboxylesterase are in close agreement with those of the esterase A₂ (Kettermann et al., 1992). Glycosylation of esterase B₂ with glucose and mannose residues was insignificant, as the enzyme did not bind to concanavalin A–Sepharose. N-terminal sequencing of the esterases A₂ and B₂ was not possible, owing to a blocked N-terminus for both enzymes.

Influence of effectors and the inhibitor confirmation of enzyme classification

The influence of several metal ions and known carboxylesterase effectors on the carboxylesterase B₂ are presented in Table 1.

Enzyme activity was inhibited only by CuCl₂, FeCl₃ and HgCl₂ of the metal ions studied. The inhibition by HgCl₂ was also observed for esterase A₂ (Kettermann et al., 1992), implicating the involvement of a thiol group for the stability of these mosquito carboxylesterases and the importance of the presence of dithiothreitol in the purification and storage of these enzymes. A phosphotriester hydrolase, purified from the insect *Heliothis virescens*, a cotton pest, also showed complete inhibition with Hg²⁺, but slight inhibition with Cu²⁺ and no inhibition with Fe³⁺ at 1 mM concentrations (Konno et al., 1990).

Partial inhibition by 0.1 µM paraoxon demonstrated the enzyme is a possible B-type serine hydrolase according to the classification of Aldridge (1953). This is in contrast with the complete inhibition observed for the esterase A₂ under the same conditions. The purified mosquito esterase B₂ was inhibited with bis-(*p*-nitrophenyl) phosphate, which has been shown to be a specific carboxylesterase inhibitor in rats (Brandt et al., 1980), but not with the chelating agent EDTA. These results are similar to those reported for esterase A₂ (Kettermann et al., 1992). The acetylcholinesterase inhibitor eserine did not inhibit the purified esterase B₂. In contrast esterase A₂ was completely inhibited by eserine, although it showed no significant activity with commonly used cholinesterase substrates (Kettermann et al., 1992). However, any structural similarity of esterase A₂ to the target enzyme, acetylcholinesterase (AChE), does not account for any greater contribution to resistance, since both A₂ and B₂ are almost equally effective at binding various insecticides.

Substrate specificity

Michaelis constants, *K_m*, and *V_{max}* values were determined for three general esterase substrates (Table 2). Both esterases showed a higher affinity towards *p*-nitrophenyl hexanoate (C₆) than *p*-nitrophenyl acetate (C₂). When the data for the two enzymes are compared, the rate of hydrolysis is considerably lower with esterase B₂ for both substrates. Pig and rat liver carboxylesterase isoenzymes have shown *K_m* and *V_{max}* values of 30–220 µM and 66–110 µmol/min per mg respectively for *p*-nitrophenyl acetate (Heymann, 1982). 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 µmol/min per mg (Kettermann et al., 1989). The two substrates used to define esterases A₂ and B₂ originally, α- and β-naphthyl acetate, were also examined. By definition, esterase A₂ is more reactive towards α-naphthyl acetate and esterase B₂ is more reactive towards β-naphthyl acetate when both substrates are present (Raymond et al., 1987). This substrate preference could be shown with α-naphthyl acetate, but with the β-naphthyl acetate reaction the Michaelis constant and *V_{max}* could not be determined because of

Table 2 Substrate interactions of esterases B₂ and A₂

The rate of substrate hydrolysis was measured at 405 nm for *p*-nitrophenyl acetate and *p*-nitrophenyl caproate, 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.

Substrate	Esterase ... A ₂		B ₂	
	Parameter ... <i>V</i> _{max} (units/mg)	<i>K</i> _m (μM)	<i>V</i> _{max} (units/mg)	<i>K</i> _m (μM)
<i>p</i> -Nitrophenyl acetate	472.0 ± 51.4	145.8 ± 45.0	63.4 ± 0.8	140.0 ± 50.0
<i>p</i> -Nitrophenyl hexanoate	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

Table 3 Kinetic constants for the interaction of esterase B₂ 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 LD₅₀ concentrations from resistant-versus-susceptible larval bioassay experiments (for organophosphates the value represents the resistance ratio for the thionate analogue). The k_a values for the esterase A₂ are presented for comparison.

Insecticide	Esterase ... B ₂				A ₂
	Parameter ...	$10^2 \times k_2$ (min ⁻¹)	$10^4 \times k_3$ (min ⁻¹)	$10^5 \times K_a$ (M)	$10^{-5} \times k_a$ (M ⁻¹ ·min ⁻¹)
Malaoxon* (7)		16.3 ± 3.8	18.1 ± 3.7	0.43 ± 0.11	0.50 ± 0.17
Fenitrooxon* (4)		37.4 ± 15.4	13.5 ± 3.9	0.56 ± 0.21	1.73 ± 0.60
Chlorpyrifos-oxon* (37)		—	2.4 ± 1.7	—	1550 ± 140
Paraoxon* (20)		—	5.1 ± 2.0	—	170 ± 53
Propoxur† (2)		40.2 ± 10.2	10.9 ± 4.4	135.7 ± 64.8	0.0052 ± 0.0017
					0.012 ± 0.003

* Organophosphate.

† Carbamate.

the insolubility of the substrate at the concentrations necessary. However, for comparative purposes, a maximum specific activity (189.9 µmol/min per mg) for esterase B₂ and (60.61 µmol/min per mg) for esterase A₂ was measured for β-naphthyl acetate at 0.1 mM. No activity of esterase B₂ could be detected when using diethyl succinate, malathion, palmitoyl-CoA, acetylthiocholine and butyrylthiocholine as substrates. Results obtained for several possible physiological substrates were similar to those observed for esterase A₂ (Kettermann et al., 1992). The preference of these mosquito carboxylesterases for medium-chain-length mono- and di-acylglycerols suggests their involvement in lipid-fatty acid metabolism in addition to a detoxication role (results not shown).

Interaction of esterases A₂ and B₂ with insecticides

The second-order rate constant for the formation of the acylated enzyme (bimolecular rate constant), k_a , Michaelis constant for the insecticide, K_a , and k_2 were determined for several insecticides. These values together with k_3 values obtained from re-activation kinetic experiments are shown in Table 3. No significant interaction of the purified mosquito esterase B₂ with phosphorothionates could be detected at their maximum solubility limits. A slight inhibition by a few thionates at high concentrations may be attributed to a possible contamination by the highly reactive oxon analogues. Several of the kinetic constants previously reported for purified carboxylesterase A₂ were for technical-grade insecticides (Kettermann et al., 1992). Purified esterase B₂ also interacted in a similar manner with those technical-grade thionates (results not shown). Chlorpyrifosoxon and paraoxon were such potent inhibitors of B₂ that the constants k_2 and K_a could not be determined and the k_a was determined in the presence of substrate (Main and Dauterman, 1963). The k_a values for the esterase A₂ were determined in both the presence and absence of substrate and were shown to agree irrespective of the manner of determination. Neither carboxylesterases interacted with the pyrethroid lambda cyhalothrin.

As shown more clearly with the oxon compounds the important constant is the rate of binding and formation of the acylated enzyme. As expected, both enzymes have higher rates of binding for the oxons, and the B₂ esterase appears to be slightly more reactive with the studied insecticides than the A₂ enzyme. When yields of both enzymes, from the purification, are compared, it is apparent that more esterase A₂ is present than esterase B₂ in the mosquito larvae of Pel RR. Esterase A₂ is also more reactive towards general esterase substrates than esterase B₂ (Table 1);

therefore any advantage of its higher availability in relation to insecticide resistance cannot be ruled out. However, lower recoveries of esterase B₂ may be due to its higher lability in the initial steps of the purification.

The inhibition rates of esterases A₂ and B₂ can be compared with the insecticide cross-resistance spectrum shown by the Pel RR strain (Peiris and Hemingway, 1990a). If the toxicity is mainly due to oxons formed *in vivo* by oxidases, the resistance 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 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 the insecticide oxon analogues. The slight discrepancies observed in this comparison may be due to the contribution of other minor resistance mechanisms. For the carbamate, propoxur, k_a values of both enzymes are very low and compatible with the low resistance ratio shown for it. The mosquito strain Pel RR has shown a negative cross-resistance (0.75 ×) 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. Differences in the k_a values of A₂ and B₂ 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 (*Myzus persicae*), had a k_a of $1330.0 \pm 80.0 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ for paraoxon (Devonshire, 1977). In sheep erythrocyte, the bimolecular rate constant (k_a) of the target site, AChE, for paraoxon has been shown to be $11 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ (Aldridge and Davison, 1952). An insect acetylcholinesterase purified from *Lygus hesperus* (Hemiptera; Miridae) had a k_a of $9.44 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ (Zhu and Brindley, 1992). Susceptible AChE from the tobacco budworm (*Heliothis virescens*) was determined to have a k_a of approx. $0.3 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ for paraoxon (Brown and Bryson, 1992). These data suggest that the insect carboxylesterases which have the putative role of sequestration in protecting the target site, acetylcholinesterase, 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. Slow deacylation rates (k_3) indicate that the major role of these enzymes in detoxication is sequestration (Table 3). The aphid esterase E4 hydrolysed paraoxon at ten times the rate of the mosquito esterases A₂ and B₂ (Devonshire and Moores, 1982). However, for malaoxon, esterase E4 hydrolysis was 30 times the rate of the mosquito esterase B₂ and 100 times the A₂ 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 B₂, but this was 4 times less than the rate for A₂.

In a purification procedure begun with 600–800 mg of total protein, about 1–1.5 mg of pure esterases A₂ and B₂ could be obtained with a final recovery of about 40–45% esterase activity. Assuming that the esterases A₂ and B₂ are mainly responsible for the high *p*-nitrophenyl acetate activity of the homogenate, it can be estimated that they comprise about 0.4% of the total protein. A mean protein estimation of 0.115 ± 0.038 mg per fourth-instar larva could be obtained from the crude homogenates prepared from individual larvae (20 independent determinations). Since each esterase has an M_r of approx. 60000, a concentration of approx. 7.67 pmol (total) of both A₂ and B₂ esterases per mosquito larva of pel RR strain can be estimated. Therefore the calculated amount of paraoxon, for example, that can be sequestered by esterase A₂ and B₂ in a mosquito larva is about 2.1 ng. Similar to the k_3 of the pure enzymes, mosquito homogenate had a k_3 value of 0.056 h^{-1} for paraoxon. Therefore a single mosquito larva can hydrolyse about 0.118 ng of paraoxon/h (5.59% of the sequestered amount). The wet weight of a fourth-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. Concentrations of the esterase E4 in resistant aphids have been shown to be in the range of 0.85–24.7 pmol/mg of aphid (Devonshire and 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/h, which was 33.2% of the sequestered amount. For the susceptible aphid variant these values were 0.04 ng, 0.01 ng/h and 25% respectively (Devonshire and Moores, 1982). It is clear that, unlike the aphid E4, the main role in resistance of the mosquito esterases A₂ and B₂ is sequestration. The rates of interaction of the mosquito esterases with organophosphates are also apparently lower than those of E4, as shown by their respective k_a values for paraoxon. In the most resistant variant of aphid, E4 accounted for approx. 3% of the total protein of an aphid (Devonshire and Moores, 1982), whereas we estimate both esterases A₂ and B₂ to be 0.4% of the total protein in fourth-instar Pel RR larvae. The lower percentage content of these esterases in homogenate was also evident because a significant band was not observable on SDS/PAGE gels. Greater efficiency of the carboxylesterase-based resistance mechanism in aphids may have evolved primarily as an adaptation to the toxic compounds found in the plant materials on which the aphids

feed. Furthermore, the insecticide selection pressure on mosquito larvae is possibly less, as the larvae are exposed to the insecticide dissolved in water, whereas aphids are exposed directly to the much higher concentrations of the insecticides.

As observed earlier for the purified A₂ carboxylesterase, the kinetic constants for the esterase B₂ indicate that the k_a and the K_a are important in determining the B₂ enzyme's role in resistance. The rates of binding and affinities of binding of the insecticides observed support the role of both B₂ and the A₂ carboxylesterases to be mainly sequestration followed by the slow turnover of the insecticide. With the characterization of both esterases involved in insecticide resistance from this one resistant mosquito strain, Pel RR, the next goal will be to determine if qualitative changes exist in one or both enzymes between resistant strains. This may have broader implications than just insecticide resistance if it can be demonstrated that naturally occurring allelic forms of carboxylesterases possess functionally important differences.

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