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

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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 $p_l$ 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 $V_{\text{max}}$, 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; Mouches 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 (Mouches 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 A₂ and B₂ (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. Hydroxylapatite, 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). $OO'OO'$-tetramethyl-$OO$'-thiodi-p-phenylene bis(phosphorothioate) (temephos, 99.7% pure), $OO$-diethyl $O$-3,5,6-trichloro-2-pyridyl phosphorothioate (fenitrotoxin, 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 (Berkshire, 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. Oska, Japan), respectively. α-Cyano-3-phenoxybenzyl 3,2-chloro-3,3,3-trifluoroprop-1-ethyl, 2,2-dimethylcyclopropenecarboxylate (lambda cyhalothrin, 81.2% pure) were 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.

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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 μmol 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 paraaxon K_m values were determined in the presence of substrate by the method of Main and Dauter-man (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 deacetylation. The generally accepted reaction mechanism is:

$$\text{E} + 1 \rightleftharpoons \text{EI} \rightarrow \text{EI'} + P_1 \rightarrow \text{E} + P_2$$

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), paraaxon (0.1 μM and 0.1 mM), EDTA (1 mM), eserin (10 μM) 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 B_2 was purified by a further development of the previous method for esterase A_2 (Ketterman et al., 1992). Using the same procedure as for esterase A_2, chromatography was performed on Q-Sepharose and phenyl-Sepharose. The esterase B_2 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 x 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 centrifprep 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 x 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 (Mr. 18,500–160,000) using a Bio-Rad Mini-Protein 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 College, 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 B_2 enzyme was then separated from esterase A_2 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 A_2 and 3 % final recovery and 50-60-fold purification for esterase B_2. With p-nitrophenyl acetate as the substrate, final specific activities for esterase A_2 were approx. 360 μmol/min per mg and for esterase B_2 were approx. 52 μmol/min per mg. By using the outlined procedures, approx. 800 μg of purified esterase A_2 and 200–300 μg of purified esterase B_2 can be obtained from 15 g wet weight of larvae. For esterase B_2 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.
Characterization of mosquito esterase \( B_2 \)

### Table 1: 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.

<table>
<thead>
<tr>
<th>Effector</th>
<th>Concentration</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuCl(_2)</td>
<td>1.0 mM</td>
<td>105.9</td>
</tr>
<tr>
<td>CuCl(_3)</td>
<td>1.0 mM</td>
<td>4.8</td>
</tr>
<tr>
<td>FeCl(_2)</td>
<td>1.0 mM</td>
<td>3.5</td>
</tr>
<tr>
<td>HgCl(_2)</td>
<td>1.0 mM</td>
<td>11.7</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1.0 mM</td>
<td>113.1</td>
</tr>
<tr>
<td>MnCl(_2)</td>
<td>1.0 mM</td>
<td>96.7</td>
</tr>
<tr>
<td>ZnCl(_2)</td>
<td>1.0 mM</td>
<td>102.3</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.0 mM</td>
<td>104.1</td>
</tr>
<tr>
<td>Eserine</td>
<td>100.0 ( \mu )M</td>
<td>103.4</td>
</tr>
<tr>
<td>Bis-(( p )-nitrophenyl) phosphate</td>
<td>0.1 mM</td>
<td>5.9</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>0.1 ( \mu )M</td>
<td>51.3</td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Enzyme activity was inhibited only by CuCl\(_2\), FeCl\(_3\) and HgCl\(_2\) of the metal ions studied. The inhibition by HgCl\(_2\) was also observed for esterase \( A_2 \) (Ketterman et al., 1992), implicating the involvement of a thiol group for the stability of these mosquito carboxylesterases and the importance of the presence of diithiothreitol in the purification and storage of these enzymes. A phosphorotriester hydrolase, purified from the insect *Heliothis virescens*, a cotton pest, also showed complete inhibition with Hg\(^{2+}\), but slight inhibition with Cu\(^{2+}\) and no inhibition with Fe\(^{2+}\) at 1 mM concentrations (Konno et al., 1990).

Partial inhibition by 0.1 \( \mu \)M 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 \( A_2 \) under the same conditions. The purified mosquito esterase \( B_2 \) 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 \( A_2 \) (Ketterman et al., 1992). The acetylcholinesterase inhibitor eserine did not inhibit the purified esterase \( B_2 \). In contrast esterase \( A_2 \) was completely inhibited by 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 enzyme, acetylcholinesterase (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.

### Physical characterization

The relative monomeric mass of the purified esterase \( B_2 \), estimated from SDS/PAGE was 62800 ± 2400. On gradient-gel PAGE native carboxylesterase had an estimated \( M_r \) of 55700 ± 3700. The pl of 5.0 was determined by isoelectric focusing on Ampholine PAGEplates. This pl 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 pl of this carboxylesterase are in close agreement with those of the esterase \( A_2 \) (Ketterman et al., 1992). Glycosylation of esterase \( B_2 \) with glucose and mannose residues was insignificant, as the enzyme did not bind to concanavalin A-Sepharose. N-terminal sequencing of the esterases \( A_2 \) and \( B_2 \) 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 \( B_2 \) are presented in Table 1.

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 \( B_2 \) and \( A_2 \) was confirmed on non-denaturing PAGE gels stained for esterase activity.

### Substrate specificity

Michaelis constants, \( K_r \), and \( V_{\text{max}} \) values were determined for three general esterase substrates (Table 2). Both esterases showed a higher affinity towards \( p \)-nitrophenyl hexanoate (\( C_6 \)) than \( p \)-nitrophenyl acetate (\( C_4 \)). When the data for the two enzymes are compared, the rate of hydrolysis is considerably lower with esterase \( B_2 \) for both substrates. Pig and rat liver carboxylesterase isoenzymes have shown \( K_r \) and \( V_{\text{max}} \) values of 30–220 \( \mu \)M and 66–110 \( \mu \)mol/min per mg respectively for \( p \)-nitrophenyl acetate (Heymann, 1982). For the same substrate two purified human liver carboxylesterases have shown \( K_r \) values of 190 and 300–870 \( \mu \)M and \( V_{\text{max}} \) values of 13.3 and 67.3–142.7 \( \mu \)mol/min per mg (Ketterman et al., 1989). The two substrates used to define esterases \( A_2 \) and \( B_2 \) originally, \( \alpha \)- and \( \beta \)-naphthyl acetate, were also examined. By definition, esterase \( A_2 \) is more reactive towards \( \alpha \)-naphthyl acetate and esterase \( B_2 \) is more reactive towards \( \beta \)-naphthyl acetate when both substrates are present (Raymond et al., 1987). This substrate preference could be shown with \( \alpha \)-naphthyl acetate, but with the \( \beta \)-naphthyl acetate reaction the Michaelis constant and \( V_{\text{max}} \) could not be determined because of

### Table 2: Substrate interactions of esterases \( B_2 \) and \( A_2 \)

The rate of substrate hydrolysis was measured at 405 nm for \( p \)-nitrophenyl acetate and \( p \)-nitrophenyl caproate, and at 235 nm for \( \alpha \)-naphthyl acetate at 22 °C. A unit corresponds to the hydrolysis of 1 \( \mu \)mol of substrate in 1 min under the assay conditions used.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_r ) (( \mu )M)</th>
<th>( V_{\text{max}} ) (units/mg)</th>
<th>( K_r ) (( \mu )M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p )-Nitrophenyl acetate</td>
<td>145.8 ± 45.0</td>
<td>140.0 ± 50.0</td>
<td>170 ± 6.0</td>
</tr>
<tr>
<td>( p )-Nitrophenyl hexanoate</td>
<td>35.4 ± 9.7</td>
<td>183 ± 9.8</td>
<td>17.0 ± 6.0</td>
</tr>
<tr>
<td>( \alpha )-Naphthyl acetate</td>
<td>30.5 ± 6.1</td>
<td>200 ± 15.0</td>
<td>172.5 ± 40.6</td>
</tr>
<tr>
<td>Parameter</td>
<td>( V_{\text{max}} ) (units/mg)</td>
<td>( K_r ) (( \mu )M)</td>
<td></td>
</tr>
</tbody>
</table>

...
the insolubility of the substrate at the concentrations necessary. However, for comparative purposes, a maximum specific activity (189.9 \( \mu \)mol/min per mg) for esterase B2 and (60.61 \( \mu \)mol/min per mg) for esterase A2 was measured for \( \beta \)-naphthyl acetate at 0.1 mM. No activity of esterase B3 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 (Ketterman 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 detoxification 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 \( k_s \) were determined for several insecticides. These values together with \( k_s \) 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 (Ketterman et al., 1992). Purified esterase B2 also interacted in a similar manner with those technical-grade thionates (results not shown). Chryoprifosoxon and paraoxon were such potent inhibitors of B2 that the constants \( k_s \) and \( K_s \) could not be determined and the \( k_a \) was determined in the presence of substrate (Main and Dauterman, 1963). The \( k_s \) 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 that 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 \( k_s \) and the insecticide cross-resistance spectrum. The greatest resistance ratios shown for chryoprifos (37) and parathion (20) may be due to the greater interaction (as shown by higher \( k_s \) values) of these esterases with the insecticide oxon analogues. The slight decrepencies observed in this comparison may be due to the contribution of other minor resistance mechanisms. For the carbamate, propoxur, \( k_s \) 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 \( \times \)) 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 \( k_s \) 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 (\textit{Myzus persicae}), had a \( k_a \) of \( 1300 \pm 80.0 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1} \) 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 \( 11 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1} \) (Aldridge and Davison, 1952). An insect acetylcholinesterase purified from \textit{Lyygus 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 (\textit{Heliothris virescens}) was determined to have a \( k_a \) of approx. \( 0.3 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1} \) 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 \( k_s \) and the insecticide resistance is also reflected by the respective \( k_a \) value (Michaelis constant for the insecticides). The constants \( k_s \) and \( k_a \) do not have any

### Table 3 Kinetic constants for the interaction of esterase B2 with the insecticides

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Esterase ( \ldots ) B2</th>
<th>A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaoxon* (7)</td>
<td>( 16.3 \pm 3.8 \times 10^3 )</td>
<td>( 0.17 \pm 0.07 )</td>
</tr>
<tr>
<td>Fenitrooxon* (4)</td>
<td>( 37.4 \pm 15.4 \times 10^3 )</td>
<td>( 0.91 \pm 0.37 )</td>
</tr>
<tr>
<td>Chlorpyrifos-oxon* (37)</td>
<td>( \quad )</td>
<td>( 145.3 \pm 59.7 )</td>
</tr>
<tr>
<td>Paraoxon* (20)</td>
<td>( \quad )</td>
<td>( 170.0 \pm 47.7 )</td>
</tr>
<tr>
<td>Propoxur† (2)</td>
<td>( 40.2 \pm 10.2 \times 10^3 )</td>
<td>( 0.012 \pm 0.003 )</td>
</tr>
</tbody>
</table>

* Organophosphate.
† Carbamate.
apparent relationship with the insecticide cross-resistance spectrum of the mosquito strain. Slow deacylation rates ($k_a$) 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 A$_4$ and B$_4$ (Devonshire and Moores, 1982). However, for malaoxon, esterase E4 hydrolysis was 30 times the rate of the mosquito esterases B$_4$ and 100 times the A$_4$ 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 4 times less than the rate for A$_4$.

In a purification procedure begun with 600–800 mg of total protein, about 1–1.5 mg of pure esterases A$_4$ and B$_4$ could be obtained with a final recovery of about 40–45% esterase activity. Assuming that the esterases A$_4$ and B$_4$ 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 ng/mg of 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. 60,000, a concentration of approx. 7.67 pmol (total) of both A$_4$ and B$_4$ 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 A$_4$ and B$_4$ in a mosquito larva is about 2.1 ng. Similar to the $k_a$ of the pure enzymes, mosquito homogenate had a $k_a$ 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 A$_4$ and B$_4$ 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 A$_4$ and B$_4$ 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 A$_4$ carboxylesterase, the kinetic constants for the esterase B$_4$ indicate that the $k_a$ and the $K_a$ are important in determining the B$_4$ enzyme’s role in resistance. The rates of binding and affinities of binding of the insecticides observed support the role of both B$_4$ and the A$_4$ 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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