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Molecular and biochemical mechanisms of organophosphate resistance in laboratory-selected lines of the oriental fruit fly (*Bactrocera dorsalis*)

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ABSTRACT

Genetic and biochemical factors leading to resistance to various organophosphate (OP) based insecticides were studied in lines selected for OP-resistance in the oriental fruit fly *Bactrocera dorsalis*. Lines were separately selected for resistance to naled, trichlorfon, fenitrothion, fenthion, formothion, and malathion. Overall, these lines showed increased resistance ratios ranging from 13.7- to 814-fold relative to a susceptible (S) line. Also, in these newly selected lines the same three point mutations in the *ace* gene, previously identified in resistance studies and designated as I214V, G488S and Q643R, were found. As expected, the enzyme from the resistant lines showed lower overall activity and reduced sensitivity to inhibition by fenitrothion, methyl-paraoxon and paraoxon compared to the wild type acetylcholinesterase (AChE) enzyme. The apparent V_{max} values for esterase from the resistant lines were 1.2–3.69 times higher than that of the S line. Although only the naled-, trichlorfon- and fenthion-r lines showed lower esterase affinities (based on apparent K_m values) compared with the S line, all of the V_{max}/K_m ratios were higher in the resistant lines compared to that of the S line. The OP-resistant lines also displayed an overall similar pattern of isozyme expression, except for one additional band found only in the naled-r line and one band that was absent in the trichlorfon-, malathion-, and fenthion-r lines. Our results also show that overall, multiple examples of high OP resistance in selected lines of *B. dorsalis* exhibiting the same genetic alterations in the *ace* gene seen previously resulted in different effects on esterase enzyme activity in relation to various OP compounds.

1. Introduction

Insects have been found to develop resistance to almost every chemical class of insecticide [1]. The most prevalent biochemical mechanisms of insecticide resistance in insects include modifications of esterases, mixed-function oxidases, and glutathione S-transferases [2]. At the DNA level, much of the insensitivity of the acetylcholinesterase (AChE) enzyme to organophosphate (OP) insecticides is caused by point mutations in the *ace* gene [3–11]. The resistance to insecticides in many insect species has been ascribed to this type of insensitivity of AChE or to increases in the rate of metabolism of the insecticides by carboxylesterases [12–14].

Because the oriental fruit fly, *Bactrocera dorsalis*, is considered to be one of the most prominent quarantine pests in the world as well as one of the most economically damaging pests in Taiwan, measures taken to control this pest often include the use of insecticides. In the field for the past 40 years a number of OP insecticides, namely naled, trichlorfon, fenitrothion, fenthion, formothion, and malathion, have been used against the oriental fruit fly [15]. In Taiwan, the insecticides used include pyrethroids (cyfluthrin, fenvalerate, and deltamethrin) as well as spinosad [16].

In part because of these extensive pesticide applications around the world, many fruit flies, including *B. oleae*, *B. dorsalis*, and *Ceratitis capitata* (Medfly) have been reported to develop high levels of resistance against various OPs in the field [7,11,17,18].

The main mechanisms of OP resistance in these flies involve modifications of esterases and AChE insensitivity [7,19, 20]. For the gene (*ace*) that produces the AChE enzyme, three point mutations and one deletion have been shown to be involved in resistance to OPs in *Bactrocera* spp. and in the Medfly [8,10,11,21]. Two of the point mutations in the *ace* gene occur in laboratory strains of *Bactrocera* spp. selected for resistance to different insecticides belonging to the same general OP classification [8,10]. One of our goals here is to ascertain whether lines selected for resistance to different OPs, but carrying the same point mutations in the *ace* gene, react differently to various carboxylesterases. These results could be used to design strategies for preventing or managing resistance in this pest. The point mutations in the *ace* gene are readily identifiable using molecular techniques [10], and the ability

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to define different mechanisms of OP resistance in fruit flies may make it easier for the management of OP resistance through the rotation of others classes of insecticides used in control programs.

For this study of the mechanisms of OP resistance in *B. dorsalis* we established six different OP-resistant lines using selection protocols based on (1) an organophosphate (naled), (2) a phosphonate (trichlorfon), (3) two phenyl organothiophosphates (fenitrothion and fenthion), (4) an aliphatic organothiophosphate (malathion), and (5) an aliphatic amide organothiophosphate (formothion) in the laboratory. In these selected lines we used α-naphthyl acetate as a substrate to estimate several different parameters representing classical kinetics of the carboxylesterases and to investigate their possible roles in OP resistance. We also report on cross-resistance to naled and fenthion in these various OP-resistant lines and discuss the possible roles of carboxylesterases and AChE insensitivity for the observed mechanisms of OP resistance. Finally, we sequenced *ace* gene variants found in each of these lines to identify mutations and determine their possible role in OP resistance.

2. Materials and methods

2.1. Fly lines

An insecticide-susceptible (S) line of the oriental fruit fly, *B. dorsalis*, was established in the laboratory from flies collected from central Taiwan in 1994. This laboratory line was reared with an artificial diet maintained without any exposure to insecticides. Six OP-resistant lines (naled, trichlorfon, fenthion, fenitrothion, malathion and formothion) were respectively selected from this line and the susceptibilities (LD_{50}) of the flies – to varied doses of their own selected insecticides – were assayed every four generations, using the topical application as described in Hsu et al. [19]. The ratios of LD_{50} (resistant line)/LD_{50} (susceptible line) were used to calculate resistance levels.

The treatment for establishing resistant lines was as described in Hsu et al. [19]. Briefly, we established the six resistant lines through selection of more than 70% mortality expectably. Survivors of each insecticide were rinsed from the cups 24 h post-treatment and placed in a new cage. The treatment dosages were similar for four serial generations, and then changed for the next four serial generations according to the same principle (>70% mortality). After 100 generations of selection the resistant lines were treated with insecticide every other generation. The naled-, trichlorfon-, fenitrothion-, fenthion-, formothion-, malathion-resistant lines established are abbreviated as nal-r, tri-r, fenit-r, fen-r, for-r, and mal-r, respectively.

Together with the resistant lines, the susceptible (S) line was also treated with each insecticide for calculation of resistance ratios (RR) based on LD_{50} values.

2.2. Chemicals

Six analytical grade insecticides, namely: naled (97% purity), trichlorfon (96%), fenitrothion (96%), fenthion (97%), formothion (98%), and malathion (97%) were tested. Formothion was obtained from Chem Services Inc, and the other insecticides were obtained from Fluka Chemie GmbH (Switzerland). The stock solutions of the insecticides were prepared to a concentration of 10 mg/ml in acetone.

The chemicals, including bovine serum albumin (BSA), eserine hemisulfate (eserine), ethylene diamine tetraacetic acid disodium salt dehydrate (EDTA-Na_2), α-naphthol, α-naphthyl acetate, sodium dodecyl sulfate (SDS), Fast blue RR salt, Fast Blue BB, acetylthiocholine iodide (ATC), 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), and triton X-100, were obtained from the Sigma chemical company (USA). Methyl-paraoxon (97%) and paraoxon (97%) was obtained from Fluka Chemie GmbH (Switzerland).

2.3. Bioassays

The six OPs were assayed to determine the levels of susceptibility in various lines. The working solutions were diluted with acetone according to insecticide toxicity and 1 μL was dropped onto the thoracic tergum of 3–5-day-old adult flies (with free insecticide as control) as described by Busvine [22]. Post-treatment, the flies were transferred to 250 ml ice cream cups and reared with a few drops of liquid food sucked in cotton wool (sugar, yeast, and water, 4:1:5). All treated flies were maintained for 24 h at 24 ± 2 °C and 12:12 (L:D) photoperiod before mortality counts were made. Data was also analyzed by probit analysis as adapted [23]. The significant differences between the lethal dose (LD) values were defined as a 95% non-overlap of their fiducial limits (FL).

2.4. Cross-resistance bioassays

To examine the possibility of cross resistance to various OPs in the resistant lines of *B. dorsalis*, all six OP-resistant and S lines were tested with naled and fenthion around the 80th generation (nal-r, tri-r fenit-r, fen-r, and mal-r lines at 84th generation and for-r line at 82nd generation). Cross resistance was measured by calculating resistance ratios using the LD_{50} (resistant line)/LD_{50} (susceptible line) for naled and/or fenthion, respectively, for each of the lines.

2.5. AChE study

2.5.1. AChE activity and insensitivity to inhibition

To measure enzyme activity, thirty heads were obtained by shaking flies under liquid nitrogen. The heads were homogenized in 5 mL of 100 mM sodium phosphate buffer (pH 7.0) using a hand-held homogenizer for 2 min in an ice bath. The homogenates were filtered through two layers of cheesecloth and then was used as the enzyme solution to measure the AChE activity. Lines used for the AChE assays were at generations 51, 50, 51, 55, 51, 49, and 50 for the S, nal-r, tri-r, fenit-r, fen-r, for-r, and mal-r lines, respectively. The resistance ratios of the various OP-resistant lines were 5, 10, 54, 25, 56, and 20 times of nal-r, tri-r, fenit-r, fen-r, for-r, and mal-r lines, respectively, compared to the S line.

AChE activity was determined by the method of Ellman et al. [24]. Briefly, 200 μL of supernatant (containing 160 μg total proteins) was pre-incubated for 5 min with 50 μL of each inhibitor at 25 °C. Two replications were done at each of 4–6 different concentrations of fenitrothion, methyl-paraoxon, paraoxon or buffer only before the AChE activity assay. After exposure to the inhibitor, 0.2 ml of the enzyme solution (with 80 μg total protein) was added to a total volume of 3 mL for the assay mixtures containing DTNB (0.32 mM) and ATC (0.50 mM) as a substrate. For this study, the range of inhibitors concentrations were 0.236–60.3 μM for fenitrothion, 6.54 nM to 1.67 μM for methyl-paraoxon, and 0.255 nM to 1.04 μM for paraoxon.

The change in light absorbance at 415 nm was recorded for 5 min in a Lambda 45 UV/VIS spectrophotometer (PerkinElmer Instruments). AChE activity is expressed in n mole ATC/min/mg protein. The inhibition concentration (I_{50}) for each inhibitor was determined based on log-concentration vs. log-% inhibition regression analysis.

Together with the resistant lines, the S line was also treated with each inhibitor to calculate the inhibition ratios (IR) based on their I_{50} values.