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Mutations in the acetylcholinesterase gene of *Bactrocera dorsalis* associated with resistance to organophosphorus insecticides

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Abstract

Mutations in the gene encoding the enzyme acetylcholinesterase (AChE) of the oriental fruit fly, *Bactrocera dorsalis,* associated with resistance to an organophosphorus insecticide have been characterized. Three point mutations producing nonsynonymous changes in the predicted amino acid sequence of the product of the *B. dorsalis ace* gene in resistant vs. susceptible flies have been identified. One of these changes is unique to *B. dorsalis* while the other two occur at sites that are identical to mutations previously described for another *Bactrocera* species. Although the precise role of the third mutation is not clearly established, the independent origin of two identical alterations in these two species strongly supports the idea proposed previously that molecular changes associated with insecticide resistance in key genes and enzymes such as AChE are largely constrained to a limited number of sites. The results obtained here also suggest that the widespread use of organophosphorus insecticides will likely lead to a predictable acquisition of resistance in wild populations of *B. dorsalis* as well as other pest species. For surveys of *B. dorsalis* populations that may develop resistance, diagnostic tests using PCR-RFLP based methods for detecting the presence of all three mutations in individual flies are described.

Keywords: Fenitrothion; *Ace* gene; Acetylcholinesterase; Insecticide resistance; *Bactrocera dorsalis*

1. Introduction

The phenomenon of insecticide resistance is a potentially major impediment for effective control of a number of pest species of agricultural and medical importance. In many cases involving insecticide applications an initial failure to effectively control a pest has resulted in a huge resurgence of a population exhibiting resistance. For example, fenitrothion, an organothiophosphate-based insecticide, has been widely used for control of agricultural pests. The development of resistance, resulting in a reduction in effectiveness of fenitrothion and other organophosphate-based insecticides, has been observed in several insect species (Konno and Shishido, 1989; Kozaki et al., 2001; Vontas et al., 2001; Hsu and Feng, 2002). In part because of this, the prospects for the future of the chemical control have often been called into question. A better understanding of the mechanisms by which resistance occurs may significantly delay the onset of problems and limitations associated with this control approach.

The enzyme acetylcholinesterase (AChE, EC 3.1.1.7) is known to be the target of many organophosphorus and carbamate insecticides. In most cases, the acquisition of resistance corresponds to a measurable alteration of AChE activity. These alterations, in almost all cases, arise from point mutations in the gene (designated *ace*) encoding this enzyme that produce amino acid substitutions in regions corresponding to the active site of the AChE enzyme. Specific mutations in *ace* genes affecting enzyme activity in association with resistance phenomena have been described in the house fly *Musca domestica* (Kozaki et al., 2001), several mosquito species (Vaughan et al., 1997; Kozaki et al., 2001; Weill et al., 2004), the Colorado potato beetle

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(Zhu and Clark, 1995), the olive fruit fly (Vontas et al., 2002), the cotton aphid *Aphis gossypii* (Andrews et al., 2004; Toda et al., 2004) along with other hemipteran species (Javed et al., 2003), and *Drosophila melanogaster* (Fournier et al., 1992; Mutero et al., 1994). Also as described by Weill et al. (2004), many mosquito species appear to have two distinct *ace* genes (designated *ace1* and *ace2*) while other insects, including tephritids such as the *Bactrocera* species, appear to have only one *ace* gene. The single *ace* gene found in these species appears to be an ortholog of the *ace2* gene of mosquitoes (Hawkes et al., 2005).

In the case of the oriental fruit fly, *Bactrocera dorsalis* (Hendel), an agriculture pest species which causes serious financial losses to orchards globally, the development of even subtle resistance can have a significant impact on the effectiveness of organophosphorus insecticides such as fenitrothion (Hsu and Feng, 2000). In this study, laboratory colonies of this species exhibiting resistance and susceptibility to fenitrothion were identified and characterized in terms of activity of the AChE enzyme and molecular changes in the structure of the gene encoding this enzyme. The results obtained here show that some of the specific structural changes of the *B. dorsalis ace* gene associated with the development of resistance are identical to changes reported in other species exhibiting insecticide resistance. The independent origin of identical gene specific changes in different species strongly supports the idea put forward by ffrench-Constant et al. (1998) that at the molecular level, resistance phenomena result from a limited set of changes in key genes such as the *ace* genes. This also suggests that the acquisition of resistance to organophosphorus insecticides will occur in a predictable manner in wild populations of this species, as well as in a wide range of other agricultural and medical pest species, where these agents are used for control purposes.

2. Materials and methods

2.1. Colonies

A colony of the oriental fruit fly, *B. dorsalis,* was established in our laboratory from specimens collected from central Taiwan (1994) and maintained without any exposure to insecticides as the susceptible colony.

2.2. Selection and biochemical assays for resistance

To establish the resistant colony, adult flies of 3–5 days old were taken from the susceptible colony. Stock solutions of insecticides were prepared in 10 mg/ml of acetone for topical application assay and 10 mM for the acetylcholinesterase-insensitivity study. Working dilutions were made from these stock solutions prior to use. The susceptibility of the flies to specific doses of different insecticides was assayed using topical application.

Individual adult flies, both from fenitrothion-resistant and fenitrothion-susceptible colonies, were used in this part of the investigation. The heads were used for biochemical tests and the bodies were used to isolate total RNA as described in the next section. For AChE activity assays with acetylthiocholine iodide (ATChI, 0.50 mM) as a substrate, 100 mg of flies' heads were homogenized for use. AChE activity is expressed in terms of nmoles ATChI/min/mg protein. Solutions of fenitroxon, methyl-paraoxon, and paraoxon (ethyl) were used as inhibitors, and for each inhibitor, 6-10 concentrations were tested. Four replicates of each experiment were done and the values are displayed as mean $\pm$ SD. The inhibition concentration (I_{50}) for each inhibitor was determined based on *log*-concentration vs. *log*% inhibition regression analysis. The insensitivity factor is the ratio of I_{50} of the resistant type enzyme to that of the susceptible colony. The plot of the *log* of residual activity against time was linear for a given inhibitor concentration and was used to calculate the bimolecular rate constant (*Ki*).

A resistance ratio (RR) was calculated as the value of the resistant LD_{50}/the susceptible LD_{50} value for the insecticide treatment. For the inhibition assays, fly heads were homogenized in 0.1 mL of 100 mM sodium phosphate buffer (pH 7.0) containing 1% Triton X-100 by volume. The homogenate was centrifuged at 5000 *g* for 1 min and the supernatant was employed for an AChE activity enzyme assay. The supernatant was pre-incubated for 10 min with 230 nM fenitroxon or buffer only at 37 $\pm$ 1 °C before the sensitivity of AChE to fenitroxon was tested. The inhibition of the enzyme was expressed as the mean inhibition activity as a percentage of the original activity. Additional details regarding the establishment of the resistant lines and experimental procedures are given by Hsu et al. (2004).

2.3. Synthesis of cDNA

Using adults from the susceptible colony, total RNA was extracted from the heads of 15 flies using a microscale total RNA extraction kit (RNeasy^{R} Mini kit, Qiagen Gmbh). The extract was treated with DNase (Qiagen Gmbh). One picogram to 5 μg of total RNA was used for the first strand synthesis of cDNA in 20 μl of total volume using the ThermoScript^{TM} reverse transcription reaction a cDNA synthesis system (Invitrogen, Inc.), according to the manufacturer's instructions. Poly dT (20) was used as the reverse primer.

2.4. PCR amplifications, cloning and DNA sequence analysis

A partial cDNA of the *ace* gene of *B. dorsalis* was amplified by PCR using degenerate primers designed from comparisons of conserved regions of published cDNA sequences of *ace* genes. The sequences used were from *D. melanogaster* (Accession no. X05893), *Apis mellifera*