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Amino-acid substitutions in acetylcholinesterase 1 involved in insecticide resistance in mosquitoes

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ABSTRACT

In natural populations of mosquitoes, high level of resistance to carbamates (CX) and organophosphates (OP) is provided by insensitive acetylcholinesterase (AChE1). Different alleles conferring resistance have been identified at the *ace1* locus. They differ from the wild-type by only one amino-acid substitution. The comparison of the biochemical characteristics of mutated recombinant proteins and AChE1 in resistant mosquito extracts confirmed the role of each substitution in insensitivity. Selection of these different resistant alleles in field populations depends likely on the insecticides used locally. Theoretical modelling studies are initiated to develop novel strategies of mosquito control.

1. Introduction

Mosquitoes are among the most important vectors of human disease. The use of insecticide to control them has led to selection of resistant populations and hence to control program failures. In many arthropods, selection of insensitive insecticide target is a common resistance mechanism. This is the case of acetylcholinesterase (AChE, EC 3.1.1.7), the target of OP and CX insecticides. In Diptera, two AChE genes are usually present (*ace-1* and *ace-2*), and *ace1* codes the synaptic enzyme [1]. In a few Diptera, such as true flies, only the *ace-2* gene is present and codes the synaptic AChE [2]. In mosquitoes, a single amino-acid change, located near the active site of the synaptic AChE1, is sufficient to provide a high resistance level. To date, only three single amino-acid substitutions have been described: G119S in *Culex pipiens*, *C. vishnui*, *Anopheles gambiae* and *A. albimanus*; F290V in *C. pipiens* and F331W in *C. tritaeniorhynchus* (according to *Torpedo californica* AChE nomenclature), suggesting a very high structural constraint of the AChE1 enzyme [3–5]. These substitutions have been shown to provide insensitivity by means of in vitro experiments with mutated recombinant enzymes [3,4,6,7]. Our aim is to understand how resistance-associated mutations are selected in natural populations in order to delay its occurrence and its spread. The present study focuses on characterization of mutated recombinant AChE1 proteins and their biochemical properties. Preliminary data are presented.

2. Materials and methods

2.1. Mosquito samples

Two *C. pipiens* reference strains were used: the susceptible one SLAB [8] and the resistant homozygous G119S SR [9]. ACE-R strain homozygous for the F290V mutation was established from the Mitsero population collected in Cyprus in 1987 [10]. *C. tritaeniorhynchus* larvae harboring the F331W AChE1 were sampled in China in 2003 [6].

2.2. Site-directed mutagenesis

Expression vectors pAc5.1/V5-His (Invitrogen) containing *C. pipiens* WT and G119S AChE1 complete coding

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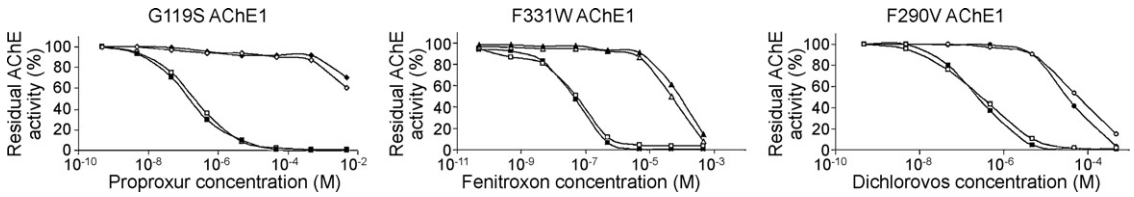

Fig. 1. Comparison of residual AChE1 activities of recombinant enzymes (black symbols) and mosquito head extracts (empty symbols) in presence of increasing doses of insecticides. For each mutated AChE1, only one insecticide is represented. Residual activity of susceptible head extract and WT recombinant AChE1 are included as control (squares).

sequences were already described [3]. Mutations were introduced into the WT vector by means of a PCR-based strategy [4,6].

2.3. Production of wild-type and mutated AChE1 in Drosophila S2 cells

S2 cells (20×10^6) were transfected using Fugene6 (Roche), in OptiMEM medium, according to the manufacturer protocol. Cells were maintained in serum-free Schneider's medium to prevent AChE activity from foetal bovine serum. Four days after transfection, cells were collected by centrifugation at $250 \times g$ for 3 min and homogenised in 500 μl phosphate buffer (0.25 M, pH 7.0) containing 1% Triton X-100. The homogenate was centrifuged for 10 min at $9000 \times g$ and the supernatant was used as source of enzyme, after dilution in phosphate buffer.

2.4. AChE1 inhibition characteristics

The relative AChE1 activity was determined spectrophotometrically at room temperature, as described by Ellman et al. [11], using 100 μl of supernatant containing the recombinant AChE1 WT, G119S, F331W or F290V. Inhibition curves were performed by incubating samples (100 μl) for 15 min with 10 μl of inhibitor solutions at various concentrations. We then added 100 μl of 1.6 mM substrate and residual AChE1 activity was estimated by measuring changes in optical density at 412 nm for 15 min. We analysed three to five replicates for each assay. Replicates were performed with distinct batches of production.

The irreversible inhibition reaction is pseudo-first order and the residual activity follows the equation $[E]/[E_0] = e^{-k_i t[I_0]}$, when inhibitors are in excess compared to enzyme. k_i is the bimolecular rate constant, t represents time of incubation and $[I_0]$ is the initial inhibitor concentration. Insensitivity ratios were calculated by dividing the k_i of wild-type (WT) recombinant AChE1 by the k_i of mutated recombinant AChE1.

2.5. Three-dimensional modelling

Three-dimensional structures of AChE1 were created by automated homology modelling using *T. californica* and *D. melanogaster* AChE as previously described [12]. RMS deviation is 1.1 Å on 528 carbon atoms.

3. Results and discussion

Different insensitive AChE1 were characterized on individual mosquitoes from different field samples using the biochemical TPP test [13]. The cDNA of *ace1* were then sequenced from homozygous resistant individuals and compared with susceptible ones. Once identified, the mutations were introduced into an expression vector containing the wild-type *ace1* cDNA to produce the mutated recombinant enzymes in *Drosophila* S2 cells. Then, sensitivity to 10 insecticides was investigated to compare the recombinant AChE1s with the enzyme extracted from resistant mosquito heads (Fig. 1). The superimposition of inhibition patterns of recombinant and mosquito extracted enzymes for all insecticides (data not shown) confirmed the role of each AChE1 mutation in resistance. The different mutated AChE1 displayed a strong insensitivity to specific insecticides (Table 1), and there was a good relationship between the highest insensitivities (resistance ratios) and the insecticide(s) used locally in control programs. Thus, F290V allele was observed in resistant *C. pipiens* sampled in Cyprus Island where the main insecticide used is dichlorvos. F331W is the resistant allele found in *C. tritae-*

Table 1
Bimolecular velocity constant (k_i) and resistance ratio to organophosphates and carbamate insecticides observed in wild-type (WT) and mutated AChE1

Pesticide class	Insecticide	k_i (1/mol/s)				Resistance ratio (WT/mutant)		
		WT	G119S	F290V	F331W	G119S	F290V	F331W
Carbamates	Aldicarb	167	50	7	N/A	3	25	–
	Propoxur	2895.4	0.029	58	49.4	99624	50	59
	Eserine	48135.2	948.5	8852	1006.7	51	5	48
Organophosphorous	Dichlorvos	1711.5	373.9	25	11.2	5	68	153
	Malaoxon	3209.01	43.03	372	60.6	75	9	53
	Paraoxon-ethyl	4557.2	37.9	917	381.3	120	5	12
	Paraoxon-methyl	6261.5	26.5	N/A	217.6	237	–	29
	Fenitroxon	17114.7	252.5	181	16.2	68	95	1053
	Chlorpyrifos-oxon	54236.9	89.9	14531	3500.7	604	4	15