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Highly-substrate active isoenzyme acetylcholinesterase-II, in rosy eye mutant of *Aedes aegypti* mosquito

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Insecticide bioassays were carried out on larvae and adults of rosy eye mutant and wildtype strains of *A. aegypti*. Both the strains were equally susceptible to DDT, malathion and deltamethrin. Biochemical assays showed an increase in acetylcholinesterase enzyme (AChE) activity in all the stages of mutant strain with both the substrates i.e. acetylthiocholine iodide and S-butyrylthiocholine iodide. However, there was no difference in the percent inhibition of enzyme activity with propoxur in these two strains. Polyacrylamide gel electrophoresis performed in native conditions on the homogenates of adults of rosy eye mosquitoes showed that AChE-II allele was highly active with the substrate acetylthiocholine iodide as compared to wildtype strain. Frequency of the highly active AChE-II allele in the mutant strain was about 68%, whereas it was about 5% in the wildtype strain.

Organophosphate (OP) and carbamate insecticides are neurotoxins, and they exert their effect by inhibiting the enzyme acetylcholinesterase (AChE). Many insects have developed resistance to these compounds due to structural modification of these enzymes. The AChE enzyme from resistant individuals is far less sensitive to inhibition than that from susceptible individuals[1]. A number of simple biochemical assays are available to detect increased activity of several detoxifying enzymes and reduced sensitivity of the target enzymes[2]. These assays determine the enzyme activity based on their reaction with the substrate.

The rosy eye mutant strain of *Aedes aegypti* was found refractory to Chikungunya (CHIK) virus[3]. Further studies were initiated to determine if there are any other biochemical and physiological differences with the wildtype strain. Initial biochemical studies carried out on this mutant showed that it was a unique example among *Aedes* mosquitoes where there was an increased activity of this enzyme due to expression of the highly active AChE-II allele but it was not associated with insecticide susceptibility in this strain of mosquitoes. The present communication reports the qualitative and quantitative assays of AChE enzyme in this mutant strain and its expression at different ages of mosquitoes.

Mosquitoes—Mosquitoes employed for the experiment as wildtype (black eye, +) *Ae. aegypti* were from a laboratory colony maintained at this institute since 1995.

Rosy eye (ry)—Isolation and establishment of the rosy eye mutant colony has been reported earlier[3]. The mosquitoes were reared in an insectary maintained at 28° ± 2°C and 70-80% RH and purity of the strains was checked in every generation.

Larval bioassay—Ethanol concentrations of DDT, deltamethrin and malathion were prepared from the technical grade of insecticides which were procured from Sigma Chemical Company, USA, Roussel India Ltd and Greyhound Chromatography and Allied Chemicals, UK, respectively. The WHO method was followed for larval bioassay[4]. Probit analysis was applied to calculate the sublethal concentration LC_{50} for different mosquito strains[5].

Adult bioassay—One or two days old, 20-25 unfed female mosquitoes were exposed to DDT and deltamethrin insecticide impregnated papers obtained from WHO, for 1 hr. Malathion impregnated papers were prepared by spreading 0.7ml of insecticide solution of the required concentrations of 0.8% in olive oil on 12 x 15 cm rectangles of Whatman's no.1 filter paper. This was made up at the WHO

recommended discriminating dosages for *Ae. aegypti* mosquitoes[6]. Tests were performed as per the protocol outlined by WHO[7]. The dosage of DDT, deltamethrin and malathion were 4, 0.025 and 0.8% respectively. After exposure, the mosquitoes were maintained in the insectary at 28°±2°C and 80-90% RH. The percent mortality count was done 24 hr after exposure. Cotton pads soaked in 10% glucose solution were provided during the recovery period of 24 hr.

Enzyme assays (Quantitative)—Assays were performed on a large number of male and female mosquitoes collected after one day of eclosion. Mosquitoes were homogenised with the help of plastic pestle (Kontes) in microfuge tubes in distilled water containing 0.09 *M* Tris (*p*H 7.4), and centrifuged at 10,000 *g* for 10min. Normal and propoxur-inhibited AChE activity was determined as per ffrench-Constant and Bonning[8] on 30 µl aliquots of supernatant of mosquito homogenate. This test was performed with both the substrates i.e. acetylthiocholine iodide and S-butyrylthiocholine iodide. The method differed from that published only in the propoxur concentration used. In these assays the final concentration of propoxur was 0.2 m*M*, as the higher concentration used earlier gave total inhibition of AChE activity in the *Aedes aegypti*. The protein content was estimated in supernatant fluid from each individual homogenate as per Lowry *et al* [9]. A reference standard protein curve was prepared using Bovine Serum Albumin fraction V.

Enzyme assays (Qualitative)—The mini-protein II dual slab cell, (Bio-Rad, USA), was employed to perform polyacrylamide gel electrophoresis (PAGE) to determine isoenzyme profile of different strains. Mosquitoes were homogenised in buffer (Sucrose 20%, 10 m*M* Tris, 1m*M* EDTA and 0.4% Triton X-100, *p*H 8) 25µl/individual. After centrifugation at 4000 *g* for 10 min, 15µl supernatant was used for PAGE. Electrophoresis was performed in native conditions at 4°C. Gels were of 6% concentration; procedure followed was similar as shown by Munstermann[10]. Gels were stained for 2-3 hr in 50 ml of 0.5*M* phosphate buffer (*p*H 6) containing 12m*M* Tri-sodium citrate, 4.5 m*M* cupric sulfate, 1.2 m*M* potassium hexacyanoferrate and 3.5 m*M* acetylthiocholine iodide as described by Hemingway[11].

Insecticide bioassay performed on this mutant showed that it was as susceptible as the wildtype susceptible colony strain. Adults of both the strains showed similar percent mortality to diagnostic concentrations of all the three groups of insecticides. The diagnostic concentrations are double of the dose, which normally gives cent percent mortality. Larvae were exposed to varying concentrations of these three insecticides to determine if concentrations lower to diagnostic dosages of insecticide show any difference in the mortality. The probit analysis performed on the mortality data showed that there was no difference in LC_{50} of these two strains (Table 1).

However, when biochemical assays were performed on the mutant strain using substrate acetylthiocholine iodide, it showed about 1.25 and 2-fold increase in the enzyme activity in adults and larvae respectively as compared to the wildtype strain. Similarly an increase in enzyme activity of 8.5 and 12.8-fold in larvae and adults of mutant strain was noticed when S-butyrylthiocholine iodide was used as substrate (Table 2). AChE enzyme inhibition assays were performed to determine whether this enzyme from mutant strain show any difference in it's binding to propoxur. Results showed that there was no

Table 1—Comparative susceptibility of *A. aegypti*, wild type and rosy eye mutant to different insecticides

	% Mortality in adults				
Strains	DDT	Deltamethrin	Malathion	OC	OP
	(4%)	(0.025%)	(0.8%)	Control	Control
Wild type	98.88	100.00	100.00	0.00	0.00
	(69/70)*	(50/50)	(75/75)	(0/25)	(0/25)
Rosy eye	98.57	100.00	100.00	0.00	0.00
	(69/70)	(50/50)	(75/75)	(0/25)	(0/25)
		LC_{50} (larvae)			
Wild Type	0.0586	0.000717	0.0719		
	(0.0497-0.069)**	(0.0006-0.0008)	(0.0632-0.0819)		
Rosy Eye	0.0595	0.000687	0.0687		
	(0.0505-0.0702)	(0.00058-0.0008)	(0.0601-0.0785)		

* = (Number dead / Number tested); ** = (Fiducial limits, n=240 in each case)

difference in the percent inhibition of enzyme activity by propoxur on these strains (Table 2).

Since, there was an increased enzyme activity in quantitative assays in mutant strains, PAGE was performed on the homogenates of adults of both the strains to determine if there is any qualitative difference in the enzyme pattern of these two strains. Same amount of protein was loaded on the gels and these were stained using acetylthiocholine iodide as substrate. It was interesting to note that the AChE-II band was densely stained in the rosy eye mutant strains as compared to wildtype. It was dense in both the sexes of rosy eye strain. However, when homogenates of heads alone were used, no difference among these two strains could be seen. *In situ* staining using S-butyrylthiocholine iodide as substrate showed only faint band of AChE-I in both the strains of mosquitoes. Hence expression of this isoenzyme was studied in larval stages using normal substrate i.e. acetylthiocholine iodide which showed AChE-I band in both the strains and a faint AChE-II band in the mutant strain. However, in pupae AChE-I band was visible in both the strains but showed slightly different mobility while AChE-II band was visible in both the strains, it was dense in mutant strain (Fig. 1). In view of the above, PAGE analysis was performed to determine the frequency of occurrence of AChE-I and AChE-II allele in the adults of these two strains. It was interesting to note that about 68% adults of mutant strain showed occurrence of AChE-II band while wildtype strain it was about 5%.

It is known that AChE is the target enzyme for the toxicity of organophosphates and carbamates in

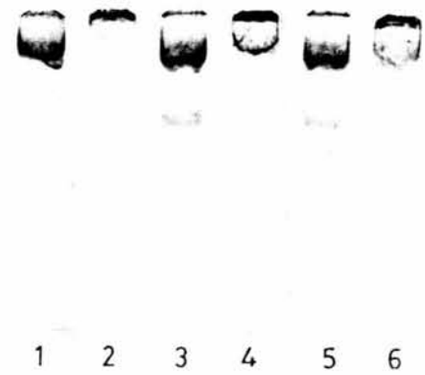

Fig. 1—Expression of isoenzyme acetylcholinestarases in the different strains of *A. aegypti*: [Adults: (Lane-1), rosy eye; (Lane-2) wildtype; Larvae: (Lane-3) rosy eye; (Lane-4), wildtype; Pupae: (Lane-5), rosy eye; (Lane-6) wildtype].

Table 2—Quantitative assay of acetylcholinesterase enzyme with different substrates in *A. aegypti*, wild type and rosy eye mutant (Activity/mg protein).

		Males	Females	Larvae	Pupae
		Wild type with Triton-X 100			
A	Activity	1749 (73)	1861 (133)	94 (4.7)	130 (3.7)
	% Inhibition	90	91	93	97
B	Activity	263 (67)	166 (38)	25 (4)	12 (6)
	% Inhibition	100	100	100	100
		Rosy eye with Triton-X 100			
A	Activity	2341 (130)	2159 (111)	188 (5)	201 (18)
	% Inhibition	90	94	93	90
B	Activity	218 (14)	134 (25)	15 (10)	12 (4)
	% Inhibition	100	100	91	100
		Wild type without Triton-X 100			
A	Activity	1547 (92)	1651 (54)	77 (10)	119 (11)
	% Inhibition	91	92	93	79
B	Activity	93 (9)	98 (17)	50 (17)	33 (8)
	% Inhibition	92	92	82	80
		Rosy eye without Triton-X 100			
A	Activity	1865 (94)	1918 (102)	148 (27)	241 (23)
	% Inhibition	90	95	89	95
B	Activity	111 (30)	84 (6)	76 (11)	47 (33)
	% Inhibition	98	97	87	100

›ubstrate acetylthiocholine iodide; B= Substrate S-butyrylthiocholine iodide.*= Percent inhibition of AChE activity with 0.2m*M* ›xur. SD in parenthesis.

mosquitoes. This is a unique case where there was 1.25 to 2-fold increases in the enzyme activity but no increase in tolerance to any of the insecticides tested. It was thought that, sublethal dosages may have differential effect on the fecundity of this strain, however, no such effects were found[12].

In the mosquitoes, altered/increased AChE is normally associated with insecticide resistance/ tolerance. It is interesting to note that although this mutant strain had increased enzyme activity due to highly substrate active AChE-II alleles; it did not show any difference in the propoxur inhibition capabilities. To our knowledge, so far there is no report of resistance to organophosphates and carbamates in *Ae. aegypti* due to increased/altered AChE. Addition of Triton-X in the homogenising solution showed an increased enzyme activity. However, the relative increase in the two strains remained unaltered, indicating that most of AChE enzyme is membrane bound in both the strains. Higher activity of AChE-II allele in the adults supports that the increase in enzyme activity noticed during quantitative assays was due to this particular allele.

Higher expression of an AChE-II isoenzyme in a mutant is perhaps due to the selection of a mutant strain from a single female from the wildtype strain, which had highly substrate active AChE-II isoenzyme. This is evident from the high frequency of this allele in the mutant strain then the parent strain.

This mutant strain is found to be comparatively refractory to CHIK virus by oral route as compared to the wildtype strain. It is interesting to note that refractivity to oral infection to CHIK virus was attributed to midgut barrier, when this barrier was bypassed and mosquitoes were inoculated intrathoracically the multiplication of virus in the heads was similar to susceptible strain[13]. In the present study, there was no qualitative difference in this enzyme in the heads of both the strains of mosquitoes. Literature review revealed no information about role of AChE enzyme in the multiplication of arboviruses.

At this juncture, it is difficult to associate these finding with the susceptibility trait of mosquitoes to viruses. The present information only support that there are some biochemical differences associated with this mutant stain which is also refractory to CHIK virus. Each mosquito population comprises homozygous virus-susceptible, virus-refractory and heterozygous mosquitoes. Presence of about 5% individuals in wildtype population which express AChE-II alleles, indicates that probably spontaneous mutation must have occurred in one such individual which was responsible for rosy eye phenotype and by cross mating it was isolated and colony could be established. Linkage of the AChE, if any to the viral refractivity/susceptibility needs to be investigated using RFLP technique. This would serve as a useful tool among other parameters available in studies on *Ae. aegypti* with different viruses.

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References

1 Soderlund D M & Bloomquist J R, 1990, Molecular mechanisms of insecticide resistance, in Pesticides resistance in Arthropods edited by B E, Tabashnik & R T, Roush, (Chapman & Hall; New York) 1990, 58.

2 Brown T M & Brogdon W G, Improved detection of insecticide resistance through conventional and molecular techniques, *Ann Rev Entomol*, 32 (1987) 145.

3 Mourya D T, Gokhale M D, Bhat H R & Banerjee K, Effect of sublethal dosages of insecticides on Chikungunya virus susceptible and refractory strains of *Aedes aegypti*, *Southeast Asian J Trop Med Public Health*, 25 (1994) 536.

4 World Health Organisation, Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides, *WHO/VBC/81.807* (1981).

5 Finney D J, Probit analysis [Cambridge University Press, Cambridge] 1971, 21.

6 World Health Organisation, *Technical report series*, 818, Vector Resistance to Pesticides, 15th Report of WHO Expert committee on Vector Biology & Control (1992).

7 World Health Organisation, Instructions for determining the susceptibility or resistance of adult mosquitoes to organochlorine, organophosphate and carbamate insecticides-Diagnostic test, *WHO/VBC/81.806* (1981).

8 french-Constant RH & Bonning BC, Rapid microtitre plate test distinguishes insecticide resistant acetylcholinesterase genotypes in the mosquitoes *Anopheles albimanus*, *An. nigerrimus* and *Culex pipiens*, *Med Vet Entomol*, 3 (1989) 1.

9 Lowry O H, Rosebrouh N J, Farr A L & Randall R J, Protein measurement with the folin phenol reagent, *J Biol Chem*, 193 (1951) 265.

10 Munstermann LE, Isoenzymes of *Aedes aegypti*: Phenotypes, linkage, and use in the genetic analysis of two sympatric subspecies population in East Africa, *Ph.D. Thesis*, University of Notre Dame, (1979).

11 Hemingway J, Genetics and Biochemistry of insecticide resistance in Anophelines, *Ph.D. Thesis*, University of London, London (1981).

12 Mourya D T, Gokhale M D, Malunjkar A S, Bhat, H R & Banerjee K, Inheritance of oral susceptibility of *Aedes aegypti* to Chikungunya virus, *Am J Trop Med Hyg*, **51** (1994) 29.