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Comparative Effect of Various Insecticides on Intracellular Proteases in an Insecticide-resistant and Susceptible Strains of *Musca domestica* L.

Sohail Ahmed, [1]Richard wilkins and [1]David Mantle
Department of Agricultural Entomology, University of Agriculture, Faisalabad, Pakistan
[1]Department of Agricultural and Environmental Science, University of Newcastle upon Tyne, Newcastle upon Tyne, UK

Abstract: In order to elucidate the advantages of elevated protease activities in an insecticide-resistant strain of *Musca domestica*, changes in protease activities following insecticide treatments were noted in an insecticide-resistant strain along with the susceptible strain. The two strains behaved differently upon treatment with chemically diversified groups of insecticides viz., DDT, permethrin and dieldrin at respective, LD_{50} level. The magnitude of increase in protease activities was larger in 17bb, a DDT resistant strain, when compared with cooper, a susceptible strain. The effect of insecticides on the activity level of proteases following insecticide treatment is discussed.

Key words: Proteases, insecticides, house fly, protein metabolism

Introduction

The effect of sublethal concentrations of insecticides on the metabolic activities of an organism has been studied elsewhere. Proteolysis is considered as a major physiological event acting as a compensatory mechanisms under insecticidal stress (Saleem and Shakoori, 1987; Zeba and Khan, 1995; Nath *et al*., 1997; Sivakumari *et al*., 1997). Parallelary, the increase in protein content in organisms, subjected to sublethal and lethal doses of insecticides has also been observed that is correlated with development of resistance towards toxic effects of these insecticides (Philip and Rajasree, 1996). In both cases, proteases, from their degradative activities on proteins, play an important part in supplying free amino acids, which can be used as compensatory metabolites in energy production or used for extra protein synthesis in the form of detoxifying enzymes (Ramaswamy, 1987). We have previously reported that elevated protease activities were found in insecticide-resistant strains of house fly, *Musca dmoestica* L. It was also exhibited that protease activities showed relative changes between insecticide-resistant and susceptible strains following insecticide application (Ahmed *et al*., 1998; Wilkins *et al*., 1999). These studies were done with insecticides to which the respective strain was resistant but in order to find out whether changes in level of proteases are confined to one insecticide or insecticide of different groups could have similar effect on the level of proteases.

Materials and Methods

House fly strains: 17bb, resistant to DDT with high cytochrome P450 and glutathione S-transferase. Cooper, a reference susceptible strain.

Chemicals: The suppliers of following chemicals are given in the parenthesis after the name of the chemical (s); DDT, dieldrin, (Chem. Service, UK), permethrin, (Hockley International, UK) All the insecticides were not <98% pure.

Rearing of flies and bioassays: Rearing of flies and bioassay procedures were described by Ahmed and Wilkins (2001).

Studies with insecticides: Adult house flies aged 3 day were used in order to determine the range of toxicity (for 0 to 99% mortality). The bioassays were carried out by topical application of a 1 μl drop of insecticide in acetone to thoracic notum with a programmable arnold micro-applicators (Burkard) and a microsyringe (1ml) with canulae (G 36x3"). Total 50 flies were treated for each concentration of insecticide and these flies were replicated five times. The flies were anaesthetized with CO_2 (flow rate, 4 ml/sec) before dosing. The flies were fed with milk-sugar (1:1) solution 2 hrs before dosing to eliminate the chances of desiccation. The anaesthetized flies were held with padded entomological forceps during treatment. All the dosing (Table 1) of flies with insecticides was done at room temperature and the flies were then transferred to the breeding room (24 ± 2°C). Responses in terms of mortality were recorded after 24 hours. Concentration-mortality data were analyzed by probit analysis (POLO PC, Leora Software, 1987) to obtain LD_{50}.

Table 1: The LD_{50} levels of DDT, permethrin and dieldrin applied on 17bb and cooper strains

Strain	Dose (ng fly^{-1})		
	DDT	Permethrin	Dieldrin
17bb	2400	60	5
Cooper	30	6	0.3

The adult flies surviving each treatment were collected at 24 h following the insecticide treatment.

Proteases determination

Preparation of enzyme solution: Six house flies (males and females in equal number) were weighed and homogenised in extraction buffer using an Ultra-Turrax T25 homogenizer (2x10 sec at 15000 rpm). Whole insect / buffer homogenate (1:60 w/v) was prepared in 50 mM Tris-acteate buffer, pH 7.5 containing 1mM dithiothreitol (DTT), 0.15M NaCl and 3 mM NaN_3 for the estimation of cytoplasmic protease activities. For lysosomal proteases, the extraction medium, as above, was used except for Tris-acetate buffer, which was replaced with 50 mM acetate buffer pH 5.5. The homogenates was centrifuged at 2000 g for 10 min at 6°C and the resultant supernatant was used for determination of protease activities.

Protease assays: Enzyme (0.05 ml supernatant) was incubated with the appropriate assay medium (total volume 0.3 ml) at 37 °C for 10-120 min and the reaction terminated by addition of 0.6 ml of ethanol. The florescence of the liberated 7-amino-4-methylcoumarin (AMC) was measured by reference to a tetraphenylbutadiene fluorescence standard block (λex 370 nm, λem 430 nm). Assay blanks were run with assay medium without enzyme solution. The stock substrate solutions (2.5mM) were prepared in 10% ethanol. Assays were carried out for the following enzymes. The reaction mixture for each enzyme is given below:

Cytoplasmic proteases

Alanyl aminopeptidase: Fifty mM Tris-acetate buffer pH 7.5, 5mM $CaCl_2$,1mM DTT, 0.25mM Ala-AMC; Arginyl aminopeptidase: 50 mM phosphate buffer pH 6.5, 0.15M NaCl, 1 mM DTT, 0.25mM Arg-AMC; Dipeptidyl aminopeptidase IV: 50mM Tris-acetate buffer pH 7.5, 1mM DTT, 0.25mM Gly-Pro-AMC; Tripeptidyl aminopeptidase: 50 mM Tri-acetate buffer pH 7.5, 2mM DTT, 0.25 mM Ala-Ala-Phe-AMC; Proline Endopeptidase: 50 mM Tris-acetate buffer pH 7.5, 2mM DTT, 0.25mM CBZ-Gly-Pro-AMC.

Table 2: Protease activities in 17bb after 24 h of treatment with DDT, permethrin and dieldrin at LD_{50} level

Enzymes	Activity (μmoles h^{-1} mg^{-1} protein)			
Cytoplasmic	DDT	Permethrin	Dieldrin	Control
Ala AP	7.82 ± 0.40b	10.66 ± 0.28a	7.47 ± 0.10b	7.60 ± 0.23b
Arg AP	8.02 ± 0.05b	9.37 ± 0.02a	5.28 ± 0.16d	5.78 ± 0.10c
DAP IV	0.20 ± 0.10a	0.14 ± 0.00b	0.18 ± 0.00a	0.14 ± 0.00b
TAP	2.19 ± 0.10b	3.31 ± 0.10a	1.40 ± 0.10c	1.60 ± 0.10c
Proline EP	0.73 ± 0.00a	0.34 ± 0.00b	0.31 ± 0.10b	0.44 ± 0.00b
Protein content	23.0 ± 0.00a	18.0 ± 0.00c	20.0 ± 0.00b	16.0 ± 0.33d
Lysosomal				
DAP I	0.19 ± 0.00c	0.44 ± 0.00a	0.37 ± 0.00b	0.19 ± 0.00c
DAP II	0.26 ± 0.00b	0.56 ± 0.00a	0.24 ± 0.10b	0.16 ± 0.00b
Cathepsin B + L	0.86 ± 0.01c	3.03 ± 0.06a	2.20 ± 0.00b	0.72 ± 0.03c
Cathepsin B	0.70 ± 0.01b	1.05 ± 0.00a	1.10 ± 0.00a	0.55 ± 0.02c
Cathepsin H	1.56 ± 0.03c	2.68 ± 0.01b	4.07 ± 0.00a	1.22 ± 0.03d
Cathepsin D	270.0 ± 0.00b	450.0 ± 0.00a	480.0 ± 0.00a	200.0 ± 0.00c
Protein content	18.0 ± 0.00a	12.0 ± 0.00c	17.0 ± 0.00b	18.0 ± 0.00a

Values are means ± SE (n = 3). Difference in enzyme activities in the three treatments with control was determined by DMRT. Means with same letters in a row are not significantly different at 5% level of significance. Protein content is expressed as mg g^{-1}fly.

Table 3: Protease activities in cooper strain after 24h of treatment with DDT, permethrin and dieldrin at LD_{50} level

Enzymes	Activity (μmoles h^{-1} mg^{-1} protein)			
Cytoplasmic	DDT	Permethrin	Dieldrin	Control
Ala AP	4.51 ± 0.24a	2.53 ± 0.04b	4.69 ± 0.31a	2.71 ± 0.10b
Arg AP	5.30 ± 0.30a	3.50 ± 0.00c	4.28 ± 0.00b	3.02 ± 0.04c
DAP IV	0.07 ± 0.00a	0.06 ± 0.00b	0.07 ± 0.00a	0.02 ± 0.00c
TAP	0.83 ± 0.00a	0.66 ± 0.04b	0.60 ± 0.00b	0.50 ± 0.00bc
Proline EP	0.58 ± 0.00a	0.35 ± 0.00b	0.19 ± 0.00c	0.19 ± 0.00c
Protein content	18.0 ± 0.00c	22.0 ± 0.00b	32.0 ± 0.00a	18.0 ± 0.00c
Lysosomal				
DAP I	0.10 ± 0.00a	0.10 ± 0.00a	0.06 ± 0.00b	0.10 ± 0.00a
DAP II	0.10 ± 0.00a	0.03 ± 0.00c	0.07 ± 0.00b	0.10 ± 0.00a
Cathepsin B + L	0.37 ± 0.00a	0.24 ± 0.00b	0.36 ± 0.00a	0.36 ± 0.00a
Cathepsin B	0.33 ± 0.00a	0.22 ± 0.00bc	0.16 ± 0.00c	0.24 ± 0.00bc
Cathepsin H	0.80 ± 0.00a	0.54 ± 0.00b	0.65 ± 0.00b	0.60 ± 0.05b
Cathepsin D	120.0 ± 0.00a	60.0 ± 0.00b	70.0 ± 0.00b	140.0 ± 0.00a
Protein content	24.0 ± 0.00b	19.0 ± 0.00c	30.0 ± 0.00a	24.0 ± 0.00b

Values are means ± SE (n = 3). Difference in enzyme activities in the three treatments with control was determined by DMRT. Means with same letters in a row are not significantly different at 5% level of significance. Protein content is expressed as mg g^{-1}fly.

Lysosomal proteases

Dipeptidyl aminopeptidase I: Fifty mM actate buffer pH 5.5, 2 mM DTT, 0.25 mM Gly-Arg-AMC; Dipeptidyl aminopeptidase II: 50mM acetate buffer pH 5.5, 2mM DTT, 0.25mM Lys-Ala-AMC; Cathepsin B or Cathepsin B + L: 50mM acetate buffer pH 5.5, 2 mM DTT, 0.25 mM CBZ-Phe-AMC (Cathepsin B + L) or 0.25mM CBZ-Arg-Arg-AMC (Cathepsin B only); Cathepsin H: 50mM phosphate buffer pH 6.0, 1mM DTT, 0.5mM puromycin, 0.25mM Arg-AMC.

Assay for cathepsin D: Assay of cathepsin D activity was based on the spectrophotometric procedure of Pennington (1977): 50 mM acetate buffer pH 3.5, 1 mM DTT, 3 mg ml^{-1} acetic acid denatured haemoglobin substrate (total assay volume 0.5 ml). The reaction was terminated by addition of 0.6 ml 10% perchloric acid (PCA); the sample centrifuged at 2000g for 5-10 minutes and absorbance of acid soluble peptides was determined at 280 nm. Assay blanks were run as above.

Determination of soluble protein: Soluble protein levels in the supernatants used for assays of the above proteases were determined by the method of Bradford (Bradford, 1976).

Results

Protease activities in the flies of 17bb strain surviving DDT treatment were elevated significantly, when compared with activities of flies in the control treatment except the activities of Ala AP, DAPI and DAPII. However, cytoplasmic protease showed significantly different activities than that of flies in the control treatment in case of cooper strain.

The permethrin treated flies of 17bb similarly showed contrasting changes with flies of Cooper strain. All the protease enzymes showed the elevation in the flies of 17bb but flies of cooper strain had activity less than control values for most of the cytoplasmic and lysosomal enzymes. The flies of both strains behaved differently from each other when the dieldrin was used. The flies of both strains had different protein content between each other when three different insecticides were used. For instance, the flies of 17bb treated with DDT had more soluble protein content in the homogenate for cytoplasmic protease assays but same was not observed in cooper strain (Tables 2, 3).

Discussion

In order to elucidate the advantages of elevated protease activities in insecticide-resistant strains of *M. domestica* (Saleem *et al.*, 1994; Ahmed *et al.*, 1998), changes in protease activities following the insecticide treatment were noted in resistant strains along with susceptible strain. The data presented here suggested a metabolic difference in terms of protease activities between resistant and susceptible strains when both were subjected to insecticides at respective lethal dose levels. The resistant and susceptible strains were capable of regulating the activities following insecticide treatment, but magnitude of regulation was different between resistant and susceptible strains. Nevertheless, the strains shared broad similarity of changes for certain proteases after the treatment. High activity levels of proteases were also maintained in flies collected at 24h following insecticide treatment. This increase in activities may result from cellular damage through insecticide attaching with cells and ultimately releasing proteases stored in them. However, on whole animal basis, the increase in activity cannot be related to cellular damage, but induction of proteases following insecticide treatment can be expected.

It is known that major target site of insecticides is the nervous

system. Little is known about the cytolysis properties of insecticides at the target sites and during the course of transportation to the target sites. The cytological changes in the haemocytes of insects by the insecticides have been reported (Gupta, 1985), but how are these changes involved in detoxication mechanism is not clear (Begum and Gohain, 1996). Chanda and Roy (1986) found increase in digestive proteases like trypsin and chymotrypsin in the gut lumen and gut tissues of *Schizodactylus monstrosus* at 6 h after phosphamidon treatment. The attenuation in the activities was observed at 12, 18 and 24 h after treatment. They suggested that rise in activities was either due to synthesis of proteases and/or release of stored enzymes when phosphamidon might had ruptured the different cellular material in the digestive tract. The changes in protease activity contrast with data reported by Saleem *et al* . (1994) who reported that decreased activities for many protease types in a resistant strain of *M. domestica* surviving exposure to malathion. This may relate to the longer sampling period used in the latter study following pesticide exposure (48 h).

The data regarding the effect of permethrin on lysosomal enzymes in 171 and cooper strains does not support the above explanation of increase in activities, as a response of two strains was not uniform towards proteases and other lysosomal enzymes after 24 h of permethrin treatment. It means that variations in enzymes are associated with tissues other than nervous system in the insect body. There is differential response of tissues in the insecticide-resistant and susceptible strains. The difference of cytochrome P450 and GSTs induction in various tissues has been recognized (Lee and Scott, 1992; Scott and Lee, 1993). The distribution of intracellular proteases in various insect body regions showed that 50% of activity was present in tissues other than digestive tract of some insect species (Saleem *et al.*, 1995). Further work is needed after obtaining baseline of protease activities in tissues (gut, fat bodies and haemolymph) and then following the changes in activities after insecticide treatment.

The decrease in protease activity compared with activity in control can be explained on the basis of production of protease inhibitors to slow down the protein catabolism as a normal physiological phenomenon or an abnormality caused by stress such as observed in water stressed flies of *M. domestica* and *Sarcophaga bullata* (Bradley *et al.*, 1989).

The effect of insecticides or other stresses on proteases have been studied in higher animals (Mantle *et al.*, 1997; Saleem *et al.*, 1998). Increase in hydrolase (other than proteases) activities in serum of male rats administered with 0.1 or 0.5 of the LD_{50} of chlorfenvinphos and dichlorvos at 1 and 24 h of treatment was accompanied by a decrease in their activity in the lysosomal fraction of liver homogenate. Also the generally high levels of lysosomal enzyme activities were seen in slices of liver, kidney, muscle and in blood of rabbits which were transferred to a new cage daily during 3 months than in rabbit left in the same cage. This increase was correlated with process of adaptation to a stress (Witek *et al.*, 1994). After application of several pyrethroids, the activity of lysosomal enzymes, β-glucuronidase and β-galactonidase increased in rodents, but not in the birds (FAO, 1979). The increased activity may be involved in the degenerative changes of myelin sheath of peripheral and central nerves in rats and hamster treated with lethal doses of pyrethyroids (Ruigt, 1985). The changes in lysosomal enzymes (particularly proteases) upon permethrin treatment, on 171 and cooper strains of *M. domestica* were not similar in both strains, hence these changes were not related to nerve damage.

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