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Intracellular Proteases in a DDT-Resistant Strain of *Musca domestica* L. Following Insecticide Application

SOHAIL AHMED[1], RICHARD M. WILKINS† AND DAVID MANTLE†
Department of Agricultural Entomology, University of Agriculture, Fasialabad–38040, Pakistan
†*School of Biology, Newcastle University, King George VI Building, Newcastle upon Tyne NE1 7RU, UK*
[1]corresponding author E-mail: saha786@fsd.paknet.com.pk

ABSTRACT

The changes in intracellular proteases following DDT application at LD_5 and LD_{20} level in a DDT-resistant strain of *Musca domestica* L. showed that at 3 h of the treatment, the cytoplasmic enzymes except Proline Endopeptidase showed a non-significant increase in activities. The activities of all cytoplasmic enzymes further increased at LD_{20} level, suggesting the dose-dependence of activities, but protease activities at 24 h with both doses showed non-significant difference with control activities. The treatment of fenitrothion to DDT-resistant flies (at LD_{50} level) could not bring a significant change in protease activities. The relationship of these alterations in level of intracellular proteases and insecticide resistance / toxicity is discussed.

Key Words: DDT; Resistant strain; Fenitrothion; Intracellular proteases; Sublethal dose

INTRODUCTION

The widespread use of insecticides has amounted to the biochemical/physiological changes which may be of adaptive significance to the life of an animal. Among these changes, alteration in xenobiotics metabolizing enzymes (i.e., mixed function oxidases, esterases and glutathione S-transferases) has dominated the toxicological literature. Parallelarly, the effect of insecticide on protein metabolism has been observed in aquatic environment (Sivakumari *et al*., 1997). One area of relevance to the above which has received little attention is the potential effect of insecticides on intracellular proteases. These proteases with diversified nature of functions are important in regulating proteins in animals. The decline in proteins level with increase in transaminase activities suggests the mobilization of amino acids during insecticide stress to meet the energy demands (Saleem & Shakoori, 1985; Saleem & Shakoori, 1987; Zeba & Khan, 1995; Reddy & Bashamohideen, 1995; Begum & Vijayaraghavan, 1996; Bhavan & Geraldine, 1997; Oruc & Uner, 1998) but nothing substantial has been reported for the intracellular proteases. The intracellular proteases of rat liver showed inhibition in an acute poisoning test with pirimiphos-methyl (an organophosphorus insecticide). There was no insecticide-induced inhibition of protease activities in heart, kidney or brain tissues, however, lysosomal enzymes (dipeptidyl aminopeptidase I, cathepsin B+L and cathepsin D) showed significantly increased activities in these tissues of rat (Mantle *et al*., 1997). *In vivo* alteration of intracellular proteases merits attention as the change in protein level can have adverse effect on the normal functioning of cells in a tissue. We have already reported the alteration in the levels of intracellular proteases in insecticide resistant and susceptible strains of house fly, *Musca domestica* L. and these studies were done when adult flies were dosed with various insecticides at LD_{50} level to find out relation between proteases and ability to resist the high insecticide doses (Ahmed *et al*., 1998; Wilkins *et al*., 1999; Ahmed & Wilkins, 2001). In order to further elaborate this relationship, the present attempt will show two types of studies (a) Effect of sublethal doses of DDT on intracellular proteases in 17bb (b) Effect of fenitrothion on intracellular proteases in 17bb. Note that 17bb has shown low ~20 fold resistance to fenitrothion.

MATERIALS AND METHODS

House fly strains. 17bb, A DDT-resistant strain obtained from Danish Pest Infestation Laboratory, Lyngby, Denmark.

Toxicity test. For obtaining LD50 of DDT and fenitrothion in 17bb strain, individual adult flies were treated with a series of DDT and fenitrothion concentrations in a group of 10 flies each time and replicated five times and sublethal concentration were determined in a probit analysis program POLO-PC (LeOra software Inc.). The survivors of the treatment at LD_5 and LD_{20} level of DDT and LD_{50} level of fenitrothion at 3 and 24 h exposure were collected and kept at -40°C for subsequent protease assays.

Protease activities. Six houseflies (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 50mM Tris-acetate buffer, pH 7.5 containing 1 mM dithiothreitol (DTT), 0.15 M 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 5.3. The homogenates were centrifuged at 3000 g for 10 min at 6°C and the resultant supernatant was used for determination of proteolytic enzyme activities.

Proteolytic enzyme assays. The quantification of proteolytic enzymes in various tissues/species reported by Ahmed *et al.* (2001) was followed. Enzyme preparation (0.05 mL supernatant) was incubated with the appropriate assay substrate and medium (total volume 0.3 mL) at 37°C for 10-120 min and the reaction terminated by addition of ethanol (0.6 mL). The fluorescence of the liberated aminoacyl 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 substrate/medium without enzyme homogenate. The stock substrate solutions (2.5 mM) were prepared in 10% ethanol. Soluble protein levels in supernatants used for assays of the above proteases were determined by the method of Bradford (1976).

RESULTS AND DISCUSSION

Effect of sublethal doses of DDT on intracellular proteases in 17bb. Effect of sublethal doses of DDT on surviving flies of 17bb (DDT-resistant) strain at 3 and 24 h after treatment is shown in Table I. At 3 h of the treatment, the cytoplasmic enzymes except Proline EP showed a non-significant increase in activities. The activities of all cytoplasmic enzymes further increased at LD_{20} level, suggesting the dose-dependence of activities. The lysosomal enzyme activities were unaffected with dose at 3 h, but showed a non-significant increase with LD_{20} for Cathepsin B and Cathepsin H. Protease activities at 24 h with both doses showed non-significant difference with control activities. However, cytoplasmic protease activities were low while lysosomal protease activities were high as compared with control activities.

Effect of fenitrothion on intracellular proteases in 17bb. Table II shows that protease activities of both types (cytoplasmic and lysosomal) were low as compared with activities in control flies. Although the difference was non-significant at both the time points, i. e. 3 and 24 h after treatment.

In order to elucidate the advantages of elevated protease activities in insecticide-resistant strains of *M. domestica*, the changes in protease activities following insecticide treatment were noted in resistant strain. A metabolic difference in terms of protease activities between resistant and susceptible strains when both were subjected to insecticides at respective lethal and sublethal dose levels has been observed (Ahmed *et al.*, 1998; Wilkins *et al.*, 1999; Ahmed & Wilkins, 2001). The increase in proteases activity may result from cellular damage through insecticide attaching with cells and ultimately releasing stored enzymes 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 (Chanda & Roy, 1986).

Table I. Protease activities in 17bb strain treated with sublethal doses of DDT

Enzymes	Activity (μmoles h^{-1} mg^{-1} protein)		
17bb (3 h)	**LD_5**	**LD_{20}**	**Control**
Cytoplasmic			
Ala AP	1.55±0.3	5.23±1.3*	1.11±0.1
Arg AP	3.12±0.4	4.54±2.2	2.06±0.4
TAP	0.21±0.02	0.62±0.04*	0.15±0.08
Proline EP	0.27±0.06	0.43±0.06	0.33±0.06
Protein content	52±9	49±9	36±4
Lysosomal			
Cathepsin B+L	0.27±0.02	0.22±0.07	0.25±0.05
Cathepsin B	0.21±0.05	0.25±0.02	0.12±0.02
Cathepsin H	0.26±0.05	0.42±0.06	0.29±0.12
Protein content	45±16	52±23	26±1
17bb (24 h) cytoplasmic	**LD_5**	**LD_{20}**	**Control**
Ala AP	4.51±1.2	4.76±1.4	4.91±1.8
Arg AP	4.61±1.4	3.10±0.8	6.88±3.1
TAP	1.56±0.5	0.97±0.3	2.08±1.0
Proline EP	1.62±0.5	0.63±0.02	1.39±0.5
Protein content	22±7	34±1	31±13
Lysosomal			
Cathepsin B+L	0.66±0.22	2.31±0.75*	0.33±0.11
Cathepsin B	0.40±0.12	0.26±0.03	0.08±0.03
Cathepsin H	0.44±0.19	0.80±0.21	0.36±0.11
Protein content	15±5	28±4	28±3

Values are means±SE (n=3). Difference in enzyme activities between treated and control flies was determined by One Way of ANOVA. * significantly different from control at P<0.05. Protein content is expressed as mg g^{-1} fly; Ala AP (Alanyl aminopeptidase), Arg AP (Arginyl aminopeptidase), TAP (Tripeptidyl aminopeptidase), Proline EP (Proline endopeptidase)

Table II. Protease activities in 17bb strain after fenitrothion treatment

Enzymes	Activity (μmoles substrate hydrolyzed h^{-1} mg^{-1} protein)		
	3h	**24h**	**Control**
Cytoplasmic			
Ala AP	3.23±0.3	2.31±0.2	4.10±0.7
Arg AP	2.11±0.6	2.75±0.4	3.06±0.4
TAP	0.57±0.1	0.80±0.07	0.84±0.16
Proline EP	0.21±0.06	0.19±0.03	0.36±0.06
Protein content	43±4	36±4	50±3
Lysosomal			
Cathepsin B+L	0.24±0.02	0.10±0.00	0.34±0.12
Cathepsin B	0.21±0.05	0.10±0.01	0.20±0.15
Cathepsin H	0.38±0.13	0.22±0.01	0.40±0.13
Protein content	31±3	33±5	34±3

Values are means±SE (n=3). Difference in enzyme activities between treated and control flies was determined by One Way of ANOVA * significantly different from control at P<0.05. Protein content is expressed as mg g^{-1} fly; Ala AP (Alanyl aminopeptidase), Arg AP (Arginyl aminopeptidase), TAP (Tripeptidyl aminopeptidase), Proline EP (Proline endopeptidase)

The changes in protease activities vary with insecticides in different strains. The treatment of fenitrothion to DDT-resistant flies (at LD_{50} level) could not bring a significant change in protease activities. The non-significant change in protease activities after 24 h of

treatment cannot be explained on the basis of biochemical resistance mechanism. The nature of fenitrothion resistance in 17bb strain is not known. The possibility of mechanism other than enhanced degradation by enzymes cannot be ruled out. Enhanced degradation may depend on induced activity of detoxifying enzymes and induction of these enzymes is supplemented by the increase in proteases, ultimately increased protein degradation to amino acids. For this reason, the increase in protease activities was not observed with fenitrothion treatment.

In order to know whether changes in protease activities of resistant strains were due to treatment of insecticide at LD_{50} level. The effect of sublethal doses of DDT on DDT-resistant strain was noted. In present study, such significant increase at 3 h of LD_{20} treatment was in cytoplasmic proteases. The activities of lysosomal enzymes were unaffected except for Cathepsin H at LD_{20}, indicating conservation rather than degradation of specific proteins. The LD_5 did not bring a significant change in protease activities after 3 h of treatment. However, lysosomal enzymes were maintaining high activity levels at LD_5 and LD_{20} (significantly high for Cathepsin B and Cathepsin B+L, respectively) as compared with activities in control which suggest the dominance of one pathway of protein catabolism. The contradiction with previous result (Ahmed *et al*., 1998; Wilkins *et al*., 1999; Ahmed & Wilkins 2001) at 24 h with LD_{50} level may be due to heterogeneous response of strain towards different doses of DDT. The significant increase of some of lysosomal proteases at 24 h at LD_5 and LD_{20} level explain the need of proteases at a later stage of intoxication as DDT is slow acting poison. It is, therefore, suggested that further investigations on alterations in proteases with different doses of insecticides, at various time periods after insecticide treatment on insecticide-resistant and susceptible strain should be carried out.

There is an interaction between insecticide treatment and change in the protease activities and this interaction varies with strain, dose of insecticide, time after the treatment. In view of the above, it is suggested that the metabolic advantage conferred by increased protease activities in resistant strain is in providing an increased supply of precursor amino acids from proteolytic degradation products (peptides and proteins), prior to *de novo* synthesis of detoxifying enzymes such as cytochrome P450, esterases etc. It should also be borne in mind while describing the changes in protease activities that protease activities by convention are expressed in terms of µmoles h^{-1} mg^{-1} protein. It is, therefore, possible that apparent changes in protease activities under particular experimental conditions may be artifact resulting from changes in overall tissue protein levels, rather than genuine changes in enzyme activities. Although, the levels of total protein vary somewhat during the various experimental time points in the present investigation, such changes are of insufficient magnitude to explain observed changes in protease activities (when taken as mg ml^{-1}), in addition, the protease activities are determined via specific and sensitive assay procedures, whereas the determination of total protein levels by comparison involves a relatively non-specific and insensitive assay procedure. It is hence argued that changes in protease activities described above represent true changes in enzyme activities, rather than an artifact associated with changes in overall tissue protein level.

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