

In vivo* Effects of Cyfluthrin on Proteolytic Enzyme Activities of Malathion-resistant and Susceptible Strains of *Tribolium castaneum

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Abstract.- To elucidate whether insecticide toxicity in insects involves insecticide - induced abnormalities of the intracellular protein catabolic process, we have determined the *in vivo* effect of a synthetic pyrethroid, cyfluthrin on the activities of representative protein catabolising cytoplasmic and lysosomal proteases (responsible for the various stages of the protein degradation cascade and essential for normal cell functioning) in resistant and susceptible strains of *Tribolium castaneum*. Effect of cyfluthrin was determined at LC₅₀ after 48 hour of treatment both in the live and dead adult beetles and compared with controls. In treated live beetles, cyfluthrin decreased alanyl aminopeptidase (18%), arginyl aminopeptidase (6%), leucyl aminopeptidase (22%), tripeptidyl aminopeptidase (12%), proline endopeptidase (43%) and dipeptidyl aminopeptidase I (23%) and increased cathepsin H (42%), while in dead beetles, almost all cytoplasmic proteases as well as cathepsin H manifested further decreasing trend. On the other hand in treated resistant strain live beetles, all lysosomal proteases and leucyl aminopeptidase were considerably decreased ranging from 19% to 58% of control activity and this decreasing trend was further intensified in treated dead beetles, which ranged from 30% to 96%. We conclude that the effect of cyfluthrin on proteolytic enzyme activities induced inhibition of proteases and could be important in the development of insecticide resistance in *T. castaneum*.

Key words: Cyfluthrin, malathion-resistant strain, cytoplasmic and lysosomal proteases, aminopeptidases, endopeptidases, cathepsins, *Tribolium castaneum*, red flour beetle.

INTRODUCTION

During the decade 1971-80, a new class of agricultural pesticides, synthetic pyrethroids has emerged and complimented the earlier groups of insecticides such as organochlorines, organophosphates and carbamates (Elliott and Janes, 1973; Elliott, 1977). These developments became imperative as the insects became resistant to earlier insecticides (Saleem and Shakoori, 1989; Saleem *et al.*, 2000). The synthetic pyrethroids possess many suitable properties such as high toxicity to mammals and birds *i.e.* non-target organisms and application at low doses (Elliott, 1977; Shakoori *et al.*, 1988). It is, therefore, appropriate to continue search for new pyrethroids that have practical application (Breese, 1977; MacCuaig, 1980; Saleem *et al.*, 1994a,b).

Cyfluthrin is one of the recently developed synthetic pyrethroids which is non-systemic and acts on the nervous system, with rapid knockdown and

long residual activity. It is effective against many insect pests especially Lepidoptera, Coleoptera, Homoptera and Hemiptera on cereals, cotton, fruits, and vegetables, in public health situations, stored products, domestic use and animal health. (Tomlin, 2000).

Keeping in view the importance of red flour beetle, *Tribolium castaneum* (Herbst.) (Coleoptera : Tenebrionidae), we have reported from our laboratory that it has developed resistance to malathion in Pakistan (Saleem and Shakoori, 1989) besides several other similar reports from other laboratories (Dyte and Blackman, 1970; Champ and Dyte, 1977). In order to elucidate biochemical mechanism responsible for the development of insecticide resistance in insects, we determined normal levels of a wide variety of cytoplasmic and lysosomal proteases in resistant and susceptible strains of *T. castaneum* (Saleem *et al.*, 2000) and *Musca domestica* (Saleem *et al.*, 1994a, b), as well as effect of insecticides including synthetic pyrethroids such as cypermethrin (Saleem and Shakoori, 1986; 1993); permethrin and malathion

(Saleem and Shakoori, 1987a; Shakoori and Saleem, 1989); Dimilin and Ambush (Saleem and Shakoori, 1987b); fenprothrin (Shakoori *et al.*, 1988) and lambda-cyhalothrin (Wilkins *et al.*, 1995) etc. in *T. castaneum*. To further elucidate the possible involvement of the process of intracellular protein catabolism in the development of insecticide resistance, we have determined in this study the effect of another member of synthetic pyrethroids, cyfluthrin, on the proteolytic enzyme activities of resistant and susceptible strains of *T. castaneum*. It is expected that the findings of the present study will provide insight regarding involvement of a comprehensive range of cytoplasmic and lysosomal proteolytic enzyme activities in development of resistance in insects to insecticides.

MATERIALS AND METHODS

Rearing of beetles

Two strains *viz.* susceptible strain (FSS-II) and resistant strain (CTC-12) of *T. castaneum* were used in this study. The FSS-II strain was described by Lloyd and Ruczkowski (1980). It has remained in culture at the Department of Agriculture and Environmental Science, Newcastle University, Newcastle upon Tyne, UK, without exposure to insecticides. The CTC-12 is multiple organophosphorous resistant strain which was first reported by Champ and Campbell-Brown (1970).

The laboratory culture of *T. castaneum* was maintained in an incubator at $30\pm 1^\circ\text{C}$ with $60\pm 5\%$ relative humidity. The new cultures were prepared every third day *i.e.* approximately twice a week in empty jam jars (300 ml). Whole wheat flour was used as culture medium. The culture medium was also placed in an oven at 60°C for 60-90 minutes to provide maximum reduction of contamination. After cooling the jars to room temperature, they were filled $\frac{1}{4}$ th with culture medium and about 100 adult beetles were added for egg laying. Ten days old beetles developing from these eggs were used for this study.

Insecticide and other chemicals

Technical grade of a synthetic pyrethroid, cyfluthrin [Baythroid; *RS*-5-cyano-4-fluoro-3-phenoxybenzyl (1 *RS*, 3 *RS*; 1 *RS*, 3 *SR*) -3 - (2, 2-

dichlorovinyl) -2, 2-dimethylcyclopropanecarboxylate] was obtained from Messers Bayer AG. All other reagents including protease assay substrates were obtained from Messers Sigma Chemical Co. Poole, UK.

Bioassay procedure

Calculated quantities of cyfluthrin were dissolved in acetone, which were then serially diluted to prepare five different concentrations. Each concentration was applied in the middle of Petri dish and was then spread uniformly by rotating the dish. For a Petri dish with an area of 130 cm^2 , 1.3 ml of insecticide solution was sufficient to spread as a thin film on the entire surface. After evaporation of acetone and drying of dishes at room temperature, forty five healthy insects of similar size and development stage were released in these dishes. Three replicates were used for each dose. Mortality counts were made after 48 hours exposure to insecticide and percentage kill was corrected by Abbott's formula for any control mortality (Abbott, 1925). The criterion for death was the one described by Lloyd (1969). Results were subjected to probit analysis. The LC_{50} values for susceptible and resistant strains of *T. castaneum* adult beetles were calculated as outlined by Busvine (1971) and described by Finney (1971).

Resistance ratio was calculated by dividing the LC_{50} for the resistant strain with LC_{50} for the susceptible strain according to the method described elsewhere (Saleem and Wilkins, 1983; Saleem and Shakoori, 1989).

For *in vivo* determination of the effect of cyfluthrin on proteases, sufficient number of adult beetles were exposed to LC_{50} values of susceptible and resistant strains. Thereafter, the concentration of proteases were determined from both live and dead beetles of both resistant and susceptible strains and compared with their respective controls.

Isolation of proteases from beetles

Approximately 50 adult beetles were weighed and homogenized in extraction buffer using an Ultra-Turrax homogenizer (2x10 sec at 15000 rpm). Whole insect homogenate (1:40, w/v) was prepared in 50 mM Tris-acetate buffer, pH 7.5 containing 1 mM dithiothreitol (DTT), 0.15 M NaCl and 3 mM

NaN₃ for estimation of cytoplasmic protease activities. For lysosomal proteases, the same extraction buffer 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 x g for 20 min at 4°C and the resultant supernatants were used for determination of proteolytic enzyme activities.

Proteolytic enzymes assays

The quantification of proteolytic enzyme activities in various tissues/species has been reported previously from our laboratories (Mantle *et al.*, 1992; Blanchard *et al.*, 1993; Faiz *et al.*, 1994; Saleem *et al.*, 1994a,b). In the present study, 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 fluorescence of the liberated aminoacyl 7-amino-4-methylcoumarin (AMC) was measured with reference to a tetraphenylbutadiene fluorescence standard block (λ_{ex} 380 nm, λ_{em} 440 nm). Assay blanks were run in which the enzyme was added to the medium immediately before ethanol addition. Assay conditions were modified for samples with high enzyme activity such that the extent of substrate utilization never exceeded 15%. Stock substrate solutions (2.5 mM) were prepared in 10% ethanol.

Assays were carried out for the following enzymes, with the corresponding reaction mixture for each enzyme given below:

Alanyl aminopeptidase: 50 mM Tris-acetate buffer pH 7.5, 5 mM CaCl₂, 1 mM DTT, 0.25 mM Ala-AMC.

Arginyl aminopeptidase: 50 mM phosphate buffer pH 6.5, 0.15 M NaCl, 1 mM DTT, 0.25 mM Arg-AMC.

Leucyl aminopeptidase: 50 mM glycine-NaOH buffer pH 9.5, 5 mM MgCl₂, 1 mM DTT, 2 mM Leu-AMC.

Dipeptidyl aminopeptidase-I: 50 mM Tris-acetate buffer pH 5.5, 2 mM DTT, 0.25 mM Gly-Arg-AMC.

Dipeptidyl aminopeptidase-II: 50 mM acetate buffer pH 5.5, 2 mM DTT, 0.25 mM Lys-Ala-AMC.

Dipeptidyl aminopeptidase-IV: 50 mM Tris-acetate buffer pH 7.5, 2 mM DTT, 0.25 mM Gly-Pro-AMC.

Tripeptidyl aminopeptidase: 50 mM Tris-acetate buffer pH 7.5, 2 mM DTT, 0.25 mM Ala-Ala-Phe-AMC.

Proline endopeptidase: 50 mM Tris-acetate buffer pH 7.5, 2 mM DTT, 0.25 mM CBZ-Gly-Pro-AMC.

Cathepsin B or cathepsin B + L: 50 mM acetate buffer pH 5.5, 2 mM DTT, 0.25 mM CBZ-Phe-AMC (cathepsin B + L) or 0.25 mM CBZ-Arg-Arg-AMC (cathepsin B only).

Cathepsin H: 50 mM phosphate buffer pH 6.0, 1 mM DTT, 0.5 mM puromycin, 0.25 mM Arg-AMC.

Assay of cathepsin D activity was based on the spectrophotometric procedures of Pennington (1977) and Pluskal *et al.* (1978). The reaction mixture comprised 50 mM acetate buffer pH 3.5, 1 mM DTT, and 3 mg/ml acid-denatured haemoglobin substrate (total assay volume 0.5 ml). The reaction was terminated by addition of 0.5 ml 10% PCA. The samples were centrifuged at 2000 xg for 10 min, and the absorbance of acid soluble peptides determined at 280 nm. Assay blanks were run as above.

Supernatant protein levels were determined by the method of Lowry *et al.* (1951) with BSA as standard.

RESULTS

Proteases of susceptible strain

Table I shows the effect of cyfluthrin at LC₅₀ dose level after 48 hours treatment on cytoplasmic and lysosomal proteolytic enzyme activities in live as well as dead adult beetles of susceptible strain of *T. castaneum*, while percent increase or decrease in live and dead beetles of susceptible strain following cyfluthrin treatment than control is shown in Table-II.

In surviving susceptible strain adult beetles, cyfluthrin treatment considerably decreased alanyl aminopeptidase by 18%, leucyl aminopeptidase by 22%, proline endopeptidase by 43% and dipeptidyl aminopeptidase I by 23%, but increased cathepsin H by 42%. The remaining proteases *viz.* arginyl aminopeptidase, dipeptidyl aminopeptidase IV, tripeptidyl aminopeptidase, dipeptidyl aminopeptidase II, cathepsin B, cathepsin L and cathepsin D manifested minor fluctuations.

In dead adult beetles of susceptible strain cyfluthrin further decreased most of the proteolytic enzymes. Thus alanyl aminopeptidase was decreased by 42%, arginyl aminopeptidase by 41%, leucyl aminopeptidase by 63%, tripeptidyl aminopeptidase by 21%, proline endopeptidase by

Table I.- *In vivo* effects of cyfluthrin on proteolytic enzyme activities (nmol substrate hydrolyzed/h/mg soluble extract protein) of malathion-resistant and susceptible strains of *Tribolium castaneum*.

Protease types	Susceptible			Resistant		
	Control	Treated live beetles	Treated dead beetles	Control	Treated live beetles	Treated dead beetles
Cytoplasmic proteases						
Alanyl aminopeptidase	30.41	24.79	17.62	93.88	94.54	96.08
Arginyl aminopeptidase	8.58	8.07	5.03	33.50	35.22	32.03
Leucyl aminopeptidase	0.32	0.25	0.12	0.97	0.75	0.65
Dipeptidyl aminopeptidase IV	18.72	19.97	16.99	28.76	32.32	31.38
Tripeptidyl aminopeptidase	14.33	12.62	11.33	17.19	18.77	15.88
Proline endopeptidase	1.36	0.77	0.70	3.97	3.94	4.39
Lysosomal proteases						
Dipeptidyl aminopeptidase I	17.54	13.52	18.38	55.06	39.50	32.58
Dipeptidyl aminopeptidase II	4.24	4.76	5.03	15.05	10.03	7.97
Cathepsin B	264.80	258.32	257.30	645.39	451.48	373.64
Cathepsin L	196.90	174.21	183.79	215.12	90.30	8.69
Cathepsin H	5.66	8.01	4.38	37.14	30.10	26.07
Cathepsin D	316.28	346.09	341.37	406.84	324.23	284.52

Table II.- Percent increase (+) or decrease (-) following cyfluthrin treatment in malathion-resistant and susceptible strains of *Tribolium castaneum*.

Protease types	Susceptible		Resistant	
	Treated live beetles	Treated dead beetles	Treated live beetles	Treated dead beetles
Cytoplasmic proteases				
Alanyl aminopeptidase	- 18.48	- 42.06	+ 0.70	+ 2.34
Arginyl aminopeptidase	- 5.94	- 41.38	+ 5.13	- 4.39
Leucyl aminopeptidase	- 21.88	- 62.50	- 22.68	- 32.99
Dipeptidyl aminopeptidase IV	+ 6.68	- 9.24	+ 12.38	+ 9.10
Tripeptidyl aminopeptidase	- 11.93	- 20.94	+ 9.19	- 7.62
Proline endopeptidase	- 43.38	- 48.53	- 0.76	+ 10.58
Lysosomal proteases				
Dipeptidyl aminopeptidase I	- 22.92	+ 4.79	- 28.26	- 40.83
Dipeptidyl aminopeptidase II	+ 12.26	+ 18.63	- 33.36	- 47.04
Cathepsin B	- 2.45	- 2.83	- 30.05	- 42.11
Cathepsin L	- 11.52	- 6.66	- 58.02	- 95.96
Cathepsin H	+ 41.52	- 22.61	- 18.96	- 29.81
Cathepsin D	+ 9.42	+ 7.93	- 20.31	- 30.07

49% and cathepsin H by 23%. The remaining proteases such as dipeptidylaminopeptidases I, II and IV and cathepsins B, L and D showed minor deviations.

Proteases of resistant strain

The effect of cyfluthrin at LC₅₀ dose level after

48 hour treatment on cytoplasmic and lysosomal proteolytic enzymes in surviving and dead resistant strain beetles of *T. castaneum* is shown in Table I, whereas Table II shows percent increase or decrease in live and dead beetles of resistant strain following cyfluthrin treatment than those of corresponding controls.

In live resistant strain adult beetles, cyfluthrin decreased only one of the cytoplasmic proteases and all of the lysosomal proteases: leucyl aminopeptidase by 23%, dipeptidyl aminopeptidase I by 28%, dipeptidyl aminopeptidase II by 33%, cathepsin B by 30%, cathepsin L by 58%, cathepsin H by 19% and cathepsin D by 20%. All the remaining cytoplasmic proteases *viz.* alanyl aminopeptidase, arginyl aminopeptidase, dipeptidyl aminopeptidase IV, tripeptidyl aminopeptidase and proline endopeptidase exhibited minor changes.

In dead resistant adult beetles, cyfluthrin further decreased the activities of proteases: leucyl aminopeptidase decreased 33%, dipeptidyl aminopeptidase I 41%, dipeptidyl aminopeptidase II 47%, cathepsin B 42%, cathepsin L 96%, cathepsin H 30% and cathepsin D 30%. Other proteases (alanyl aminopeptidase, arginyl aminopeptidase, dipeptidyl aminopeptidase IV, tri-peptidyl aminopeptidase and proline endopeptidase), however, remained unchanged in dead beetles after cyfluthrin treatment.

DISCUSSION

With regard to biochemical mechanism of resistance in insects to insecticides, previous researchers have mainly focussed on induction of detoxication enzymes such as mixed function oxidases, carboxylesterases and glutathion-S-transferases (Terriere, 1984; Ishaaya, 1993). It is well established that these enzymes are significantly elevated in the resistant strains of insect than in susceptible strains (Terriere, 1984; Wilkins *et al.*, 1995). One area of investigation, which received very little attention is the possible involvement of the process of intracellular protein catabolism in the development of insecticide resistance and effects of commonly used insecticides including synthetic pyrethroids on the activities of a comprehensive range of cytoplasmic and lysosomal proteolytic enzymes in resistant strains compared with susceptible strains of various pest insects in general and *T. castaneum* in particular.

Keeping these objectives in view, from our laboratories we have already reported that there was different overall and relative levels of range of protease activities in head, thorax, abdomen and gut in larvae of *Spodoptera littoralis* and *Pieris*

brassicae and adults of *Periplaneta americana*. However, broadly similar patterns of protease activities were noted in corresponding body compartments of the two larval forms which differed markedly from the adult form (Saleem *et al.*, 1995). It was also reported that the range of cytoplasmic and lysosomal proteases were considerably higher in the resistant strain than in susceptible strain of *T. castaneum* (Saleem *et al.*, 2000), and *Musca domestica* (Saleem *et al.*, 1994b; Ahmad *et al.*, 1998). The trend was maintained even after treatment with deltamethrin, lambda-cyhalothrin, pirimiphos-methyl, malathion, gamma-hexachlorocyclohexane and fenitrothion and some insecticide synergists on various proteases in resistant and susceptible strains of *T. castaneum* and *M. domestica* (Saleem *et al.*, 1994a, b; Wilkins *et al.*, 1999; Saleem *et al.*, 2000; Ahmed *et al.*, 2001).

From the results of the present study it was concluded that cyfluthrin at LC₅₀ depleted alanyl and leucyl aminopeptidase, proline endopeptidase and dipeptidyl aminopeptidase I and increased cathepsin H in live susceptible strain of *T. castaneum* beetles, further decreased these proteases alongwith arginyl aminopeptidase and tripeptidyl aminopeptidase in dead treated beetles of susceptible strain, decreased all lysosomal proteases and leucyl aminopeptidase of cytoplasmic proteases in live treated resistant strain and further decreased the same proteases in dead treated beetles.

These results indicate the potential role of proteolytic enzymes in the development of resistance in insects to insecticides. However, these results are in conformation with those already reported from our laboratories (Saleem *et al.*, 1994a,b; 2000). The most obvious mechanism of development of resistance involves the inhibition of the most of key cytoplasmic and lysosomal proteases especially in resistant strain of *T. castaneum* followed by their further depletion in dead treated beetles. This reveals possible disruption of protein degradation cycle resulting in cell death.

Above-mentioned findings of this study lead to accepting the hypothesis of involvement of cytoplasmic and lysosomal proteolytic enzyme activities in insecticide resistance mechanism. It also leads to the conclusion that a wide range of cytoplasmic and lysosomal intracellular proteases

are considerably affected by the synthetic pyrethroid, cyfluthrin after 48 hours of treatment. These results are in agreement with our previous findings already reported from our laboratories (Saleem *et al.*, 1994a, b; 2000; Ahmad *et al.*, 2001).

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