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Comparative Effects of Cyfluthrin on Intracellular Protease Activities in Insecticide-Resistant and Susceptible Strains of *Musca domestics* L.

Mushtaq A. Saleem, Richard M. Wilkins and A. R. Shakoori

Department of Agriculture Entomology, University College of Agriculture, B. Z. University, Multan, Pakistan (MAS), Department of Agriculture and Environmental Science, University of Newcastle upon Tyne, NEI 7RU, England (RMW) and Biological Sciences, Punjab University, New Campus, Lahore, Pakistan (ARS)

Abstract.- To elucidate whether insecticide toxicity in insects involves insecticide-induced abnormalities of the intracellular protein catabolic process, we have determined *in vivo* effect of one of the commonly used 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 M*usca domestica.* Effect of cyfluthrin was determined at LC50 dose level after 48 hour of treatment in both live and dead adult flies, compared with controls. In susceptible strain, cyfluthrin increased all cytoplasmic proteases in surviving flies (ranging from 16 to 148%) and their elevation was further intensified in dead flies (ranging from 24 to 177%). Of lysosomal proteases only cathepsin H (+18%) and D (-26%) were affected in surviving flies while in dead flies cathepsin B and L were additionally affected. Conversally in resistant flies, cyfluthrin produced mixed responses of increase or decrease in cytoplasmic and lysosomal proteases of surviving flies and this affect was further increased or it adopted the route of readjustment in dead flies. We conclude that cyfluthrin affected almost all proteases and such changes in proteases could play an important role in the development of resistance in *M. domestica* to insecticides.

Key words.- Cufluthrin, *Musca domestica,* insecticide-resistance, cytoplasmic proteases, lysosomal proteases, aminopeptidases, endopeptidases, cathepsins

INTRODUCTION

According to the reports of Central Plant Protection Department, Pakistan has been importing a large quantities of synthetic organic insecticides to control insect pests of important crops such as cotton, sugarcane, rice etc. It is estimated that pesticides more than value of Rs. 12.0 billion are imported and used on such crops every year (Saleem and Ashfaq, 2004). This figure is increasing steadily with the rapid growth of population. Of the total import, about 80% is consumed on cotton crop. The extensive and indiscriminate use of insecticides has resulted development of resistance in various insects to insecticides. For instance a high level of resistance has been developed in *Helicoverpa armigera* and *Bemisia tabaci* against cypermethrin and methamidophos, respectively (Ahmad *et al.*, 1995). Development of insect resistance to insecticides has emerged as important problem in this country and the world over. Likewise *Musca domestica* has also developed resistance to commonly used insecticides such as DDT and malathion etc. Thus it has become imperative to study biochemical resistance mechanisms in various insect pests to the related insecticides (Saleem *et al*., 1994a,b).

It is now well established that insecticide detoxification enzymes such as mixed function oxidases, carboxylesterases and glutathione-S-transferases are mainly responsible for development of resistance in insects to various insecticides. According to review of the literature, these detoxification enzymes are inducible and such increased enzymatic activities may be due to alteration in gene regulation *e.g.* increased transcription and translation or post-translational modification of protein (Bresnick, 1980; Terriere, 1984; Brown and Brogdon, 1987). Hence extensive research studies have been focused on induction of detoxification enzymes in various insects followed by insecticidal treatments (Terriere, 1984). On the contrary very little attention has been paid regarding possible role of proteolytic enzyme activities in mechanism of resistance development in insects to insecticides, although much work has been

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conducted on proteases in higher animals (Pennington, 1977; Faiz *et al*., 1994; Mantle *et al*., 1997; Saleem *et al*., 1998). Importance of proteolytic enzymes in normal growth and development of different insects particularly during digestion and moulting has also particularly been established (Law *et al*., 1977; Murdock *et al*., 1987; Christetter *et al*., 1992; Purcell *et al*., 1992).

Most of the previous studies have been based on the use of non-specific protease assay procedures and focused primarily on proteases associated with the gastrointestinal tract such as the identification of cysteine type and serine type proteases as the major digestive enzymes in the gut of many coleopteran insects (Murdock *et al.,* 1987) and lepidopteran pests (Johnson *et al*., 1990; Christetter *et al*., 1992). Proteolytic enzymes have also been reported to be important in the general process of intracellular protein catabolism essential for normal functioning of cells in all tissues (Pennington; 1977, Turner, 1986; Anonymous, 1990), and may have additional specialized functions.

Of all enzymes, proteases are the most widely distributed in all living cells. Protein metabolism constitutes a major physiological resource that can act as a compensatory mechanism under pesticidal stress. The degradation products of intracellular protein can be re-utilized in protecting the cell from stress. From our laboratories we have already reported high intracellular protease activities in insecticide-resistant strains of adults of *Musca domestica* (Saleem *et al*., 1994a, b; Ahmed *et al*., 1998; Wilkins *et al*., 1999), suggesting a potential role of these enzymes in the development of insectcicide resistance. In order to further elaborate this hypothesis, the objective of the present investigation was to (i) compare changes in the levels of a range of cytoplasmic and lysosomal proteases (representative of the two major pathways of intracellular protein degradation) in insecticide-resistant strain of *M. domestica* than susceptible strain and (ii) effect of a synthetic pyrethroid, cyfluthrin at LC_{50} dose level on proteases of surviving and dead flies of both strains. This study is therefore expected to provide insight regarding role of proteases in resistant strains of insects to insecticides.

MATERIALS AND METHODS

Rearing of flies

Cooper, a susceptible strain of housefly, *M. domestica* (Chapman and Morgan, 1992) was used in this study while a resistant strain, which was highly resistant to malathion was obtained from Roussel Uclaf Environmental Health Limited, Berkhamsted, U.K. The stock cultures were maintained at 25ºC in a mesh cage (50x30x30 cm). The adults were provided with granulated sugar as well as cotton wool soaked in milk-sugar solution as food, in Petri dishes. Adult female flies laid eggs in a food cup (250 ml) having larval food (yeast, milk powder and bran, 1:2:10, meshed in 500 ml (distilled water). After 24 hours, food cups containing eggs were removed from the ovipositor cage and placed in another cage at the same temperature for hatching of larvae and emergence of flies. Five day old adult flies were used in the experiments.

Insecticide and other chemicals

Technical grade of a synthetic pyrethroid, cyfluthrin [Baythroid; RS-α-cyano-4-fluoro-3-phenoxybenzyl (1 *RS,* 3 *RS;* 1 *RS,* 3 *SR)*-3-(2, 2-dichlorovinyl)-2- dimethylcyclopropanecarboxylate] (Tomlin, 2000), was obtained from Bayer AG. All other reagents including protease assay substrates were obtained from 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 130² cm, 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 ten healthy five day adult flies of similar size and development stage were used for topical bioassay with the help of Arnold micro applicator and then released in the Petri dish. 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 and Ruczkowski (1980). Results were subjected to probit analysis. The LD_{50} values for susceptible and resistant strains of *M. domestica* adult flies were calculated as outlined by Busvine (1971) and described by Finney (1971).

Resistance ratio was calculated by dividing the LD_{50} for the resistant strain with LD_{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 beta-cyfluthrin on proteases, sufficient numbers of adult flies were exposed to LD_{50} values of susceptible and resistant strains, respectively. Thereafter analyses of proteases were determined from both live and dead flies of both resistant and susceptible strains and compared with their respective controls.

Isolation of proteases from beetles

Approximately 6 adult flies were weighed and homogenized in extraction buffer using an Ultra-Turrax homogenizer (2x10 sec at 15000 rpm). Whole insect homogenate (1:60, w/v) was prepared in 50 mM Tris-acetate buffer, pH 7.5 containing I mM dithiothreitol (DTT), 0.15 M NaC1 and 3 mM $NaNO_3$ for estimation of cytoplasmic protease activities. For lysosomal proteases, the same extraction buffer as that 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 4ºC 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 ($lambda_{ex}$ 380 nm, $lambda_{em}$ 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 $CaC1_2$,1 mM DTT, 0.25 mM Ala-AMC.

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

Leucyl aminopeptidase: 50 mM glycine-NaOH buffer pH 9.5, 5 mM MgCl,, 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 of 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 x g 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 bovine serum albumin (BSA) as standard.

RESULTS

In vivo *effect of cyfluthrin on cytoplasmic proteases of susceptible strain*

Table I shows *in vivo* effects of cyfluthrin at LD_{50} dose level after 48 hours treatment on cytoplasmic and lysosomal proteolytic enzyme activities in live as well as dead adults of susceptible strain of *M domestica,* while percent increase or decrease in surviving and dead adult flies of susceptible strain following cyfluthrin treatment than their, controls is shown in Table II.

In surviving susceptible strain adult flies, cyfluthrin increased all cytoplasmic proteolytic enzyme activities when compared with their corresponding controls. Thus alanyl aminopeptidase was increased by 53%, arginyl aminopeptidase by 73%, leucyl aminopeptidase by 16%, dipeptidyl aminopeptidase IV by 17%, tripeptidyl aminopeptidase by 148% and proline endopeptidase by 111%. Likewise cyfluthrin increased soluble protein content by 8% in treated surviving flies than controls.

In dead susceptible strain adult flies, cyfluthrin also raised all cytoplasmic proteases like those of surviving susceptible strain adult flies. Intensity of elevation was much higher in case of dipeptidyl aminopeptidase IV (by 308%), leucyl aminopeptidase (by 48%), tripeptidyl aminopeptidase (by 19%), and soluble protein (by 100%).

In vivo *effect of cyfluthrin on lysosomal proteases of susceptible strain*

Of lysosomal proteases, cyfluthrin affected only two cathepsins viz cathepsin H and cathepsin D in surviving adult flies, while the remaining lysosomal proteases including soluble protein showed minor fluctuations when compared with their corresponding controls. Thus cathepsin H was increased by 18% while cathepsin D was decreased by 26% (Table I and II).

On the other hand, almost all lysosomal proteases were found considerably affected in dead flies following cyfluthrin treatment. Thus elevated activities were found in cathepsin L (by 128%), cathepsin H (by 54%), cathepsin D (by 771%) and soluble protein (by 16%) whereas depleted activities were detected in dipeptidyl aminopeptidase I (by 17%), dipeptidyl aminopeptidase II (by 10%) and catheprill B (by 43%) (Table I and II).

In vivo *effect of cyfluthrin on cytoplasmic proteases of resistant strain*

The effect of cyfluthrin at LD_{50} dose level after 48 hour treatment on cytoplasmic and lysosomal proteolytic enzyme activities in surviving, and dead resistant strain adults of *M. domeslica* is shown in Table I whereas Table II shows percent increase or decrease in proteases of surviving and dead resistant strain adult flies following cyfluthrin treatment when compared with their corresponding controls.

In treated living resistant strains adult flies, cyfluthrin resulted elevated activities of dipeptidyl aminopeptidase IV by 11%, tripeptidyl aminopepetidase by 66%, proline endopeptidase by 183% and soluble protein by 20% while arginyl aminopeptidase and leucyl aminopepetidase activities were depleted by 34% and 71%, respectively. Aanyl aminopeptidase activity was left undisturbed.

Cyfluthrin produced almost similar response of either increase or decrease in cytoplasmic proteases in dead flies of resistant strain as it was found in surviving flies. Thus tripeptidyl aminopeptidase, proline endopeptidase and soluble proteins were increased by 58%, 61% and 24%, respectively while activities of arginyl aminopeptidase and leucyl aminopeptidase were decreased by 37% and 72%, respectively. Alanyl aminopeptidase and dipeptidyl aminopeptidase IV activities were not disturbed.

Table I.- ***In vivo*** **effects of cyfluthrin on proteases in insecticide-resistant and susceptible strains of** ***Musca domestica*** **adults.**

Protease Types	Susceptible strain			Resistant strain		
	Control	Live adults	Dead adults	Control	Live adults	Dead adults
Cytoplasmic proteases						
Alanyl aminopeptidase	707.78	1082.09	1061.67	1150.99	1167.84	1086,36
Arginl aminopeptidase	508.72	877.92	767.82	2133.55	1413.09	1335.32
Leucyl aminopeptidase	39.63	45.94	48.98	60.83	17.52	17.92
Dipeptidyl aminopeptidase IV	25.80	30.06	43.18	37.43	41.52	38 98
Tripeptidyl aminopeptidase	117.96	292.64	325.45	205.87	342.57	321. 40
Proline endopeptidase	38.71	81.66	75.83	70.18	198.53	113. 16
Soluble protein (nighnl)	1.32	1.43	1.54	1,04	1.25	1.29
Lysosomal proteases						
Dipeptidyl aminopeptidase I	101.37	98.84	84.22	218.25	159.36	191 94
Dipeptidyl aminopeptidase 11	68.94	76.03	61.99	137.75	108.27	125.71
Cathepsin B	665.02	608.25	378.99	1373.93	1561.99	l3416 26
Cathepsin L	405.50	440.98	926.41	729.90	248.14	113.54
Cathepsin H	313.59	370.02	481.92	415.04	233.57	443.35
Cathepsin D	85.00	63.02	740.38	200.74	122.00	283.33
Soluble protein	0.90	0.96	1.04	0.68	0.90	1.00

Table II.- **Percent increase** (+) or **decrease** (-) **in insecticide-resistant and susceptible strains** ***of*** *Musca domestica* **following cyfluthrin treatment.**

Protease types	Susceptible strain		Resistant strain	
	Live flies	Dead flies	Live flies	Dead flies
Cytoplasmic proteases				
Alanyl aminopeptidase	+52.89	+50.00	+1.46	-5.62
Arginyl aminopeptidase	+72.57	+50.93	-33.77	-37.41
Leucyl aminopeptidase	+15.92	+23.59	-71.20	-71.72
Dipeptidyl aminopeptidase IV	+16.51	+67.36	+10.93	+4.14
Tripeptidyl aminopeptidase	+148.08	+175.87	+66.40	+57.58
Proline endopeptidase	+110.95	+95.89	+182.89	+61.24
Soluble protein	+8.33	+16.67	+20.19	+24.04
Lysosomal proteases				
Dipeptidyl aminopeptidase I	-2.50	-16.92	-26.98	-12.05
Dipeptidyl aminopeptidase II	+10.28	-10.08	-21.40	-8.74
Cathepsin B	-8.54	-43.01	+13.69	-2.01
Cathepsin L	+8.75	+128.46	-66.00	-84.44
Cathesin H	+17.99	+53.68	-43.72	+6.82
Cathepsin D	-25.86	+771.03	-39.22	+41.14
Soluble protein	+6.67	+15.56	+32.35	+47.05

In vivo effect of cyfluthrin on lysosomal proteases of resistant strain

In treated surviving resistant strain adult flies, cyfluthrin affected all lysosomal proteolytic enzyme activities than their control groups. Hence capthesin B and soluble proteins manifested elevated activities by 14% and 32%, respectively while dipeptidyl aminopeptidase I, dipeptidyl aminopeptidase II, cathepsin L, cathepsin H and cathepsin D were depleted by 27%, 21%, 66%, 44% and 39%, respectively.

On the other hand in treated dead resistant strain adult flies, some of the lysosomal proteases manifested adjustments where as others exhibited either increase or decrease following cyfluthrin treatment. Thus cathepsin L, cathepsin D and

soluble protein revealed considerable disturbance (by -84%, +41% and +47%, respectively) while all the remaining lysosomal proteases analysed in the this study such as dipeptidyl aminopeptidase I, dipeptidyl aminopeptidase II, cathepsin B and cathepsin H demonstrated normalization than those of surviving treated adults of *M. domestica.*

DISCUSSION

Of all enzymes, proteases are the most widely distributed in all living cells in insects as well as higher animals. Insect like other living organisms, require proteases for degrading proteins and peptides. Intracellular proteases are either exopeptidases, which act at the amino- or carboxyl-terminal or endopeptidases, which are capable of clearing peptide bonds in the central region of polypeptides. Exopeptideases acting on amino- or carboxy-termini of the amino acids are known as aminopeptidases or carboxy peptidases, respectively. The exopeptidases act on short chain peptides that are produced by the action of endopeptidases, thus acting as polymer terminal enzymes as part of a cascade effect in protien catabolism. The multitude of processes in which cellular proteases participate has made it difficult to identify the functions of these proteases adequately. Nevertheless, the cellular proteases function to (i) create biologically active molecules or (ii) destroy biological active proteins and peptides (Bond and Butler, 1987; Rivett, 1990). In insects, proteases are involved in specialized functions related to their particular physiology as well as in digestion of dietary proteins, in embryogenesis and reproduction, and is growth and development (Law *et al*., 1977). Besides proteases are involved in digestion, reproduction and development, intercellular proteases (cytoplasmic and lysosomal) responsible for general protein turnover also form an important feature of an insect life. However, the study of intracellular proteases in insect physiology is very fragmentary, but first discovery of this kind was reported by Saleem *et al*. (1995) and subsequently confirmation by other workers from our laboratories (Ahmed *et al*., 1998, 2001; Wilkins *et al*., 1998) have highlighted the relationship of proteases with insecticide resistance in insects.

Likewise knowledge of the effects of insecticides on intracellular proteases is limited. Saleem *et al*. (1994a,b, 2000) first of all reported the effect of certain insecticides such as lambda-cyhalothrin, malathion, deltamethrin, pirimifos-methyl and gamma-hexachlorocyclohexane on proteolytic enzyme activities of insecticide-resistant and susceptible strains *of M. domestica* and red flour beetle, *Tribolium castaneum.* In this study, efforts have been made to determine *in vivo* comparative effects of one of the commonly used synthetic pyrethroids, cyfluthrin, on proteolytic enzyme activities of insecticide-resistant and susceptible strains of *M. domestica.* Effects of cyhalothrin was compared between treated surviving and treated dead adults flies as well as than their corresponding control groups.

The results of the present study revealed that cyfluthrin considerably increased all cytoplasmic enzymes in both treated surviving, (ranging from 16% to 148%) and treated dead (ranging from 24% to 176%) adults flies. However intensity of elevation for most of the enzymes was further intensified in dead than surviving flies. Of lysosomal proteases, cathepsin L, cathepsin H and soluble proteins also exhibited further increased activities in daed than living *M. domestica.* On the other hand cathepsin D was depleted in living flies but exhibited drastically raised levels in dead flies while the remaining lysosomal proteases were left undisturbed except cathepsin B which was decreased further in dead than living flies.

In contrast to susceptible strain, cyfluthrin produced mixed response by either increasing or decreasing the proteases in resistant strain, although most of them exhibited depleted levels. Thus arginyl aminopeptidase, leucyl aminopeptidase, dipeptidyl aminopeptidase I, dipeptidyl aminopeptidase II, cathepsin L, cathepsin H and cathepsin D manifested depleted activities while tripeptidyl aminopeptidase and proline endopeptidase activities were raised in surviving treated flies. In dead flies these proteases manifested mixed response by showing further decreasing trend (such as arginyl and leucyl aminopeptidases and cathepsin L), increasing trend (such as soluble proteins) or adopted the rout of adjustment before death (such as tripeptidyl aminopeptidase, proline endopeptidase,

dipeptidyl aminopeptidase I and 11, cathepsin B, H and D).

In a similar study Ahmed *et al*. (1998) reported that following *in vivo* topical treatment with DDT of a DDT-resistant strain M. *domestica,* alanyl aminopeptidase and arginyl aminopeptidase activities increased significantly and rapidly at 1,2,3 and 24 hour after treatment compared with control activities. After 24 hour, the activities of most proteases returned to that of control. The dead flies 24 hour after treatment had lower activities than the live ones in all cases. They further reported that in insects exposed to fenitrothion, the pattern of activity over the subsequent 24 hour period varied depending on the individual proteases. However, corresponding enzymes shared broadly similar pattern of activity changes in the both the resistant and susceptible insect strains.

Both resistant and susceptible strains of *M. domestica* exhibited increased protease levels upon lethal insecticide treatment. There was a trend in the magnitude of the increase, that was larger in the susceptible strain compared to the resistant, which had a high level. Moreover, cytoplasmic proteases increased more than lysosomal proteases in insect treated with cyfluthrin. This suggested possible more role for cytoplasmic proteases in protein turnover, which provide the precursor amino acids for protein synthesis. Following cyfluthrin lethal treatment, lysosomal proteases returned to baseline (on lower) values after a period of elevated or decreased activities in surviving flies.

Above mentioned findings of this study lead to accepting the hypothesis of involvement of involvement of cytoplasmic and lysosomal proteases in metabolic insecticide mechanisms. It also leads to the conclusion that a vide range of cytoplasmic and lysosmal intercellular proteases are considerably affected either directly or indirectly followed by cyfluthrin treatment. We, therefore, suggest that it must be the combination of intrinsically higher protease levels (prior to pesticide exposure), together with the capacity to further increase protease activities following insecticide exposure, which is important in the mechanism by which proteases may confer survival advantages in insecticide resistant insects. We, therefore, speculate that this mechanism may involve increased supply to precursor amino acids from proteolytic degradation products to the intercellular pool, prior to *de novo* synthesis of detoxifying enzymes following insecticide exposure.

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