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HISTOCHEMICAL LOCALIZATION OF PROTEINS AND ALKALINE PHOSPHATASE IN DIFLUBENZURBON TREATED INSECTS

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The proteins and alkaline phosphates were localized histochemically through electrophoresis, in diflubenzuron (DFB) treated *Musca domestica* (L.) (PCSIR strain) larvae and *Blattella germanica* (L.) (Karachi University strain) nymphs, respectively. In *M. domestica* DFB at 0.03125% inhibited proteins to some extent while at a higher dose (0.5%) the inhibition was negligible. The alkaline phosphatase was inhibited by DFB with little variation in the two doses. In *B. germanica* at 0.03125% dose DFB was more active than at 0.0625%, and inhibited a number of protein bands and showed a regular inhibition of enzymes with the increase in dose.

Key words: Protein, Alkaline phosphate, Diflubenzurbon.

INTRODUCTION

Houseflies and german cockroaches are well known for their filthy habits and serve as carries for various pathogens. For their control conventional pesticides like DDT have been used generously, but in view of environmental pollution, entomologists started thinking to adopt new methods of pest-control. In this connection Knipling [1] suggested the control of insects by sterilization technique. This gave birth to the era of "Neopesticides", which include chemosterilants. Insect growth regulators (IGR's) chitin inhibitors and hormonal pesticides (JHA's). Some of these compounds are more or less insect-specific, readily decompose and do not contribute much to environmental pollution.

Spraying of DDT and now malation (570% EC.) for the control of mosquitoes by Malaria Eradication Board in Karachi (Pakistan) is resulting in the development of resistance in mosquitoes and also polluting the atmosphere. Inview of this, IGR, diflubenzaron was tested against household pests and its effect on alkaline phosphatase was also investigated.

Although much work has been done on the role of enzymes in the degradation of pesticides by various workers but not much work has been done on this aspect in the case of neopesticides. Moreover inhibition of esterases by colorimetry and electrophoresis due to the use of pesticides has been shown by some workers [2-13]. To provide positive or negative evidence to the colorimetric findings reported by Shafi *et. al.* [14], present work was undertaken. This will clarify some aspects of IGR degradation with reference to alkaline phosphatase in the body of insects and perhaps will encourage the use of neopesticides in insect control.

MATERIALS AND METHODS

Rearing of *M. domestica* (L.) (PCSIR susceptible strain) and *B. germanica*(L.) (Karachi University strain) was done according to Ashrafi *et. al.* [15] with some modifications in the laboratory of Zoology, Department of Karachi University.

The 1% stock, solution of dimilin or DFB was prepared by dissolving 2.0 g of DFB 25% W.P. in 50 ml acetone. For the treatment of larvae and nymphs, 0.5% concentration of the compound was made by diluting 5 ml of 1% stock solution in 5 ml of acetone. Further dilutions (0.25%, 0.125%, 0.0625%, 0.03125%, 0.015625%, 0.0078125%, 0.00390625% and 0.001953125%) were made according to formula $C_1 \times V_1 = C_2 \times V_2$.

Method of treatment. Injection method was adopted by using manual microapplicator. The volume was kept constant at 8 µl and the dose variation was obtained by using different concentrations. In *M. domestica* the check batches were dosed first with acetone alone. For each concentration, control and check a batch of 10 larvae (3rd instar) were taken. Mortality counts were observed after 24 hours. Similar treatment was applied to *B. germanica* nymphs (5th instar nymphs).

Preparation of homogenate. The larvae and nymphs were crushed in 2 ml bi-distilled water with pestle and mortar. They were then homogenized in Teflon Pyrex tissue grinder for 5 minutes at 1000 rpm. Further they were centrifuged in "Labofuge 15000" at 3500 rpm for 15 minutes. Supernatants were taken in separate tubes to be used for histochemical localization of proteins and enzymes. During experiments the homogenate and reaction mixtures were kept in ice at 2° approximately.

Histochemical localization. Histochemical localization of protein and enzyme bands was done in polyacrylamide gels according to Maurer [16], with slight modifications.

(i) Electrophoresis for protein bands. It was done in electrophoresis apparatus prepared by PCSIR Laboratories, using tubes of 85x5 mm size. The gels were prepared by small and large pore solutions. Following reagent solutions were prepared for this purpose.

Solution A. It was made by mixing 48 ml 1N NHCl 36.6 g tris (hydroxymethyl) methylamine and 0.46 ml of N' N' N' N' — tetramethyl -1, 2-diaminoethane (Merck). The volume

was completed to 100 ml by double distilled water and the it's pH was adjusted at 8.9.

Solution B. It was made by mixing 48 ml of 1 N HCl with 5.98 g tris and then completing the volume upto 100 ml by double distilled water.

Solution C. It was prepared by dissolving 10 g of acrylamide and 2.5 g of N'N' -methylenebis acrylamide in double distilled water and completing the volume upto 100 ml.

Solution E. It consisted of 0.004% riboflavin dissolved in double distilled water.

Solution F. (Ammonium persulphate solution (1.0%) It was made by dissolving 0.028 g of ammonium persulphate in 2 ml double distilled water.

All the solutions were filtered by Whatman filter paper No. 1 and kept in refrigerator at 2°.

(ii) Small pore solution. It consisted of 2 parts of solution A, 4 parts of solution C, 1 part of solution F and 2 parts of double distilled water. After mixing the solution was degased.

(iii) Large pore solution. It contained 2 part of solution B, 2 parts of solution D, 1 part of solution E and 4 parts of double distilled water. After mixing the solution was degased.

(iv) Electrode buffer solution. It was made by dissolving 6.0 g of tris and 28.8 g glycine in double distilled water and completing the volume upto one litre. The solution served as stock and it was diluted to 1:10 strength by distilled water keeping the pH constant at 8.5 pH.

(v) Staining solution. For preparing this solution, 0.125g of amido black was dissolved in 227 ml of 50% methanol, followed by the addition of 23 ml glacial acetic acid. It was used for staining the gels.

(vi) Destaining solution. It was made by mixing 50 ml methanol and 75 ml glacial acetic acid, completing the volume upto one litre. The gels were kept in it for removing unbound stain.

(vii) Preservative solution. It consisted of a mixture of 5 ml ethanol and 2.5 ml glacial acetic acid. The volume was completed upto 100 ml with double distilled water. It served for keeping the gels in it for preservation.

PROCEDURE

The tubes of 85x5 mm size were cleared and fitted in a stand with lower ends closed. They were first filled with small pore solution leaving 2 cm on the top. To make the upper surface of the gel uniform a few drops of distilled water were added gently. Later on after polymerisation the water was removed and 0.5 cm large pore solution was added followed by few drops of water. The gel were kept in UV light for polymerissation and then taken out before use. The gels were fitted in the upper buffer tank of the apparatus and the samples were added to them gently. Each sample contained 200 µl of the protein sample, 60 µl of 20% sucrose solution and 20 µl of 0.5% bromophenol blue (prepared in 2% glycerine solution with distilled water). The sample was gently poured on the top of the gels. The tubes were then filled by distilled water to avoid disturbance of the sample layer during the addition of buffer solution. The buffer was poured in the upper and lower chamber gently, so that the two ends of the gel tubes remained in the solution to facilitate the current flow through the gels. Air bubbles were removed. After connecting the two electrodes the apparatus was switched on. The output control knob was adjusted for passing 2 milliamperes current per gel for 5 minutes, which was doubled after that. The running time was from 5 to 7 hours. As soon as the bromophenol blue layer migrated nearly to the lower ends (5-10 mm above) the current was stopped, tubes were removed and the gels were taken out. The flow distance was measured.

To stain the protein bands the tubes were placed in amido black for 20 minutes. After that the gels were taken out, washed with distilled water and kept in destaining solution for removing the unbound stain. The measurements were taken for calculating mobility rate of each band, before and after staining and the position of bromophenol blue was also noted. These values were converted on 100 mm scale for comparison.

2. Electrophoresis for enzyme localization. Same procedure of the preparation of gels was followed as with the protein bands. But instead of staining them will amido black the gels were incubated with substrate solutions for histochemical localization of proteins as described by Naqvi [17]. Electrophoresis for protein and enzyme bands was done simultaneously under similar conditions.

Histochemical localization of alkaline phosphatase. Following reagents were used for its estimation.

(a) Incubation solution was prepared by equal quantities of 0.5 M tris buffer, 0.06 M HCl and 0.5 M $MgCl_2$, readjusting the pH at 8.0.

(b) Naphthol As-Mx phosphate solution. Fresh 0.5% solution was made by the addition of 4 ml naphthol As-Mx phosphate (Sigma Chemical Co., pH 8.6) to 96 ml bidistilled water.

(c) *Fast blue RR salt solution.* Its fresh 0.1% solution was prepared by dissolving 25 mg fast blue RR salt (diazotate-4-benzylamine -2-5-dimethyl aniline. Sigma Chemical Co.) into 25 ml of bidistilled water. This solution was used as diazonium salt in the histochemical reaction.

(d) *Incubation solution of the gels.* Each gel was immersed in a solution containing equal volume of the incubation solution (Napthol As-Mx phosphate solution and fast blue RR salt solution). After that the gels were incubated at 37° in water bath (Karl Kolbs D. 6072) for about 2 and 4 hours for *M. domestica* and *B. germanica*, respectively. Blue-violet bands were developed at the position of alkaline phosphatase. These bands were compared with the protein band. During all the experiments treated, untreated (control) and check (acetone) samples were run simultaneously and were compared to see the effect of the compounds on the enzyme activity.

RESULTS

Polyacrylamide gel-electrophoresis was performed and localization of protein and enzyme bands was done simultaneously. Homogenate from the treated and untreated larvae and nymphs were run through the gels for protein separation, four gels were run for the test compound, two for check (acetone) and two for control, during each experiment, separately. Half of the gels were stained with amido black for developing protein bands and the rest were incubated for alkaline phosphatase activity as described. Enzyme active bands in normal were compared with treated ones.

Proteins bands. In *M. domestica* larva both control and check (acetone) gels and similar intensities of protein bands (bands D.G high; E moderate; F, H low; B, C poor and A, I absent). But DFB at 0.03125% inhibited proteins to some extent (bands G moderate and E, low), while at 0.5% the inhibition was negligible (Fig. 1).

In case of *B. germanica*, as usual the gel containing control and check had similar intensities of protein bands but DFB at 0.03125% inhibited more bands (H, moderate; C, low; A,B, G poor activity and I, traces of protein) (Fig. 2) while at 0.0625% dose DFB inhibited less (band A, low; B, I, poor) (Fig. 2).

Zymograms of *M. domestica* showed that (Fig. 3) in control and check alkaline phosphatase bands were similar while the gel treated by 0.03125% dose of DFB has band G with moderate and D with poor activity. While at 0.5% the gel revealed band G with moderate and D with low activity.

In *B. germanica* the zymograms displayed band H low and A,G with poor activity at 0.03125% dose of DFB. At 0.0625% dose gel showed band A,H with low activity and G was absent (Fig. 4).

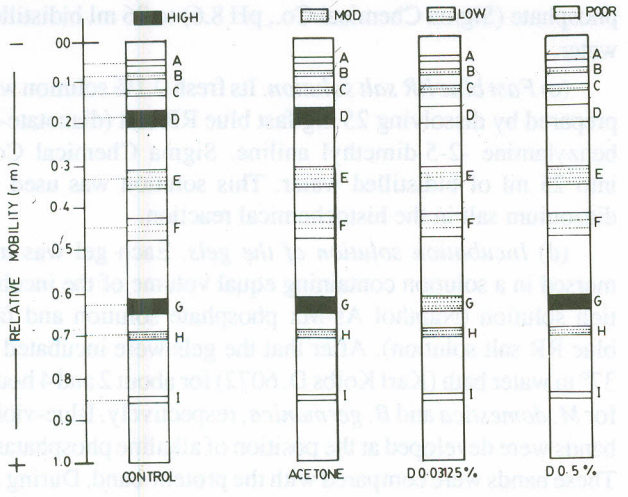

Fig. 1. Protein bands (A-I) in normal and dimilin treated *Musca domestica* larvae.

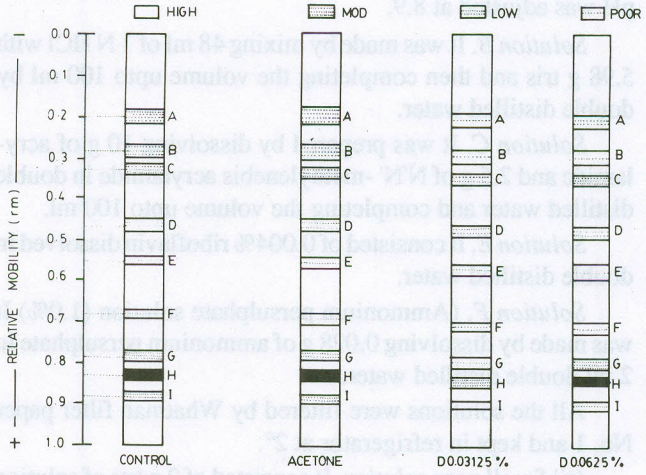

Fig. 2. Protein bands (A-I) in normal and dimilin treated *B. germanica* 5th instar nymphs.

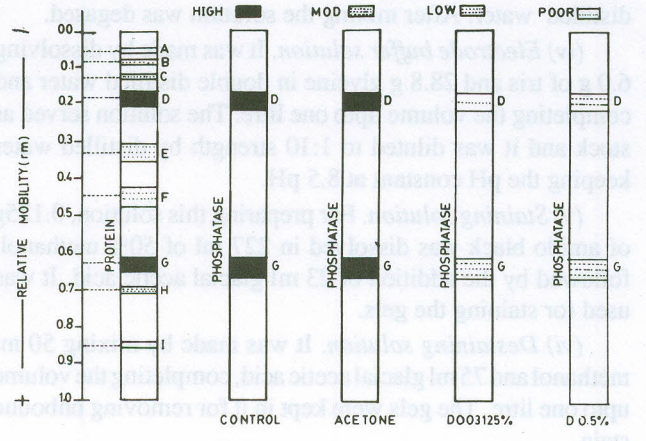

Fig. 3. Histogram showing effect of dimilin on alkaline phosphatase activity in *Musca domestica* L. (PCSIR Strain).

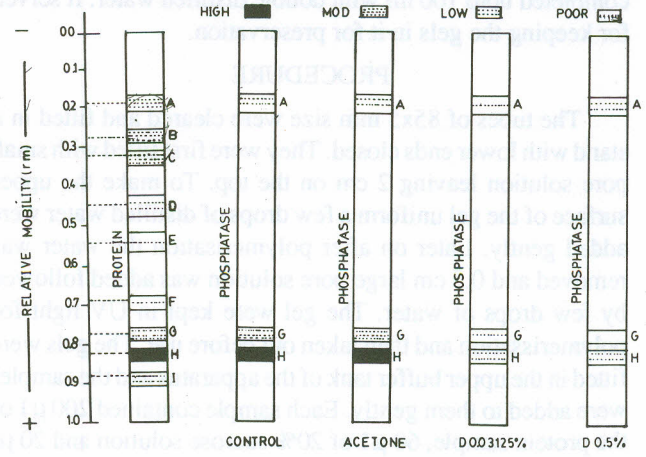

Fig. 4. Histogram showing effect of dimilin on alkaline phosphatase activity in *B. germanica* L. (KU strain).

DISCUSSION

Effect of pesticides on protein and enzyme activity have been studied by few workers. On this aspect, Ashrafi *et. al.*[18] studied the comparative effect of insecticides on the activity of high energy producing enzymes. According to them the inhibition by acetone on the phosphatase activity was significant and so were the acetone solutions of malathion parathion and petkolin. But in the present case the inhibition of the same enzyme by acetone was negligible. The above inhibition might be due to insect susceptibility or the type of insecticide used. Naqvi *et. al.* [19] reported a hormone-enzyme relationship in *Schistocera gregaria* nymphs. Such a relation might be present between enzymes and IGR because in the present study IGR (DFB) affected the metamorphosis and the enzyme-protein levels. Naqvi *et.al.*[12] determined cholinesterase, protease, acid and alkaline phsophatase in the normal and abnormal pupae of *M. domestica*. It was found that thiourea inhibited alkaline phosphatase more then tepa. Present work also support the inhibition of alkaline phosphatase by chemicals like DFB thus confirming the above report. Similarly Gorgees *et.al.* [20] by using biochemical and histochemical methods found that chemosterilants (Tepa, shikonin) decreased the activity of alkaline phosphatase in *Aedes aegypti* larvae, but ecdysone (hormone) had no effect. Similar inhibition of alkaline phosphatase was observed in *M. domestica* larvae by DFB which support the earlier findings. Carlisle and Loughton [21] reported that injection of corpora cardiaca homogenates into *Locusta* sp. caused no consistent difference in the incorporation of H-leucine into hemolymph protein, but only caused inhibition in protein synthesis. Electrophoresis of the homogenate suggested that the inhibition was due to adipokinetic hormone, thus indicating that inhibition in protein synthesis might take place by a hormone. Similar inhibition of proteins was observed during present work with a difference of the substance used.

Inhibition of proteins (E, G and H, I) and alkaline phosphatase (25-65%) as evident from gel electrophoresis indicated some hindrance in protein synthesis. Alkaline phosphatase has been correlated with growth by various workers, so if IGR, DFB retards the growth, the enzyme inhibition is logical. The present investigation indicates that there may be an enzyme-neopesticide relationship as revealed by inhibition of protein and enzyme bands during gel electrophoresis, confirming the colorimetric estimations of Shafi *et. al.* [14] in the same insect.

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