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Storage protein in the haemolymph of 6th instar larvae of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae) is increased by the ecdysone mimic, methoxyfenozide

Linvy Vincent, Sridhu Prakash, Reshma RM, Resmitha C, * Kannan Vadakkadath Meethal
Department of Zoology, University of Calicut, Kerala, India

Abstract
Spodoptera mauritia is a sporadic pest of paddy which causes, great economic loss. Insect Growth Regulators (IGRs) belong to a group of compounds which interfere with normal growth, development and reproduction in insects by disrupting hormonally regulated physiological processes. As IGRs are more target-specific, non-persistent, biodegradable and environmentally benign substances with less toxicity to non-target organisms, they are ideal for pest control programs. In this study we demonstrate the effect of methoxyfenozide, an ecdysone mimic, on the haemolymph protein profile of 6th instar larvae of *Spodoptera mauritia*. When the sixth instar day 0 larvae of *Spodoptera mauritia* was treated with different concentrations of methoxyfenozide, there was a statistically significant increase in the haemolymph protein concentration after 24 hours. The haemolymph collected after 24 hours when subjected to SDS-PAGE, there was an increase in the intensity of the major protein band of 83kDa compared to control. Storage proteins are the major proteins in the haemolymph of last instar lepidopteran larvae. Whether the increase in storage protein concentration by methoxyfenozide is related to insecticide detoxification and resistance is worth investigating and studies in this direction are ongoing.

Keywords: insect growth regulator, ecdysone analogue, methoxyfenozide, protein profile

1. Introduction
Spodoptera mauritia is a sporadic pest of paddy. It is estimated that the loss in yield caused by *Spodoptera mauritia* larval infestation range from 10 to 20%. Pest management is an integral part of any successful agriculture. Conventional pesticides pose great threat to human health and environment. Development of more eco-friendly pest management approaches is of great importance for food security and human health. Hormones play crucial role in insect growth and development. Insect Growth Regulators (IGRs) belong to a group of compounds which interfere with normal growth, development and reproduction in insects by disrupting hormonally regulated physiological processes. Several such compounds are known and their effects on metamorphosis and reproduction in a number of insect species have been worked out [1]. Use of IGRs for pest management is an attractive alternative as they are more target-specific, non-persistent, and environmentally benign substances, with less toxicity to non-target organisms. Many IGRs are Juvenile Hormone (JH) or Ecdysone agonists or antagonists. Ecdysone is a steroidal prohormone of the major insect molting hormone 20-hydroxyecdysone. Ecdysone along with JH regulates the process of moulting and many other metabolic processes. Ecdysone agonists exert their toxicity by binding to the ecdysone receptor as does the natural insect molting hormone, 20-hydroxyecdysone [2, 3].

Diacylhydrazines (DAHs) are potent non-steroidal ecdysone agonists, and four of them, tebufenozide, methoxyfenozide, chromafenozide, and halofenozide, have been developed as insecticides. Although these compounds exert toxic effects on insects, they are relatively safe for mammals and are environmentally benign. Their action on insects is also selective; the first three are effective against Lepidoptera but weakly active or inactive on Diptera and Coleoptera. On the other hand, halofenozide is effective on Coleoptera but mildly active on Lepidoptera [4]. The most common effect of ecdysone agonist treatment is precocious lethal moult [5].

Hemolymph protein levels generally increase during each instar but decline during moulting. Hexamerins are the major haemolymph storage proteins and their subunits have masses in the range of about 80 kDa, with a native molecule of about 500 kDa [6]. Typically two to four physico-chemically distinct storage protein species occur. Storage proteins are amino acids reserve for the production of adult proteins [7, 8]. The last larval instar of holometabolous insects has been characterized by active synthesis of arylphorin (aromatic amino acids bearing storage proteins) and pupal storage proteins [9]. During metamorphosis larval plasma proteins were hydrolyzed to free amino acids and major part being incorporated into new adult proteins. Structural studies showed the presence of several papain cleavage sites on *Bombyx mori* arylphorines [10] indicating the role of such proteases in its degradation. Thus haemolymph proteins are crucial for insect development. Studying the effect of IGRs on the haemolymph protein profile of *Spodoptera mauritia* will be helpful in understanding the modulation of haemolyph protein by IGRs which can be exploited for the management of pests. In this study we investigated the effect of Ecdysone mimic, methoxyfenozide on the larval haemolymph protein profile of *Spodoptera mauritia* Boisd. and identified a methoxyfenozide-responsive protein.

2. Methodology

2.1 Collection and rearing of *Spodoptera mauritia* larvae

The moths were attracted by fluorescent light traps during night. They were kept in glass chimneys closed at both ends with muslin cloth and fed with 10% solution of honey. The adults were allowed to lay eggs. The caterpillars were fed with fresh, tender leaves of grass *Ischaemum aristatum*. Larvae were maintained at room temperature (28^{o}C) with a relative humidity of 70-80%.

2.2 Treatments

Fifth instar larvae with moulting marks were separated on previous day to get sixth instar larvae (day 0). Larvae were treated with different amount of methoxyfenozide in acetone (0.005µg to 1 µg in 5µl acetone) by topical application of along the dorsal midline of meso and metathorax and to the abdomen using a Hamilton Micro-Syringe. Control larvae received an equal volume of acetone.

2.3 Collection of Haemolymph

After 24 hours of treatment larvae were anesthetized in a specimen tube using diethyl ether. One of the prolegs of larvae excised with a pair of sterilized scissors and the exuded haemolymph containing haemocytes from each larva was drawn into separate centrifuge tubes and stored at -20^{0}C.

2.4 Estimation of Haemolymph Protein concentration.

Concentration of SDS-solubilized protein in the haemolymph was estimated by modified Lowry's method [11] using bovine serum albumin (BSA) as standard.

2.5 Electrophoretic Analysis of Haemolymph proteins

The protein samples were subjected to SDS-PAGE under reducing conditions using 10% acrylamide in a mini slab gel according to the method as described by the Laemmli [12].

3. Results

3.1 Effect of methoxyfenozide on haemolymph protein concentration in 6th instar larvae

We found that after 24 hours of the treatment of 6th instar larvae of *S. mauritia* with sub lethal concentrations of methoxyfenozide on day 0, led to increase in total haemolymph protein concentration compared to control as shown below (Table 1). As the methoxyfenozide concentration increased, the haemolymph protein concentration also increased indicating that the effect is concentration dependant. The increase in protein concentration was statistically significant (P< 0.05) for 1.0, 0.5 & 0.1µg methoxyfenozide/larvae. But for lower concentrations of methoxyfenozide (0.005 and 0.05 µg/larvae) difference was not statistically significant (P> 0.05) compared to control.

Table 1: Quantitative changes in haemolymph protein of 6th instar larvae of *S. mauritia* treated with methoxyfenozide and untreated.

Sl. No.	Amount of Methoxyfenozide (µG) /Larvae	Haemolymph Protein Concentration (µg/µl) ± Se
1	0.0 (Control)	10.84±0.31
2	0.005	11.62±0.34
3	0.05	12.33±0.59
4	0.1	13.63±0.63
5	0.5	14.50±0.46
6	1.0	16.43±0.89

3.2 Effect of methoxifenozide on haemolymph protein profile

When protein profile of the haemolymph of the methoxyfenozide treated larvae were analyzed by SDS-PAGE, there was an increase in intensity of an 83 kDa band in the treated compared to control after 24 hour of exposure to methoxyfenozide (Fig. 1). With increase in concentration of methoxyfenozide there was an increase in intensity of the 83 kDa band at higher concentrations of methoxyfenozide.

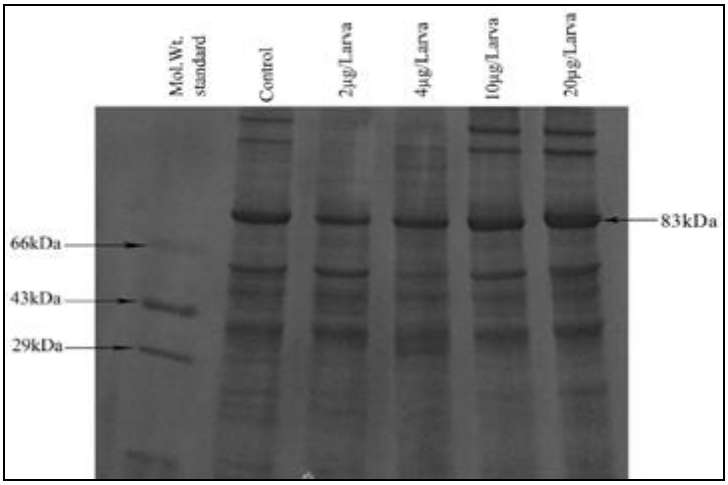

Fig 1: Polyacrylamide gel (10%) electrophoresis of haemolymph of 6th instar larvae of *S. mauritia* treated with different concentrations of methoxyfenozide.

4. Discussion

Haemolymph protein of the 6th instar larvae when treated with sub lethal concentrations of methoxyfenozide on day 0, led to increase in haemolymph protein concentration compared to control (Table 1). As the protein concentration increases with increase in methoxyfenozide concentration, the effect is concentration dependant. The increase in protein concentration was statistically significant ($P< 0.05$) for 1.0, 0.5 & 0.1µg methoxyfenozide/larvae. But for lower concentrations of methoxyfenozide (0.005 and 0.05 µg/larvae) difference compared to control was not statistically significant ($P> 0.05$) indicating that a minimum threshold level is required for significant effect. Thus the protein level in the haemolymph is increased by methoxyfenozide treatment at or above 0.1µg/larvae. Treatment of 5th instar larvae of *Spodoptera mauritia* with sub lethal concentration of the JH mimic, pyriproxyfen, also led to an increase in the haemolymph protein level as well as the size of the larvae [13].

When protein profile of haemolymph of 6th instar (day 0) larvae of *Spodoptera mauritia* treated with different concentration of methoxyfenozide was analyzed by SDS-PAGE, there was an increase in intensity of an 83 kDa band with increase in concentration of methoxyfenozide (Fig.1). This indicates that effect of methoxyfenozide on 83 kDa haemolymph protein is concentration dependent. Thus methoxyfenozide may be affecting the synthesis/degradation of the major haemolymph protein. The most affected polypeptide (83 kDa) is the major protein in the haemolymph of larvae of *S. mauritia.* Storage proteins represent the predominant proteins in the haemolymph of last instar lepidopteran larvae. The most abundant storage proteins that accumulate in the hemolymph or fat body are composed of six subunits and thus are also called hexamerins. Hexamerins are mainly synthesized by the fat body during larval development, stored in the hemolymph, and serve as sources of nitrogen and amino acids for pupae and adults during metamorphosis and reproduction [14]. Three storage proteins named SL-1, SL-2 and SL-3, the former two being synthesized only in the last larval instar, were purified from haemolymph of the common cutworm, *Spodoptera litura* [15]. All these three storage proteins have molecular sizes between 400 and 450 kDa, and are composed of subunit(s) which range in size from 70 to 80 kDa. SL1 and SL-2 are methionine rich and SL-3 is an arylphorin. Thus the methoxyfenozide- responsive polypeptide appears to be a member of the storage proteins as they constitute the most abundant polypeptide in the haemolymph. Vadakkadath Meethal *et al* reported that treatment of 5th instar larvae of *S. mauritia* with JH analogue, Pyriproxifen, led to a similar increase in the level of an 83 kDa protein in the haemolymph [13]. Alteration in protein profile is also reported for methoxyfenozide treated *Bomboxi mori* larvae [16]. Storage hexamerins are composed of six identical or similar subunits of ~80 kDa with a native molecular weight around 500 kDa [6]. They represent the most abundant storage proteins that accumulate in the hemolymph or fat body of insects. Thus increase in haemolymph storage protein appears to be a more general effect of the IGR mimic exposure to the larvae.

As storage proteins are crucial for insect development they may be targeted for developing better insect control strategies. In addition, the general effect of over expression of the storage protein makes it an ideal target protein as it may be involved in the response to multiple insecticides/toxicants. Whether the over expression of the protein is a mechanism to resist the effect of insecticide/toxicant needs to be explored and studies in this direction are ongoing in our laboratory.

5. Conclusions

Treatment of sixth instar larvae of *Spodoptera mauritia* with methoxyfenozide led to a statistically significant increase in the haemolymph protein concentration after 24 hours of treatment. Also the treatment resulted in an increase in the intensity of the major protein band of 83kDa in the haemolymph compared to control. Storage proteins are the major proteins in the larval haemolymph of many lepidopterans including *S. mauritia* and it will be worth investigating whether this protein has any role in the detoxification/resistance to the insecticide.

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