IGR, Lufenuron, alters chitin and total soluble protein content of Aedes aegypti larvae during development

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Abstract

The chitin synthesis inhibitor, Lufenuron (Match) is newly introduced synthetic Insect Growth Regulator used as a crop protection product (pesticide) worldwide for variety of crops. When II and IV instar larvae of Aedes aegypti were treated with sub-lethal concentrations (LC20 and LC40) of Lufenuron through the culture medium for 48 hrs to investigate chitin and total soluble protein content in the larval tissues during development, it was found that for all concentrations tested, there was a significant reduction in chitin and total soluble protein content of the treated larvae as compared to that of control. At LC20 and LC40, there was a progressive increase in the protein: chitin ratio as a function of increase in age of the larvae. Thus sub-lethal concentrations of Lufenuron alters the chitin and total soluble protein content of Aedes aegypti larvae during development thereby resulting in developmental abnormalities as observed earlier (Salokhe et al., 2010). SDS-PAGE analysis revealed that there were quantitative differences in subunits of molecular weights 44, 50, 70 and 98 kDa proteins.

Keywords: chitin synthesis inhibitor, lufenuron, Aedes aegypti

Introduction

Lufenuron, an acylurea IGR, interferes with biosynthesis of chitin, a vital and almost indestructible part of the insect exoskeleton. Chitin is a polymer of N-acetyl-D-glucosamine bound with the protein. Cross-linking of chitin and protein to form cuticle plays a crucial role in the development of insect. Suppression of chitin deposition in treated insects often causes mortality during molting when the procuticle is subjected to the stresses of ecdysis and cuticular expansion (Dean et al., 1998). Among the changes which occur in the physiology of insects, those involving chitin and protein should yield the most information since they are very closely associated with growth and reproduction processes (Muzzarelli, 1977). It was found that benzoylphenylureas affect a cascade event involved in chitin biosynthesis (Post et al., 1974; Marks and Sowa, 1974). The disturbance of the formation of cuticular tissue by benzoylphenylureas led to biochemical studies on the possible effects on the chitin and protein constituents of the cuticle. Effect of sub-lethal concentration of Lufenuron on biological parameters (survival and metamorphosis) of A. aegypti larvae was reported for the first time by us (Salokhe et al., 2010). The present study is the continuation of the earlier one with an emphasis on biochemical changes as an effect of sub-lethal
concentrations of Lufenuron on the larval tissues of A. aegypti.

Materials and Methods

Maintenance of culture

A culture of Aedes aegypti was maintained at 25 ± 2º C and 80 ± 5 % relative humidity. Adults were maintained in 1.5×1.5×1.5 feet wire mesh cages with aluminium frame work. Adult male mosquitos were provided with 5% sucrose solution and females with Rabbit blood. Moist filter paper strips were provided for egg laying. Eggs strips were dipped in deoxygenated water for hatching. Larvae were maintained in enamel pans and fed a pinch of powdered dog biscuits + Brewer’s Yeast (1:1). Each larval stage was maintained in separate pans so as to facilitate easy harvest of required stages for bioassay.

Chitin extraction and estimation

The effects of sub lethal concentrations of lufenuron on chitin content of the larvae were determined by standard WHO method by releasing II and IV instar larvae of A. aegypti in water treated with sub-lethal concentrations of Lufenuron (LC$_{20}$ and LC$_{40}$) for 48 hrs. Three replicates of the 400 larvae each were prepared. After the treatment, 250 larvae of II instar and 120 larvae of IV instar were weighed and homogenized in homogenizing buffer (100mg/ml), containing Tris (5mM), Glycine (38 mM), pH 8.4. The homogenate was centrifuged at 10,000 rpm for 15 min and the precipitate was dissolved in 6N HCl. The dissolved precipitate was transferred into hydrolysis tubes which were sealed under vacuum and the material was hydrolyzed at 100ºC for 16 h in a temperature controlled heating block. On completion of hydrolysis, the solution was centrifuged at 10,000 rpm for 10 min. The supernatant was collected and neutralized with 30% NaOH. N-Acetylglucosamine content was estimated according to the method of Ressig et al. (1955). Entire experiment was repeated thrice on different occasions.

Glucosamine 0.1-1 µmole was taken in a capped test tube. To this was added 1.5% acetic anhydride (v/v) prepared in acetone and 1ml potassium tetraborate (100 mM), pH 8.5. The tubes were sealed tightly, incubated in boiling water bath for 3 min and cooled immediately in an ice bath. Subsequently 3 ml of DMAB [10% DMAB prepared in acetic acid containing 12.5% 10 N HCl (v/v)] diluted with acetic acid (1:9) was added to the solution in the tubes. The tubes were incubated at 37ºC for 20 mins. Absorbance of the mixture was measured at 585 nm in UV-visible spectrophotometer.

Protein extraction and estimation

A. aegypti II and IV instar Larvae were introduced in control and treated (with sub lethal concentration of lufenuron, LC$_{20}$ and LC$_{40}$) water. Three replicates of 400 larvae each were made for each concentration. Forty eight hours after the treatment, 250 larvae of II instar and 150 larvae of IV instar were weighed and homogenized in protein extraction buffer (250 mg/ml) containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM PMSF, and 0.1% Triton X-100, followed by centrifugation at 10,000 rpm at 4ºC for 20 min. Protein content of the supernatant was determined by the Bradford’s method (1976). The entire experiment was repeated thrice on different occasions.

SDS-PAGE

Samples containing equal amount of protein, as determined by the Bradford’s method from control and treated larval tissue extracts were denatured in a
sample buffer for 3-5 min and analyzed on 10% SDS-PAGE along with the molecular weight marker proteins. Electrophoresis was carried out at a constant current (25 mA) at room temperature and the gel was stained by silver staining.

Results

Effect of Lufenuron on chitin and total soluble protein content of A. aegypti larvae

It was observed that in Lufenuron treated II and IV instar larvae of A. aegypti there was reduction in chitin and protein content as compared to that of control (Figs. 1 and 2). Further it was found that the reduction in chitin content was dose dependent in II and IV instar larvae (Figs.1a, 2a). In II instar larvae, though treatment with LC<sub>20</sub> and LC<sub>40</sub> of Lufenuron resulted in decreased in total protein content as compared to that of control, there was no significant difference in reduction in protein content of LC<sub>20</sub> and LC<sub>40</sub> treated larvae (Fig. 1b). However in IV instar larvae decreased in protein content was dose dependent (Fig. 2b). When the results were expressed as the protein: chitin ratio, it was observed that the ratio was significantly reduced in LC<sub>20</sub> and LC<sub>40</sub> treated II instars larvae as compared to that of control. However, there was no significant difference in the ratio of LC<sub>20</sub> and LC<sub>40</sub> treated II instar larvae. In the IV instar larvae the ratio increases at LC<sub>20</sub> and decreases at LC<sub>40</sub> as compared to that of the control (Table 1).

Effect of Lufenuron on protein profiles (SDS-PAGE) of A. aegypti larvae

SDS-PAGE analysis revealed that, though there were no significant differences between the protein profiles of control and treated samples in general. However, there were quantitative differences in some subunits (molecular weight 50-98 kDa). In II instar the protein of 70 kDa increased considerably in Lufenuron (LC<sub>20</sub> and LC<sub>40</sub>) treated larvae as compared to that of the control larvae while protein of 50 kDa decreased considerably in treated larvae as compared to that of control (Fig. 3A). In IV instar larvae proteins of molecular weight 44-98 kDa showed variations. The protein of 70 kDa increased considerably in Lufenuron (LC<sub>20</sub> and LC<sub>40</sub>) treated larvae as compared to that of the control. The 98 kDa protein decreased considerably in Lufenuron treated larvae as compared to that of the control. Further, the protein of 44-50 kDa decreased considerably in Lufenuron treated larvae as compared to the control (Fig. 3B).

Fig. 1. Effect of the sub-lethal concentration of Lufenuron on II instar larvae of A. aegypti

a) On Chitin content

![Chitin content](chart1.png)

b) on protein content

![Protein content](chart2.png)

Discussion

Sub-lethal concentrations of Lufenuron alter chitin and total soluble protein contents of II and IV instars larvae of Aedes aegypti. Reduction in the chitin
content of the II and IV instars larvae was in proportion with the concentration of Lufenuron. Similar results were found in also, quantitative analysis of protein revealed that there was decrease in the total soluble proteins in Spodoptera littoralis larvae treated with Flufenoxuron (Sammour et al., 1996) Lufenuron treated larvae as compared to that of control. Further we observed variation in protein:chitin due to treatment with the sub-lethal concentrations of Lufenuron. Similar observations were made in house fly larvae treated with TH6040 (Ishaaya and Casida, 1974). Since cross-linking of chitin and protein to form cuticle plays important role in the development of insect, variation in the ratio in Lufenuron treated larvae of A. aegypti affected growth and development as reported earlier (Salokhe et al., 2010).

**Fig. 2.** Effect of the sub-lethal concentration of Lufenuron on IV instar larvae of A.aegypti

![Graph of protein and chitin content](image)

**Table 1.** Ratio of protein: chitin content of A. aegypti larvae

<table>
<thead>
<tr>
<th>Dose</th>
<th>II instar larva</th>
<th>IV instar larva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.69</td>
<td>2.86</td>
</tr>
<tr>
<td>LC₂₀</td>
<td>4.27</td>
<td>2.98</td>
</tr>
<tr>
<td>LC₄₀</td>
<td>4.39</td>
<td>2.52</td>
</tr>
</tbody>
</table>

**Fig. 3A.** SDS-PAGE analysis of total soluble protein from control and treated (LC₂₀ and LC₄₀) II instar larvae of A. aegypti. M: Marker, L1:LC₂₀, L2:LC₄₀, C: Control. Arrows indicates the polypeptides showing variation in their pattern.

![SDS-PAGE II instar larvae](image)

**Fig. 3B.** SDS-PAGE analysis of total soluble protein from control and treated (LC₂₀ and LC₄₀) IV instar larvae of A. aegypti. M: Marker, L1:LC₂₀, L2:LC₄₀, C: Control. Arrows indicates the polypeptides showing variation in their pattern.

![SDS-PAGE IV instar larvae](image)

The SDS-PAGE protein analysis revealed quantitative variation in 70 kDa protein in Lufenuron treated II and IV instar larvae. These are the stress...
proteins which play a major role in protection and maintenance of many fundamental cellular functions (Fink, 1999; Naideau et al., 2001). Induced expression of these proteins probably provides protection against insect growth regulatory stress at LC20 and LC40 of Lufenuron. Also we found the variation in 50 kDa protein in treated larvae which is major protein involved in insect development. Deficiency of such proteins was found to interfere with cuticle formation (Marcu et al., 1998). Decrease in the quantity of these proteins in lufenuron treated larvae possibly resulted in the development of abnormal adults as reported earlier (Salokhe et al., 2010). However, more specific analysis would be required to identify these proteins and thereby conclude on the effect of such changes on development of larvae.

References


