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Humoral immunity of the adult desert locust, *Schistocerca gregaria* developed following bacterial infection

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ABSTRACT

The haemolymph plasma of adult desert locust, *Schistocerca gregaria* was analyzed in order to detect the induction of antibacterial activity following injection with a sublethal dose of *Bacillus thuringiensis israelensis* (*Bti*). A slight activity was demonstrated in the normal locust plasma, while a significant increase of this activity was appeared in both control and immune plasma, when tested by the agar-disk diffusion method. The maximum activity was attained after 24 h post-injection. Analysis of proteins by SDS polyacrylamide gel electrophoresis showed disappearance of some protein bands and appearance of other new ones in the injected adult locusts. Some of these new proteins may affect adult immunity. The phenoloxidase (PO) activity was significantly higher at 6 and 12 h after injection with *Bti*, but at 24 and 48 h, the activity was significantly lower than that of the controls. However, no significant changes in lysozyme activity were detected at all time intervals post-injection compared with the control insects.

Keywords: *Schistocerca gregaria*, *Bacillus thuringiensis israelensis*, antibacterial activity, haemolymph proteins electrophoresis, phenoloxidase and lysozyme.

INTRODUCTION

Insect tissues can control infection by several mechanisms. The haemolymph, for instance, plays the major role. It is essential for the recognition and defense against foreign invaders (Bogaerts *et al.*, 2009), controls bacteria by phagocytosis and nodule formation (Barakat *et al.*, 2002), and generates antibacterial compounds (Boman *et al*., 1991). The fat body might also be involved in infection control (Hultmark, 1993). Since the fat body is a source of haemolymph proteins (Faye and Wyatt, 1980), it could be the source of antibacterial compounds found in the haemolymph (Hoffmann *et al*., 1995).

Haemolymph usually contains, in addition to a large amount of water, protein which is the main nitrogenous constituent of all living materials. Pathogenic infection, e.g., bacteria, produces drastic changes in the haemolymph protein content of the infected host (Barakat and Meshrif, 2007). Hultmark (1993) mentioned that the invading bacteria into the insect`s hemocoel resulted in the synthesis of antibacterial proteins appear in the haemolymph within a few hours after infection and display a broad spectrum of antibacterial activity. Such molecules act as agglutinins, lysins, precipitins, opsonins, compliment-like and microbicides (Fries, 1984). The precise mechanisms whereby they inactivate bacteria are poorly characterized, but Otvos (2000) noted that they act through disintegrating the bacterial membrane or interfering with membrane assembly.

Several comprehensive reviews covering the humoral immunity discussed a number of immune molecules that were induced by the injection of bacteria (Boman *et al.*, 1981; Götz and Boman, 1985; Boman and Hultmark, 1987; Hultmark, 1993). However, little information on Phenoloxidase (PO) and lysozyme as an inducible humoral components of humoral defense mechanisms in insects is known. Thus, a considerable attention has been paid to the characterization of haemolymph proteins and understanding their role in defense reactions.

Phenoloxidase (PO) occurs together with its substrates in the haemolymph, as well as other tissues of many insects. It becomes active only under special circumstances; for example, at sites of integument injury, in bleeding, and in newly secreted cuticle, when this undergoes tanning (Ratcliffe and Rowley, 1979). The absence of PO activity at other times has been attributed to the occurrence of the enzyme in an inactive precursor or prophenoloxidase (proPO). The existence of proPO was first suggested by (Bodine and Allen, 1937). Activation of the proenzyme appears to be achieved by proteolytic cleavage of proenzyme (Ashida and Dohke, 1980). The proPO of various insects can be activated experimentally by a variety of treatments; heating (Dularay and Lackie, 1985) and exposure to denaturating agents such as detergents (Ashida and Soderhall, 1984). It could also be activated by admixture with lipid soluble components (Preston and Taylor, (1970) and one or more pertinacious factors from the same organisms (Ashida and Yoshida, 1988).

Phenoloxidase produces indole groups, which are polymerized to melanin. During melanogenesis, a set of intermediate products were found such as quinones, diphenols, superoxide, hydrogen peroxide, and reactive nitrogen intermediates, which are important during defense against bacterial, fungal, and viral agents (González-Santoyo and Córdoba-Aguilar, 2012). Insects possess an antioxidant defense system of enzymatic components, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione- S-transferase (Karmabeer *et al*., 2013), and non-enzymatic components consist of small organic molecules such as reduced glutathione and vitamin C (Krishnan *et al*., 2009).

Lysozyme is one of the antimicrobial proteins with which insects respond to the challenge of bacteria (Jiang *et al*., 2011). Lysozymes are of several classes (Callewaert and Michiels, 2010) and have a muramidase activity, i.e., they are able to decompose the β-1,4-glycosidic linkage between the alternating linked residues of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) of peptidoglycan, which is the main bacterial cell wall polymer, leading to their lysis (Vocadlo *et al*., 2001). They are widely spread throughout nature; occurring in insects, vertebrates, plants and microorganisms (Jollès and Jollès, 1984). Lysozymes generally exhibit greater antibacterial activity against Gram positive than Gram-negative bacteria (Wang *et al*., 2009). Insect haemocytes have reported to synthesize and release lysozymes into the haemolymph (Lemaitre and Hoffmann, 2007). However, other tissues, such as epidermis, muscles and mid gut cells may also participate in lysozymes production (Hultmark, 1996).

The desert locust, *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae) represents a relatively important group of plant-feeding insects. Aside from their strong immune responses against bacteria was previously studied by Meshrif, and Barakat (2002) and Barakat *et al.* (2002). This locust is often used as a laboratory model for studies concerned with the immune response. Furthermore, *Bacillus thuringiensis israelensis (Bti)* is a bacterium of great agronomic and scientific interest. It is an endospore-

forming bacterium characterized by the presence of a protein crystal within its cytoplasm. The proteins within this crystal are toxic to insects and this explains the extensive use of *Bti* as a biological insecticide (Rudd *et al.*, 2001).

The aim of the present work is to investigate and clarify the appearance of antibacterial activity in the haemolymph of *Bti* injected *S. gregaria* adult locusts, the influence of pathogenic bacteria on the plasma native proteins, and the time course in the changes of PO and lysozyme activities as inducible humoral components. The expected results may lead to understanding of the physiology of infected insects, and hence the improvement of using entomopathogenic agents in biological control measures or in integrated pest management.

MATERIALS AND METHODS

Experimental design:

In the present study, *S. gregaria* were reared according to methods of Hassanein (1965). Adults (both sexes being within 2–4 days after the final molt) were used and divided into three groups, each of 20-30 adults. The first group was intrahaemocoelic injected with 10 ul of *B. thuringiensis israelensis* (*Bti*) adjusted to a sublethal concentration of 3×10^4 CFU/ml. the second group was injected only with equivalent volumes of saline solution (0.5% NaCl), and considered as a positive control. The third group was left uninjected and considered as a negative control. The haemolymph was collected from the bacteria-injected and control locusts after 6, 12, 24 and 48 h post-injection by amputation at the arthropodial membrane of the hind coxa with fine scissors. The collected haemolymph was centrifuged at 1200 rpm for 10 min at 4 ºC. The resulting supernatant was stored at -4°C in Eppendorf tubes until use.

Estimation of protein in the haemolymph:

The total protein content was determined according to Bradford (1976) using Coomassie Brilliant Blue G-250 (CBB) reagent. 20 ul of test sample was made up to 1 ml with distilled water and 5 ml of the CBB reagent were added, mixed thoroughly, allowed to stand at room temperature for 10 min and the intensity of the colour developed was read at 595 nm in 3 ml cuvettes against a reagent blank prepared from 1 ml of distilled water and 5 ml of CBB. Values are calculated from the standard calibration curve using and bovine serum albumin (BSA) as the standard protein solution.

Demonstration of antibacterial activity in the haemolymph:

The antibacterial activity against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria was tested using agar disk-diffusion technique. The antibacterial drug Ciprofar was used as positive control for G +ve bacteria and Curisafe for G-ve bacteria (obtained from Farco B International, Egypt). This technique was performed according to the method developed by Gingrich (1964) and modified by Walters and Ratcliffe (1983). Heated nutrient agar was pipetted into sterile Petri dishes. After cooling, a cotton swab, heavily socked with 2.2 x 10^9 CFU/ml of *Bti* was streaked over the agar surface. These preparations were incubated at 30 ºC for 18 h. To carry out the test, a standard filter paper disks (6 mm diameter) soaked in plasma collected from uninjected and injected insects with 2.33 x 10^3 CFU/insect at different time intervals post-injection (6, 12, 24, and 48 h) were placed over the agar surface. The preparations were further incubated at 30 ºC for 18 h. When the dish was held obliquely to transmitted light, a distinct clear zone was seen surrounding any paper disk that contained immune plasma. The

actual zone width was measured as the following:

Zone width = zone diameter - disk diameter.

The titer of any particular plasma tested by the agar-well method was determined by the measurement of the width of the inhibition zone. The measurements were replicated five times for each time interval.

Phenoloxidase (PO) assay

The PO activity was determined by measuring formation of dopacrome according to the method of Ashida and Soederhaell (1984). 20 µl of plasma were added to 780 µl of 0.01 M ice-cold phosphate buffered saline (pH 7.4) and mixed in an Eppendrof tube. Another 800 µl of 20 mM L-DOPA (Sigma) was added to each sample and the mixture was incubated at 25 ± 2 ºC. The absorbance was measured at 490 nm after 20 min. The PO activity is expressed as PO unit per ml of plasma; one unit is the amount of enzyme required to increase the absorbance by 0.001 min^{-1}. This test was replicated 5 times for each determination.

Lysozyme assay

To determine lysozyme activity in plasma of adult *S. gregaria*, enzymatic assay was employed according to Sigma instructions that based on Shugar (1952). The test sample, was containing 1 ml *Micorcoccus lysodeiktus* cell suspension (Sigma) (0.015% w/v) in potassium phosphate buffer (66 mM, pH 6.24) and 40 µl enzyme solution. Blank contained 40 µl buffer instead of enzyme solution. The solutions were mixed by inversion, and then the decrease in absorbance at 450 nm was recorded for approximately 5 min. One unit was calculated as a ΔA_{450nm} of 0.001 per minute at 25 ± 2 °C and pH 6.24 using a suspension of *M. lysodeikticus* as substrate.

Data analysis:

Data were expressed as mean ± standard error (SE), and compared using Student's "t test" for paired samples. The level of significance for each experiment was set at $P < 0.05$ and was corrected using Bonferroni's method for multiple comparisons.

RESULTS

The locust`s total haemolymph proteins

In uninjected locusts, the total haemolymph proteins was 52.89 ± 2.03 mg/ml (Fig. 1). In *Bti*-treated insects, there was a significant increase ($P \leq 0.025$) at 6 h post-injection followed by significant decrease thereafter, as compared with the saline-treated insects. At the 48 h, a significant increase was observed again (Fig. 1).

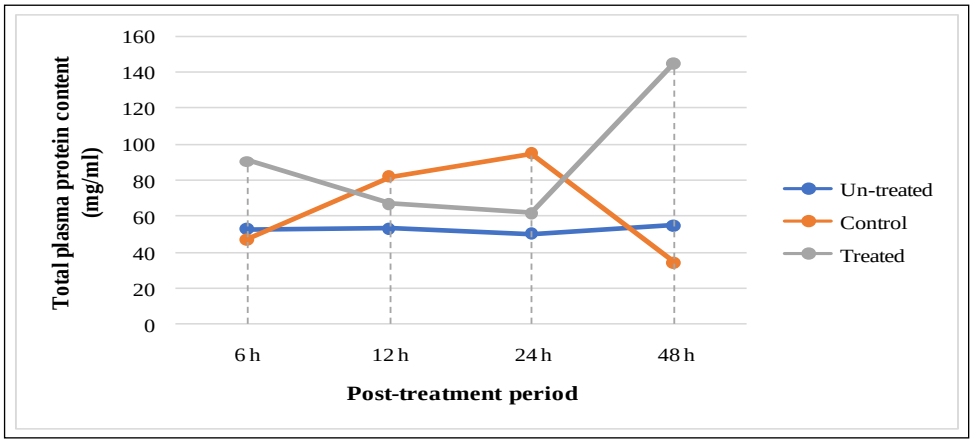

Fig. 1: Total plasma proteins of adult *S. gregaria* determined at different time intervals post-treatment with saline (control) and *Bti*.

Demonstration of antibacterial activity:

Very weak antibacterial activities against Gram-positive bacteria, *S. aureus* were observed in all the plasma tested at all time intervals. Against the Gram-negative bacteria, *E. coli* was a weak activity in both plasma of healthy and control insects at all post-injection intervals, however a slight increase ($P \geq 0.05$) in the antibacterial activities at all time intervals was observed in immune plasma collected from *Bti*-injected locusts. The increase was significant ($P \leq 0.025$) at 24 h post-injection (Fig. 2 and Plate 1).

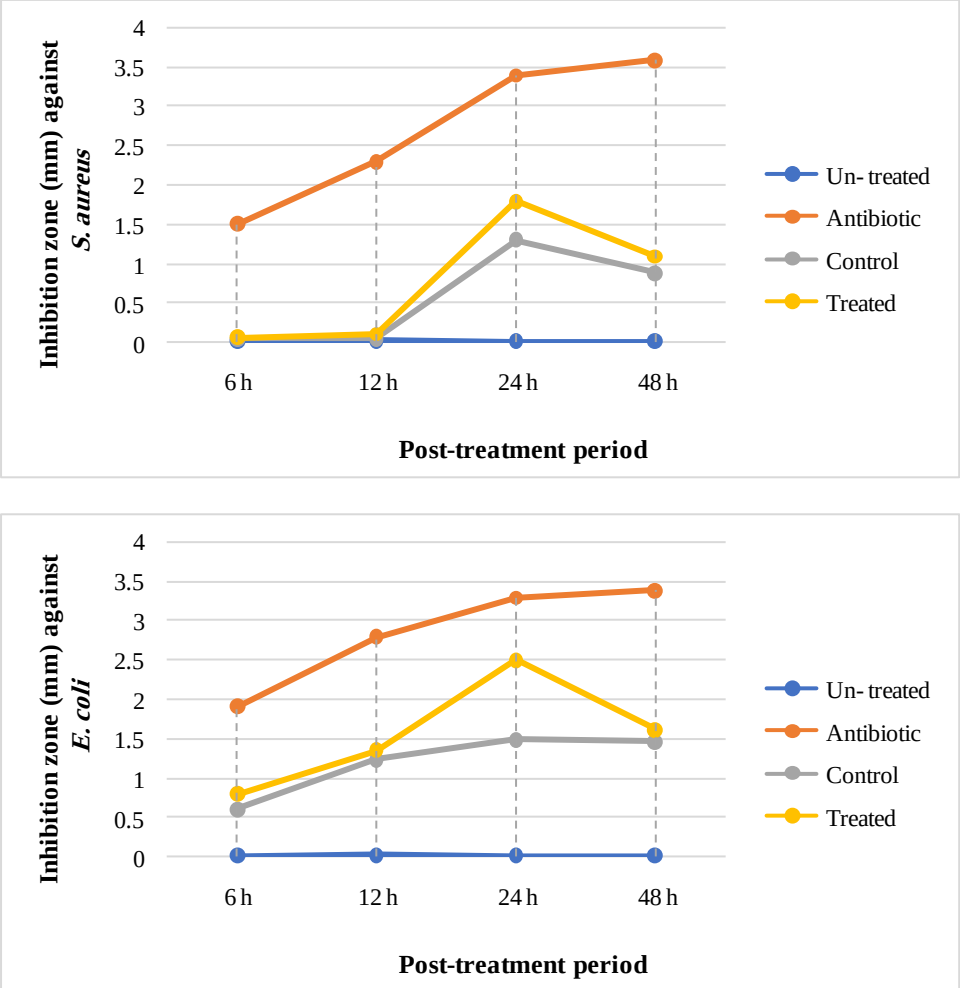

Fig. 2: Inhibition zone (mm) of antibacterial activity of plasma from adult *S. gregaria* collected at different time intervals post-treatment with saline (control) and *Bti* against both G +ve *S. aureus* and G -ve *E. coli*.

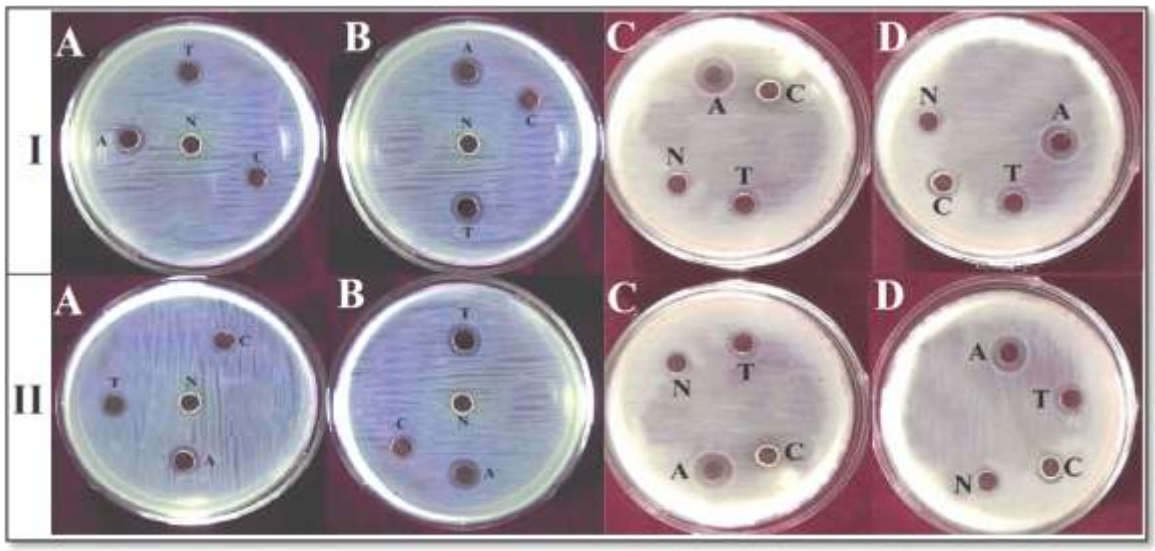

Plate I. Photomicrographs showing the inhibition zone of antibacterial activity of plasma from adult *S. gregaria* against both G +ve *S. aureus* (I) and G -ve *E. coli* (II) collected at different time intervals; A: 6 h, B: 12 h, C: 24 h and D: 48 h, post saline and *Bti*-injection. A: (antibiotic); N: (normal plasma); C: (plasma of control larva); and T: (plasma of treated larvae).

Phenoloxidase activity

The PO activity was higher (P<0.01) at 6 and 12 h after challenge with *Bti* compared to the controls, but at 24 and 48 h, the activity was significantly lower than that of the controls (Fig. 3).

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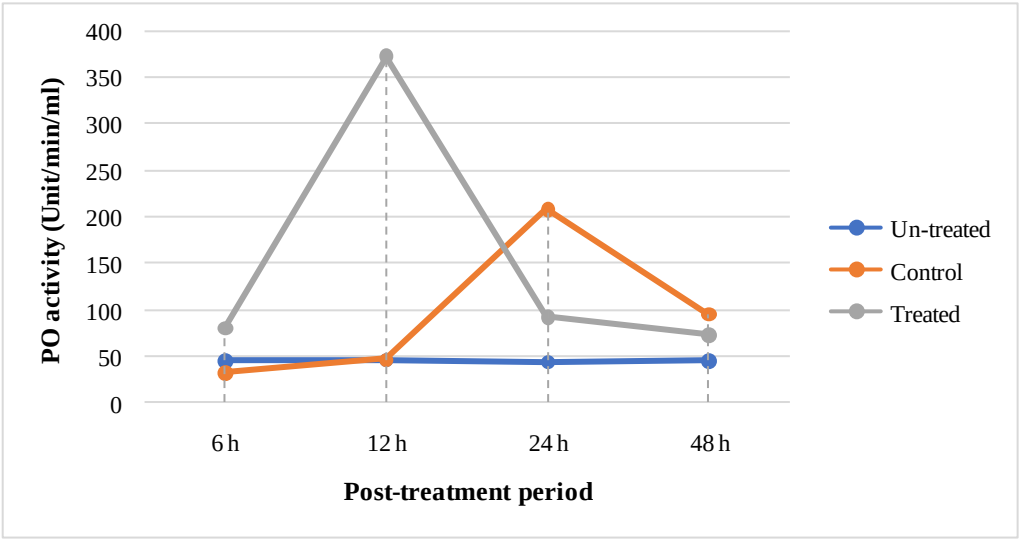

Fig. 3: Plasma phenoloxidase (PO) activity of adult *S. gregaria* determined at different time intervals post-treatment with saline (control) and *Bti*.

Lysozyme activity

No significant changes (P≥0.025) in lysozyme activity were detected at all time intervals post-injection compared with the control insects (Fig. 4).

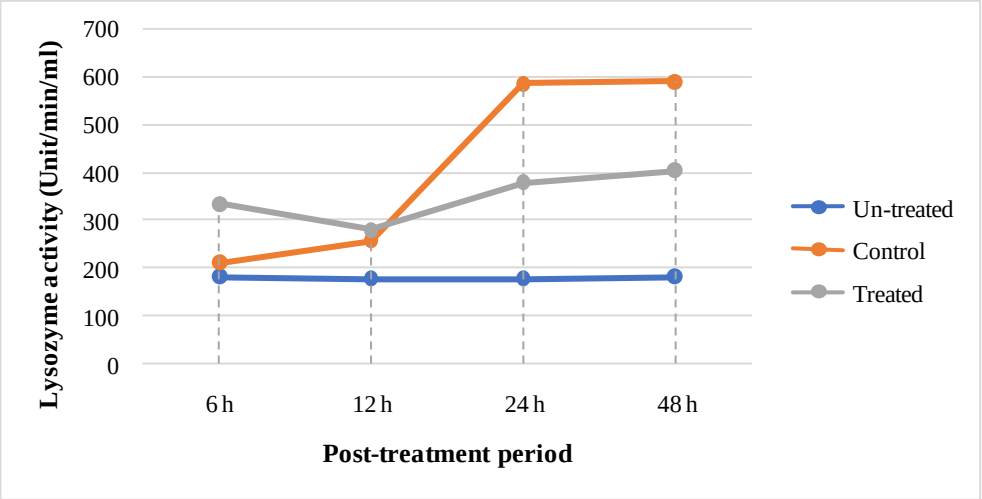

Fig. 4: Lysozyme activity of adult *S. gregaria* determined at different time intervals post-treatment with saline (control) and *Bti*.

DISCUSSION

Results obtained in the present study from testing plasma of the uninjected locusts, proved that the normal insects attained a weak antibacterial activity to virulent bacteria without they had received antigenic challenge. This result is supported by the findings of Heimpel and Angus (1959) who found antibacterial activity in the haemolymph of some insects as a result of naturally occurring antimicrobial substances, which are contained in the food. The fat body may have a specific mechanism for regulating the size of the symbiont population within the insect. In addition, the fat body is the source of haemolymph proteins that may contain the antibacterial substances (Faye and Wyatt, 1980). Another explanation suggested by Mirsch (1960) who mentioned that several substances such as fatty acids, lipids, certain peptides and aliphatic amines are known to exert antibacterial action *in vitro*. One or more of these substances as integral parts of cells could be liberated to act on bacteria upon destruction of the cells or a change in the cell's natural environment. Accordingly, the amount of antibacterial activity in the haemolymph of normal insect should be proportional to the amount of cell destruction or to the degree in which the environment was altered.

Injection of saline or a sublethal dose of *Bti* into adult *S. gregaria* resulted in the increase of antibacterial activity in the haemolymph of insects. This indicates that the humoral defense reactions needed to some extent for newly synthesis and release of the antibacterial substances (Barakat *et al*., 2002). These results are close to those of Gingrish (1964) on *Oncopeltus fasciatus* injected with *P. aeruginosa* and Abu El-Magd *et al*. (1994) on *Spodoptera littoralis* larvae injected with *Bt.* The bactericidal activity in the injected insects was supported by the work of Boman *et al*. (1991) who demonstrated that the injection of *Hyalophora cecropia* pupae with the live bacterial cells of *Enterobacter cloacae* induced the production of about 10 immune proteins. Six haemolymph native protein bands of antibacterial activity were separated from the haemolymph of *Bombyx mori* larvae after injection of live or heat-killed bacteria (Morishima *et al*., 1988).

The overall results suggest that *S. gregaria* could be immunized against *Bti* by a previous injection of low dose of the same bacterium. Hoffmann (1980) reported that the antibacterial activity in the plasma of *Locusta migratoria* induced by low dose of living cells of *Bt, Escherichia coli, Pseudomonas aeruginosa* and non-bacterial substances. The traces of antibacterial activity found in the haemolymph suggested that the response to specific and non-specific substances is probably identical in nature but different in intensity.

In the light of the present findings, there are several possible explanations of this immune response provoked in the haemolymph of *S. gregaria* against the injected materials. Some factors may exhibit certain degree of specificity *in vivo* and be more active against *Bti* than saline. The relative amounts of these produced factors may depend on the type of the antigen injected. Different antigens may produce different factors with different properties in the haemolymph.

However, data obtained from agar-desk diffusion test is not satisfactory, so it is necessary to analyze and characterize the haemolymph proteins to show if these proteins play a role actually in the humoral defense in *S. gregaria* or not and are these proteins specific to the bacteria or to the injection generally.

In insects, PO activity is an important part of innate immune system. It is a key enzyme in cellular and humoral immune responses as well as its active role as recognition factor of non-self-molecules (Leonard *et al*., 1985; Kanost *et al*., 2004). For these reasons, the measurement of PO activity in the haemolymph has often been used as an estimate of immune status and disease resistance. Mahmoud *et al.* (2015) found that PO from *S. gregaria* is most probably a tyrosinase-type calcium-containing mono enzyme, which functions, not only as a catalytic enzyme in melanin production in locusts, but perhaps also as a humoral factor in host defense via melaninization as in other insects.

In adult locusts, the PO activity increased sharply at 6 h post-bacterial injection. This increase might be due to degranulation or rupture of coagulocytes and granular haemocytes in the haemolymph. This scenario was supported with the observed reduction in these cell types after infection (unpublished data). Jiang *et al*. (1997) and Ling and Yu (2005) detected PO activity in oenocytoids and granulocytes. Moreover, Vergas-Albores and Plascencia (2000) found that upon infection, glucan binding protein reacts with β-glucans and a complex induces degranulation and the activation of proPO). The significant decrease of PO at 24 h might be attributed to the consumption of the PO during nodule formation that has been detected at 24 h post-injection and further to the expiry of antigenicity due to eradication of bacterial infection.

Lysozyme is powerful tool of the humoral defense in insects and considered to be the other partner of PO that enable us to estimate disease resistance (Adamo, 2004). The mechanisms behind the decrease of lysozyme activity in insects injected with *Bti* at 24 h is unknown, but may be a sign of immune redistribution, in which resources within the immune system are shifted to augment immunity against a different class of invaders as stated by Bidochka and Khachatourians (1987) and Braude *et al*. (1999). This assumption could be supported by our observation that the nodule formation was the highest at the same time interval (Mo`men *et al*., 2010).

The significant decrease in lysozyme presumably attributed to the absence of antigenicity because the bacteria may be able to avoid the induction of host lysozyme. Parasitoids coat themselves with a layer of soluble host proteins or by non-antigenic secretion (Asgari and Schmidt, 1994). Another explanation is that at the end of struggle between the bacteria and haemocytes, the

exhausted haemocytes are unable to recognize the non-self-molecule anymore.

CONCLUSION

In conclusion, infection with *Bti* had drastic effect on adult *S. gregaria*, particularly as they reduced haemolymph nutrients (e.g. plasma proteins). The capacity of bacterial pathogen to consume host nutrients is considered one of the virulence factors. Plasma PO and lysozyme may be an important enzymatic defense against bacteria, associated with attempted clearing of bacteria from the hemolymph.

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Humoral immunity of the desert locust, *Schistocerca gregaria* following bacterial infection

المناعة الخلطية للجراد الصحراوي شيتوسيركا جريجاريا ، *Schistocerca gregaria* بعد العدوى البكتيرية

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المستخلص

تم تحليل بلازما الدم في الجراد الصحراوي البالغ ، *Schistocerca gregaria* من أجل الكشف عن تحريض النشاط المضاد للبكتيريا بعد الحقن بجرعة شبه مميتة من {(*Bacillus thuringiensis israelensis* (*Bti*)}. تم إثبات نشاط طفيف في بلازما الجراد الطبيعية ، بينما ظهرت زيادة كبيرة في هذا النشاط في كل من بلازما التحكم والبلازما المناعية ، عند اختبارها بطريقة انتشار قرص الأجار. بلغ أقصى نشاط 24 ساعة بعد الحقن. أظهر تحليل البروتينات بواسطة الرحلان الكهربائي للهلام متعدد الأكريلاميد SDS اختفاء بعض العصابات البروتينية وظهور عصابات جديدة أخرى في الجراد البالغ المحقون. قد تؤثر بعض هذه البروتينات الجديدة على مناعة البالغين. كان نشاط الفينولوكسيديز (PO) أعلى بشكل ملحوظ في 6 و 12 ساعة بعد االحقن مع *Bti* ، ولكن في 24 و 48 ساعة ، كان النشاط أقل بكثير من نشاط عناصر التحكم. ومع ذلك ، لم يتم الكشف عن أي تغييرات معنوية في نشاط الليزوزيم في جميع الفترات الزمنية بعد الحقن مقارنة مع الحشرات الضابطة