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# Activation of Prophenoloxidase during Bacterial Injection into the Desert Locust, *Schistocerca Gregaria*

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***Abstract*—**The present study has been conducted to characterize the prophenoloxidase (PPO) system of the desert locust, *Schistocerca gregaria* following injection of *Bacillus thuringiensis kurstaki* (Bt). The bulk of PPO system was associated with haemocytes and a little amount was found in plasma. This system was activated by different activators such as laminarin, lipopolysaccharide (LPS) and trypsin suggesting that the stimulatory mechanism may involve an enzyme cascade of one or more associated molecules. These activators did not activate all the molecules of the cascade. Presence of phenoloxidase activity (PO) coincides with the appearance of protein band with molecular weight (MW) 70.154 KD (Kilo Dalton).

***Keywords*—**Schistocerca gregaria, haemolymph, proteins, prophenoloxidase system, phenoloxidase

## I. Introduction

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, as previously shown by [1]-[2], this locust is often used as a laboratory model for studies concerned with the immune response.

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 [3]. The absence of PO activity at other times has been attributed to the occurrence of the enzyme in an inactive precursor or prophenoloxidase (PPO). The existence of PPO was first suggested by [4]. In the wax moth, *Galleria mellonella* (L.), PPO and its activating system was studied by several workers [5],[6],[7],[8]. Prophenoloxidase activity has also been identified in the haemolymph of a variety of insect species [9]. Activation of the proenzyme appears to be achieved by proteolytic cleavage of proenzyme [10]. The PPO of various insects can be activated experimentally by a variety of treatments; heating [11],[12] and exposure to denaturating agents such as detergents [13]. It could also be activated by admixture with lipid soluble components [14] and one or more pertinacious factors from the same organisms [15].

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The present work forms a part of a wide study that aims to describe the role of PPO and its activating system in immune defense mechanisms of the desert locust, *S. gregaria*. Whether this system is present in haemocytes and/or in plasma is a subject of controversies. It is necessary to know the exact location in this insect species. Activation of the PPO itself is also controversial, thus the present work also describes the activating ability between the blood cells and blood plasma.

## II. Materials And Methods

### *A. Insects*

The desert locust, *Schistocerca gregaria* (Forskal), was obtained from the Locust and Grasshopper Research Department, Plant Protection Research Institute, Agricultural Research Center, Egypt, and reared for several generations in Faculty of Science, Ain Shams University and had shown no infectious diseases. Methods used for rearing and maintaining locusts were those reported by [16].

### *B. Bacteria*

The bacterium, *Bacillus thuringiensis kurstaki* (Bt) (3200 IU/mg, AGERIN- wettable powder) produced by The Agricultural Genetic Engineering Research Institute (AGERI). Bt was grown at 28 ± 2˚C in nutrient broth tubes for 48 hr, and harvested by suspending in sterile distilled water. Bt suspension was adjusted to a concentration of $2.5X10^9$ cells/ml.

### *C. Injection technique*

Injection of insects was made with a 10 µl Hamilton micro- syringe fitted with a 26-gauge needle. Ten µl of Bt and/ or laminarin/Bt were injected into each insect. Uninjected insects were used as control.

### *D. Activation of the phenoloxidase (PO)*

For investigating the influence of pathogenic infection after PO activation, different concentrations of laminarin, lipopolysaccharide (LPS) and trypsin (Sigma) ranging from 0.01 to 1.0 mg/ml, was pre-injected into the haemocoel of locusts, prior to the injection of Bt. Results of these experiments were compared with those of Bt-injection without PO activation.

### *E. Collection of haemolymph and preparation of plasma (P) and haemocytes lysate (HL)*

Haemolymph was collected and immediately centrifuged at 1200 rpm for 10 min. Supernatant [= plasma (P)] was removed from the haemocyte pellet. The resulting pellet was washed twice with 2ml of 0.01M sodium cacodylate buffer (SCB)containing 0.25M sucrose pH 7. The pellet was then homogenized and centrifuged at 1200 rpm for 10 min. The supernatant is referred to as haemocytes lysate (HL).

*F. Assay for phenoloxidase (PO) activity*

In order to measure PO activity, we recorded the formation of dopachrome from L-dihydroxy phenylalanine (L-DOPA) spectro-photometrically at 470 nm, according to [17] with some modifications. A solution of L-DOPA (2mg/ml) was made in a sodium phosphate buffer (0.01 M, pH 5.9). Aliquots (20µl) of (HL or P) and a possible activator (laminarin or LPS or trypsin) , or sodium cacodylate buffer (SCB) in case of the control, were added to 2ml of DOPA solution, after which the formation of dopachrome (reddish brown pigment) was recorded each minute for 5 min, to make sure the linear increase of the optical density. Prior to activation, HL or P was diluted (v/v) in a SCB. The PO activity was expressed as PO unit, where one unit is the amount of enzyme activity required to produce an increase the absorbance by 0.001 min/mg protein.

*G.Estimation of the total haemolymph proteins*

Protein concentrations of HL or P were assayed according to [18], with bovine serum albumin mg/ml (Sigma) as a standard. Data are given as mean values ± SD (n = 6). Significance was calculated with the Student *t*-test. The significance level was set up at $p \leq 0.05$.

*H.Electrophoretic analysis of the haemolymph proteins:*

Ten µl of HL and P was diluted with three volumes of treatment buffer (0.125 M tris-HCl pH 6.8, 4% Sodium dodecyl sulphate (SDS), 20% glycerol, 10% 2-mercaptoethanol), and then denatured by heating at 95 ºC for one min. Then, the denatured sample was analyzed by SDS-polyacrylamide gel slabs using P9DS apparatus from Owl separation systems. Electrophoresis conditions and procedures were as described by [19]. Electrophoresis was carried out at 100 volts for 5 hr at room temperature. After electrophoresis, the gels were removed and fixed for one hr in fixing solution , then were stained for 2 hr in Coomassie Blue R-250. They were socked in destaine solution for 4 hr and then further destained for 24 hr with excess of destaining solution or until bands were visible.The gel was scanned with gel documentation system by using a scanner (Scan tack, Sport Technology) and then, the bands were analyzed by using software: Gel-Pro Analyzer, version 3.1 for windows 95/NT, from Media Cybernetics (1993-1997) U.S.A. The location of a band was expressed as relative mobility (Rm) and was defined as the distance that the given band migrates from the origin / the distance travelled by the marker.

## III. Results And Discussion

*A. Activation of PPO system and determination of PO activity*

Spectrophotometric measurements of the PO activity are presented in figures (1)and (2),they showed that the pre-treatment of P and HL with laminarin , LPS and trypsin at different concentrations ranging from 0.01-1.0 mg/ml caused a significant increase ($P<0.05$) in the PO activity compared with the buffer control (SCB). However, the HL preparations were found to exhibit considerable activating ability over P preparations. At the same time both P and HL preparations pre-treated with laminarin; showed a significant increase of PO activity compared with other activators. The greatest enzyme activity was recorded at the concentration (0.5 mg/ml) of laminarin. This concentration was used in the subsequent experiments as PPO activator. Our results indicated that, the pre-activation of the haemolymph of *S. gregaria*, caused PO activity, and the enzyme is confined mostly to the haemocytes (HL). This supports the contention that the haemolymph PO is normally present in the haemocytes [20]. The difference in protein content evidenced in (HL and P) preparations could account for some of the discrepancies. Reference [21] attributed this to the differences in protein composition between the two fractions. The protein of haemocytes possibly contains a greater portion of aromatic amino acids than does the plasma proteins [22].

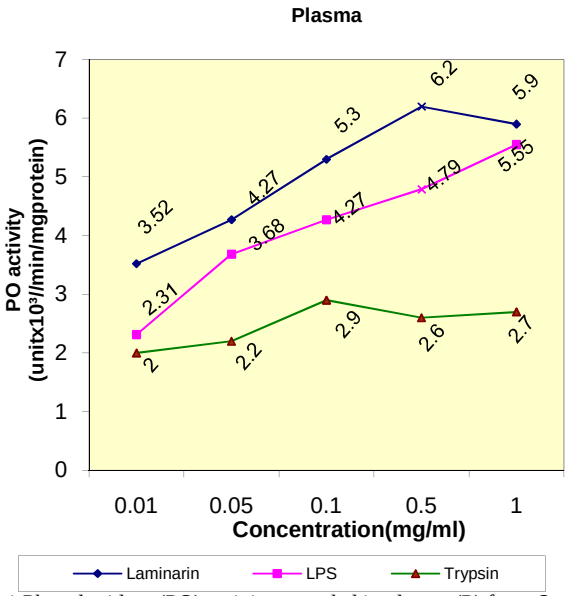


Fig. 1 Phenoloxidase (PO) activity recorded in plasma (P) from *S. gregaria* after activation by laminarin, LPS and trypsin at different concentrations

*B. Effects of bacterial injection on total haemolymph Protein content*

In Bt-injected locusts, there was a significant decrease ($P<0.05$) in the total plasma proteins at all time intervals post-injection compared with the uninjected control .Laminarin/Bt-injected plasma showed significant increase ($P<0.01$) in the total plasma proteins at all times post-injection, compared with bacterial-injected insects (table 1). There was a significant increase ($P<0.01$) in total HL protein content at all experimental periods, except at 6 hr post-injection compared with the control.

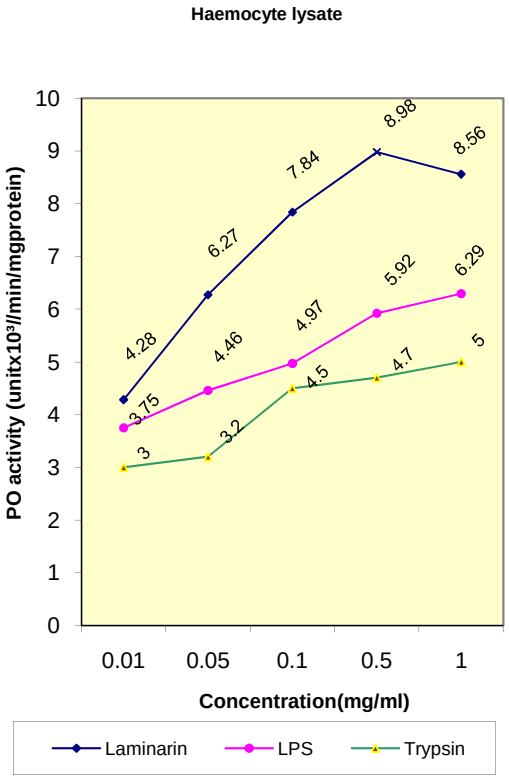


Fig. 2 Phenoloxidase (PO) activity recorded in haemocytes lysate (HL) from *S. gregaria* after activation by laminarin, LPS and trypsin at different concentrations

Total HL protein content increased significantly ($P<0.01$) in laminarin/Bt-injected insects at all periods post-injection, with a peak at 24 hr compared with Bt-injected insects (table 1). The levels of total (HL) protein contents mostly higher than those of (P) proteins at all times post injection of Bt or laminarin/Bt-injection. Following Bt-injection, the total plasma proteins decreased while the total HL proteins increased at all experimental periods. We can attribute the decrease due to intensive consumption of plasma proteins during growth and multiplication of bacteria. In addition to cessation of feeding observed in the injected locusts. These data agreed with the results of [23],[24].

Results obtained in the present study from testing activated haemolymph (Laminarin/Bt-injection) showed a rapid increase in the total P and HL proteins at all periods following injection. This result is supported by the findings of [25] who attributed such increase to the synthesis of new proteins associated with immunity. Total HL protein contents of *S. gregaria* mostly higher than those of P proteins at all times of post injection of Bt and laminarin/Bt , this may be attributed to the greater activity of PPO associated with haemocytes, this finding is explained by high levels of naturally occurring inorganic ions in plasma which prevent developing full PO activity.

*C. Electrophoretic analysis of the haemolymph proteins:*

The Electrophoretic protein profiles of both haemolymph preparations (P and HL) before (fig 3) and after activation (fig 4-7) of PPO system at 6, 12, 24 and 48 hr post-injection was done. SDS-Polyacrylamide gel electrophoresis (PAGE) has been extensively used as an excellent tool for the separation of proteins, lipoproteins and glycoproteins from both plant and animal sources [26]. Our results demonstrated that the number and kinds of protein fractions evidenced in haemocytes and plasma are highly variable, this observation supported by the work of [21]. These proteins may function in the immune responses, reproduction, storage components, or metabolic processes [27]. The haemolymph plasma (P) of the normal (uninjected) insects was separated electrophoretically into 15 protein bands with MW ranging from 200.416 – 36.624 KD (fig. 3). Bt-injection induced the synthesis of 20 protein bands; the band with MW 219.300 kD is a specific band for bacterial injection (fig .4). The haemocyte lysate proteins (HL) of normal (uninjected) insects resulted in the separation of 13 protein bands ranging from 197.015 - 42.112 kD (fig .3).After bacterial injection, only one new band with MW 85.578 kD, is a specific band for bacterial-injection. (fig. 6),

After laminarin/Bt-injection, the band with MW 70.154 KD is a specific band for laminarin/Bt-injection in plasma (fig 5). While, in HL, the band with MW 70.154 kD is as specific band for laminarin/Bt-injection (fig 7).

The disappearance of some haemolymph proteins after injection may be attributed to their involvement in the immune reactions. This is in agreement with the findings of [28] on the larvae of *Pobilia japonica* infected with *B. popilliae*, and [29] on the eri silkworm, *Philosamia ricini* infected with a flacherie disease.

The synthesis of new immune proteins may be a result of simultaneous induction of injected bacteria [30] and a fast rate of transcription [31]. This was confirmed by the study of [32] on *Drosophila* infected with bacteria. The author observed that a number of immune genes are activated at the level of transcription leading to the synthesis of antimicrobial peptides. Activators (figures 5 & 7 ) were found capable of changing the profile of proteins qualitatively; in plasma , bands with MW of 200.416, 63.251, 55.000 kD completely disappeared. While, in HL, bands with MW ranging from 127.520 - 51.155 KD completely disappeared. Disappearance of these bands may be attributed to injury and their involvement in the immune reactions or existence of PPO system. At the same time, one specific band was recorded in both haemolymph (P & HL) preparations after activation, this band with MW of 70.154 kD, and may be a result of simultaneous induction and formation of active PO. This indicates that this band is prominent amongst proteins synthesized during formation of PO and that it may contain PO. This protein band approximates the band suggested by [10] of 70 kD for PO formation from plasma (not from haemocytes) of *Bombyx mori*.

In conclusion, our results suggest that, the disappearance of different bands from P & HL after activation may indicate that the activation system of PPO is different in plasma than in HL.

TABLE I
TOTAL PLASMA (P) AND HAEMOCYTES LYSATE (HL) PROTEIN CONTENT (MG/ML) OF *S. GREGARIA* DETERMINED AT DIFFERENT TIME INTERVALS POST-INJECTION WITH BT, AND LAMINARIN/BT

| Hours post-injection | Total protein (mg/ml) (mean ± SE) | | | |
|---|---|---|---|---|
| | Plasma | | Haemocytes lysate | |
| | Bt | laminarin/Bt | Bt | laminarin/Bt |
| 6 | 28.1* ±0.5 | 29.5 ±1.04 | 33.8 ±0.80 | 50.3 ±2.35** |
| 12 | 28.6 ±0.2* | 40.9 ±0.6** | 44.5 ±1.60** | 64.8 ±1.66** |
| 24 | 31.8 ±0.7* | 45.1 ±1.5 ** | 50.7 ±2.9** | 70.1 ±3.49** |
| 48 | 28.2* ±1.6 | 39.2 ±1.8** | 41.5 ±1.7** | 60.0 ±1.15** |
| Control ( Uninjected) | 40.0±2.577 | | 32.2±1.56 | |

* Significance (P <0.05), ** Significance (P <0.01)

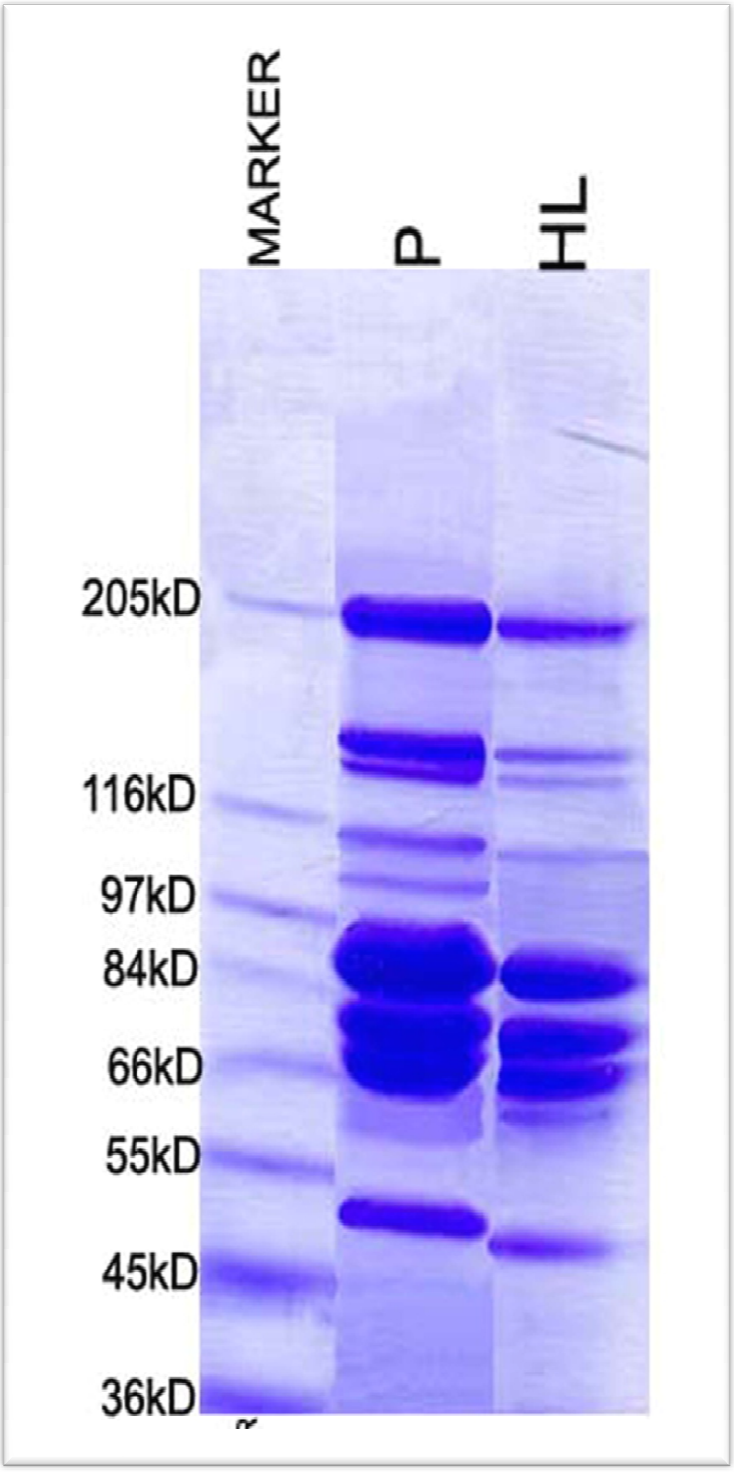


Fig. 3 Haemolymph protein banding patterns of uninjected (control) *S.gregaria* separated using SDS-PAGE technique.(where P:Plasma , HL: haemocyte lysate)

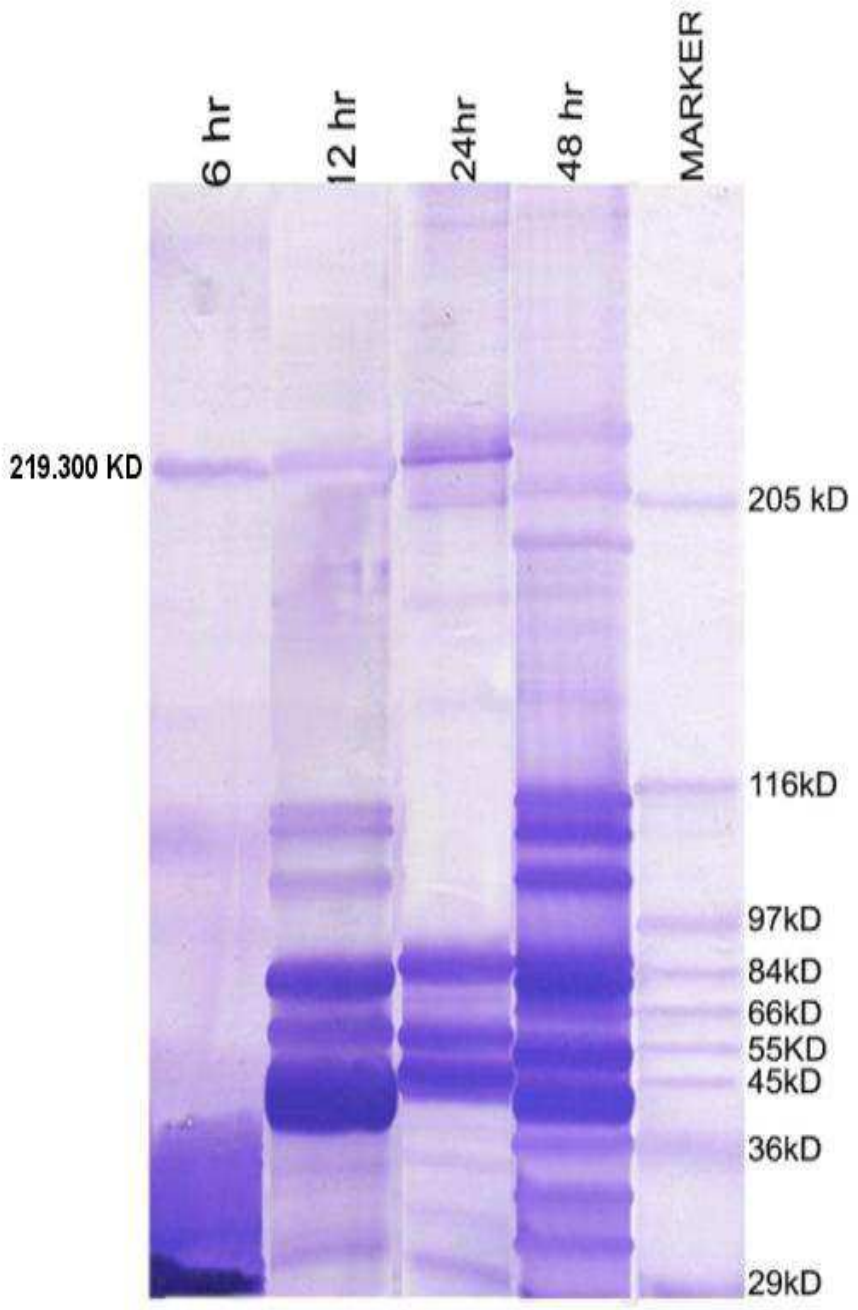


Fig. 4 Changes in the plasma (P) protein banding patterns of *S. gregaria* separated using SDS-PAGE technique post-injection of Bt at 6, 12, 24, and 48 hr

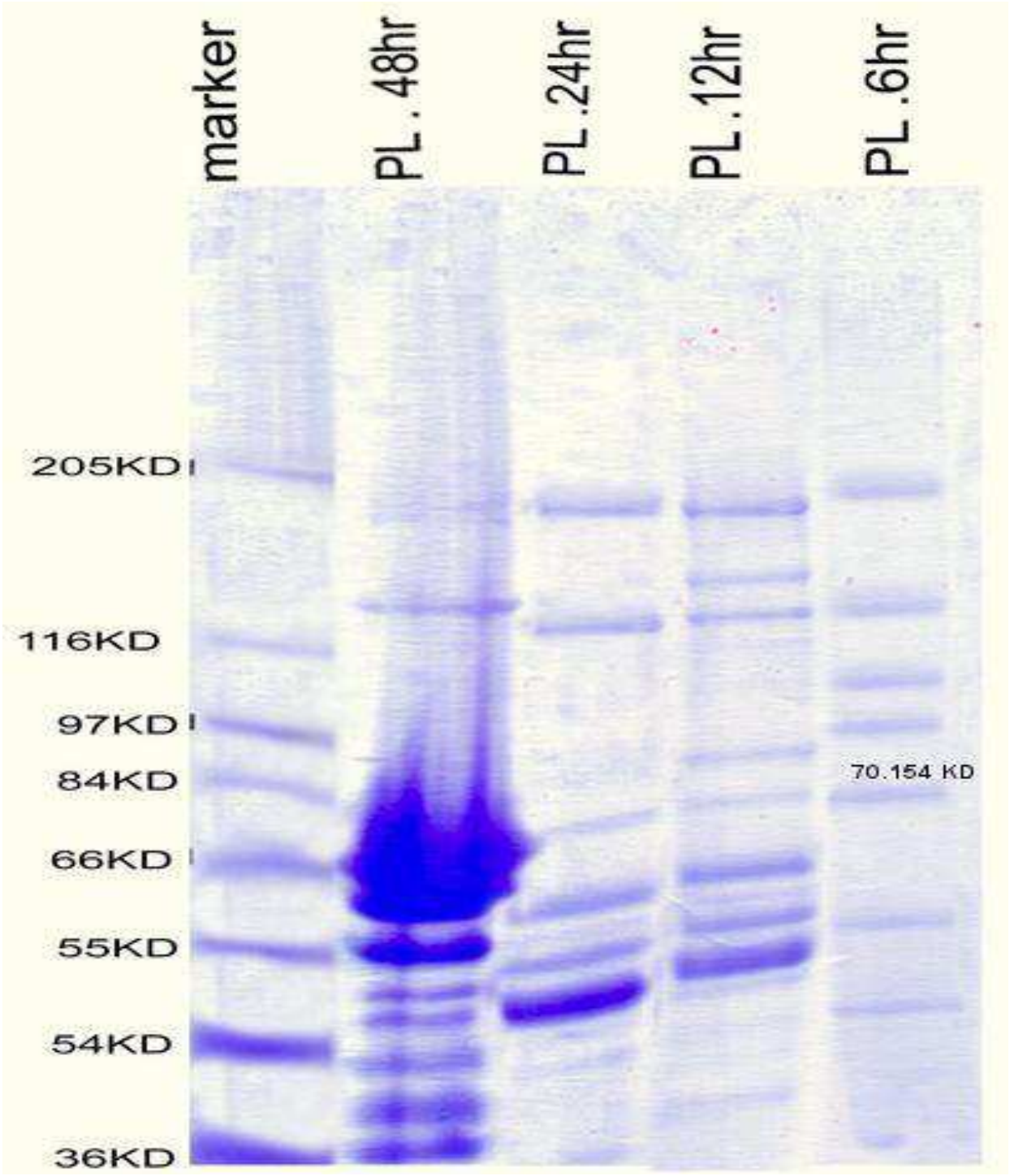


Fig. 5 Changes in the plasma(P) protein banding patterns of *S. gregaria* using SDS-PAGE technique post-injection of laminarin/Bt at 6, 12, 24, and 48hr

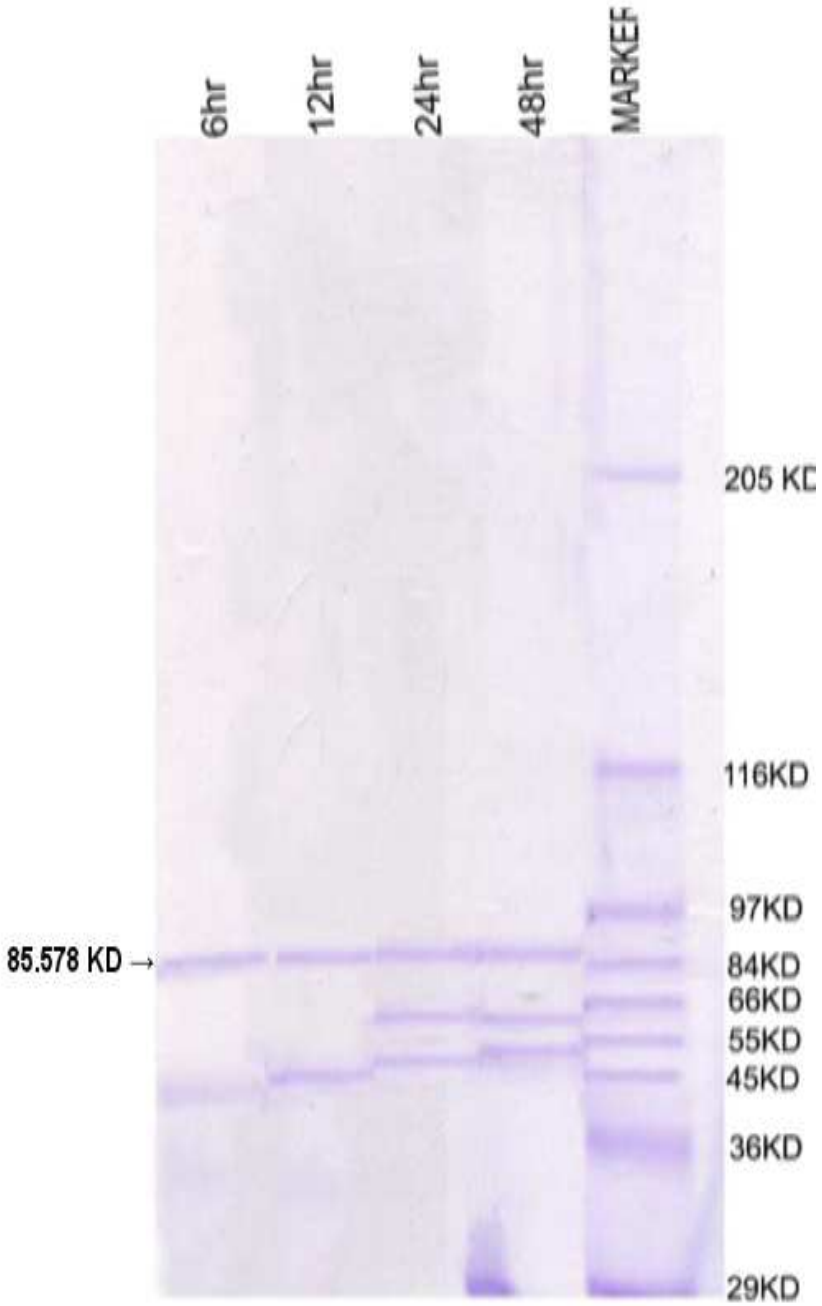


Fig. 6 Changes in the haemolymph lysate ( HL) protein banding patterns of *S. gregaria* separated using SDS-PAGE technique post-injection of Bt at 6, 12, 24, and 48 hr

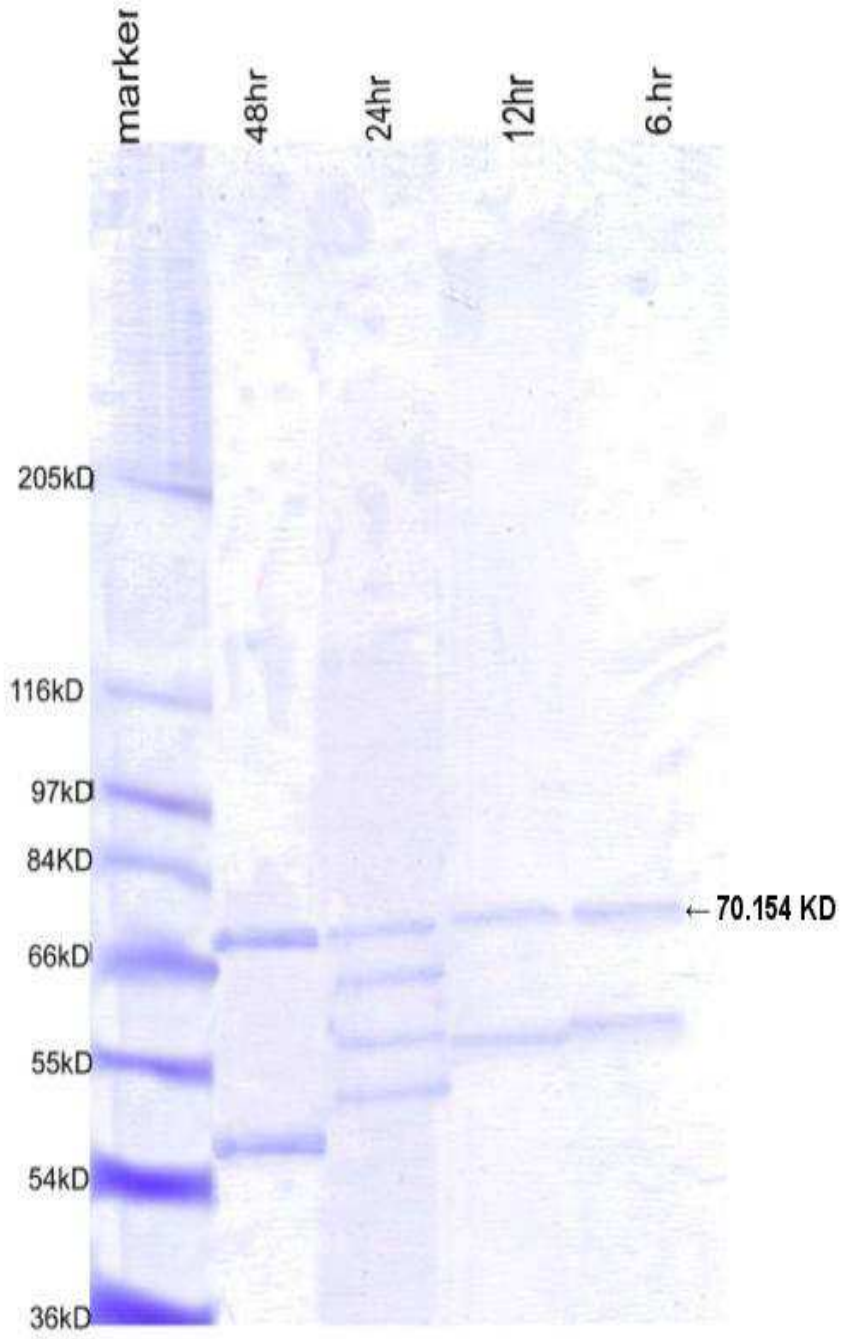


Fig. 7 Changes in the haemocyte lysate (HL) protein banding patterns of *S.gregaria* separated using SDS-PAGE technique post-injection of laminarin/Bt at 6, 12, 24, and 48hr

## REFERENCES

[1] Meshrif, W.S. and Barakat, E.M.S. (2002). Cell mediated immunity in the Locust, Shistocerca gregaria against the bacterium, Bacillus thuringiensis. J. Egypt. Acad. Soc.Environ. Develop., 2(1): 117-130.
[2] Barakat, E.M.S.; Meshrif, W.S. and Shehata, M.G. (2002). Changes in the haemolymph of the desert locust, Schistocerca gregaria after injection Bacillus.
[3] Ratcliffe, N.A. and Rowley, A.F. (1979). Role of hemocytes in defense against biological agents. In Insect Hemocytes: Development, Forms, Functions and Techniques, ed. AP Gupta, pp. 331–414. London: Cambridge Univ. Press (GR & PLS phagocytosis).
[4] Bodine, J.H. and Allen, T.H. (1937). Enzymes in ontogenesis (Orthoptera): XV. Some properties of protyrosinase. J. Cell. Physiol., 18, 151-160.
[5] Ratclifffe, N.A.; Leonard, C. and Rowley, A.F. (1984). Prophenoloxidase Activation: Non-self recognition and cell cooperation in insect immunity. Sci., 226(2): 557-559.
[6] Leonard, C.; Ratcliffe, N.A. and Rowley, A. F. (1985). The role of prophenoloxidase activation in non-self recognition and phagocytosis by insect blood cells. J. Insect Physiol., 31(2): 789-799.
[7] Brookman, J.L.; Ratcliffe, N.A. and Rowley, A.F. (1988). Optimization of a monolayer phagocytosis assay and its application for studying the role of the prophenoloxidase system in the wax moth, Galleria mellonella. J. Insect Physiol., 34: 337-345.
[8] Anggraeni, T. and Ratcliffe, N. A. (1991). Studies on cell-cell cooperation during phagocytosis by purified haemocyte populations of the wax moth, Galleria mellonella. J. Insect Physiol. 37(6): 453-460.
[9] Söderhäll, K. and Aspan, A. (1993).Prophenoloxidase activating system and its role in cellular communication. In: Insect Immunity (Ed.J.P.N.Patak) pp.113-129.Oxford & IBH Publishing Co.Ltd.
[10] Ashida, M., Dohke, K., 1980. Activation of prophenoloxidase by the activating enzyme of the silkworm Bombyx mori. Insect Biochem. 10: 37–47.
[11] Dularay, B. and Lackie, A.M. (1985). Haemocytic encapsulation and the prophenoloxidase-activation pathway in the locust Schistocerca gregaria Forsk. Insect Biochem. 15: 827-834.
[12] Brehélin, M., Drif, L., Baud, L. and Boemare, N. (1989). Insect haemolymph: Cooperation between humoral and cellular factors in Locusta migratoria. Insect Biochem. 19(3): 301-307.
[13] Ashida, M. and Soderhall, K. (1984). The prophenoloxidase activating system in crayfish. Comp. Biochem. Physiol., 77(B): 21-62.
[14] Preston, W. F. and Taylor, R. L. (1970). Observation on the phenoloxidase system in the haemolymph of the cockroach Leucophaea maderia. J. Insect Physiol., 16: 1729-1744.
[15] Ashida, M. and Yoshida, H. (1988). Limited proteolysis of prophenoloxidase during activation by microbial products in insect plasma and effect of phenoloxidase
[16] Huxham, I. M.; Samuel, K.D.Z.; Heale, J.B. and MacCorkindale, N.J. (1989). In vivo and in vitro assays for pathogenicity of wild type and mutant strains of Metharhizium anisopliae for three insect species. J. Invertebr. Pathol. 53: 143 – 151.
[17] Aso, Y.; Karmer, K.; Hopkins, T. and Lookhart, G.L. (1985). Characterization of haemolymph protyrosinase and cuticular activator from Manduca sexta. Insect Biochem., 15: 9-17.
[18] Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem., 72, 248–254.
[19] Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Natur., 227:680–68
[20] Söderhräll, K. (1982). Prophenoloxidase activating system and melanization – a recognition mechanism - of artropods ? A review. Dev. Comp. Immunol., 6: 601-611.
[21] Brehélin, M. (1979). Haemolymph coagulation in Locusta migratoria: Evidence for a functional equivalent of fibrinogen. Comp. Biochem. Physiol., 62(B): 329-334.
[22] Evans, J. J. T. (1968). The distribution of prophenoloxidase and its activator in pupa of the Chinese oak silkmoth, Antheraea pernyi. J. Insect Physiol., 14: 107-119.
[23] Sabbour, M. (2001). Biochemistry of haemolymph of Earias insulana larvae treated with Bacillus thuringiensis and Beauveria bassiana. J. Egypt. Ger. Soc. Zool. 36(E): 19-27.
[24] Meshrif, W.S.(2008) Defense reactions and biochemical changes in the haemolymph of Spodoptera littoralis (Boisduval) (Lepidoptera: Noctuidae) following infection with entomopathogenic hyphomycete fungi Ph.D Thesis, Sci. Fac., Tanta Univ..
[25] Hoffman, D.; Hultmark, D. and Boman, H.D. (1981). Insect immunity : Galleria mellonella and other Lepidoptera have cercopin-p9- like factors active against Gram negative bacteria. Insect Biochem., 11: 573-548.
[26] Zacharius, R.M.; Zell, T.E., Morrison, J.H. and Woodlock, J.J. (1969). Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem., 30: 148-152.
[27] Miranpuri, G.S., Bidochka, M.J. and Khachatourians, G.G. (1991). Morphology and chemistry of haemocytes and analysis of haemolymph from Melanoplus sanguinipes (Orthoptera: Acrididae). J. Econ. Enotomol. 84(2): 371-378.
[28] Bread, R.L. (1945). Studies on the milky disease of Japanese beetle larvae. Conn. Agn. Stn. Bull., 491: 505-583.
[29] Poonia, F. S. (1979). Haemolymph protein in 5th instar of eri silkworm, Philosamia ricini after infection with a flacherie disease. Indian J. Seric. 18(1): 43-47.
[30] Rasmusom, T. and Boman, H. G. (1979). Insect immunity. V. Purification and some properties of immune protein P4 and haemolymph of Hyalophora cecropia pupae. Insect Biochem. 9: 259-264.
[31] Sun, S.-C. (1992) Ceoropia immunoresponsive factor, an insect immunoresponsive factor with DNA-binding properties similar to nuclear factor NF-kB Eur.J.Biochem., 204,885-892.
[32] Engström, Y. (1999). Induction and regulation of antimicrobial peptides in Drosophila. Dev. Comp. Immunol., 23: 345-358.