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Haemolymph Proteins on Stressed Cockroach *Periplaneta Americana*

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Abstract

Immune responses in insects are humoral and cell mediated and the haemolymph proteins are found to be involved in humoral immune responses. In this experiment cockroaches were divided into two groups as control and wounded and the haemolymph samples were collected at different time intervals and subjected for quantitative analysis of proteins, proteolytic activity and protein profile. Haemolymph protein concentration reduced initially after injury then they recovered after 24 hrs. Proteolytic activity was found to be the highest during the first 24hrs of the wounded insects. Seven protein bands were observed in PAGE gel electrophoresis of the wounded and control cockroaches. A 115 kDa protein band was found to appear in the wounded *P.americana* after 8 hours is an important finding of this experiment. Some of the protein bands were reduced in the wounded cockroach and another important finding was protein retaining to normal level in the haemolymph after 24hrs and 80 and 76kDa protein bands were thicker in this group. Cockroach recovering after wounding is revealed after 8hrs after wounding.

Key words: haemolymph proteins, insect proteins, wound healing, Cockroach.

1. Introduction

Insects, the most widespread metazoans on earth, can withstand extreme conditions of climates and pathogens. They have a well-developed innate immune system that allows general and rapid responses to infectious agents while they lack an acquired immune system. The primarily barriers that act against pathogens are cuticle, gut, trachea and the tissues that are difficult to be penetrated and their immune response is originated from the fat body and haemocytes.

Fat body the largest organ of the insect body cavity is the major site for the production and secretion of antimicrobial peptides (Hofmann, 2003). Haemolymph are the fluid fraction that transport nutrition, hormones and metabolic waste and contains elements of the immune system while the cellular components are haemocytes (Gillot, 1995). Haemocytes that circulate in the insect haemolymph are derived from the stem cells that differentiate into specific lineages and haemocytes types are not common in all insects and differ among species (Charalambidis *et al.*, 1995; Meister and Lagueux, 2003). Different types of haemocyte have important role in the protection of insects against invading pathogens and parasitoids.

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The humoral immune responses is based on the products of characterized immune genes induced by microbial infection and encode antimicrobial peptides, which are synthesized predominantly in fat body and released into haemolymph (Hofmann, 1955; Gillespie *et al*., 1997; Nakatogawa *et al*., 2009; Shia *et al.*, 2009). Haemocytes and epithelial layers of the integuments and the gut are also sites for the synthesis of such molecules. These genes are activated only after infection (Hofmann, 1995; Engstrome, 1998).

In addition, humoral immune responses include activation of enzymic cascades that regulate coagulation and melanization of haemolymph, and production of reactive oxygen and nitrogen species (Gillespie *et al*., 1997; Bogdan *et al*., 2000; Nappi and Vass, 2001; Hofmann, 2003; Mavrouli *et al*., 2005). Cellular responses are performed by haemocytes and include phagocytosis, nodulation and encapsulation (Schmidt *et al*., 2001; Nappi *et al*., 2004; Lamprar *et al*., 2005; Mavrouli *et al*., 2005, Sideri *et al*., 2007).

Antimicrobial peptides play an essential role in fighting against invading pathogens in insects, especially those that lack an adaptive immunity (Toke, 2005). Normally due to microbial infection, antimicrobial peptides are synthesized in fat body or certain haemolymph cells of insects or body injury, and then rapidly released into haemolymph to kill microorganisms (Brivio *et al*., 2006; Yu *et al.*, 2010; Yakovlev 2011).

Insects are remarkably resistant to bacterial infections by detecting of bacteria, a complex genetic cascade is activated, which eventuates in the production of a series of antimicrobial peptides and is released into the haemolymph (Eleftherianos *et al*., 2006 and Eleftherianos *et al.*, 2007). These antimicrobial peptides are mostly small amphipathic, cationic molecules (Gao and Zhu, 2013). They have an effect on membrane of microbial cell changing permeability or by breakdown bacteria membrane (Toke, 2005;Dai *et al*., 2008 and Huang *et al*., 2008). In addition, insect peptides may affect the synthesizing of DNA or protein as well as the protein folding of the bacteria (Otvos, 2000; Huang *et al*., 2008; Shen *et al*., 2010 and Bang *et al*., 2012).

Haemolymph of insects contain proteins which are of immunological importance for example some insects can synthesize inducible antibacterial peptides such as lysozyme which is also constitutive like lipopolysaccharide (LPS) - binding protein which was isolated from the haemolymph of the American cockroach (*Periplaneta americana*) (Ha lee *et al*., 2007; Fiolka, 2008). This protein acts as an opsonin (Jomori and Natori 1992; Hashimoto *et al*., 2009; Kim *et al.*, 2010). Generally, five major groups of antibacterial peptides have been introduced (Hultmark, 1993) including cecropins, insect defenis, attacin-like (glycine-rich) proteins, proline rich peptides and lysozymes. The mechanisms of some these peptides have been studied extensively (Sawa and Kurahashi 1999, Innler and Bulet 2005, Wang *et al*., 2009).

Insects are fascinating in their ability to induce antimicrobial proteins, creating chemotherapeutic compounds in their haemolymph in response to outside stimuli (Natori, 1994). Antimicrobial peptides are important in the first line of host defense system of many animal species (Boman, 1995). As a preliminary study on proteins role in defense mechanism on wound healing with relation to time was made on cockroach *Periplanata americana*.

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2. Materials and Methods

2.1 Haemolymph Collection

Adult male *P. americana* cockroaches were collected during night from the kitchen, bathrooms etc. They are reared in a container at laboratory condition. And the haemolymph were collected after 1, 4, 8, 12, 24 hrs. Haemolymph samples were collected by cutting the antenna using the sterilized razor blade / legs and centrifuge by keeping them upside down. A sample of five cockroaches was used per set of experiment for collecting haemolymph. Equal volume of extraction buffer was added. The protein content were analyzed from the collected samples by following Lowry *et al.* (1951) method and BSA used as standard. proteolytic activity (Briegel and Lea, 1975) estimated using 1% casein as substrate incubated with the extract for 1hrs at 37ºC and L-tyrosine used as standard. Protein bands were analyzed on SDS PAGE following Laemmli, (1970) method. Statistical analysis the experimental results were subjected to ANOVA and significant of the values were indicated at 0.05 level.

3. Results

The haemolymph concentrations as well as the protein profile bands changed in the stressed cockroaches *P. americana* when compared with control groups. A sudden decrease in the haemolymph protein level was observed on stressed cockroaches immediately after injury that is 1hr after wounding (Fig 1). The mean protein values of control cockroaches was found to be 0.6 g % and after one hour wounding the protein value drastically reduced to 0.2 g %. A similar change was also noticed during fourth hour of wounding and they were statistically significant. After 8 hrs the wounded cockroach haemolymph showed the proteins volume as 0.5 g%. A significant increase in the haemolymph protein was noted 12hrs after injury; the increase was upto 0.8g %. After 24hrs the protein concentrations matches with the control as 0.6 g%.

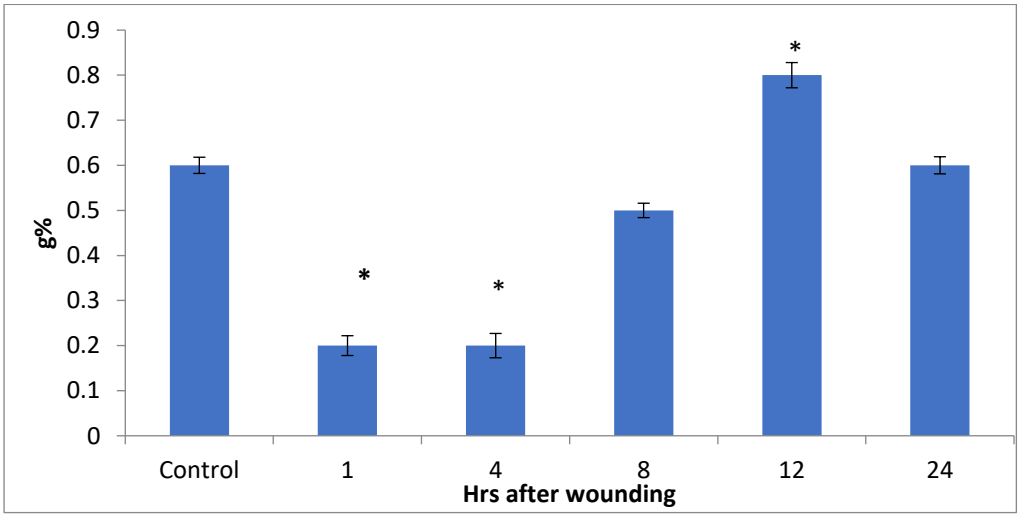

Figure-1. Protein in g% at different hours after wounding in the haemolymph of *P.americana.*

Mean ±S.D, * $p<0.05$

The haemolymph samples were analyzed for proteolytic activity, after 1 hr the control and wounded cockroach showed the rate of proteolytic activity as 0.2g%/ hr. After 4hrs the rate of

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activity remained decreased and it was statistically significantly. A rise in the rate of activity was noted after 8 hrs (0.2g %/hr.). A shift in 12hrs after wounded was observed and it was a drastical decrease compared to control. The rate of enzyme activity rose to 0.3g% /hrs during 24hrs after injury (Figure-2).

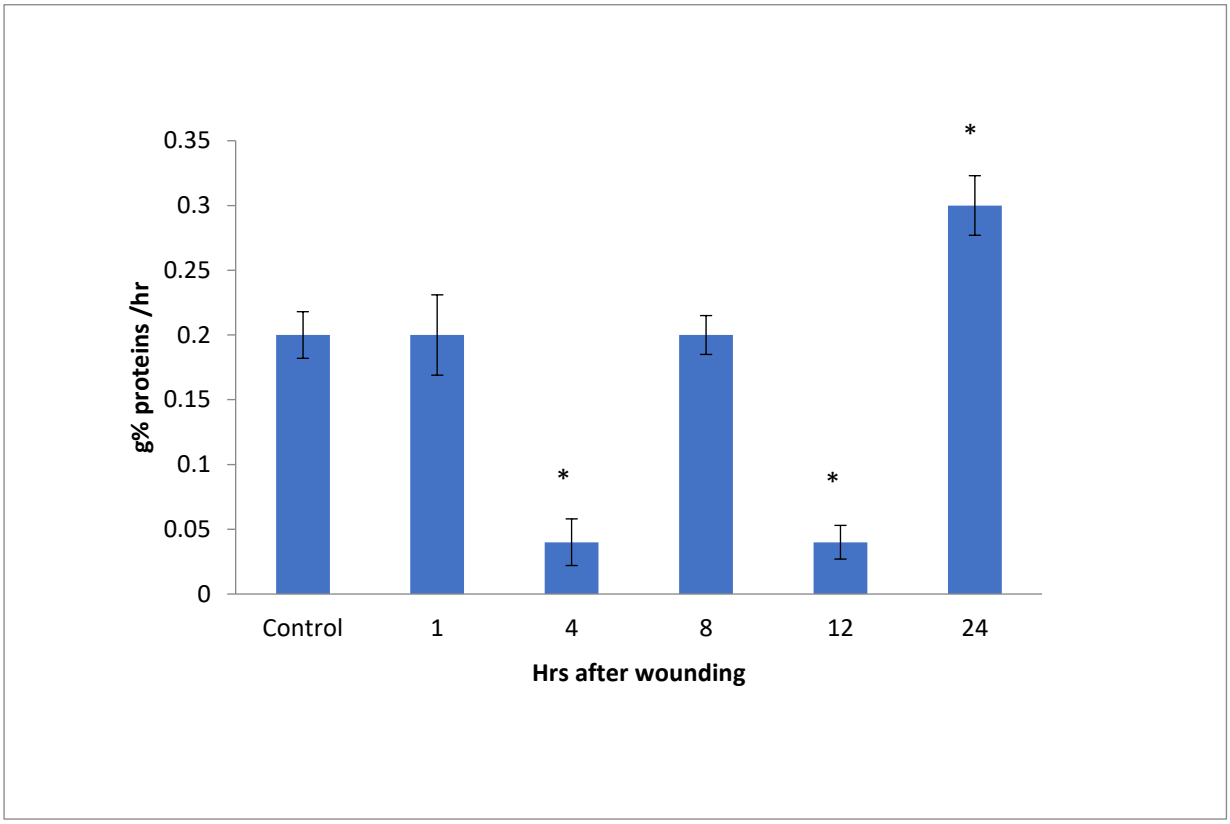

Figure- 2 Proteolytic enzyme activity (g%/hr) in the haemolymph of *P. americana* at different hours after wounding. Mean ±S.D, * p<0.05

3.1 Protein Profile

The protein PAGE profile of control and wounded *P. americana* showed seven prominent bands stained with coomassive brilliant blue (Fig 3). The prominent bands of both wounded and control proteins are 35, 76, 80, 102, 160, 200, 240 kDa proteins. 76, 80 kDa bands were found as doublets and they were the prominent one of all other protein bands. 35 kDa protein was found in all the groups which was the lowest molecular weight protein bands observed. A thin band of 102, 160, 200, and 240 was also observed in all the samples. 1 hr (lane 2) after wounded the haemolymph protein bands were thin compared to other samples. Similarly, lane 5 also showed thinner bands, especially 76 and 80 kDa proteins. In lane 4 a thin band of 115 kDa protein band appeared 8hr after wounded, which is not detected in other experimental or control groups. In lane 6 that is after 24hrs 80 and 76kDa protein bands were more prominent and it was thicker and compared to all other groups.

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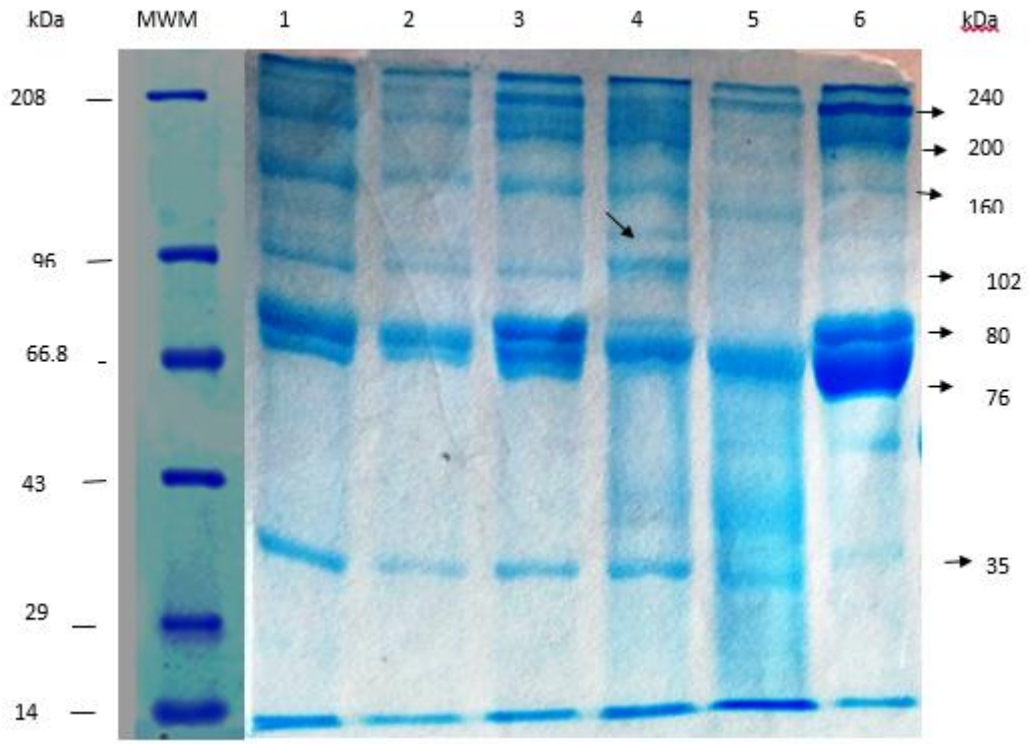

Figure- 3. The protein profile of the cockroach *P. americana* haemolymph. MWM molecular weight marker. Lane 1 control, Lane 2 1hr, Lane 3 4hr, Lane 4 8hr, lane5 12hr and Lane 6 24 hrs after wounding. kDa kilodalton. Arrow mark 115kDa protein band.

4. Discussion

The protein profile of the cockroach *P.americana* haemolymph shows seven different haemolymph protein bands of molecular weight 240, 200, 160, 80, 72, 35 kDa respectively in both control and wounded. Similar protein bands were analyzed by George *et al*, (1987) on American cockroaches as 220, 162, 115,102, 95 and 45kDa.

Stress related alteration in the haemolymph protein bands in the wounded cockroach is evident from the reduction in the amount of protein immediately after wounding. This change in the haemolymph protein pattern might be due to protective nature of immune system of the animal against injuries, pathogen and healing process. Antibacterial proteins from cockroaches were isolated from their haemolymph by Bassori *et al*, (2016) and the molecular weight of the proteins was determined as 72kDa and 62 kDa. In this study 80 and 72 kDa protein was found to be reduced after wounding and this protein may be related with wound healing as well as active concerning with antibacterial activity, since the bands were prominent after 24hrs as they recover. Because, insects antimicrobial peptides are found to play an important role against invading pathogens (Toke, 2005). .

The haemolymph protein reduction in the wounded cockroaches (1, 4 and 8 hrs after wounding) can be correlated with healing process because the cells and the lymphs could have rushed towards the wounded site for healing purpose. Peptides act as antibacterial molecules, small peptides of 20-50 residues kDa proteins actively involved in antimicrobial activity was isolated

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from the large precursor proteins of insects, similarly 34-51 peptide residues are reported as insect defensin (Yu *et al*., 2014).

Natural microbial infection takes place in all living organisms when they are exposed to atmospheric air on open wounds. In higher animals complicated immunological reactions takes place to overcome such microbial invasions. Among insects, the antimicrobial peptides formed after infection are synthesized in fat body or in the haemocytes and released into haemolymph to destroy the microbes (Brivio *et al.,* 2006; Yu *et al.*, 2010; Yakavlev, 2011). George *et al*, (1987) showed 102 molecular weight proteins the most likely candidate for immune mediators was reported in cockroach.

Appearance 115 kDa protein band during 8 hrs after wounding may be such a kind of proteins involved in healing or act as antibacterial agent. 115 kDa protein band was reported in cockroach when immunized with soluble proteins (George *et al* 1987). 80 and 72 kDa protein consistently increased in 24 hrs after wounding, similarly haemolymph proteins were also found to increase during these hours. This can be interpreted that the increase in the concentration of this proteins due to increase in their rate of production. A reduction in 80 kDa protein and appearance of 115 kDa peptides can be correlated that 80 kDa peptides production may be reduced or stopped instead of the production of 115 kDa proteins surged for healing purpose. A similar 115 kDa peptide was found to be exhibited only on immunized female cockroaches (George *et al*., 1987). In *P. americana* two LPS-binding proteins called C- type lectins which has a carbohydrate recognition domain and act as opsonin was identified to be 186, 146 kDa peptides (Jomori *et al*., 1990 and Marmaras *et al*., 1994).

102 kDa protein which was the best active factor of immune functions could be used by the insects during 12 and 24 hrs because the band was not found during those hours. The absence or reduction in the amount of protein can also be related with proteolytic activity, and was found to be maximum during 24 hrs after wounding. The proteolytic enzyme may be associated with the immunologic function like wound healing in this insect. Because this enzyme not only associated with digestive process but also in the activation of pro enzymes and liberation of physiologically active peptides, complement activation and inflammation processes (Neurath, 1984).

The insect may utilize this enzyme for their survival because the enzyme involvement in immune response in Diptera and Lepidopterans was reported (Ashida and Yamazaki, 1990). Gregoire (1974) stated that during wound healing haemocytes aggregate at wound sites forming clot formation. The reduction in the protein after wounding might be the haemocytes along with haemolymph got aggregated near the wounded site (Kotani *et al*., 1995 and Gregoire, 1974). Because the damaged epidermal cells were found to release a partially purified protein called haemokinin that induces rapid aggregation of injury haemocytes in *H. cecropia* (Cherbas *et al*., 1973). In medfly *Ceratitis capitate*, a protein with molecular mass of 47 kDa secreted by hemocytes was found to be involve in nodule formation and aggregation to entrap bacteria (Marmaras *et al*., 1994). So this low molecular weight protein (35kDa) which was found to reduce after wounding may be involved in nodule formation.

` Proteolytic enzymes were found to be involved in wound healing in animals. Wound healing can be accelerated by removing necrotic tissue (Mekkes, 1998). Proteolytic enzymes when activated serve many functions in normal as well as pathological situations. In

particular they are involved in the regulation of cell maturation and multiplication; collagen synthesis and turnover; the development and removal of the perivascular fibrin cuffs found in venous insufficiency and leg ulceration as well as the removal of dead tissues following inflammation in higher animals (Sinclair and Ryan, 1994). Seemingly the wound healing process started well after 12 hr and the level of proteolytic enzyme increased significantly after 24hr. Insect immune system involving haemolymph proteins in wound healing as one of the immunological functions is evident in cockroach *P.americana*.

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