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RESEARCH ARTICLE

INNATE IMMUNE RESPONSE OF *BOMBYX MORI* AGAINST BACTERIAL INFECTION

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ABSTRACT

In this study, we elevate the innate immune response of *Bombyx mori* against *Staphylococcus aureus* infection. The fifth instar *Bombyx mori* larvae was used and infected by intrahaemocoelic injection of bacterial sample. The haemolymph was collected from the infected and control group larvae at 24 hours of post infection and stored at -4°C in eppendorf tubes to use. Phenoloxidase a key component of the insect innate immune system.

The activity of phenoloxidase was increased in the haemolymph of infected larvae at 24 hours of post infection. The total protein concentration in the haemolymph was determined according to the Lowry method and total haemocyte population in the haemolymph was estimated by trypan blue test. Protein content and total haemocyte population increased in infected larvae when compared to control larvae at 24 h post infection with bacteria. The haemolymph was placed directly over a sterilized glass slide and slides were prepared by Giemsa staining method. Giemsa stain confirmed that aggregation of haemocytes in the haemolymph of infected *Bombyx mori* larvae.

INTRODUCTION

Insects possess immune system to eliminate invading pathogens and parasites (Lemaitre and Hoffmann, 2007). There are two types of immune systems in insects, first one is non-specific immunity, which consists of structural and passive barriers like cuticle, gut physio-chemical properties and peritrophic membrane (Narayanan, 2004). The second one is specific immune system involving cellular and humoral immunity, which includes the activation of phenoloxidase cascade, phagocytosis, nodulation, encapsulation especially with reference to, bacteria, fungi, protozoa including nematode invaders (Gillespie and Kanost, 1997). Antimicrobial proteins appear to be multi components of the innate mechanisms existing in *Bombyx mori* (Kangayam and Minoru, 2002), most of which are produced in the fat body and haemocytes released into the haemolymph of the insect, synergistically to kill the invading microorganisms (Hoffmann *et al*., 1995).

The injection of bacteria into insects haemolymph resulted in the synthesis of antibacterial proteins, many antibacterial proteins have been isolated from various insects and at least four types (cecropin, attacin, lebocin and moricin) have been identified in *B. mori* (Kangayam and Minoru, 2002). Injection of bacteria into the haemolymph, granulocytes release sticky material and the haemocytes and bacterial cells clump together, resulting in the formation of nodules (Ratcliffe and Gagen 1977; Rowley and Ratcliffe 1981). Plasmatocytes and granular cells are the two types of haemocytes most often observed in phagocytosis, encapsulation and nodule formation (Gillespie *et al* 2000, Togo *et al*. 2000). Nodule formation consists of microaggregates of haemocyte entrapping large numbers of bacteria within a mucopolysaccharide matrix. The process ends with the melanization, which leaves darkened nodules attached to various organs of the insect (Cleonor and Silva, 2002).

Activation of pro-phenol oxidase (proPO) in insects and crustaceans is important in defense against wounding and infection and kill pathogens and can also be used for synthesis of melanin to seal wounds and encapsulate parasites (Haobo *et al*., 1998). Phenoloxidase produces indole groups, which are subsequently polymerized to melanin. The enzymatic reactions in turn produce a set of intermediate products such as quinones, diphenols, superoxide, hydrogen peroxide, and reactive nitrogen intermediates, which are important during defense against bacterial, fungal, and viral agents (Isaac and Alex, 2012). Insects possess an antioxidant defense system that consists of both enzymatic and non enzymatic components. The enzymatic components are superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase. (Karmabeer *et al*., 2013). The non-enzymatic

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components consist of small organic molecules such as reduced glutathione and vitamin C (Krishnan *et al*., 2009; Zhao and Shi, 2009; Buyukguzel *et al*., 2010). Earlier studies on the reactive oxygen species and antioxidant defense mechanism in insects suggested that there exists a regulatory mechanism for balancing prooxidants and antioxidants (Ahmad 1992). Reactive oxygen species modulate immunity against pathogens (Molina *et al*., 2008). Bacterial infection caused intestinal hydrogen peroxide (H_2O_2) and nitric oxide (NO) levels to increase significantly by 8 and 16 h post-infection. Insect gut epithelial cells produce reactive oxygen species (ROS) and antimicrobial peptides (AMPs) to protect hosts from pathogenic microorganisms (Zhang *et al*., 2015).

MATERIALS AND METHODS

Experimental design

In the present study 5th instar *Bombyx mori* larvae were used and divided in to two groups, each group consisting of 20-30 larvae. One group was infected with the bacterium (3×10^5 cfu/ml to 1×10^8 cfu/ml) by intrahaemocoelic injection of bacterial sample. A similar number of larvae were injected with distilled water and considered as a control. Both control and infected larvae were reared under room temperature. The time of infection was recorded and the haemolymph was collected from the infected and control group larvae by cut were made on the proleg cuticle at 24 hrs post infection. The collected haemolymph was centrifuged at 2000 rpm for 15 min at 4 ºC. The resulting supernatant was stored at -4°C in eppendorf tubes until use.

Phenol oxidase assay

The phenoloxidase activity was determined by the method of Horowitz and Shen, (1952). The reaction mixture consisted of 1ml of 0.02 M, 4-di hydroxyphenylalanine (DOPA), 3.9 ml of 0.1M phosphate buffer, pH 6.0 and 0.1 ml of enzyme solution. After incubation at 30^o C for 5 minutes the color intensity of dopachrome was measured at 490 nm filter. One unit of enzyme was defined as the amount causing increase in absorbance of 0.01 under the above condition.

Estimation of protein in the haemolymph

The total protein content was determined with Folin Ciocalteau's reagent according to the Lowry *et al*., (1951). Aliquot of test sample was made up to 1.0 ml with distilled water and 5.0 ml of alkaline solution was added, mixed thoroughly and allowed to stand at room temperature for 10 min. Then 0.5 ml of Folin-Ciocalteau's reagent was added rapidly with immediate mixing and the intensity of the colour developed was read at 750 nm after 30 min. Values are calculated from the standard graph.

Determination of haemocyte viability

The haemocyte suspension (0.2 ml) was mixed with 0.3 ml of PBS and 0.5 ml of trypan blue in a small test tube. An aliquot is then placed on hemocytometer and count number of viable cells was made under the microscope. The plasma membrane of the viable cells does not permit the entry of electrolyte dye substance. This phenomenon is used to distinguish dead cells from living haemocytes.

Giemsa staining

Silkworms were dipped into hot water (60˚C for 1 to 10 min) and the posterial leg was cut and a drop of haemolymph was placed directly over a sterilized glass slide and subsequently a cover slip was placed over it to make a thin film. The film was air dried before staining. Air dried film was immersed in Giemsa solution for 20 min to 2 h. The dried films were rinsed with distilled water and then immersed briefly in water to which few drops of lithium carbonate were added. Further the thin film of slide was rinsed in distilled water again and to which a few drops of diluted HCL were added. Immediately the haemocytes present on thin film appear as blue particles. Again the slide was rinsed in distilled water and blotted. The dried slide was mounted in Canada balsam

RESULTS AND DISCUSSION

Phenoloxidase (PO) activity

The phenol oxidase (PO) activity increased in the infected group (0.66 U/µl/min) when compared with control group (0.46 U/µl/min) at 24 hours post infection (fig 1). PO is an important innate immunity protein in the defense mechanism of insects. PO is the one of the key enzyme activated in the haemolymph of many invertebrates in response to immune challenge or wounding and including pathogen-associated molecular pattern molecules like bacterial peptidoglycan via prophenoloxidase (PPO) cascade (Gillespie *et al*., 1997). PPO converted in to Phenoloxdiase (PO), PO is involved in the conversion of phenols to quinones and the subsequent production of melanin (Gurmeet Bali and Sanehdeep, 1997). PO is an enzyme that can induce melanization around invading pathogens (Lemaitre and Hoffmann, 2007). Intermediates produced in the melanization process can kill bacteria directly (Zhao *et al*., 2007). Injection of bacteria into the hemocoel stimulated the activation of the proenzyme and increased the level of the phenoloxidase activity. Phenoloxidase bind to the surface of bacteria and increase the binding of haemocytes to the bacteria to in vitro (Silva *et al*. 2000).

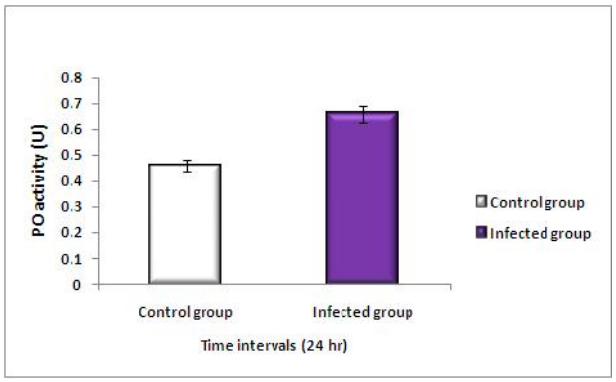

Fig 1 PO activity in the haemolymph of control and infected group.

Estimation of protein in the haemolymph

The protein concentration increased in the haemolymph of infected group (34.65 mg/ml) when compared to control group (15.3 mg/ml) after 24 h infection with *S. aureus* (fig 2). During infection profound biochemical changes occur in the haemolymph, in the particularly concentration of proteins. The production of antimicrobial substances such as lectin, defensin and attacin with the entry of foreign bodies as part of the defense mechanism may be the reason for the elevation of protein content during bacterial and viral infection (Wago, 1995). The initial enhancement of protein content in inoculated silkworm may be due to elicit humoral as well as cellular response to encounter microbial inoculation (Rajitha *et al*., 2013).

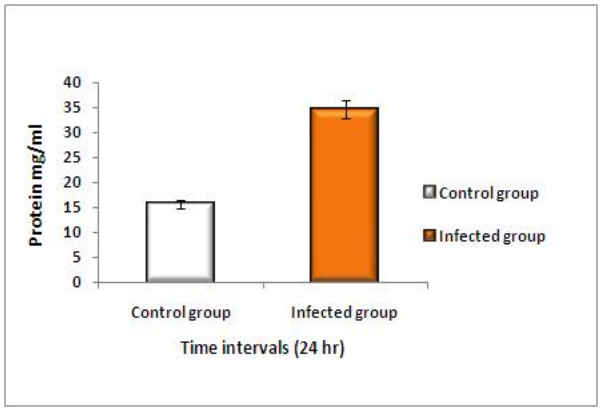

Fig 2 Protein concentration in the haemolymph of control and infected group.

Determination of haemocyte viability

In this study we investigated the proliferation of haemocytes after infection with bacteria. The total number of haemocytes count increased in the silkworm larvae infected with *S. aureus* when compared to control group (fig 3). Insect haemocytes are key components of immune system and they defend against foreign bodies via innate immune responses. In the present investigation, there was a significant change in total haemocyte count in 5th instar larvae after 24 hours of infection with *S. aureus.*

The results from the present study are in agreement with the earlier investigation that the number of haemocytes may increase (Bala *et al*., 2001) or decrease (Gilliam and Shimanuki, 1967) to counter foreign body when infected. In lepidopteran like *Bombyx mori* they are five types of haemocytes was identified and classified as prohaemocytes, granulocytes, plasmatocytes, spherulocytes and Oenocytoids (Tan *et al*., 2013; Liu *et al*., 2013). The major function of haemocytes includes phagocytosis of small particles, encapsulation of large foreign materials, haemolymph coagulation and storage and distribution of nutritive materials (Anandakumar and Sandhya, 2011). The cellular responses in insects are mediated by haemocytes and include phagocytosis, nodulation and encapsulation (Ribeiro and Brehelin, 2006). Haemocytes respond to infection by trapping parasites in nodular structures and phagocytosis the parasites (Thiago *et al*., 2013).

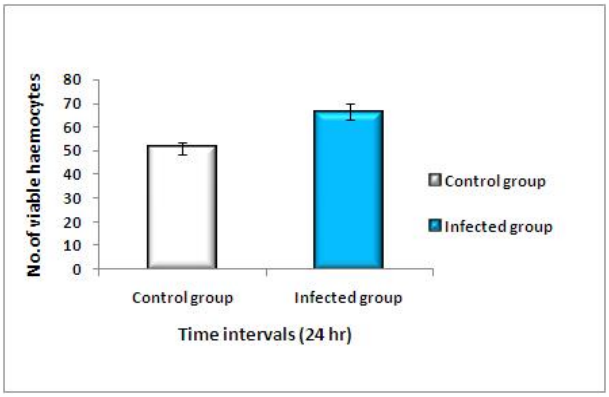

Fig 3 Changes of total no. of viable haemocytes count in the healthy (control) and infected group.

Giemsa staining

Aggregation of haemocytes was observed in the haemolymph of infected *Bombyx mori* (fig 4). Haemocytes, complex of several types of circulating cells found in the haemolymph of insects, provide defense against foreign bodys. The insect immune response may be categorized in two types the humoral and cellular responses (Ferrandon *et al*., 2007). The humoral response includes the activation of complex enzymatic cascades that regulate haemolymph coagulation, melanization and pathogen recognition (Dushay, 2009; Lemaitre and Hoffmann, 2007). The cellular responses, in turn, are mediated by haemocytes and include phagocytosis, nodulation and encapsulation (Ribeiro and Brehelin, 2006). Nodulation refers to the binding of multiple haemocytes to aggregate pathogens (Schmidt *et al*., 2001). This response has been described to act against a wide range of pathogens, such as bacteria and fungi (Ribeiro and Brehelin, 2006). In the hematophagous insects, *haemocytes* form nodules and aggregate the parasite when the parasite colonizes the insect haemocoel (Takle, 1988).

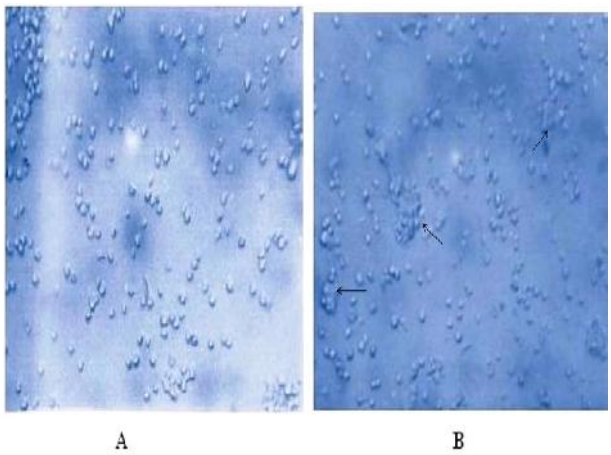

Fig 4 (A) Viable haemocytes from the healthy *Bombyx mori* larvae. **(B).** Bacteria induced haemocyte aggregation in the infected *Bombyx mori*.

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