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RESEARCH ARTICLE

Analysis of proteins profile and antibacterial activity in haemolymph of Eri silkworm, *Samia cynthia ricini* after bacterial inoculation

Ravindar G, Ragamalika G and Nagaraja Rao P

Molecular Entomology Lab, Department of Zoology, Osmania University, Hyderabad -07, India

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Corresponding Author

Ravindar G and Nagaraja Rao P

Abstract

The current study was designed based on proteomic approaches to understand the immune response of Eri silkworm after 24 hour post bacteria inoculation. Analysis of the proteins profile of non inoculated haemolymph, sterile injured haemolymph and bacterial inoculated haemolymph of Eri silkworm after 24 hour post bacterial inoculation by polyacrylamide gel electrophoresis for quantitative and qualitative changes after protein separation. Bacterial inoculation caused haemolymph protein lyses as shown by changes in number of proteins after post inoculation i.e more dominant protein bands were appeared at 60 - 90 KDa and 14 - 20 KDa, as well as specific low density protein or peptide bands at 21-30 KDa and 6-13 KDa were also observed in both gram +ve and gram –ve inoculated insect haemolymph. The antibacterial activity significantly increased in Eri silkworm haemolymph after inoculation with *Escherichia coli* and *Micrococcus luteus*. Therefore, we conclude that, this study revealed that the induced to produce antimicrobial substances in the haemolymph of Eri silkworm larvae after the bacterial inoculation and increase the quantity of antimicrobial substances may contribute to the antibacterial activity in haemolymph as an immune response. Finally, we might be concludes that induced antimicrobial substances are play major role insect innate immunity and defence system either destroy or invading, elimination of pathogens of host insect.

INTRODUCTION

Invertebrate, like all organisms can experience invasion by bacteria, fungi and other pathogens. The first line of defense for an insect is physical barrier in the form of a hard exoskeleton such as paritrophic matrix of the midgut and tracheae lined with cuticle (Dunn, 1990; Lavine and Strand, 2002; Iwanaga and Lee, 2005; Jiravanchpaisal et al., 2006). If the invaders survive and pass through these barriers into the hemocoel, the insects must rely on their innate immunity systems as they lack adaptive immunity. Insect innate immunity can be divided into two categories i.e. cellular and humoral responses. Cellular responses are mediated by haemocytes which includes phagocytosis, encapsulation and nodulation (Marmaras and Lampropulou, 2009; Tsakas and Marmaras, 2010). Humoral responses include the production of reactive intermediate oxygen or nitrogen species (Cerenius et al., 2010).

The humoral immune response is mediated by four signaling pathways, i.e. Toll, Imd, JAK/STAT and JNK, the signaling mechanisms are best studied in the Drosophila melanogaster that elicit expression of AMP's after inducement (Delaney and Stoven 2006; Lamaitre and Hoffmann,2007). The Imd pathway mainly regulates the response to gram-ve bacterial infection and some gram+ve bacterial infection also where as the Toll pathway primarily for the response to infection by fungi and other gram+ve bacteria (Tanji et al ., 2007). Upon bacterial infection, the signaling pathways are rapidly activated to induce the synthesis of large amount of AMP's resulting

finally in strong antimicrobial activities of haemolymph to kill the invasive microorganisms in all the insects (Ragan et al., 2009).

The Non mulberry Eri silkworm, *Samia cynthia ricini* has been raised for more than 100 years in Asian countries and it is a major economic resource for many families (Fujimoto et al., 2001; Singh et al., 2010). Currently, the silkworm is not only a domesticated insect used for silk production but it is also a model lepidopteron for biologically pest control studies. This insect is often used as a model organism in biochemical studies involving innate immunity due to its large size, sufficient haemolymph volume, relatively short life cycle and ability to be reared year round on fresh caster leaves (Jiang et al., 2010). Kanost et al., (2004) said that Individual 5^{th} instar caterpillars can contain 0.5-1 mL of haemolymph and 10^6 haemocytes, which make Eri silkworm an ideal candidate for studies involving haemocytes and haemolymph proteins. Using Eri silkworm for further the understanding of the mechanisms behind innate immunity can enhance our understanding immune systems in other insects

Here, we were investigated that the identification of novel antimicrobial components and their induced innate immunity of Eri silkworm against bacterial inoculation. The observed that antimicrobial activity and synthesis of low molecular weight proteins and peptides of Eri silkworm haemolymph significantly increased upon microbial infection, and were greatly induced after bacterial infection, especially in fifth instar larvae. This insect immunity study could help us better understand the human immune response and evolutions of innate immunity.

Material and Methods:

Insect collection and rearing:

Eri silkworm was obtained from the Regional Eri Research Center at Kammadhanam, Mahabhabunagar district, Telangana State. Eri silkworm larvae were reared on an fresh castor leaves at 28° C under relative humidity of 60-80 % and photoperiod of 12 hours light and 12 darkness. Insect at fifth instar larvae stages were collected for further experiments.

Maintaining of Bacteria culture:

Two different bacteria strains were used in this study, *Escherichia coli* (MTCC *106), gram negative and *Micrococcus luteus* (MTCC 1687), gram positive were procured from Microbial Type Culture Center (MTCC) Chandigarh and maintaining culture as per MTCC instructions.

Inoculation and collection of Haemolymph:

E.coli and *M.luteus* bacteria were cultured freshly in the LB media and Nutrient media and diluted with 0.9% Normal Saline into 3.6×10^5 for *E.coli* and 2.4×10^5 for *M.luteus* cells/µl. Fifth instar day 0 larvae of Eri silkworm was collected from the same batch and were divided into four groups, and inoculated them with above concentration of *E.coli* and *M.luteus* respectively.

After bacterial inoculation of larvae they were bled by puncturing the abdomen with fine tipped calibrated glass capillary. The free flowing haemolymph was transferred to sterilized effondrof tubes containing 1µl of mixture of Phenylthiourea (PTU) to prevent melanization and concentration of 0.1mg/ml Aprotinin, a protease inhibitor and in each group 6-10 larvae used for in each time period, haemolymph was kept at -20° C until further analysis.

Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Haemolymph of post inoculation (24 phi) and non inoculation and sterile injured were electrophoresis by SDS-PAGE using a discontinuous gel system by Laemmalli method (1970). In briefly, after staking, separating and simultaneously pouring gel, the comb was inserted by a slop way. The haemolymph protein (40ug/weel were treated with the reducing buffer 12% SDS containing 0.7 M 2-mercoptoethanol, 5% glycerol and 0.001% bromophenol blue) in the ratio of 1:2. The treated proteins were immersed in a boiling water bath for 2 min to ensure protein denaturation. After 2 hour polymerization of the gel and removing of the comb, unstained protein molecular weight marker (Sigma) and the treated protein were loaded in the wells. A voltage of 100 v. was applied until the bromophenol blue had reached the bottom of the gel. The gel was then stained with 0.025% commassie blue (0.25g L^{-1}) at room temperature, over night. To visualize the protein bands, the gel was washed several times with destaining solution (45% methanol, 5% glacial acetic acid and 50% distilled water) until the back ground become completely clear. Finally, the gel image was photographed by gel doc.

Antibacterial activity assay:

For antibacterial activity assay by Hultmark et al., (1983), haemolymph collected from bacterial inoculation Eri silkworm larvae and non inoculated Eri silkworm larvae and boiled for 10 min to remove most high molecular weight proteins and then centrifuged at 12000 rpm for 10 min. The test bacteria were either *E.coli* (Gram negative) or *M.luteus* (Gram positive), an aliquot (0.1ml) of fresh overnight culture was spread onto agar plates (9cm) containing NB and LB medium, respectively. After solidification, 5µl or 10µl of haemolymph supernatant was applied as a droplet onto the plates with a pipette tip. After 24 hour incubation in an incubator at 37° C, the diameter

of the clear zone of inhibition was measured and documented by photography. The antibacterial activity is defined as the square of the difference between the radius of inhibition zone and radius of loading well.

Estimation of total Protein

Estimation of total protein was made according to Bradford, (1976) method, Bovine Serum Albumin (1mg/ml) as a standard and absorbance was measured at 595 nm.

Results and Discussion:

Analysis of protein profiles, variation of quantity of total protein and antibacterial activity were carried out in all the groups of Eri silkworm haemolymph, such as Control, 0.9% NaCl injured, Gram –ve (*E.coli*) and Gram +ve (*M.luteus*) bacteria challenged groups of Eri silkworm larvae Graph-1 showed that the total protein concentration increased at 24 hour post inoculation (hpi). Injured larval haemolymph had shown only 10% increase of protein concentration at 24 hr post injection. However, the results revealed that after post bacterial challenged larval haemolymph had showed significantly ($P<0.05$) elevated protein concentration levels when compared with those of control and injured haemolymph samples collected from the larvae.

The larval haemolymph showed higher antibacterial activity in gram –ve and gram +ve bacterial challenged haemolymph when compared to other groups i.e. controls and normal saline injured. Fig 1a and Fig 1b showed highest zone of inhibition 3.85 mm in *E.coli* challenged and 3.3 mm in *M.luteus* challenged haemolymph samples at 24 hour post inoculation. Whereas, injured larval haemolymph had shown only 1 mm of inhibition zone in post injection insect larval haemolymph. However, the antibacterial activity studies revealed that significantly higher activity in both bacterial challenged haemolymph when compared control and injured. The order of bacterial zone inhibition was control insect, sterile (0.9% NaCl) injured, *M.luteus* challenged and finally with higher activity with *E.coli* challenged haemolymph.

Fig 2 showed that the protein profile of haemolymph of bacterial inoculated, sterile injured and control Eri silkworm by using SDD-PAGE gel electrophoresis method. The visual analysis of proteins and peptide bands showed that the *E.coli* inoculated haemolymph consists of 16 bands (ranged from 220 - 3.5 KDa), *M.luteus* inoculated haemolymph could separate into 16 bands range from 220 - 3.5 KDa, sterile injured haemolymph could separate into 10 protein bands and control haemolymph separated into 8 protein bands. Protein band of 66 KDa was detected as more dominant band in both *E.coli* and *M.luteus* bacteria inoculated and less dominant band appeared in sterile injured, control group of haemolymph. Protein band of 14.4 KDa has been observed in Gram +ve and Gram –ve bacteria haemolymph. In the protein band 66 KDa to 6.5 KDa between few more low density protein bands appeared in gram +ve and gram –ve bacteria inoculated haemolymph after 24 hour post hour inoculation while compared to control and sterile injured insect groups. In general it could be notice that high molecular weight proteins (84-220 KDa) revealed that no changes in bacterial inoculated haemolymph when compared to control and sterile group haemolymph. However, the most observed changes were seen in low molecular weight (31-6.5 KDa) protein or peptide in both gram +ve and –ve bacteria inoculated haemolymph sample.

The quantitative protein analysis of haemolymph showed an increased protein concentration in both gram –ve, (*E.*coli) and gram +ve, (*M.luteus*) bacteria challenged Eri silkworm larvae. Similar findings were recorded in non mulberry silkworm haemolymph after non pathogenic bacterial injection (Sharma et al., 2005). Our results were also corroborated with earlier reports on *Galleria mellonella* larvae where haemolymph proteins level showed higher during bacterial infection (Jarosz, 1995). The protein concentration of insect haemolymph is generally were higher than that of the internal fluids of other invertebrates (Florkin and Jeuniaux, 1974). Adamo, (2004) stated that bacterial injected haemolymph have high protein concentration because of induced proteins formed against bacteria for self defense and survivability due to immunity. Seufi, (2011) showed that the various bacteria such as *Staphylococcus aureus, Streptococcus* and *E.coli* bacteria challenged *Spodoptera litura* larvae had variations in the protein profile of its haemolymph. Studies with *Bombyx mori* and *Maduca sexta* haemolymph had revealed that the presence of a variety of proteins formed in response to injury or bacterial challenge (Abraham et al., 1995; Dickinson et al., 1988). Gajandra et al., (2011) reported that the greater synthesis of protein profile from fat body and release into the haemolymph of silkworm, *Antherae mylitta* larvae during bacterial infection. Similarly situation might have occurred in our experiments also.

The bacterial inoculated haemolymph of Eri silkworm, antibacterial activity was observed higher after post inoculated larval haemolymph obtained from E.coli and *M.luteus* bacteria challenged insects. Sharma, et al., (2005) also noticed that the increased level of antibacterial activity in muga silkworm haemolymph after bacterial

challenge. However, Chapelle et al., (2009) and Seufi et al., (2011) suggested that the increased level of antibacterial activity was time dependant in *E.coli* and *M.luteus* challenged haemolymph of *Spodoptera litura* and *Spodotera fruguperda*. Similarly, Wojda et al., (2009) also recorded an increase of antibacterial activity level in cell free haemolymph obtained from *Galleria mellonella* larvae (Jarosz, 1995). In the *Spodoptera frugiperda* larval unchallenged and sterile injured larval haemolymph had antibacterial activity against gram positive *M.luteus* bacteria and it was reported by Chapelle et al., (2009). Variations noticed in antibacterial activity of Eri silkworm haemolymph at different time intervals as noticed in the present investigation may be because of species variation, food and habitat of present test insect when compared with other insects including Lepidoptera or insects from other orders (Freitak et al., 2007).

So far, little information is available about the immune response of silkworm, *Bombyx mori* larvae but no one has done in Eri silkworm larvae. We have challenged Eri silkworm larvae by injection of normal saline and gram+ve and gram –ve bacteria respectively, and analyzed the proteins and peptides pattern by gel electrophoresis of haemolymph samples collected 24 hour post inoculation. Our results reveal the induction of six immune proteins and peptides i.e. ApoL P-I &II, Transferrin, Defensin, lebocine and MRJP (Fig 2). None of these Antimicrobial peptides (AMP) were detected in haemolymph samples collected from noninfected larvae. Similar, our results are corroborated with Randolt, et al., (2008). They noticed that the immune related proteins induced in the haemolymph after aseptic and septic injury in honey bee larvae. Earlier, similar finding were reported by in the Evans (2004) who studied the defense reaction of honey bee to pathogen paenibacillus larvae. Sribnaya et al., (2010) revealed that the haemolymph got separated into three proteins and they were high, medium and low molecular weight proteins. Peptides were observed in *Galleria mellonella* larvae during microbial infections with various time intervals.

In accordance with this observation, no antibacterial activity was measured in control and sterile injured samples by zone of inhibition assay, suggesting that no or very low amounts of AMP's are constitutively produced. We hope that this work could provide the basis for further study of the induction mechanism of AMP in important agriculture pest, and even in other insects and higher animals. The acquired knowledge might contribute to the sources of biological control programs of various serious insect pests. Who's immunity plays an important role in making them not susceptible or susceptible to bio control agents. Thus, in turn may lead to the development of new pest control measures as well as improvement in the health of beneficial insects. Few novel inducible antibacterial proteins were observed, which requires further purification and characterization. These inducible haemolymph proteins or peptides were involved in the insect defence mechanism against bacterial infection.

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Graph 1: Protein concentration of the larval haemolymph of Eri silkworm control, injured (0.9% NaCl) and bacteria (*E.coli* and *M.luteus*) challenge groups

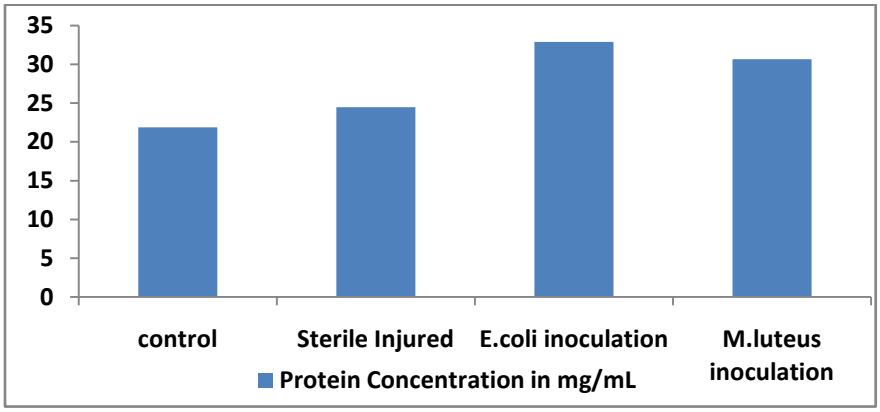
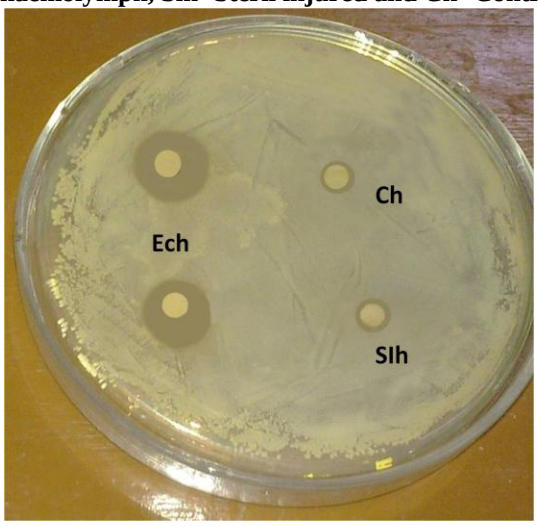
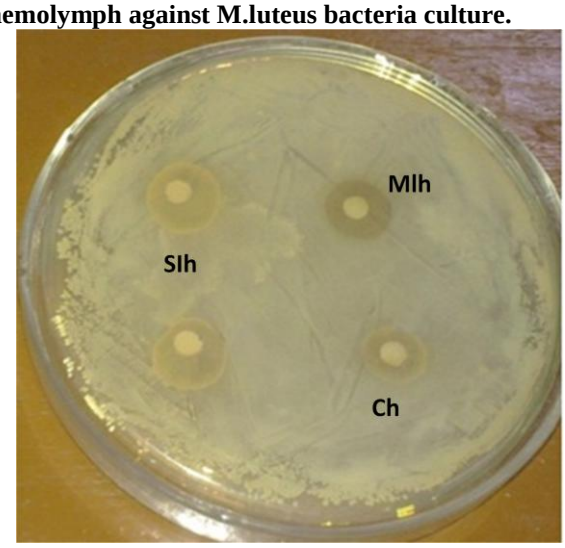


Fig: Anti bacterial activity in *E.coli, M.luteus* bacterial challenged, sterile injured and control haemolymph of Eri silkworm larvae after 24 post inoculation, Fig 1a indicates Ecoli bacterial growth inhibition zones in Ech-Ecoli challanged haemolymph, Sih-Steril injured and Ch-Control haemolymph against Ecoli bacteria culture and Fig 1b indicate M.luteus bacterial growth inhibition zones in Mlh- M.luteus challnged haemolymph, Sih- Steril injured and Ch- Control haemolymph against M.luteus bacteria culture.

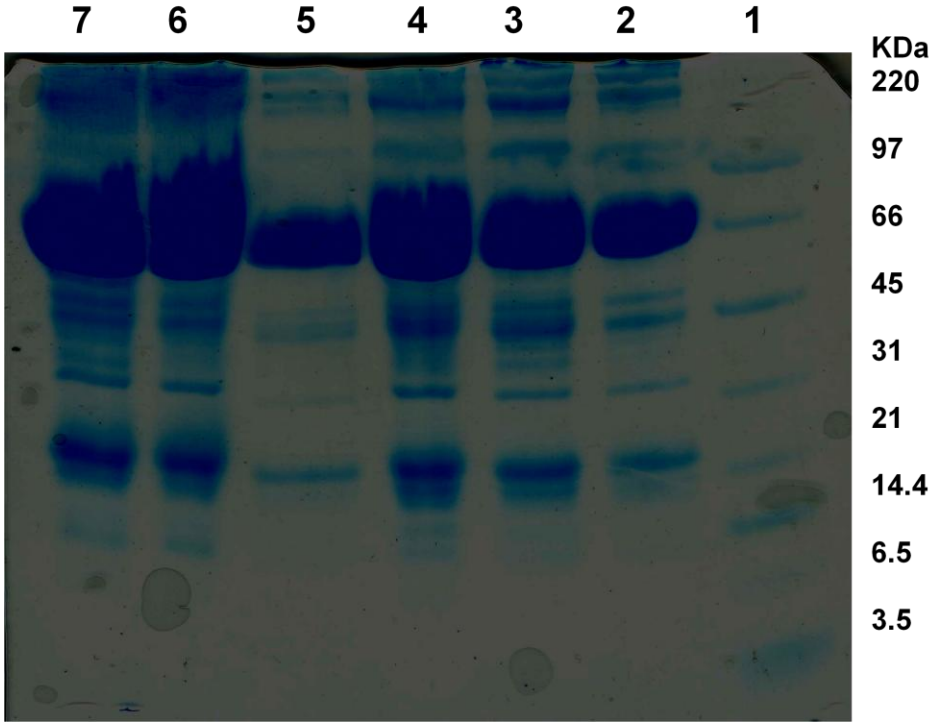


1a



1b

Fig 2: Gel Electrophoretic analysis of haemolymph proteins of *E.coli*, *M.luteus* bacterial inoculation, sterile injured and (non inoculated) control. Lane 1, represent molecular weight marker, Lane 5 represents conrol, Lane 2, represents sterile injured, Lane 3,4 represent *E.coli* inoculation and Lane 6,7 represents *M.luteus* inoculated haemolymph sample.



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