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Research Article

CERTAIN BIOCHEMICAL CHANGES IN HAEMOLYMPH OF ERI SILKWORM, *SAMIA CYNTHIA RICINI* AFTER INOCULATION WITH BACTERIA

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Abstract

The insect immune system exists to protect the host from pathogenic invaders and from other harmful insults. Upon bacterial infection insect haemolymph may lead to fatal consequences and it plays a most important role in transport and storage of nutrients, amino acids and free amino acids concentrations are marked changes for substrates by the immune system, these substrates are provide energy and precursors for the synthesis of new cells, effecter molecules, and protective molecule. We have been studied the certain biochemical changes in bacterial challenged Eri silkworm haemolymph at various time intervals. Results shows that the proteins and amino acids levels were significantly elevated and 18 individual free amino acids were found and that are quantitatively variation in the haemolymph after challenge with gram –ve and gram +ve bacteria when compared and control and sterile haemolymph. We may conclude that these were directly involved in the antimicrobial immune response of Eri silkworm innate immunity.

Key words: Biochemical changes; Proteins; Amino acids; haemolymph; Eri silkworm; *samia cynthia ricini*

Introduction

Invertebrates also experience and recognize invasion of bacteria, fungi and other pathogens. The first line of defense for an insect is its physical barrier (Gillespie *et al.*, 2000b) and it is the hard exoskeleton made up of chitin, the peritrophic membrane matrix of the midgut and tracheae lined with cuticle (Iwanaga and Lee, 2005; Jiravanichpaisal *et al.*, 2006). Insects are one of the most successful animal groups and live in every habitat on Earth (Ratcliffe, 1985; Vilmos and Kurucz, 1998). Hence, they have been developed effective defense strategies against invading microorganisms (Duvic *et al.*, 2012). The places from where a microbe can invade, insects rely solely on innate defense mechanisms, and adaptive immunity is totally absent (Hoffman, 1995). A major task of the insect immune system is the defense against multicellular microorganisms and macro organism such as parasites, parasitoids etc (Schmid-Hempel, 2005; Lazzaro and Little, 2009). Innate immune response of insects has many apparent similarities to those of mammals (Gillespie *et al.*, 2000a). Particularly, cell mediated innate immunity through haemocytes and biochemical based humoral innate immunity through cell free haemolymph proteins (Lavine and Strand, 2002).

Firstly, haemolymph proteins were discovered from lepidopteron insects and understanding of their innate immune responses has potential practical significance (Jiang *et al.*, 2010). Moth larvae have proven to be extremely useful for experiments providing insights on the innate immune system of insects (Hoffman, 1995; Broderick *et al.*, 2010). Earlier, the researchers work on lepidopteron immunity has made use of large moth species, including the tobacco hornworm, *Manduca sexta* and wild silk moths such as *Hyalophora cecropia* (Ragan *et al.*, 2009). The wax moth, *Galleria mellonella* was one of the earliest species used for current research in insect immunity (Mukherjee *et al.*, 2011). In addition, most research work on immune systems was carried out on moths whose larvae were the most destructive agricultural pests worldwide, especially Noctuidae and Saturnidae family members (Kanost *et al.*, 2004). The domestic silk moth, *Bombyx mori* has also provided significant discoveries about immunity in moths. The silkworm not only brings economic benefit, but also provides important insight on insect immunity (Tomita *et al.*, 2003; Tanaka and Yamakawa, 2011).

Eri silkworm is not only one of the first domesticated economic insect, but also one of the excellence model organisms for classical genetics research (Siddiqui *et al.*, 2000). An attentive immune surveillance system of Eri silkworm is able to detect and neutralize the invading microbes effectively and also protects from microbial invasion, multiplication and infection during with wound healings. Using Eri silkworm to understand the innate immunity mechanisms will enhance in understanding other

insects immune system also. It is one of the lepidopteron and similarly to other family have developed a powerful innate immune response mechanism i.e. *Bombyx mori*, *Spodoptera* species, *Achea janata* etc

The major of aim of the studies to understand how a microbial infection in insects triggers immune response against nonpathogenic bacteria and an insect overcome it through humoral factors. In this view we have been attempt to induce the immune system of *Samia cynthia ricini* by inoculating with *E. coli* (garm -ve) and *M. luteus* (gram +ve) bacteria. We were majorly interested in a aspects of biochemical components such as Proteins, amino acids free amino acids and lipids with reference to Eri silkworm larvael humoral innate immune response. These biomoleculs are may play a major role in primary innate immunity during intra hemoceolomic bacterial challenge and are involved cascade of insect immune system.

Materials and Methods

Eri silkworm 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 fresh castor leaves at 28° C under relative humidity of 60-80 % and photoperiod of 12 hours light and 12 hours darkness. Insect at fifth instar larvae stages were collected for further experiments.

Bacteria Culture maintains and Challenging

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 MTCC Chandigarh and maintaining culture as per MTCC instructions. Bacteria 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 larvae were divided into four groups, and inoculated them with above concentration of *E. coli* and *M. luteus* respectively. We have been taken 50 larvae in each group.

Haemolymph collection

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.

Estimation of total protein

Estimation of total protein was made according to Bradford, 1976 method. The Bradford protein assay is a simple procedure for determination of total protein concentration in solution that depends upon the change in absorbance in Coomassie Brilliant Blue G-250 (CBB- G - 250) which basically depending upon binding proportion of protein. In briefly, Haemolymph, 250µl of Bradford reagent has been added to 5µl of haemolymph in a poly propylene tube and incubated for 10 Min at room temperature (RT) and the absorbance was measured at 595 nm.

Total amino acids

Amino acids were determined spectrophotometrically according to the method of Lee and Takahashi (1966). In briefly, 0.1 ml of test samples of haemolymph added 1.9 ml of ninhydrin citrate glycerol mixture (0.5 ml of 1% ninhydrin in 0.5 M citrate buffer, pH 5.5: 1.2 ml of glycerol and 0.2 ml of citrate buffer) and the final pH of the reaction mixture was adjusted to 6.0. After mixing and shaking well, the test tubes were placed in a boiling water bath for 12 min and then cooled to a room temperature under running tap water. The test sample which had developed colour was read spectrophotometrically at 750nm against blank test tubes in which distilled water replaced the haemolymph sample and L-leucine (1mg/ml) as a standard.

Sample preparation for free amino acids analysis

To100 µl of haemolymph (Cell-free plasma) 1µl of 5% TCA (Trichloric Acid) was added and vortex for 5 min to precipitate protein. Samples were centrifuged at 12000 rpm for 10 min at 4°C. Supernatant transferred into the new eppendorf tube and again 5% TCA was added into the residue for getting clear sample from protein for three times. Then pooled all the supernatants, added to 3ml of ether, shaken for 30 sec and the aqueous layer was concentrated in a low pressure rotary evaporator under 40 °C to A fairly highly viscous fluid. Then diluted it with deionized double distilled water for amino acids analysis followed by method of Lee-Shing *et al*., (1992).

Amino acids analysis

Haemolymph sample was made up of 0.5µl with 95.5 µl Lithium citrate buffer from which 40µl sample was taken and injected into amino acid analyzer (Biochrome -30) as per the instructions given in operation manual of the manufacture procedure and the flow rate 20ml/hr (100 µl/3 min)and column temperature was maintained at 34-72°C.

Results and Discussion

Biochemical analysis were carried out in bacterial (*E. coli* and *M. luteus*) challenged, injured (0.9% NaCl injected) and control larval haemolymph of Eri silkworm, *Samia cynthia ricini*. Variation in the quantity of total protein, amino acids, free amino acids of the larval haemolymph were determined in all the groups, such as Control, 0.9% NaCl injured, Gram –ve (*E. coli*) and Gram +ve (*M.luteus*) bacteria challenge of Eri silkworm larvae groups during different time points (1 hr, 6 hr, 12 hr, 24 hr, 48 hr and 72 hr). These investigations revealed that the bacterial challenged haemolymph had higher levels of total amino

acids and free amino acid contents than obviously in control and injured groups at all the time points.

Quantitative estimation of total protein content in the larval haemolymph

The results **(**Fig 1) showed that the total protein concentration moderately increased (23-28% respectively) during 1hr ,6hr and 12 hr and the it reached to maximum (50%) at 24 hpi. Whereas, total protein concentration was slightly decreased in the haemolymph (13-17%) at 48 and 72 hpi in both *E. coli* (Gram -ve) and *M. luteus* (Gram +ve) bacteria challenged larvae. Injured larval haemolymph had shown 10% increase of protein concentration at 24 hr post injection and no significant change was observed at the remaining time points. However, the results revealed that at all the time intervals of 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.

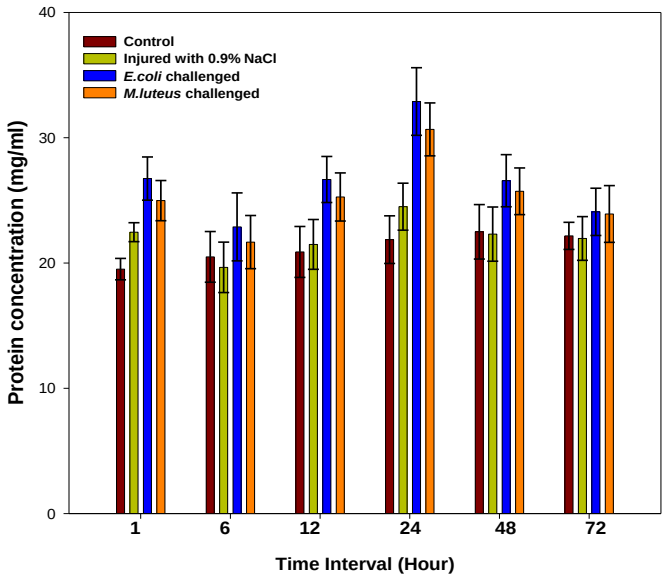

Fig 1: Total Protein concentration of the larval haemolymph of Eri silkworm control, injured (0.9% NaCl) and bacteria (*E. coli* and *M.luteus*) challenge groups at different time interval

[The values represent in replicates and bar indicate Mean ± SD, *significance variation in $P<0.05$]

Quantitative estimation of total amino acids and free amino acids in the haemolymph

The results depicts that the amounts of total amino acids were found to be high (45%) at 1 hr post bacterial inoculation, steadily decline (12-18%) at 6 hr and 12 hr and it reached maximum (68%) at 24 hr post bacterial inoculation. The amino acid availability level was slowdown (20-28%) during 48 hr and 78 hr in during its larval haemolymph post inoculation of gram –ve (*E. coli*) bacteria. Whereas in *M. luteus* (gram +ve) bacterial challenged larval haemolymph, the amino acid levels have shown somewhat similar pattern in all the time points with respect to their each time course of bacterial challenged groups. Even though, quantity of amino acids level was showed higher in the entire time interval of bacterial challenged *Samia cynthia* larvae when compared to control and injured larvae (Fig 2).

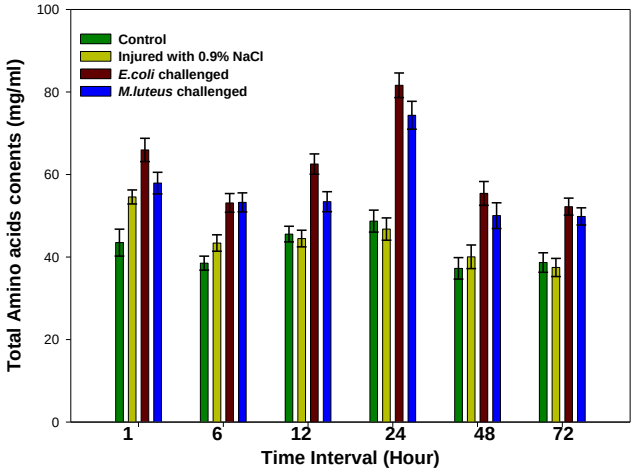

Fig 2: Total amino acid contents of the larval haemolymph of Eri silkworm of control, injured (0.9% NaCl) and bacteria (*E. coli* and *M.luteus*) challenged groups at different time interval

[The values represent in replicates and bar indicate Mean ± SD, *significance variation in $P<0.05$]

The analysis of free amino acids levels in the haemolymph of test insect *Samia cynthia* made us to know that there is a presence of 18 individual free amino acids in the haemolymph and these found to be a quantitatively variation after challenge with gram –ve and gram +ve bacteria. The free amino acids and their concentrations were Phenylalanine (70%), Argnine (50%) Glutamic acid (52%), Isoleucine (45%), Leucine (37 %), Sarcosine (32%) Valine (28%), Methione (18%), Ornithine (18%), and Tyrosine (14%). They were always higher in *E. coli* challenged insect larval haemolymph. Sarcosine (49%), Alanine, Glycine and Lysine were 12% higher in *M. luteus* challenge larval haemolymph. Other free amino acids present were Cystine, Cystathionine, Histidine and Serine levels and they showed a decline (20%) after bacterial challenged haemolymph of Eri silkworm larvae when compared with control and injured haemolymph larval groups (Fig 3).

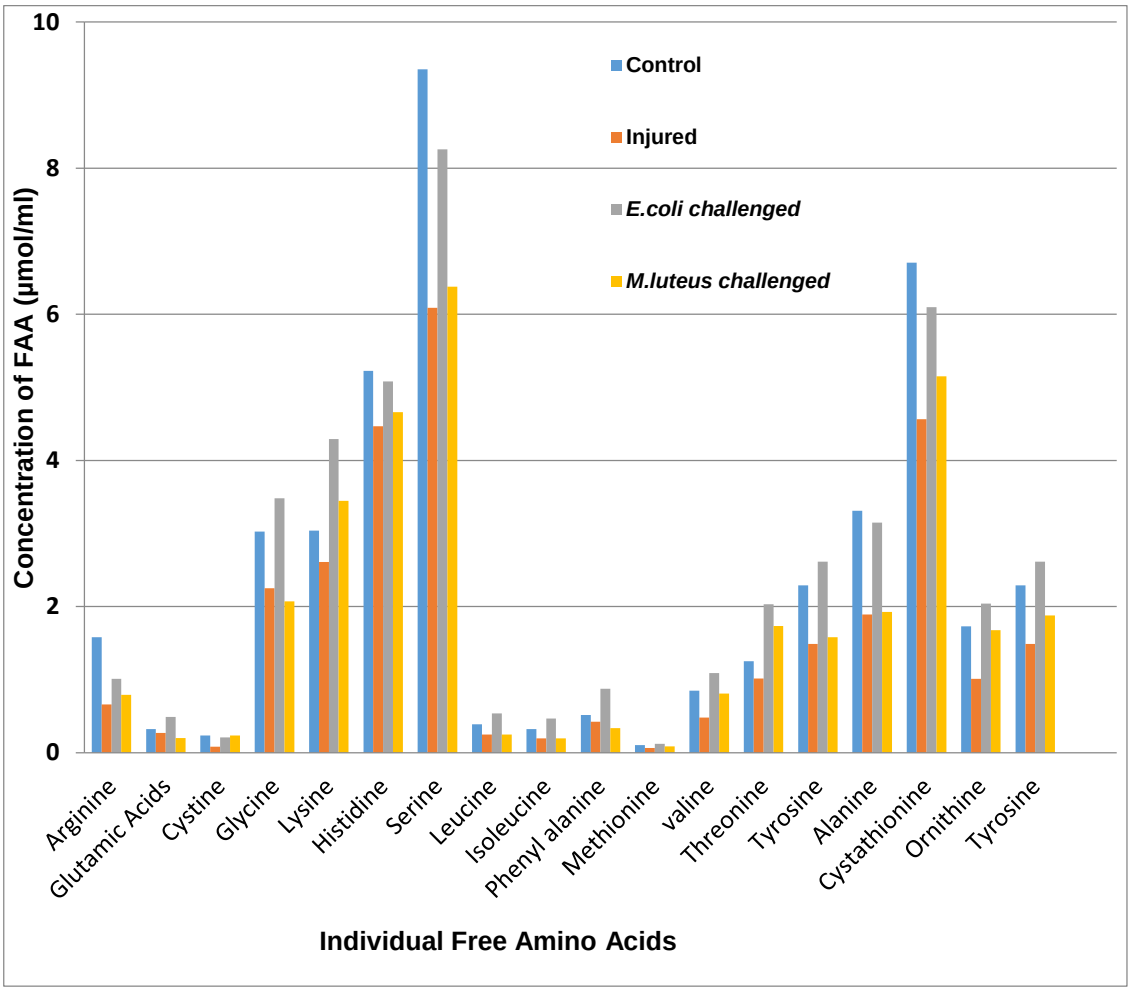

Fig 3: Individual Free Amino Acids (FAA) concentration in haemolymph of control, injured, *E. coli* and *M. luteus* bacteria challenged Eri silkworm larvae

In the present study quantitative protein analysis of cell free haemolymph showed an increased protein concentration in both gram –ve, (*E. coli*) and gram +ve, (*M.luteus*) bacteria challenged Eri silkworm larvae at all-time interval. Similar findings were recorded in non-mulberry silkworm haemolymph after nonpathogenic bacteria injection (Jyotsna *et al.*, 2005). Our results were also corroborated with earlier reports in *Galleria mellonella* larvae where haemolymph proteins level showed higher at various time intervals during bacterial infection (Jarosz, 1995). The protein concentration of insect haemolymph is generally 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 have revealed the presence of a variety of proteins formed in response to injury or bacterial challenge (Abraham *et al.*, 1995; Dickinson *et al.*, 1988). Gajendra *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.

Significantly quantitative differences in total amino acids and free amino acids were detected in the haemolymph of control and bacterial challenged Eri silkworm larvae in the present studies. Total amino acids showed a higher level during entire selected time intervals in case of both gram –ve and gram +ve bacteria challenged insect haemolymph. Salama et al (1991 and 1994) also report that quantitative changes in amino acid profiles in the larval haemolymph of *Spodoptera littoralis* insect. Narayanan (1973) found an obvious increase in the total amino acids in the larvae of *Plutella maculipennis curt* treated with *B. thuringinesis*. Similar observation was also reported by Reddy *et al.* (1994) in thyroxine treated fifth instar larvae of tasar silkworm, *Antheraea mylitta*.

Pant and Agrawal (1964) suggest that all amino acids found were likely to contribute a general pool for the synthesis of new proteins and for the products of protein breakdown, osmoregulation and buffering. They suggested that these results could be due to both protein degradation and a reduction in protein synthesis. Wyatt (1961) reviewed extensively the literature on the qualitative and quantitative

aspects of amino acids metabolism in relation to changes in food intake during the period of development and diseased conditions. Similarly, Aboul-Ela *et al.* (1991) found that the quantitative changes in the amino acids of haemolymph of *Plodia interpunctell* larvea after treatment with bacteria. However, cold stress and environment factors effected the total amino acid concentration and in its increase in Lepidoptera insects such as *Antheraea mylitta*, *Spodopter litura*, *Galleria mellonella* and *Bombyx mori* (Sinha *et al.*, 1987; Salama *et al.*, 1994; Shamitha and Rao, 2008; Singh *et al.*, 2010). Present results suggest that there is an impact on the total amino acid contents of haemolymph of test insect after bacterial inoculation and it has variations at different time intervals. The change in the total amino acid pool will directly influence the protein turnover and thus obviously reflects the physiological state of the organism (Lazar and Mohamed, 1988).

The quantitatively variation of 18 individual free amino acids was distinguished in both gram –ve (*E. coli*) and gram +ve (*M.luteus*) bacterial challenged haemolymph of Eri silkworm larvae. Haemolymph amino acids play an important role in the synthesis of cuticle constituents and in silk production. In our results, Phenylalanine, Arginine, Glutamic acid, Isoleucine Leucine and Valine amino acids levels were higher in the haemolymph of bacterial challenged Eri silkworm larvae. These amino acids are important as a stress response indicator (Tanguy *et al.*, 2005; Leroy *et al.*, 2010) and play a crucial role in managing oxidative stress also (Michaelis, 1998; Matés *et al.*, 2002). Glutamine is a major constituent of the glutathione biosynthetic pathway and Elevated glutamine levels are associated with enhanced nitrogen detoxification from protein metabolism and it was reported earlier by Chen and Chen, (2000).

Phenylalanine is one of the constituents of a protein complex which acts like metallothionein by binding to heavy metal ions (Roesijadi, 1981) and is an important component of tyrosine metabolism (Van Ruitenbeek *et al.*, 2009). Alanine and few other amino acids are believed to be involved in the cold and hardness. Storey *et al.*, (1981) and Chen (1985) stated that the insect haemolymph usually contains very high levels of total free amino acids. Similarly, Liadouze *et al.*, (1995) found a significant increase in the amounts of Lysine and Arginine and later they suggested that these amino acids were related to nitrogen excretory metabolism. The above individual amino acids are all belong to essential amino acids group, and hence cannot be synthesized by de novo method in an insect (Koštál *et al.*, 2011). The high free amino acids (FAA) content in the haemolymph can be attributed to high proteolytic activity also (Gorres and Raines, 2010).

Reduction in the levels of Cystine, Cystathionine, and Histidine were found after bacterial challenged in the Eri silkworm haemolymph. Gordon and Hahn, (2010) reported similar results and found that there is a decrease in above mentioned Cystine, Cystathionine and Histidine in *Pyrrhocoris apterus* insect during overwintering season. The amounts of FAA are Aspartic acid, Threonine, Serine, Proline, Glycine, Methionine and Tyrosine and noticed a decrease in the haemolymph. It may indicate that the possibility of active role of amino acids in Kreb's cycle and Glycolytic pathways to meet the emergency energy needs as well as their utilization in the production of some new proteins and their synthesize (Colinet *et al.*, 2007). Proline is important amino acids for many other biological functions besides energy production. The presence of proline within the peptide chain regulates structural susceptibility against protease activity which in turn controls a multitude of biological functions (Yaron *et al.*, 1993). Colinet *et al.*, (2007) and Lalouette *et al.*, (2007) stated that the above metabolic scenario links the accumulation and Pro biosynthesis of protein directly to degradation of proteins.

In the present study, it was also noticed that Hydroxyproline and Sarcosine amino acid levels were relatively higher in both *E. coli* and *M. luteus* bacterial challenged haemolymph. Similarly, its levels were relatively lower and variable. Koštál *et al.*, (2011) reported that in heteropteran insect, *Pyrrhocoris apterus* showed one of the most dramatic increases in Hydroxyproline (up to ten-fold) and Sarcosine during winter. Hydroxyproline and Sarcosine are the major components of collagens. Thus, high levels of Hydroxyproline during overwintering must be available solely from proteolysis (Francois, 1985; Gordon and Hahn, 2010). Accumulated amino acids could serve as important reserves used for the synthesis of heat shock proteins that are rapidly up-regulated following the cold exposure (Koštál and Tollarová-Borovanská, 2009). Thus, changes in free amino acids levels in haemolymph were likely used for the synthesis of compounds related to the immune response or fatty acids transport and few FAA which were probably used as energy source in gluconeosis.

In conclusions the total Proteins, amino acids and free amino acids were raised in the larval haemolymph of Eri silkworm at all the time intervals during post bacterial challenge. Upon bacterial infection insect haemolymph may lead to fatal consequences, amino acids and free amino acids concentrations are marked changes for substrates by the immune system. These substrates are providing energy and precursors for the synthesis of new cells, effecter molecules, and protective molecule. Hence, we may conclude that these were directly involved in the antimicrobial immune response of Eri silkworm innate immunity.

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