

Nitrogen Metabolism in the Haemolymph of Silkworm *Bombyx Mori* L During the Progress of Fungal Pathogen *Beauveria Bassiana* (Bals) Vuill



Zoology

KEYWORDS : *Beauveria bassiana*, *Bombyx mori*, Haemolymph, Ammonia, Uric acid, Urea

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ABSTRACT

Day to day changes in the levels ammonia, uric acid and urea were examined in the haemolymph of 5th instar silkworm Bombyx mori L during the progress of fungal pathogen Beauveria bassiana. Significant reduction of ammonia content was recorded from 3rd day of induction of fungal pathogen till the end of the instar (25.95 µg/ml to 28.84 µg/ml) in experimental larvae compared to control (29.49 µg/ml to 35.49 µg/ml). No significant change was observed in the uric acid content till the 3rd day of fungal infection (33.22 µg/ml to 38.77 µg/ml) and then significant elevation of uric acid levels was observed from 4th day to 6th day of inoculation in 5th instar silkworm larvae (41.54 µg/ml to 48.37 µg/ml) compare to control (39.33 µg/ml to 44.49 µg/ml). Significant elevation of urea content was recorded from the 4th day to 6th day of induction of fungal pathogen (16.55 µg/ml to 16.03 µg/ml) with reference to control (13.59 µg/ml to 12.74 µg/ml).

INTRODUCTION

Excretion forms an important factor for the balance of nitrogen in the body. The nitrogenous products in most insects eliminated from the haemolymph via the malpighian tubules. Ammonia is the primary end product of nitrogen metabolism, but it is highly toxic except in extreme dilutions. For most terrestrial insects water conservation is essential and loss by excretion must be reduced to a minimum. Hence it is necessary to produce a less toxic substance than ammonia so that less water is required for its safe elimination. The substrate produced is uric acid, most insects excrete 80-90% of the waste nitrogen as uric acid. Urea is commonly present, in relatively small amounts and it represents less than 10% of the nitrogen excreted. The excretory pattern depends upon a number of environmental factors such as temperature and humidity (Alexandria and Stanchion 1981 and Dhinaker 1990) and stress induced by the different types of pathogens. This paper reports adjustments in nitrogen substances due to stress induced by the fungal pathogen *Beauveria bassiana*.

MATERIALS AND METHODS

For the investigation silkworm hybrid of PM x CSR₂ was selected. Silkworm larvae were brushed and reared under laboratory conditions according to Dandin et al (2003). Immediately after fourth moult i.e. on the first day of the fifth instar, the larvae were inoculated, by dipping in fungal spore suspension (2.15 x 10⁶ spores/ ml @ 50 ml/100 worms for 45 sec). The larvae treated with double distilled water were used as control. After 24 hours of the induction of fungal pathogen haemolymph was collected into pre-chilled centrifuge tubes with a pinch of thio-

urea by clipping third pair of abdominal legs of silkworm larvae for analysis. The parameters examined and methods employed were as follows

1. Ammonia - Berg Mayer (1965) with a slight modification
2. Uric acid - Brown (1945) as given by Oser (1965)
3. Urea - Natelson (1971)

Recorded data of the study was statistical analysed by using t-test.

RESULTS AND DISCUSSION

Ammonia

The results on ammonia content are presented in Table-1 and Graph-1. The results clearly shows that the ammonia content in the haemolymph was steadily increased in control from 1st day to 5th day (22.11 µg/ml to 37.24 µg/ml) and then reduction of ammonia content (35.49 µg/ml) was recorded. The trend was similar in the experimental larvae from 1st day to 5th day (20.18 µg/ml to 31.27 µg/ml) and then reduction (28.84 µg/ml) was observed. But significant reduction of ammonia content was recorded from 3rd day of induction of fungal pathogen till the end of the instar (25.95 µg/ml to 28.84 µg/ml) in experimental larvae compared to control(29.49 µg/ml to 35.49 µg/ml).

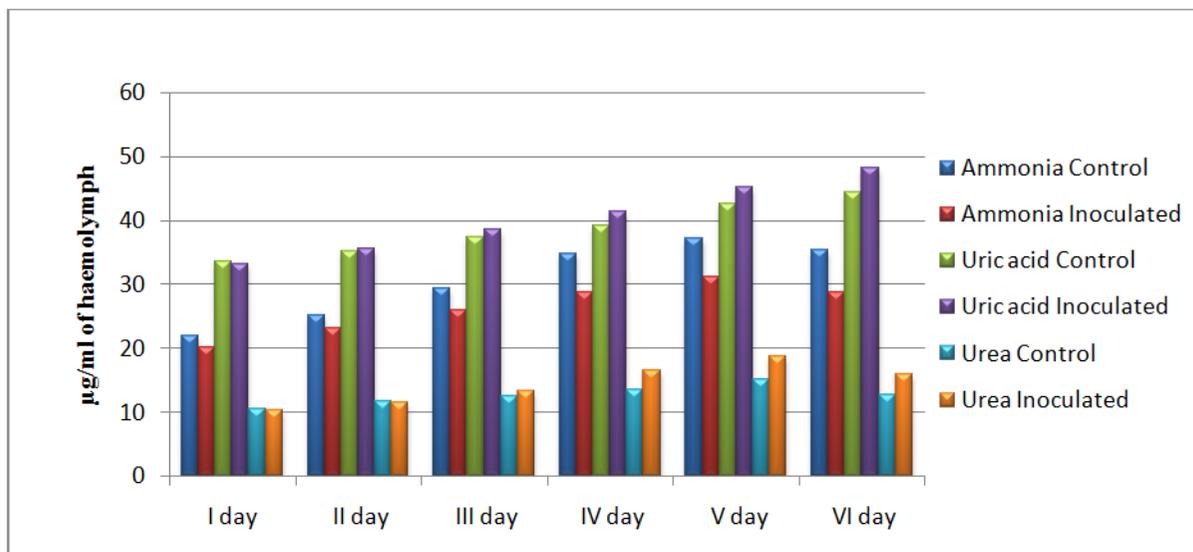
Protein catabolism leads to the production of ammonia in addition to water and carbondioxide. Ammonia must be eliminated outside the body due to its cellular toxicity. In insect, ammonia is not usually excreted but it is converted into less toxic uric acid (Chapman 1982). Reduction of ammonia in the study may be due to conversion of ammonia nitrogen to non-essential amino

Table-1: Day to day changes in ammonia, uric acid and urea levels (µg/ml) in haemolymph of silkworm *Bombyx mori* L. inoculated with fungal pathogen *Beauveria bassiana* (Bals) Vuill with reference to control during 5th instar

Ammonia						
V instar	I day	II day	III day	IV day	V day	VI day
Control	22.11±1.85	25.13±1.28	29.49±0.66	34.89±1.23	37.24±1.83	35.49±0.75
Inoculated	20.18±1.64 NS	23.22±1.25 NS	25.95±1.05 ****	28.81±1.50 ****	31.27±1.01 ****	28.84±1.48 ****
Uric acid						
Control	33.57±1.85	35.32±1.28	37.49±0.66	39.33±1.23	42.74±1.83	44.49±0.75
Inoculated	33.22±1.64 NS	35.68±1.25 NS	38.77±1.05 NS	41.54±1.50 **	45.34±1.01 **	48.37±1.48 ***
Urea						
Control	10.53±0.73	11.74±0.67	12.61±0.67	13.59±0.61	15.22±0.68	12.74±0.68
Inoculated	10.45±1.35 NS	11.63±0.73 NS	13.35±0.73 NS	16.55±0.74 ****	18.77±0.69 ****	16.03±0.97 ****

NS = Not Significant * P<=0.05, ** P<=0.02, *** P<=0.01, **** P<=0.001

Graph-1: Histogram showing day to day changes in ammonia, uric acid and urea levels ($\mu\text{g/ml}$) in haemolymph of silkworm *Bombyx mori* L. inoculated with fungal pathogen *Beauveria bassiana* (Bals) Vuill with reference to control during 5th instar



acid nitrogen (Hirayama et al 1996) and to uric acid which is less toxic than ammonia. It is very much evident from the investigation. Raghavaiah et al (1988) reported a drastic reduction of ammonia content in *Beauveria bassiana* infected silkworm larvae compared to control with the progress of the infection indicating the impairment of amino acid metabolism. A similar observation was made by Ambika (1990) and Renuka and Shamitha (2012). Barsagade and Tembhare (2004) have studied the excretory metabolism of lepidopteran larvae at different stages of larval life and concluded that the end products fluctuate enormously from day to day.

Uric acid

The results are furnished in Table-1 and Graph-1. No significant change was observed in the uric acid content till the 3rd day of inoculation (33.22 $\mu\text{g/ml}$ to 37.77 $\mu\text{g/ml}$) and then significant elevation of uric acid levels was observed from 4th day to 6th day of inoculated 5th instar silkworm larvae (41.54 $\mu\text{g/ml}$ to 48.37 $\mu\text{g/ml}$) compare to control (39.33 $\mu\text{g/ml}$ to 44.49 $\mu\text{g/ml}$). The increased trend of uric acid was observed in haemolymph of both experimental and control silkworm (33.57 $\mu\text{g/ml}$ to 44.49 $\mu\text{g/ml}$).

Uric acid is the principal nitrogenous excretory product of insects (Wyatt 1961) and also terrestrial insects (Cochran 1975). Most of the nitrogenous waste excreted by the insects at any stage of development is represented by this metabolite. Razet (1961) found that either uric acid or allantoic was a major larval excretory product of several species of Lepidoptera. Large number of lepidopteran species shown to have uric acid as the predominant excretory product of both larval and pupal stages (Cochran 1975 and Buckner et al 1990). Uric acid is the primary excretory product of nitrogen and purine metabolism in *Bombyx mori*. It has to be excreted out as and when it is formed, so that a lower level is maintained in the haemolymph which will not in any way affect the normal growth of the larvae. Uric acid have many vital functions rather than being only an excretory product or induce pigmentation. Mitlin and Muldin (1966) Reported that uric acid can possibly serve as an amino acid precursor. Timmermann and Berenbaum (1999) documented that it also can act in biological system as a powerful antioxidant and it serves a protective function not only against predators in mimicry system, but also against oxidative stress generated by the phototoxic allelochemicals. Similarly Souza et al (1997) and Becker (1993) considered uric acid as a powerful antioxidant and radical-scavenging properties.

From the results it is quite clear that, urecomia has resulted due to infection by the fungus. This may be the direct consequence

of impairment of excretory organs (Malpighian tubules). It is well supported by Govindan et al (1998) and suggested that the enhancement of protein metabolism by NPV infection may induce the formation of large quantity of uric acid in diseased larvae. As per the report by Watanabe (1971), the enhancement of protein and nucleic acid metabolism in CPV infected larvae may induce the formation of large quantity of uric acid since it is the major end product of nitrogen metabolism in silkworm. Smirnoff (1971) also noticed that infection by NPV was associated with a substantial increase in the uric acid content in the larvae of *Neodiprion swainei*. Smirnoff and Loiselle (1969) investigated the effect of infection by a densovirus on the formation of uric acid in larvae of greater wax moth *Galleria mellonella*. The uric acid content of adipose tissue was considerably greater than the uninfected larvae and this increase was a direct physiological alteration in purine and protein metabolism of the host larva. It is known that uric acid is produced principally in the cells of the fat body and released into the blood, which transports it to the malpighian tubes for excretion. Pawar and Ramakrishnan (1977) reported that uric acid level comparatively decreased in normal larvae while it increased slightly in infected larvae. The difference reflects modifications in the metabolism of uric acid provoked by NPV infection. The results are in agreement with the report of Raghavaiah et al (1988). Uric acid accumulation increased in healthy and infected larvae according to Ambika (1990) the extent of increase being high in infected *Bombyx mori* suggesting the onset of urecomia in haemolymph due to infection.

Cochran (1975) and Karowe and Martin (1989) reported that larvae of antlion, excreted smaller quantities of nitrogenous excretory products during starvation than during periods of food abundance and suggested that quality and quantity of diet have a major impact upon the concentrations of uric acid in many insects. Tarek et al (2011) reported that highest concentration of spinetoram caused dramatic changes in especially on the 2nd and 6th day of treatment compared to the control. This may be attributed to insecticidal stress of spinetoram which may alter many metabolic activities. Similar findings were reported by Ramadev and Rao (1989). Shekari et al (2008) supported this hypothesis and reported that insecticidal application caused uric acid to the extent that prevented the natural excretion of uric acid and attributed this to altered metabolic pathway after treatment. Generally, there is an adverse correlation between the amount of protein and uric acid in haemolymph of silkworm and those larvae with lowered protein have elevated uric acid. It is evident from the present study that lower level of protein content and increased uric acid content was recorded in haemolymph of inoculated larvae.

Urea

The results on urea content are presented in Table-1 and Graph-1. Increased trend of urea was observed in haemolymph of both inoculated (10.45 µg/ml to 18.77 µg/ml) and control (10.53 µg/ml to 15.22 µg/ml) from 1st day to 5th day then the decreased trend was observed in both inoculated (16.03 µg/ml) and control (12.74 µg/ml) on the 6th day of the treatment. Compared to control (13.59 µg/ml to 12.74 µg/ml) significant elevation of urea content was recorded from the 4th day to 6th day of induction of fungal pathogen (16.55 µg/ml to 16.03 µg/ml).

Urea as an excretory compound plays an important role in silkworm physiology and changes of its concentration in haemolymph of silkworm larvae are dependent on many factors such as larval stage, diet etc (Sumida et al 1993). Changes in urea content are directly related to nitrogen metabolism and amino acids (Hirayama et al 1996). Urea is a significant excretory product of insects (Cochran 1975). The occurrence of urea has been demonstrated in the haemolymph of numerous insect species (Wang and Patton 1969; Corrigan 1970; Yamada and Inokuchi 1985; Lazar and Mohamed 1989). Urea plays a vital role in controlling of blood volume in terrestrial and semiterrestrial animals. The urea accumulates in the blood and reduces the vapour pressure and thus reduces the evaporative water loss.

The urea content of haemolymph increased till fifth day and decreased suddenly on six day of fifth instar in both inoculated and control batches. The levels of haemolymph urea in infected were significantly higher from 4th day to 6th day of fifth instar compared to control. Raghavaiah (1988) noticed the gradual elevation of urea in both healthy and *Beauveria bassiana* infected larvae up to fourth day but the level of urea has dropped in the later stage. But slight elevation of urea content was noticed in experimental animal compared to control suggested that uremia has not resulted with infection.

Sumida et al (1995) reported that the time of 72 hr of the fifth instar corresponds to the period for the silkworm larvae to initiate intensive biosynthesis of fibroin and sericine in the silkworm and their secretion into the lumen contents of the tissue

for storage for the cocoon formation at the later spinning stage. It requires abundant amino acid source. The starved silkworm larvae must find an alternative amino acid source from other tissue than midgut. A major candidate is the fat body which contains stored proteins in it. Probably upon starvation, the stored proteins start to be hydrolysed and amino acids produced are either metabolized in the cells or released into the haemolymph. This may be correlated with the alterations of urea content in the study during the development of fungal pathogen which induces stress in the host organism.

In contrast to the present study Renuka and Shamitha (2012) reported that uric acid and urea in the excretory pellets of pebrine infected larvae has been decreased, with a corresponding decrease in larval and silkworm weight which may be due to poor nutrition coupled with lesser enzyme activity in the malpighian tubules and midgut which are the main regions involved in elimination of excretory material.

Nitrogenous waste materials are the end products of metabolic reactions. Ammonia, urea and uric acid are the major nitrogenous wastes excreted. These waste materials must be removed from the body, if these nitrogen materials are allowed to accumulate in the body may become toxic. In view of the significance of various nitrogen products in various metabolic cycles the study was carried out to demonstrate the day to day changes in nitrogen waste products i.e. ammonia, urea and uric acid during the progressive development of fungal pathogen *Beauveria bassiana* in 5th instar silkworm larvae. More elaborate investigations on these lines are required to convincingly establish the correlation with reference to nitrogen metabolism in silkworm larvae during the development of fungal pathogen.

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