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Hemolymph Protein and Carbohydrate Alteration during Larval Development in *Samia ricini* Infected with *Nosema*

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Abstract: ***Aim of the present investigation was to analyze the hemolymph protein and carbohydrate concentration in the last instar larva of Samia ricini inoculated with Nosema. It was found that during the development of V instar larva of Samia ricini, there is a rapid increase in the hemolymph protein concentration which attained its peak at the end of larval life irrespective of the season. During July to August it was found that hemolymph protein concentration decreased significantly compared to control on day 3 post inoculation. The decline in protein concentration continued till the last day (day 6). During September-October and November-December, no significant difference was observed in hemolymph protein concentration between control and inoculated larva till day 5 post inoculation. On day 6 post inoculation hemolymph protein concentration was found to be significantly low compared to normal control. Hemolymph carbohydrates also increased steeply in the normal V instar larva and attained its peak on the last day of larval life. After inoculation with Nosema, no significant difference was observed during day 1-5 post inoculation. However, hemolymph carbohydrates decreased significantly at the end of larval life compared to control.***

Keywords: *Samia ricini*, *Nosema*, hemolymph protein, silkworm

1. Introduction

During the larval development high molecular weight proteins are synthesized in large amount by the larval fat body and secreted into the hemolymph. During the pupation these proteins gradually accumulate in the fat body as membrane bound storage granules. Such proteins are known as major larval hemolymph proteins or storage proteins. Synthesis of such proteins is dependent on the nutritional status of silkworm during the larval development (Ramesh Babu *et al*., 2009) and environmental conditions (Benchamin and Anantharaman, 1990, Ramesha *et al*., 2010). Proteins are important for the development, metamorphosis and to maintain a number of physiological functions (Kumar *et al*., 2011, Murthy *et al*., 2014). Nutritional importance of proteins in respect to development of silkworm has been elucidated by Sumioka *et al* (1982) and Singh *et al* (2011). Carbohydrates are also important class of biomolecule playing important role as energy source and protecting silkworm during adverse condition. Silkworm larva generally suffers from various diseases causing heavy loss to silk industry. The most destructive parasitic diseases are Pebrine caused by *Nosema* and Muscardine by *Beauveria*. Even though, most of the work has been done on the breeding aspect of silkworms, not much work has been published so far on pathological aspect of this *Samia ricini*. To date, reports on cellular and biochemical changes after parasitic infection in *Samia ricini* are scanty. So far, no systematic investigation has been made on comprehensive and comparative study on protein and carbohydrate content during the larval development of *Samia ricini* infected with *Nosema*. Present investigation was carried out to understand the dyanamics of two main biomolecules, protein and carbohydrate during the development of *Samia ricini* infected with *Nosema*.

2. Material and Method

Disinfected eggs of *Samia ricini* were collected from Central Silk Production Centre, Azara, Guwahati. Rearing was done as per the sericulture manual, Directorate of Sericulture, Government of Assam. *Nosema* spores were collected from infected larvae and crushed in a glass mortal pestle with isotonic buffered saline. Crushed suspension was filtered through muslin cloth and stored in deep freezer. Spores were purified by 60% sucrose gradient method.

Inoculation of larva with Nosema spores

Newly hatched healthy V instar larva were randomly collected from stock room, starved for 6 h and divided into 4 batches, each batch with 50 larvae. 3 µl spore suspension containing $8x10^5$ spores/ml was smeared on each castor leaf. Leaves were dried in air and used to inoculate larvae of three batches. Experimental batches were reared in a separate room. Control batch was provided with leaves smeared with distilled water. The inoculation was done during 3 different seasons (July-August, September-October and November-December).

Hemolymph collection

Hemolymph was collected in a pre-chilled test tube containing a few crystals of thiourea by cutting the first proleg of V instar larva. Hemolymph was collected at an interval of 24h for 6 days from day 0 post-inoculation. Collected hemolymph was centrifuged at 3,000 rpm for 3 min and supernatant was used for estimation of total hemolymph ptoteins and carbohydrates.

Estimation of Protein and Carbohydrate

Total hemolymph protein was estimated according to Lowry *et al* (1951) using Bovine Serum Albumin (BSA)

as standard. Total hemolymph carbohydrates were estimated by Anthrone Method.

Statistical Analysis

For data analysis the statistical computer application SPSS16.0 was used. Mean average of three independent experiments was calculated. One way analysis of variance (ANOVA) was used to test the significance of mean at $p<0.05$

3. Results and Discussion

In the present study it was found that during the development of V instar larva of *Samia ricini*, there is a rapid increase in the hemolymph protein concentration which attains its peak at the end of larval life irrespective of the season. Similar observations have been recorded where protein concentration was found to be higher at the end of larval stage in the silkworms (Ito and Arai, 1963, Banno *et al*., 1993, Murthy *et al*., 2014). Nutrition plays an important role in the development and metamorphosis particularly in lepidopteran insects where adult is a non-feeding stage (Srivastava *et al*., 1982). High concentration of hemolymph proteins have been correlated with high consumption of mulberry leaves and subsequently high rate of conversion and their accumulation in hemolymph of *Bombyx mori* (Banno *et al*., 1993, Aruga, 1994). It has been proved that larval fat body is active in the synthesis and secretion of hemolymph proteins during the larval growth. When larva cease to feed these proteins are again selectively removed from the hemolymph and stored as intracellular granules for use at the time of metamorphosis (Chen, 1978). High protein concentration is an indication of greater metabolic activity. Synthesis and utilization of hemolymph proteins are conditioned by genetic and hormonal control (Hurliman and Chen, 1974). In the present study, a steady increase in the hemolymph protein concentration during the development of last larval instar in all the seasons indicates the influence of dietary proteins with no effect of season provided dietary status remain the same. Similar trend has been observed in the hemolymph carbohydrates which increased with the advancement of age of larva and reached at its peak on the last day of V instar larva. Similar observation have been recorded earlier where total carbohydrates/concentration of reducing sugars were found to increase and attaining its peak in different silkworms during late larval stage (Simex and Kodrik, 1986, Mishra *et al*., 2010, Murthy *et al*., 2014). Like glycogen and trehalose are main constituent of hemolymph and play an important role during growth, metamorphosis and diapause (Jo and Kim, 2001). It has been reported that high concentration of carbohydrates in hemolymph are maintained during larval development as energy reserve to be utilized later during metamorphosis, pupal and adult stage (Simex and Kodrik, 1986, Mishra *et al*., 2010). During metamorphosis, glycogen and trehalose supply glucose which provides an energy source and a substrate for the synthesis of pupal and adult cuticle. In preparation for metamorphosis, insect usually accumulate maximum carbohydrates as a mature non-feeding larva. At this time carbohydrates reserve is mainly glycogen in the fat body and trehalose in the hemolymph (Chippandale, 1978).

During July to August it was found that hemolymph protein concentration decreased significantly compared to control on day 3 post inoculation. The decline in protein concentration continued till the last day (day 6). During September-October and November-December, no significant difference was observed in hemolymph protein concentration between control and inoculated larva till day 5 post inoculation. On day 6 post inoculation hemolymph protein concentration was found to be significantly low compared to normal control. In contract to present findings, higher protein concentration was observed in the larva of *Antheraea mylitta* infected with polyhedrosis virus, however, on the last day the protein concentration was found to decrease significantly compared to control (Singh *et al*., 2011). Increase in the hemolymph protein concentration was related to increased synthesis and release of fat body proteins, production of antimicrobial substances such as lectins, defensins and attacins. Pombo (1998) also reported production of viral induced protein in silkworm larva infected with baculovirus. Watanbe *et al*.(1971) reported active synthesis of midgut proteins as well as polyhedron proteins induced by polyhedrosis viral infection which continued till the end of larval life.

Table 1: Hemolymph protein concentration in V instar *Samia ricini* infected with *Nosema* in three different seasons

Season	Hemolymph Protein Concentration (mg/ml)						
	Days after Inoculation						
	Treatment	1	2	3	4	5	6
July-August	Control	36.68±3.32	43.06±2.98	47.11±3.97	52.12±4.31	53.73±4.07	60.00±3.68
	Infected	35.40±3.23	41.86±4.67	33.60±2.28*	30.60±1.94*	28.20±1.39*	23.33±1.63*
September-October	Control	45.38±3.60	46.52±3.09	51.31±1.79	55.11±3.38	54.82±4.35	60.66±4.80
	Infected	43.47±3.61	45.54±2.83	49.66±2.44	53.81±3.36	52.95±4.00	53.29±2.32*
November-December	Control	44.69±5.32	47.25±4.31	52.62±1.90	52.69±1.94	55.18±2.62	61.20±3.43
	Infected	42.47±5.69	46.02±4.26	51.43±2.35	52.00±1.31	52.58±2.74	51.06±5.81*

*Significantly different $p<0.05$

Table 2: Total Hemolymph Carbohydrates in V instar *Samia ricini* infected with *Nosema* in three different seasons

Season	Total Hemolymph Carbohydrates (mg/ml)						
	Days after Inoculation						
	Treatment	1	2	3	4	5	6
July-August	Control	16.58±3.75	17.57±3.03	22.81±1.04	23.46±2.17	28.59±2.94	30.54±1.62
	Infected	15.67±3.45	16.19±2.32	21.67±1.09	20.90±2.68	22.0±1.65*	23.66±3.67*
September-October	Control	14.13±0.99	16.42±0.73	17.47±0.83	19.01±0.55	21.97±1.23	23.60±1.05
	Infected	13.27±0.40	16.67±1.00	18.20±0.60	19.48±0.30	20.48±0.90*	21.52±1.10*
November-December	Control	13.28±1.23	17.67±0.74	19.60±0.25	20.25±0.17	20.41±0.78	22.61±0.32
	Infected	11.97±1.22	17.01±0.52	18.83±1.13	20.09±0.37	20.32±0.61	21.17±0.52*

* Significantly different $p<0.05$

In another study on *Bombyx* mori larva infected with *Bassiana*, hemolymph proteins exhibited no change on the day 1 post inoculation followed by an increase on day 2 and 3 and decrease thereafter till the last day of larval life (Rajitha *et al*., 2013). It was stated that initial enhancement was due to elicit humoral as well as cellular response to encounter the microbial inoculation. The humoral factors present in the hemolymph are attributed as a lysozymal function to bacterial substance (Powning ang Davidson, 1973, 1976). In another study, Watnabe *et al*. (1968) observed hypoproteinemia in the hemolymph of silkworm inoculated with *Bombyx mori* Nucleopolyhedrosis virus (BmNPV). They attributed this to fat body which is most susceptible tissue to Nucleo polyhedrosis virus (NPV). Devdas (1991) reported a significant reduction in hemolymph and gut protein content after inoculatation of *Serratia marcescens* and correlated with the fat multiplication of bacteria.

In the present study, a decrease in the hemolymph protein concentration was observed at a later stage of inoculation. This may be due to reduced feeding by infected larva as well as due to metabolism of protein and amino acid of hemolymph by developing pathogen. Low protein content may also be due to reduced digestion/absorption from the midgut since the spores of *Nosema* have been found invading the midgut epithelial cells (unpublished observations). Similar to present observations, hypoproteinemia was observed in silkworm larva infected with Cytoplasmic Polyhedrosis Virus (CPV) only in the later stage of infection (Kawase and Hayashi, 1971). They concluded that hyperproteinemia was due to (i) reduction of protein content caused by formation of polyhedral bodies and (ii) starvation due to dysfunctioning of midgut. Kumar *et al*. (2011) studied protein profiles of various tissues of *A. mylitta* and reported decrease in hemolymph protein concentration. It was suggested that decrease was due to drastic degradation of structural proteins. But decrease in protein content failed to coincide with increase in amino acid concentration suggesting that degraded proteins might be utilized by pathogen for rapid development.

In the present study, like changes in hemolymph protein concentration, hemolymph carbohydrate concentration also decreased at the end of larval stage and may be due to compensation of deficiency of energy caused by stress.

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