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Research article

BIOCHEMICAL CHANGES DURING THE PROGRESSIVE INFECTION OF BmIFV IN THE SILKWORM, *BOMBYX MORI* L.

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ABSTRACT: Among silkworm diseases, Viral diseases are the major and most common during silkworm rearing and cause considerable damage to cocoon production. Viral flacherie in silkworm is caused by the two non-occluded viruses *viz*., BmIFV and BmDNV. BmIFV causes the highly contagious infectious flacherie disease in silkworm. In our earlier studies susceptible and tolerant silkworm breeds against BmIFV were identified from the Indian Germ plasm stock. In the present study, the changes in major organic constituent's *viz.*, total protein, total carbohydrate and total lipid content during the progressive infection of BmIFV in a susceptible breed, CSR2 was studied. As the larval age increases, there was a consistent increase in the level of organic constituents *viz*., total protein, total carbohydrates and total lipids in both control and treated batches but the level of increase in treated batches were markedly less when compared to the respective control batches. During the progressive infection of BmIFV, there was a significant decrease in total protein and total carbohydrate contents and significant increase in total lipid content in the haemolymph and mid gut tissues when compared with control batches. These changes were reflected in the expression of discernible morphological changes, disease symptoms and larval mortality.
Key words: BmIFV, Haemolymph, Mid gut, Progressive infection, Silkworm, *Bombyx mori* L., Total protein, Total carbohydrate, Total lipid.

INTRODUCTION

The success and stability of silkworm cocoon production depends upon the protection of silkworms from the diseases caused by different pathogens. Among silkworm diseases, viral flacherie is common during silkworm rearing. *Bombyx mori* Infectious flacherie virus (BmIFV) causes infectious flacherie disease in silkworm which is chronic in nature. The infected larvae show gradual reduction in size, retarded growth, reduction in body weight and flaccid condition of body followed by vomiting and death [1]. The biochemical parameters *viz*., Proteins, Carbohydrates, Lipids, Nucleic acids *etc.*, vary significantly during the life cycle of all living organisms. The quantitative variation in these biomolecules in the body of insects depends upon the nutritional status of the food and their utilization during growth and metamorphosis [2]. A large volume of literature is available on the biochemical profile of the silkworm, *Bombyx mori* L. [3, 4, 5]. The progressive multiplication of a pathogen in the host system is often reflected by specific metabolic changes coupled with corresponding biochemical changes in the infected tissues. The effect of BmDNV1 infection on the biochemical parameters in silkworm, *B. mori* L. was reported in earlier studies [6]. In our earlier studies [7], susceptible and tolerant breeds were identified from the Germplasm stock of CSR&TI, Mysore against BmIFV and CSR2 was found as most susceptible. The studies also reported the lethal doses (LC_{50}) of BmIFV to different instars of silkworm and morphological changes during the progressive infection of BmIFV in the susceptible breed (CSR2) of silkworm. However, the impact of progressive infection of BmIFV on the biochemical changes in the silkworm has not been studied. Hence, in the present paper, the changes in the selected biochemical parameters during the progressive infection of BmIFV in a susceptible breed of silkworm were studied and discussed.

MATERIALS AND METHODS

Preparation of BmIFV stock inoculum

Initial inoculum of BmIFV was received from Silkworm Pathology, Central Sericultural Research and Training Institute (CSR&TI), Mysore, India and was multiplied in a susceptible silkworm breed by *per os* inoculation through mulberry leaf. The mid guts of BmIFV infected larvae were collected pooled and 10 % homogenate was prepared in sterilized distilled water. .

The homogenate was centrifuged at 5,000 rpm for 30 min at 4°C. The supernatant was collected and filtered through 0.45 mm membrane filter. The filtrate is used as BmIFV stock inoculum and stored at -20°C for further use.

Inoculation of BmIFV

Based on the earlier works [7], BmIFV susceptible breed (CSR2) was selected for the present study. The disease free layings of this breed were received from Silkworm Germ Plasm stock of CSR&TI, Mysore and were reared following the standard method [8] up to III moult. Immediately after III moult the larvae were divided into two sets. First set of larvae were per orally inoculated with LC_{50} dose of BmIFV at $10^{-7.3}$ dilution of stock inoculum by smearing onto mulberry leaf @ 1ml/100 sq. cm leaf/100 larvae. Second set of larvae was reared without inoculation of pathogen to serve as control batches. Three replications were maintained for each treatment and larvae were reared till cocooning.

Collection of samples

From 0 day (Immediately after III moult, before inoculation of BmIFV) to 12 days of post inoculation (PI), the haemolymph and mid gut tissues were collected from both treated and control batches (6 samples each) by the following methods.

Haemolymph

The pro legs of silkworm were cut and collected the haemolymph in eppendorf tubes containing a speck of phenylthiourea. The collected haemolymph was stored at 5ºC for further estimation of different organic constituents.

Mid gut tissue

The mid gut tissue from the larvae of different treatments and control batches was collected and homogenized in sterilized distilled water to obtain 1 % tissue homogenate. The homogenate was stored at 5°C for further estimation of different organic constituents.

Estimation of Total Proteins

The total protein content in the haemolymph and mid gut was estimated by following the method of Lowry *et al.* [9]

Haemolymph

100 µl of haemolymph and 100 µl of ice-cold 20 % trichloroacetic acid were added in an eppendorf tube and the contents were mixed thoroughly and kept in refrigerator for 30 minutes. The tubes were centrifuged at 3,000 rpm for 5 minutes. The pellets were washed twice with cold 80 % acetone followed by cold diethyl ether twice. Finally, the pellet was suspended in 500µ l of 0.1N NaOH. To 100 µl of this sample, 5 ml of alkaline copper sulphate reagent (To prepare alkaline copper sulphate reagent, Reagent A: 2 % Sodium carbonate in 0.1N NaOH; Reagent B; 0.5 % Copper Sulphate and 1 % Potassium Sodium tartrate in 1:1 ratio. Reagent A and B were mixed at 50:1 ratio just before the use) was added in a test tube and the contents were mixed well and kept for 30 minutes. Later, 0.5ml of Folin phenol reagent was added to this and the contents were shaken well. After 30 minutes the colour intensity was read at 660 nm in a spectrophotometer against the blank sample which contained 5 ml alkaline copper sulphate reagent and 0.5 ml Folin-phenol reagent. The protein content was recorded from, the standard curve prepared for bovine serum albumin (10-100 µg). The protein content was expressed as mg/ml of haemolymph.

Mid gut tissue

500 µl of mid gut homogenate and 50 µl of ice –cold 20 % trichloroacetic acid were taken in an eppendorf tube and the contents were mixed thoroughly and kept in an refrigerator for 30 minutes and later centrifuged at 3,000 rpm for 5 minutes. The pellets were washed twice with cold 80 % acetone followed by cold diethyl ether twice. Finally, the pellet was suspended in 500 µl of 0.1N NaOH. To 500 µl of this sample, 5 ml of alkaline copper sulphate reagent was added in a test tube and the contents were mixed well and kept for 30 minutes. Later, 0.5 ml of Folin-Phenol reagent was added to this and the content were shaken well. After 30 minutes the colour intensity was read at 660 nm in spectrophotometer against the blank sample, which contained 5 ml alkaline copper sulphate reagent and 0.5 ml Folin-Phenol reagent. The protein content was recorded from the standard curve prepared for bovine serum albumin (10-100µg). The protein content was expressed as mg/g wet weight of tissue.

Estimation of Total Carbohydrates

The total carbohydrate content in the haemolymph and mid gut tissues was estimated by the phenol- sulphuric acid method described by Dubois *et al.* [10].

Haemolymph

100 µl of haemolymph, 0.4 ml of 5 % phenol acid were taken in an eppendorf tube and the contents were mixed well. To this 2 ml of conc. H_2SO_4 was added and the contents were mixed well again. The tubes were cooled at room temperature by keeping them in running tap water. The colour intensity was measured at 490 nm in a spectrophotometer against the blank sample b. The total carbohydrate present in each sample was calculated from a standard curve prepared by taking 20-200 µg of glucose. The total carbohydrate present in each sample was expressed as mg/ml.

Mid gut tissue

500 µl of mid gut tissue sample 0.4 ml of Phenol acid were taken in an eppendorf tube and the contents were mixed well. To this 2 ml of conc. H_2SO_4 was added and the contents were mixed well again. The tubes were cooled at room temperature by keeping them in running water. The colour intensity was measured at 490 nm in an each sample was calculated from a standard curve prepared by taking 20-200 µg of glucose. The total carbohydrates present in each sample were expressed as mg/g wet weight of tissue.

Estimation Total Lipids

Total lipid content in the haemolymph and gut samples was estimated gravimetrically following the method of Folch *et al.* [11].

Haemolymph

1ml of haemolymph, 1ml of chloroform methanol mixture (2:1 v/v) were mixed thoroughly. After 30 minutes the mixture was centrifuged at 3,000 rpm for 15minutes. the lower chloroform layer containing lipid was collected into a pre-weighed plastic vial. To the upper aqueous layer 0.5ml of chloroform methanol mixture was added and the procedures were repeated thrice. The pooled chloroform-lipid mixture was air-dried and the weight of the vial was recorded until there was no change in the weight of the vial which gave the lipid content of the sample. The quantity of lipid present was expressed in mg/ml of haemolymph.

Mid gut tissue

1ml of mid gut tissue homogenate, 1ml of chloroform methanol mixture (2:1 V/V) were mixed thoroughly after 30 minutes, the lower chloroform layer containing lipid was collected into a pre-weighed plastic vial. To the upper layer 0.5ml of methanol mixture was added and the process was repeated thrice. The chloroform-lipid mixture was air-dried and the weight of the plastic vial was taken. The difference between the initial and final weights of the vial gave the lipid content of the sample. The quantity of lipid present was expressed in mg/g wet weight of tissue.

RESULTS

Changes in the Total Proteins

The results on the total protein content in heamolymph and mid gut tissue during the course of BmIFV infection is presented in Table 1. The haemolymph and mid gut total protein content increased steadily as the age of the larvae increased both in control and BmIFV treated batches. On the day of inoculation (0 day) the haemolymph total protein content was 6.31 mg/ml and increased by 12^{th} day post inoculation (PI) to 44.11 mg/ml in the control and 22.42 mg/ml in BmIFV treated batches. In the same way, total protein content in the mid gut tissue was also increased. There was a significant reduction in the total protein content of the haemolymph and mid gut tissues of BmIFV treated batches during the progressive infection when compared to the respective controls. The percent change over the control in total protein content in the tissues during the course of BmIFV infection in silkworm is presented in Fig. 1. As the BmIFV infection progresses, the percent reduction of total protein content in both the tissues was more and incase of haemolymph it was from 3.72 to 49.16% and in mid gut it was form 7.59 to 36.48 %.

Changes in the Total Carbohydrates

The changes in the total carbohydrate content in haemolymph and mid gut tissue during the course of BmIFV infection is presented in Table 2. As the age of the larvae increased (control and treated batches) the haemolymph and mid gut carbohydrate content was also increased steadily. In control batches, the carbohydrate content in the haemolymph and mid gut tissue increased from 7.19 to 9.85 mg/ml and 11.21 mg/g to 24.08 mg/g respectively as the larval age increases from 0^{th} day to 12^{th} day while in treated batches it was 7.19 to 8.85mg/ml and 11.21 to 20.80mg/g. It is observed that the carbohydrates contents of the haemolymph and mid gut tissue have reduced in BmIFV treated batches compared to respective controls. The per cent changes over the control in total carbohydrate content in the tissues during the course of BmIFV infection in silkworm is presented in Fig. 2. As the BmIFV infection progresses, the per cent reduction of total carbohydrate content in both the tissues was increased from 2.69 to 10.19% incase of haemolymph and form 2.93 to 13.62%.

Changes in the Total Lipids

As the age of the larvae increases (IV instar to V instar) the total lipid content increased both in control and treated batches.

Table 1. Total protein content in the tissues during the course of BmIFV infection in the silkworm, *Bombyx mori* L.

Days after inoculation	Total Protein content in the haemolymph (mg/ml)			Total Protein content in the mid gut (mg/g)		
	Control	Treatment	't' value	Control	Treatment	't' value
0 day	6.31±0.08	6.316±0.08	-	12.19±0.17	12.19±0.17	-
1 day	7.28±0.10	7.01±0.06	5.905**	14.49±0.52	13.39±0.488	3.765**
2 day	12.03±0.93	8.16±0.24	1.240NS	15.55±0.36	13.80±0.30	9.189**
3 day	19.74±0.69	11.38±0.88	4.069**	18.18±0.11	15.46±0.22	26.848**
4 day	21.23±0.14	18.69±0.52	11.606 **	20.69±0.60	17.30±0.73	8.818**
5 day	18.40±2.91	16.03±2.63	1.481NS	23.531±0.52	19.46±0.29	16.796**
6 day	30.42±3.92	25.94±1.65	2.578**	27.77±0.54	22.30±0.51	18.080**
7 day	34.29±2.45	26.81±2.85	4.880**	29.63±0.56	23.38±0.49	20.451**
8 day	37.07±1.21	27.81±2.30	8.712**	33.42±1.23	24.77±1.74	9.938**
9 day	40.52±1.57	25.83±1.90	14.589**	37.32±0.96	26.79±2.78	8.764**
10 day	41.22±2.67	29.40±1.11	10.032**	42.79±0.74	27.98±0.78	33.816**
11 day	42.40±2.63	24.21±2.65	11.935**	44.13±1.53	28.32±1.329	19.164**
12 day	44.11±1.03	22.42±1.64	27.497**	48.73±0.55	30.96±1.00	38.230**

NS: Non significant; *Significant at 5% level; **Significant at 1% level

Table 2. Total carbohydrate content in the tissues during the course of BmIFV infection in the silkworm, *Bombyx mori* L.

Days after inoculation	Total Carbohydrate content in the haemolymph (mg/ml)			Total Carbohydrate content in the mid gut (mg/g)		
	Control	Treatment	't' value	Control	Treatment	't' value
0 day	7.19±0.03	7.19±0.03	-	11.21±0.02	11.21±0.02	-
1 day	7.24±0.02	7.05±0.05	8.457**	11.27±0.03	10.94±0.05	14.260**
2 day	7.27±0.04	7.04±0.10	5.264**	12.42±0.05	12.01±0.04	15.038**
3 day	7.49±0.04	7.22±0.09	6.967**	12.60±0.02	12.07±0.05	22.571**
4 day	7.75±0.09	7.37±0.05	9.484**	12.92±0.03	12.38±0.04	25.947**
5 day	7.64±0.04	7.30±0.07	10.866**	13.55±0.03	12.92±0.02	39.397**
6 day	7.89±0.06	7.52±0.02	14.542**	15.04±0.03	13.93±0.02	66.901**
7 day	8.18±0.04	7.75±0.08	12.390**	15.05±0.03	13.70±0.02	102.465**
8 day	8.78±0.03	8.23±0.07	17.841**	16.74±0.02	15.11±0.06	60.824**
9 day	8.85±0.03	8.16±0.06	26.685**	18.30±0.04	16.24±0.07	64.996**
10 day	9.00±0.12	8.27±0.14	9.955**	18.99±0.04	16.54±0.12	48.134**
11 day	9.50±0.04	8.64±0.07	26.591**	20.37±0.05	17.47±0.65	10.868**
12 day	9.85±0.14	8.85±0.07	16.027**	24.08±0.13	20.80±0.04	59.323**

Ns: Non significant; **Significant at 1% level

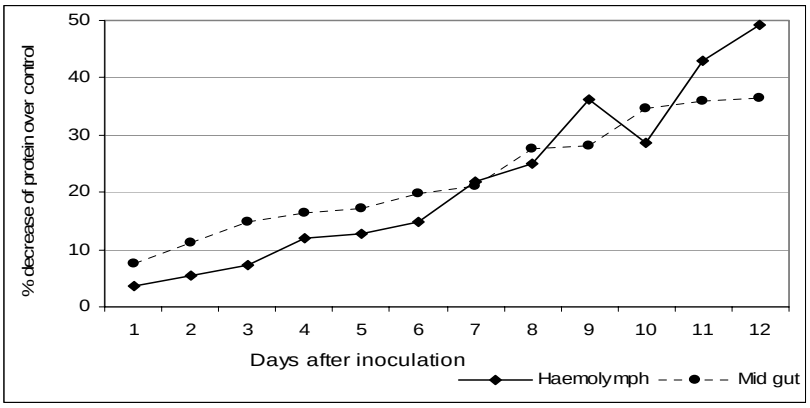

Figure 1: Percent changes in total protein content over the control in the tissues during the progressive infection of BmIFV in silkworm.

The changes in the total lipid content in haemolymph and mid gut tissue during the course of BmIFV infection is presented in Table 3. Total lipid content of the haemolymph and mid gut tissue in treated batches has significantly increased when compared to the respective control. The lipid content of the haemolymph from 0^{th} day to 12^{th} day increased from 9.89 to 29.39 mg/ml and mid gut tissue was 12.06 to 31.67 mg/g. The per cent changes in total lipid content over the control in the tissues during the course of BmIFV infection in silkworm is presented in Fig. 3. As the BmIFV infection progresses, the per cent increase of total lipid content in both the tissues was increased from 6.59 to 28.37% incase of haemolymph and form 6.89 to 30.99%.

Table 3. Total lipid content in the tissues during the course of BmIFV infection in the silkworm, *Bombyx mori* L.

Days after inoculation	Total lipid content in haemolymph (mg/ml)			Total lipid content in mid gut (mg/g)		
	Control	Treatment	't' value	Control	Treatment	't' value
0 day	9.89±1.20	9.89±1.20	-	12.06±0.83	12.06±0.83	-
1 day	18.11±2.86	19.39±1.02	2.345*	18.83±0.75	20.22±0.86	2.976*
2 day	18.61±0.77	20.56±0.91	3.989**	20.39±0.98	22.33±1.01.	3.390**
3 day	22.11±0.81	24.50±0.75	5.301**	21.61±0.65	24.06±0.85	5.588**
4 day	24.56±1.19	27.67±1.14	3.328**	25.28±1.18	28.11±1.11.	4.283**
5 day	21.11±0.95	24.17±0.96	4.628**	25.44±1.41	2833±0.84	3.478**
6 day	22.44±1.05	25.56±0.72	5.997**	23.39±0.49	26.17±0.81	7.187**
7 day	24.61±0.77	28.56±1.16	6.982**	26.22±2.20	29.67±2.01	3.380**
8 day	27.44±1.03	31.94±0.88	8.157**	31.17±0.62	38.94±0.77	19.194**
9 day	28.78±1.11	35.00±0.60	12.105**	31.94±0.65	41.17±0.62	25.140**
10 day	28.06±1.64	36.06±1.91	7.778**	32.94±1.68	43.22±0.91	13.181**
11 day	29.11±1.26	39.06±0.83	16.166**	32.89±2.33	44.06±0.88	10.962**
12 day	28.39±1.02	38.56±1.26	15.368**	31.67±1.07	45.89±1.17	21.952**

NS: Non significant; *Significant at 5% level; **Significant at 1% level

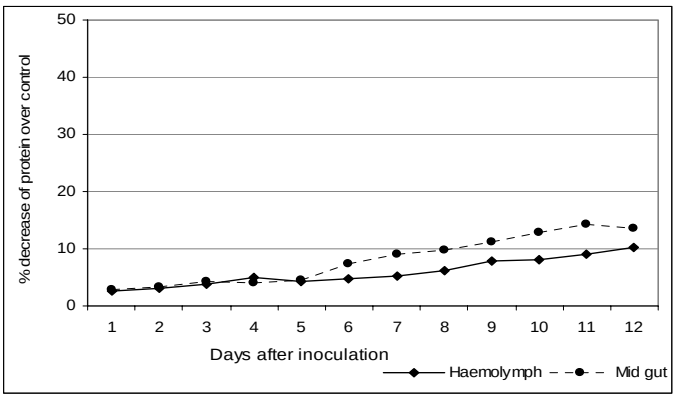

Figure 2: Percent changes in total carbohydrate content over the control in the tissues during the progressive infection of BmIFV in silkworm.

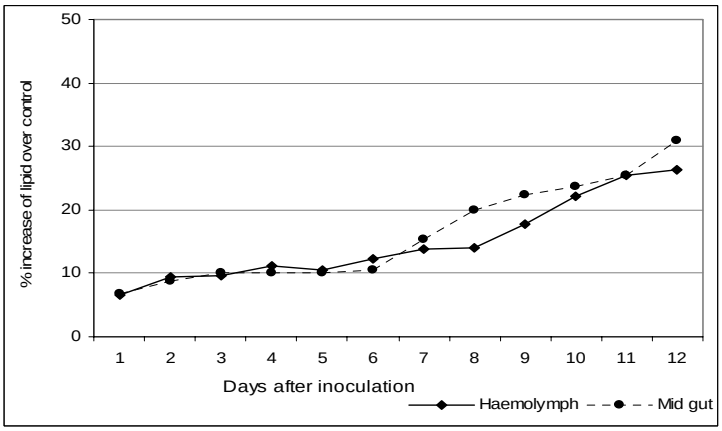

Figure 3: Per cent changes in total lipid content over the control in the tissues during the progressive infection of BmIFV in silkworm.

DISCUSSION

Pathogenic infections are reported to induce biochemical and physiological alterations in insect tissues [12, 13]. It is observed from the results of the present study that BmIFV is influencing the biochemical constituents such as protein, carbohydrate and lipid contents of silkworm. Earlier studies [6] also reported the same results with BmDNV1 infection in CSR2 breed silkworm. The proteins are derivatives of high molecular weight polypeptides, which play a vital role in the formation of structure of different organs. The decrease in total protein content in haemolymph and mid gut tissue can be attributed to the disintegration of structural organization at sub cellular level. Another reason for the decrease in protein content may be either by activated proteolysis or impaired protein synthesis in the tissues during the infection. The growth and development of larvae always depends on the active synthesis of protein in the tissues [14, 15]. The decreased protein content during BmIFV infection therefore could have inhibited the process of larval development. It is evident from the stunted growth of the larvae observed during BmIFV infection in susceptible breed. There was a significant depletion in total carbohydrate content in haemolymph and mid gut tissue during the progressive infection of BmIFV in treated larvae compared to the control. Carbohydrates serve as main source of energy to a number of insect species [16] and in the biological system. A breakdown of organic constituents mainly essential to meet the energy under stress condition [17]. The decreased carbohydrates levels in haemolymph and mid gut tissue can be attributed to excessive utilization of carbohydrates to meet the demand of energy of BmIFV infection. There was a significant increase in total lipid content in haemolymph and mid gut tissue during the progressive infection of BmIFV treated batches compared to respective control. Lipids serve as a source of metabolic energy as well as essential for structural components of cells. They also play a role in the synthesis of viral envelops [18] probably being responsible for the infectivity of virus [19].

The results of the present study revealed that the amount of lipid in the haemolymph and mid gut tissue increased markedly during the progressive infection of BmIFV. The marked increase in the lipid content of virus infected larvae had been attributed to the altered host lipid metabolism [20, 21]. The results of the present study are confirming the earlier results [1] indicated that CSR2 breed is highly susceptible to BmIFV infection. The larvae became inactive and under sized larvae were noticed in the rearing bed on 5DPI and on 7 DPI, larval mortality was recorded. As the larval age increases and the infection progresses, the disease symptoms become more discernible and diseased larvae became dull, soft and flaccid before death and developed dysentery and vomited gut juice. These symptoms are also similar to those of the reports [22].

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