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# Comparative Studies on the Midgut Proteins During Cypovirus Infection and in Mutants Raised from EMS Treated Silkworm *Bombyx mori* L.

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## ABSTRACT

*Two mulberry silkworm breeds viz., Pure Mysore and $NB_4D_2$ were selected for the present study. Two doses of cytoplasmic polyhedral inclusion bodies (CPIBs) i.e., 1.562 x $10^6$ and 3.125 x $10^6$ $ml^{-1}$ were selected based on the breed susceptibility test and inoculated in to silkworm larvae by oral injection. The control as well as CPIBs inoculated silkworm larvae were collected daily with a regular interval of 24 h for midgut tissue collection. Similarly, the $F_1$ progeny was raised from 2.5, 5 and 10 mM ethyl methanesulfonate (EMS) treated silkworms. The silkworms from control as well as EMS treated sets were collected daily with a regular interval of 24 h for midgut tissue collection. The midgut tissues of both CPIBs inoculated and the $F_1$ progeny raised from EMS treated silkworms were subjected for protein assay. Among the experimental batches, the CPIBs inoculated larvae exhibited reduction in the protein level and also there was gradual reduction as the disease progressed when compared to controls. On the other hand the silkworm batches raised from lower doses of EMS treated batches exhibited higher concentration of protein when compared to control as well as the silkworms treated with higher doses of EMS.*

***Keywords***: *Silkworm, Bombyx mori, cytoplasmic polyhedrosis virus, ethyl methanesulfonate, mutant, protein.*

## INTRODUCTION

The silkworm cocoon crops are highly unpredictable due to several factors including various diseases[1]. Of the various pathogens, the *Bm*CPV is widely distributed viruses in the sericultural belt of Karnataka State, India and causes considerable damage to silk production. In Karnataka alone the infection of *Bm*CPV accounted 27.76 %[2]. In the tissues of insect with an infectious disease, various biochemical, physiological and cytomorphological alterations can be observed[3,4,5]. Such alterations indicate the deteriorated physiological status of the host by reflecting either reduced or enhanced rate of metabolism as a part of defense mechanism. Most of the biochemical studies associated with cytoplasmic polyhedrosis in *Bombyx mori* are nucleic acid and protein alterations[6]; enzymes responsible for metabolism[7]; free amino acids in haemolymph and midgut[8]; protein metabolism[9]; amylase and succinate dehydrogenase activity levels[10]; effects of CPV and its transmission[1]; effect on haemolymph proteins[11] and haemolymph glucose level[12].

On the other hand, the silkworm, *Bombyx mori* L. is the most important insect being used for commercial production of silk in sericulture industry. Studies on the mutagenesis in silkworm, *Bombyx mori* L., have been in progress for the last few decades to synthesize new gene combinations with improved commercial qualities. Tanaka[13] was the first one to induce mutations in silkworm after the popular work of the artificial transmutation of the gene by Muller[14].

Since then, many efforts have been made by various scientists like Kogure, Tazima, Aruga, Takasaki, Chikushi and Tsujita[15] and also, numerous interesting results by Datta *et al*.[16]; Iyengar *et al*.[17]; Subramanya and Sreeramareddy [18]; Bhoopathy and Muthukrishan[19]; Mahesha *et al.*[20] ; Mahesha and Honnaiah[21]; Mahesha and Thejaswini[22] ; Mahesha and Thejaswini[23] have been reported.

The analysis of biomolecules like proteins, amylase, succinate dehydrogenase[5,10,21], alkaline phosphatase [24, 25] and alkaline protease [12] and molecular marker assisted breeding [13] will help in the silkworm breeding programme for better cocoon characters and disease resistance/tolerance. Hence, the present investigation was undertaken.

## MATERIALS AND METHODS

Two pure silkworm breeds namely, Pure Mysore and $NB_4D_2$ were selected as experimental system for the present investigation. The silkworm rearing was conducted in the laboratory following the method described by Krishnaswami[26].

The Cytoplasmic Polyhedral Inclusion Bodies (CPIBs), obtained from Sericulture Department, University of Agricultural Sciences, G.K.V.K. Bangalore, India, was *per orally* inoculated into the silkworm larvae immediately after second moult for the multiplication of virus. After 10 days of inoculation, the midgut of the silkworms which exhibited flaccid condition were dissected and the white midguts were selected. Such midguts were used for the isolation of CPIBs. Purification of CPIBs was carried out by following the method described by Balakrishnappa and Honnaiah [27]. Finally, the stock suspension was prepared which contained 6.25 X $10^6$ polyhedra $ml^{-1}$. Enumeration of polyhedra was done by following Neuber's haemocytometer. Based on the results of breed susceptibility experiment [12], the silkworm larvae immediately after 4$^{th}$ moult, *i.e.*, after first two feedings during fifth instar were inoculated with two different doses of CPIBs *viz.*, 1.562 x $10^6$ $ml^{-1}$ ($T_1$ batch) and 3.125 x $10^6$ $ml^{-1}$ ($T_2$ batch) by oral injection [20, 21, 28] and allowed to continue larval development. Each batch was consisted of 100 worms in triplicate. The midgut tissues was obtained from five larvae daily with a regular interval of 24h till the end of fifth instar by dissecting the larvae in ice cold water and the gut contents were removed. The tissues were thoroughly washed in sterile distilled water. A 1% (w/v) tissue homogenate was prepared in pre cooled sterile distilled water using mortar and pestle. The homogenate was centrifuged at 3000 rpm for 10 minutes in a cooling centrifuge at 5ºC. The clear supernatant was used for the assay of total proteins.

For rising the $F_1$ progeny three different concentrations of EMS like 2.5, 5 and 10 mM were selected after preliminary studies at chromosome level, viability of larvae, hatching percentage and viability of $F_1$ progeny[5]. Forty µl of final concentration of EMS freshly prepared in 0.75 % NaCl solution was administered separately to each worm in to the gut by 'oral injection[20, 21, 28]. The control worms received the same amount of NaCl solution only. For each concentration, 100 worms in triplicate were taken. After treatment, the larvae were allowed to continue development, spinning and pupation. Of the cocoons harvested, only uniform and healthy cocoons were selected and processed for the preparation of disease free layings. The layings of $NB_4D_2$ race were treated with hydrochloric acid to get immediate hatching[29,30, 31]. Such $F_1$ silkworm larvae from first day of fifth instar were collected daily with a regular interval of 24h till the end of fifth instar. The homogenate of the tissue was prepared as explained above.

The total soluble protein present in the midgut tissue was estimated by following the method of Lowry *et al*.[32]. Bovine Serum Albumin was used as standard protein. The values were expressed as µg of protein/mg tissue.

The data obtained from the biochemical experiments were statistically analyzed through SPSS by two way ANOVA, to determine the level of significance between experimental sets, between age groups and the interaction effect between the experimental sets and age groups [33] and Scheffe's post hoc test [34] wherever they were applicable.

## RESULTS AND DISCUSSION

Total proteins were estimated in midgut tissues of control as well as CPIBs inoculated worms of both Pure Mysore and $NB_4D_2$ breeds. In control worms of Pure Mysore breed, the concentration of total proteins showed gradual increase from 2$^{nd}$ day to 4$^{th}$ day. From 5$^{th}$ day onwards, there was a gradual decrease in protein concentration till the end of 5$^{th}$ instar.

Of the inoculated batches, $T_1$ batch showed gradual decrease from 4th day till the end of 5th instar, except 7th day when the protein concentration was found to be increased by about 12.47% as compared to that of 6th day. The batch of $T_2$ followed the pattern as in the case of control set, except 7th day when a slight increment was noticed (Table 1). The average protein concentration was the highest in the control (54.78 µg/mg) followed by $T_1$ (50.35 µg/mg) and $T_2$ worms (46.86 µg/mg). In $NB_4D_2$ breed, total protein levels of the control larvae showed gradual reduction throughout the 5th instar except 4th and 6th day, when a slight increment in the protein concentration was observed over their respective previous days. The *Bm*CPV inoculated worms exhibited a little alteration in the pattern as well as in the quantity of proteins (Table 2). When the average concentration of proteins during 5th instar was considered, the control set showed highest concentration of protein of 48.62 µg/mg tissue followed by $T_1$ worms of 42.82 µg/mg and $T_2$ worms of 41.41 µg/mg tissue.

In case of $F_1$ progeny raised from EMS treated worms, total protein levels were estimated in the control as well as of experimental sets of both Pure Mysore and $NB_4D_2$ breeds. In control worms of Pure Mysore, the concentration of total proteins showed a significant increase up to 4th day. From 5th day onwards, decreased pattern was observed till the end of fifth instar. In treated batches, the quantity of protein was increased and also the pattern was altered. The higher concentration of proteins was observed in 2.5 mM set, (59.65 µg/mg was the average during 5th instar) followed by the 5 mM set (59.10 µg/mg), 10 mM set (58.44 µg/mg) and control (56.02) (Table 3). In case of $NB_4D_2$ larvae, total protein levels in the control set showed the same pattern as in Pure Mysore, except for 3rd and 5th day. Of all the batches estimated, the 2.5 mM set showed the highest concentration of 47.41 µg/mg, followed by 5 mM set (46.60 µg/mg), control batch (46.38 µg/mg) and 105 mM set (44.59 µg/mg) (Table 4).

The *Bm*CPV infected Pure Mysore and $NB_4D_2$ larvae showed significant reduction in the protein content of midgut tissue (Tables 1-2). The decrease in protein levels was found to be high as the quantity of virus increased. In midgut tissue, lower protein level might be due to either or both of the following possibilities: owing to dysfunction of midgut tissue and/or utilization of silkworm proteins by the pathogen for the synthesis of viral and polyhedral proteins[5]. Kawase and Hayashi's[8] results also support our observations. Their results revealed that the silkworms infected with CPV showed distinct decrease of protein in the blood and also in the midgut cells. Inagaki and Suto, proposed two possibilities in CPV infected larvae. Firstly, the blood protein in the diseased larvae is decreased due to the formation of polyhedra. Secondly, the decrease of blood protein in the diseased larva is caused by starvation, because of the dysfunction of the midgut.

On the other hand, the average concentration protein in the Pure Mysore larvae raised from EMS treated worms is higher than the control worms. Mahesha[5,22] reported that lower doses of EMS might stimulate silkworm vitality and improves the commercial characters, which has been confirmed by the biochemical studies also [12, 20, 21]. Further, it might have induced mutation resulting in the changed character, which is passed on from generation to generation in *Bombyx mori*. The enhanced rate of metabolism in the treated sets clearly indicated enhanced digestion, absorption, conversion and productivity.

Hence, the present investigation clearly indicated that the *Bm* CPV inoculated silkworms showed reduced amount of protein level reflects the utilization of less food material, reduced rate of conversion and metabolism resulting in less production in the surviving silkworms as the main portion of the digestive system *i.e*., midgut is infected by virus. On the other side, the $F_1$ silkworms raised from lower doses of EMS treated larvae exhibited more protein content clearly indicated enhanced digestion, absorption, conversion and productivity. Such a basic knowledge about these biochemical aspects during cypovirus infection as well as in mutants is useful to plan detailed studies at the molecular level for identification of biochemical markers useful for developing disease resistant breeds/improved commercial qualities. Also, the information obtained from this research work contributes a lot to basic physiology of silkworm *Bombyx mori* in general.

**Table 1: Concentration of total proteins (µg/mg) in midgut tissue of Pure Mysore silkworm during cypovirus infection**

| Dose of *Bm*CPIBs | Fifth Instar Development in Days | | | | | | | Average Concentration |
|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 | 8 | |
| C | 55.06 | 56.29 (+2.23) | 59.23 (+5.22) | 57.13 (-3.54) | 55.31 (-3.18) | 53.63 (-3.04) | 46.81 (-12.71) | 54.78 |
| T1 | 54.79 (-0.49) | 56.73 (+3.54) (+0.78) | 53.57 (-5.57) (-9.55) | 51.11 (-4.59) (-10.54) | 43.14 (-15.59) (-22.04) | 48.52 (+12.47) (-9.53) | 44.61 (-8.06) (-4.70) | 50.35 |
| T2 | 49.41 (-10.26) | 50.08 (+1.36) (-11.03) | 51.57 (+2.97) (-12.93) | 48.44 (-6.07) (-15.21) | 41.84 (-13.62) (-24.35) | 45.66 (+9.13) (-14.86) | 41.01 (-10.18) (-12.39) | 46.84 |

C-Control batch; $T_1$ and $T_2$ - Live virus treated batch.
Values within parenthesis (1st row) represent percent change over previous day.
Values within parenthesis (2nd row) represent percent change over control.
The variation between control and experimental set is significant at 5% level.

**Table 2: Concentration of total proteins (µg/mg) in midgut tissue of $NB_4D_2$ silkworm during cypovirus infection**

| Dose of *Bm*CPIBs | Fifth Instar Development in Days | | | | | Average Concentration |
|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | |
| C | 50.94 | 47.94 (-5.89) | 48.90 (+2.00) | 46.02 (-5.89) | 49.31 (+7.15) | 48.62 |
| T1 | 43.00 (-15.59) | 39.32 (-8.56) (-17.89) | 46.45 (+18.13) (-5.01) | 44.13 (-4.99) (-4.11) | 41.22 (-6.59) (-16.41) | 42.82 |
| T2 | 45.34 (-10.99) | 33.46 (-26.20) (-30.20) | 42.64 (+27.64) (-12.80) | 43.07 (+1.01) (-6.41) | 42.55 (-1.21) (-13.71) | 41.41 |

C-Control batch; $T_1$ and $T_2$ - Live virus treated batch.
Values within parenthesis (1st row) represent percent change over previous day.
Values within parenthesis (2nd row) represent percent change over control.
The variation between control and experimental set is significant at 5% level.

**Table 3: Concentration of total proteins (µg/mg) in midgut tissue of $F_1$ progeny raised from EMS treated Pure Mysore silkworms**

| Conc. of EMS (mM) | Fifth Instar Development in Days | | | | | | | | Average Concentration |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | |
| C | 56.26 | 58.13 (+3.32) | 58.74 (+1.05) | 60.27 (+2.60) | 58.72 (-2.57) | 54.64 (-6.95) | 53.37 (-2.32) | 48.04 (-9.99) | 56.02 |
| 2.5 | 66.46 (+18.13) | 65.26 (-1.80) (+12.26) | 63.30 (-3.00) (+7.76) | 49.15 (-22.35) (-18.45) | 53.00 (+7.83) (-9.74) | 55.19 (+4.13) (+1.00) | 61.75 (+11.88) (+15.70) | 63.10 (+2.19) (+31.35) | 59.65 |
| 5 | 63.98 (+13.72) | 62.75 (-1.92) (+7.94) | 57.90 (-7.73) (-1.43) | 58.08 (+0.31) (-3.63) | 62.17 (+7.04) (+5.87) | 55.98 (-9.96) (+2.45) | 55.87 (-0.20) (+4.68) | 56.10 (+0.41) (+16.78) | 59.10 |
| 10 | 62.34 (+10.80) | 60.94 (-2.24) (+4.83) | 59.12 (-2.99) (+0.65) | 56.-1 (-5.26) (-7.07) | 60.16 (+7.41) (+2.45) | 55.99 (-6.93) (+2.47) | 56.12 (+0.23) (+5.15) | 56.82 (+1.25) (+18.27) | 58.44 |

C-Control batch; 2.5, 5, 10- EMS treated batches.
Values within parenthesis (1st row) represent percent change over previous day.
Values within parenthesis (2nd row) represent percent change over control.
The variation between control and experimental set is significant at 5% level.

**Table 4: Concentration of total proteins (µg/mg) in midgut tissue of $F_1$ progeny raised from EMS treated $NB_4D_2$ silkworms**

| Conc. of EMS (mM) | Fifth Instar Development in Days | | | | | | Average Concentration |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| C | 35.83 | 51.28 (+43.21) | 46.34 (-9.63) | 50.83 (+9.69) | 45.13 (-11.21) | 48.84 (+8.22) | 46.38 |
| 2.5 | 35.39 (-1.22) | 47.22 (+33.42) (-7.91) | 53.96 (+14.27) (+16.44) | 52.58 (-2.55) (+3.44) | 45.65 (-13.17) (-1.15) | 46.73 (+2.36) (-4.32) | 47.41 |
| 5 | 46.44 (+29.61) | 51.09 (+10.01) (+0.37) | 53.44 (+4.59) (+15.32) | 51.46 (-3.7) (+12.39) | 47.22 (+8.22) (+4.63) | 29.96 (+36.55) (-38.66) | 46.60 |
| 10 | 41.40 (+15.54) | 40.64 (-1.83) (-20.83) | 59.23 (+45.74) (+27.82) | 52.12 (-12.00) (+2.54) | 45.97 (+11.78) (+1.86) | 34.17 (+25.66) (-30.04) | 44.59 |

C-Control batch; 2.5, 5, 10- EMS treated batches.
Values within parenthesis (1st row) represent percent change over previous day.
Values within parenthesis (2nd row) represent percent change over control.
The variation between control and experimental set is significant at 5% level.

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