EFFECT OF NUCLEAR POLYHEDROSIS ON SOME BIOMOLECULES OF SILKWORM, BOMBYX MORI.

H.B. Mahesha, H.P. Krupa and P.H. Thejaswini

Department of Biotechnology, Yuvaraja's College, University of Mysore, Mysore - 570 005, India
Department of Biotechnology, Maharani's Science College for Women, Mysore - 570 005, India.

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The effects of infection by nuclear polyhedrosis on the haemolymph proteins, amylase, superoxide dismutase and β-esterase were studied in the silkworm, Bombyx mori L. In the diseased larvae, the quantity of total proteins was gradually decreased as the disease progressed. The electrophoretic analysis also revealed the variation in the number as well as intensity of the protein bands. Also, isoenzymes of amylase, superoxide dismutase and β-esterase exhibited variation in the R.F. and intensity, in addition to the appearance of a new fraction of superoxide dismutase. Such changes in the infected larvae, depicting the possible defense cellular adjustment of the host in response to viral attack.

Key words: Amylase, β-esterase, Bombyx mori, haemolymph, protein, superoxide dismutase.

INTRODUCTION

In the tissues of insects with an infectious disease, various biochemical, physiologica and cytomorphological alterations can be observed (Martignoni, 1964; Sujak et al., 1978; Mahesha, 1997; Mahesha et al., 2000a, 2002). Most of the biochemical studies associated with nuclear polyhedrosis in silkworm, Bombyx mori are enhanced activity of transaminases (Ramaiah and Gowda, 1970; Gowda and Ramaiah 1976); highest activity of DNA synthesis in fat body (Shigematsu and Noguchi, 1969); lowered synthetic activity of silk protein in the silk gland cells (Watanabe and Kobayashi, 1969); and increased number and survival period of haemocytes (Balavenkatasubbaiah et al., 2001). The analysis of biomolecules like proteins, amylase, succinate dehydrogenase (Mahesha, 1997; Mahesha and Honnaiah, 2002; Mahesha et al., 2002), alkaline phosphatase and alkaline protease (Lakshmikumari, 1995) and molecular marker assisted breeding (Datta and Ashwath, 2000) will help in the silkworm breeding programme for cocoon characters and disease resistance. However, studies combining enzymes like amylase, superoxide dismutase, esterase and proteins with nuclear polyhedrosis are rather scarce. Hence, the present investigation was undertaken to study the effects of nuclear polyhedrosis on qualitative analysis of few enzymes and proteins of haemolymph.

MATERIALS AND METHODS

A commercial silkworm hybrid namely, PM X CSR, was selected as experimental system in the present investigation. The silkworm rearing was conducted in the laboratory following the method described by Krishnaswami (1978).

The Nuclear Polyhedral Inclusion Bodies (PIBs) were collected from the field, confirmed by observing the symptoms and appropriate staining techniques. The crude extract from the diseased worms was per orally inoculated into the silkworm larvae immediately after third moult for the multiplication of virus. After seven days of inoculation, the worms, which exhibited white haemolymph, were taken for the collection of blood. Purification of PIBs was carried out by following the method described by Balakrishnappa and Honnaiah (1992). Finally, the stock suspension was prepared which contained 1.36 X 10³ polyhedral inclusion bodies per ml. Enumeration of PIBs was done by using Neuber’s haemocytometer.

The silkworms on the second day of fifth instar were used for inoculation. Mulberry leaves of K. variety were washed with distilled water and surface sterilized with 70 per cent ethyl alcohol using sterile cotton wad. Then the leaves were cut to square shape (10 cm²) and 0.125 ml PIBs suspension (from the stock of 1.36 X 10³ ml) was uniformly smeared, shade dried, chopped to the required size and fed to the silkworms. Two such leaves (20 cm²) smeared with PIBs suspension were made use for 50 worms. Suitable untreated batches were also
maintained. All experimental batches were maintained in triplicate. Later, the worms were allowed to complete larval stage, spinning, pupation and moth emergence. The control and live virus treated batches are mentioned as C and L respectively. The haemolymph was used for all studies.

The larvae from third day of fifth instar were collected daily with a regular interval of 24 h. The haemolymph was collected (Mahesh et al., 2000a & b, 2002, Mahesh and Honnaiah, 2002) and preserved in a deep freezer at -20°C as stock, and it was used whenever required.

The total protein present in haemolymph was determined by following the method of Bradford (1976). Bovine serum albumin was used as standard protein. The results were expressed as μg of protein/μl of haemolymph.

The qualitative analysis of total protein was carried out according to Laemmli (1970) by 10% sodium dodesil sulphate poly acrylamide gel electrophoresis with slight modifications (Mahesh et al., 2000 a & b). A uniform quantity of protein (100 μg) from each batch was loaded to each slot of the gel. Molecular weight markers (both high and low) from Pharmacia were also used in a slot to compare the molecular weight of the proteins separated from that of the sample. After appropriate destaining, the gels were scanned, analysed and photographed in a gel documentation unit with Biogene software (Vilber Laurmat Bioprofil image analysis system).

The qualitative analysis of isozymes of three enzymes viz., amylase, esterase and superoxide dismutase was carried out in Native Poly Acrylamide Gel Electrophoresis (PAGE) with the discontinuous buffer system (Rothe, 1994). The vertical slab gel apparatus was used.

The haemolymph collected was mixed with equal volumes of 0.5 M Tris-HCl buffer (pH 6.8), 10% glycerol and 0.006% bromophenol blue, and then centrifuged at 3000 rpm for 5 minutes. The supernatant was used as sample. A 8% separating gel and 4% stacking gel were used for separation of isozymes. Tris-Glycine buffer 0.05 M, pH 8.3 was used as tank buffer. A uniform quantity of protein (150 μg) from each batch was loaded to each slot.

Activity staining of amylase: The gels, soon after the removal, were washed in running distilled water followed by the incubation in 1% starch in 40mM phosphate buffer pH 7.0 at 37°C in a rotary shaker for 30 min. After incubation, the gel was placed in iodide solution (5mM \( \text{I}_2 \)-KI in distilled water) for 15 min or until negative bands appeared (Rothe, 1994). Then the gels were photographed.

Activity staining of superoxide dismutase (SOD, EC 1.15.1.1): The gels, soon after the removal, were washed in running distilled water followed by the incubation in 100 ml 50 mM sodium phosphate buffer pH 7.8 containing 50 mg NBT, 1 mg riboflavin and 0.326 ml TEMED for 30 min in dark. Then the solution was poured off, gels were placed in distilled water and illuminated under a fluorescent lamp for 15 min or until the desirable transparent (achromatic) bands clearly appeared in a dark blue background. After the appearance of bands the gels were photographed (Zhengjun Xia, 2000).

Activity staining of β esterase (EC 3.1.1.1): Solution A: 25 mg β naphthyl acetate in 1 ml of acetone followed by the addition of 1 ml water and 12.5 ml of 0.5 M sodium phosphate buffer pH 5.9.

Solution B: 25 mg fast blue RR/B salt in 2 ml of solution A followed by the addition of 12.5 ml 0.1 M sodium phosphate buffer pH 6.5.

Solution C: Mix solution A and B.

The gels, soon after the removal, were washed in running distilled water followed by the incubation in staining solution C for 20 min at 37°C in dark. After the appearance of bands the reaction was stopped by the addition of 2-3 ml glacial acetic acid. After the appearance of bands, the gels were photographed (Halatma et al., 1999). Then the gels were scanned and analysed using the gel documentation unit with Biogene software (Vilber Laurmat Bioprofil image analysis system) for R.F., volume, height and area of the band. The experimental data obtained was subjected to students t-test (Zar, 1984) at P=0.05 level of significance.

RESULTS AND DISCUSSION

Total haemolymph protein levels were estimated in control and live virus treated batches of the hybrid silkworms. In control worms, haemolymph proteins showed a significant increase in their levels at every 24 hours till the end of fifth instar. This pattern of haemolymph proteins was observed even in the treated batches (Table 1). The higher concentration of proteins was observed in control set (27.132 μg/μl) was the average during 5th instar) followed by the live virus treated set (25.074 μg/μl). The variation between the
Table 1: Concentration of total proteins (μg/μl) in haemolymph of Pure Mysore X CSR2 larvae infected with BmNPV

<table>
<thead>
<tr>
<th>Dose of PIBs</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; day</th>
<th>4&lt;sup&gt;th&lt;/sup&gt; day</th>
<th>5&lt;sup&gt;th&lt;/sup&gt; day</th>
<th>6&lt;sup&gt;th&lt;/sup&gt; day</th>
<th>7&lt;sup&gt;th&lt;/sup&gt; day</th>
<th>8&lt;sup&gt;th&lt;/sup&gt; day</th>
<th>9&lt;sup&gt;th&lt;/sup&gt; day</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>9.430* (+22.70)</td>
<td>12.200 (+38.89)</td>
<td>19.966 (+18.83)</td>
<td>24.600 (+24.46)</td>
<td>32.569 (+23.05)</td>
<td>42.330 (+11.79)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-1.06) (+2.17)</td>
<td>(-19.53) (-4.34)</td>
<td>(-3.28) (+4.79)</td>
<td>(-13.31)</td>
<td></td>
<td></td>
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</table>

C-Control batch; L-Live virus treated batch.

Values within parenthesis (1<sup>st</sup> row) represent percent change over previous day.

Values within parenthesis (2<sup>nd</sup> row) represent percent change over control.

The variation between control and experimental set is significant at 5% level.

*Not significant.

Experimental sets was significant at 5% level.

The protein profiles of haemolymph of Pure Mysore X CSR<sub>2</sub> silkworms of control and live PIBs treated sets are presented in the figure 1. On the 3<sup>rd</sup> day of fifth instar, four protein bands with the molecular weight of 456, 285, 163 and 103 kDa were found to be more prominent in live PIBs treated sets. On 4<sup>th</sup> day, a protein band of 456 kDa was paler in live PIBs inoculated set when compared to control set. Again on 8<sup>th</sup> day, two protein bands of 456 and 89.8 kDa were paler in the treated sets. On 9<sup>th</sup> day two new protein bands with 84.458 and 75.068 kDa were appeared only in the live PIBs treated sets. The quantitative analysis of proteins of diseased larvae showed reduced amount of proteins when compared to control sets. The qualitative analysis of haemolymph proteins by SDS-PAGE clearly indicated five types of changes, viz., the intensity of some protein bands either increased or decreased when compared with control set. Besides, some of the fractions disappeared while a few bands appeared. Some of the protein bands increased in their intensity only in the selected treatments. Such a metabolic change might help in improving the defense mechanism of the host. Disappearance of protein bands during different periods of larval development indicates either the non production or utilization of blood proteins. The hydrolysis of proteins might have occurred during the larval period to form amino acids, which in turn, might be utilized to form silk proteins. The hydrolysis of blood proteins to form amino acids are in agreement with the results of Beadle and Shaw (1950), who reported the hydrolysis of proteins during the larval life of the *B. mori* for the maintenance of amino acid concentration in the blood. Florkin (1937) has reported that the free amino acids in the blood of *B. mori* are the efficient precursors for the production of silk.

The zymogram of amylase of control and live PIBs treated sets are presented in the figure 2. The amylase is one of the digestive enzymes, however the role of haemolymph amylase is not yet known (Tanaka and Kusano, 1980). According to Wyatt (1967), it may participate in the degradation of glycogen in haemolymph. Two major isozymes, Rf 0.136 and 0.159 were observed in control sets on third day. On 4<sup>th</sup> day, three bands with Rf 0.136, 0.159 and 0.278 were observed in control set as well as inoculated sets. In the live PIBs treated sets, slight variation was observed. In the beginning of the pathogenesis both the fractions in live PIBs inoculated sets are more prominent when compared to control sets. On 8<sup>th</sup> and 9<sup>th</sup> day, four bands were observed in both the sets. This indicates the interference
of pathogen on the metabolism of the host and/or depicts the altered metabolic status of the host to provide necessary energy source for both the host as well as pathogen for their mutual benefits.

The zymogram of beta esterase of haemolymph of control and live PIBs treated silkworms are presented in the figure 3. Also, in case of esterase significant visible variation was recorded in tested sets. In the initial stages of pathogenesis the isozymes of esterase in live PIBs inoculated set were more prominent; however, at the later stages it was reduced to a significant level. Esterase A appears to be closely related to the stimulation of embryogenesis in B. mori (Kai and Nishi, 1976). Probably, during polyhedrosis this might encourage to maintain healthy status of the host. As the disease progressed, the intensity of bands was darker in diseased worms when compared to control; this might be due to stimulation by the viral attack.

The zymogram of superoxide dismutase of control and live PIBs treated sets are presented in the figure 4. Superoxide dismutase is an essential component in the defense mechanism against the effects of the superoxide radicals, O$_2^-$ (Gupta et al., 1993). One major fraction with an Rf of 0.470 was noticed on third day and same was noticed in the PIBs treated sets. However, the live PIBs inoculated sets exhibited increased intensity and a new fraction with Rf 0.518. This clearly indicated that the activity of superoxide dismutase might be triggered by the live PIBs treatment, indicating efficiency of the host to resist viral attack.

Hence, the present investigation clearly indicated that the BmNPV inoculated silkworms showed both qualitative and quantitative variation in haemolymph proteins; qualitative variation in isozymes of amylase, esterase and superoxide dismutase reflects the utilization of less food material, reduced rate of conversion and metabolism resulting in less production in the surviving silkworms. Such a basic knowledge about these biochemical aspects during nuclear polyhedrosis is essential to plan detailed studies at the molecular level for identification of biochemical markers useful for developing disease resistant breeds. Also, the information obtained from this research work contributes to basic virology in general.

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![Figure 1. SDS-PAGE analysis of total proteins from haemolymph of PM X CSR2 silkworms](image)

Lanes, C - Silkworms of control batch.
L - Silkworms treated with live polyhedral inclusion bodies.
Figure 2. Zymograms of amylase from haemolymph of PM X CSR2 silkworms.
Lanes, C - Silkworms of control batch.
L - Silkworms treated with live polyhedral inclusion bodies.

Figure 3. Zymograms of esterase from haemolymph of PM X CSR2 silkworms.
Lanes, C - Silkworms of control batch.
L - Silkworms treated with live polyhedral inclusion bodies.
REFERENCES


