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Research Article

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Studies on isozymes of amylase, superoxide dismutase and esterase during induction of tolerance against nuclear polyhedrosis in silkworm *Bombyx mori* L.

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ABSTRACT

To study the effects of induced tolerance in silkworm Bombyx mori L. against nuclear polyhedrosis virus a hybrid silkworms were used. The silkworms were fed with known amount of inactivated nuclear polyhedral inclusion bodies (PIBs) thrice at various time intervals for induction of tolerance followed by the challenge inoculation with known amount of live PIBs. The silkworm larvae of the control, induced, induced followed by live PIBs inoculated and live PIBs inoculated batches were collected daily with a regular interval of 24 h for haemolymph collection. The collected haemolymph was subjected for qualitative analysis of amylase, superoxide dismutase and beta esterases. Among the experimental batches significant variation was observed. Such changes in the experimental larvae, depicting the possible defense cellular adjustment of the host in response to killed as well as live viral inoculum. Also, the results pave light towards use of induced tolerance in silkworms against the dreaded nuclear polyhedrosis.

KEYWORDS: *Nuclear polyhedrosis, tolerance, amylase, superoxide dismutase, esterase.*

INTRODUCTION

The silkworm, *Bombyx mori*, lost its natural resistance against varieties of ailments because of continuous domestication since time immemorial. These pathogens caused an annual crop loss up to 40%, which runs to the loss of several crores of rupees. Of all silkworm diseases, the crop loss due to NPV has been reported to an extent from 32.9 to 55.3 %[1] and most common in summer season[2]. Though the insects exhibit both humoral and cellular immune response against various pathogens, no immune system is effective against viral infections[3], some attempts have been made to immunize insects with NPV[4,5,6,7]. Also, in the tissues of insects various biochemical, physiological and cytomorphological alterations can be observed with an infectious disease,[8,9,10,11,12] as well as after induced tolerance[13,7]. Most of the biochemical studies associated with nuclear polyhedrosis in silkworm *Bombyx mori* is limited to activity of transaminases[14,15]; highest activity of DNA synthesis in fat body[16]; lowered synthetic activity of silk protein in the silk gland cells[17]; and increased number and survival period of haemocytes[18]; effects of NPH on biomolecules[19] and effect of induced tolerance on haemolymph proteins, amylase and succinate dehydrogenases[7]. However, studies combining induction of tolerance with biomolecules are rather scarce. Hence the present investigation was undertaken in an attempt to acquire information on the effects of induced tolerance against nuclear polyhedrosis on isozymes of amylase, superoxide dismutase and esterases.

MATERIALS AND METHODS

A commercial hybrid (♀PM $XCSR_2$♂) silkworm was selected for the present investigation. Disease free layings of pure breeds were obtained from the Germplasm, Department of Studies in Sericultural Sciences, University of Mysore, Mysore, India and the hybrid was prepared in the laboratory[20]. The

silkworm rearing was conducted in the laboratory[21]. The Nuclear Polyhedral Inclusion Bodies (PIBs) were collected from the field, confirmed, multiplied in the laboratory and purified[22]. Finally, the stock suspension was prepared which contained 7.125 X 10^6 polyhedral inclusion bodies per ml. Enumeration of polyhedral inclusion bodies was done by following Neuber's haemocytometer. For the induction of the tolerance, polyhedral bodies were treated in 2% formaldehyde solution for 24h at 30 $\pm$ 1 ºC twice and used.

The mulberry leaves of M_5 variety were washed with sterile distilled water and surface sterilized with 70 per cent ethyl alcohol using sterile cotton wad. Then the leaves were cut to square shape (10 cm^2) and 0.1 ml inactivated PIBs suspension (from the stock of 0.835 x 10^7/ml PIBs suspension in sterile distilled water) was uniformly smeared, shade dried and chopped to required size and fed to the silkworms. Such type of inoculation was carried out thrice *i.e.*, at the age of third instar second day, again at the age of fourth instar second day and also, at the age of fifth instar first day. This was followed by treatment with 0.125 ml of live PIBs (from the stock of 1.36 X 10^7/ml) on second day of fifth instar. Suitable untreated batches were also maintained. All experimental batches were maintained with 50 worms in triplicate. The control batches, induced batches, induced followed by live virus treated and live virus treated batches are mentioned as C, A, AT and L respectively.

The larvae during fifth instar were collected daily with regular interval of 24 h. The abdominal legs were punctured and the haemolymph was collected in a clean, pre cooled 1.5ml mciro centrifuge tubes containing 0.1mM thiourea to prevent oxidation[23], centrifuged at 3000 rpm for 5 minutes in a cooling centrifuge at 5°C 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[24]. Bovine serum albumin was used as standard protein.

The qualitative analysis of isozymes of three enzymes *viz.*, amylase, superoxide dismutase and beeta esterase was carried out in Native Poly Acrylamide Gel Electrophoresis (PAGE) with the discontinuous buffer system. A uniform quantity of protein (150 µg) from each batch was loaded to each slot.

Activity staining of α-amylase (α-1, 4-glucan-4-glucanohydrolases; EC 3.2.1.1): The gels, soon after the removal, washed in running distilled water followed by the incubation in 1% starch in 40mM phosphate buffer pH 7.0 at 37º C in a rotary shaker for 30 min. After incubation, the gel was placed in Iodide solution (5mM I_2-KI in distilled water) for 15 min or until negative bands appeared[25].

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[26].

Activity staining of β esterase (EC 3.1.1.1): The gels, soon after the removal, washed in running distilled water and incubated in the following solution C in a rotary shaker at 37ºC in dark for 20 min or until the bands appeared. After the appearance of bands the reaction was stopped by the addition of 2-3 ml glacial acetic acid.

Solution A was prepared by dissolving 25 mg 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 was prepared by dissolving 25 mg fast blue RR 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 was prepared by mixing solution A and B.

After the appearance of bands, the gels were scanned, analyzed and photographed in a gel scanner (Vilber Laurmat Bioprofil image analysis system).

RESULTS AND DISCUSSION

The electrophoretic separation of isozymes of amylase, superoxide dismutase and esterase exhibited variation in Rf. volume, height and area of the bands. The results of amylase, superoxide dismutase and

esterase gel analysis for Rf values are presented in table 1. The amylase zymogram of control, induced, induced followed by live PIBs inoculated and live PIBs inoculated batches are presented in the figures 1 and 2. The amylase is one of the digestive enzymes; however the role of haemolymph amylase is not yet known[27]. According to Wyatt[28], it may participate in the degradation of glycogen in haemolymph. Two major isozyme fractions were observed in control as well as attenuated sets with almost same Rf value when the mean value was considered. In the attenuated followed by live PIBs inoculated sets and 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 remaining experimental sets. However, as disease progressed the intensity of the same was gradually reduced. In case of the attenuated and attenuated followed by live PIBs treated batches also exhibited variation in the intensity of the bands. This indicates the interference of pathogen on the metabolism of the host/depicts the altered metabolic status of the host to resist the multiplication of the virus.

Table 1: Results of Amylase, Superoxide diamutase and Esterase Gel Analysis for Rf value

Experimental Batches	Band No.	3rd day	4th day	5th day	6th day	7th day	8th day	9th day
AMYLASE								
C	1	0.265	0.268	0.255	0.262	0.168	0.157	0.172
	2	0.295	0.299	0.285	0.292	0.200	0.198	0.196
A	1	265	0.262	0.265	0.258	0.168	0.169	0.163
	2	295	0.299	0.295	0.292	0.214	0.204	0.196
AT	1	0.265	0.255	0.255	0.258	0.149	0.154	0.166
	2	0.289	0.285	0.289	0.292	0.198	0.199	0.193
L	1	0.258	0.255	0.262	0.258	0.157	0.157	0.163
	2	0.289	0.297	0.295	0.289	0.213	0.191	0.190
SUPEROXIDE DISMUTASE								
C	1	0.438	0.495	0.505	0.509	0.509	0.585	0.600
A	1	0.466	0.491	0.509	0.512	0.572	0.592	0.604
AT	1	0.484	0.491	0.509	0.505	0.577	0.600	0.604
L	1	0.488	0.495	0.509	0.495	0.585	0.600	0.611
	2	-	-	-	-	-	-	0.592
ESTERASE								
C	1	0.265	0.258	0.223	0.212	0.221	0.205	0.228
	2	0.458	0.466	0.424	0.388	0.397	0.381	0.375
	3	0.527	0.527	0.477	0.436	0.442	0.436	0.433
A	1	0.265	0.250	0.216	0.221	0.212	0.231	0.237
	2	0.477	0.458	0.413	0.391	0.385	0.381	0.375
	3	0.525	0.500	0.470	0.442	0.442	0.436	0.429
AT	1	0.261	0.231	0.212	0.221	0.208	0.231	0.240
	2	0.473	0.443	0.405	0.391	0.401	0.375	0.375
	3	0.519	0.504	0.470	0.442	0.446	0.433	0.436
L	1	0.258	0.231	0.208	0.221	0.212	0.231	0.208
	2	0.473	0.436	0.398	0.391	0.385	0.372	0.375
	3	0.519	0.477	0.462	0.446	0.436	0.433	0.439

Superoxide dismutase is an essential component in the defence mechanism against the effects of the superoxide radicles, O_2^- [29]. The zymograms of superoxide dismutase are presented in figures 3 and 4. One major fraction with an Rf of 0.438 was noticed on third day and the same was recorded increased Rf as the development progressed and similar trend was noticed in the remaining experimental sets. However,

in case of attenuated and attenuated followed by live PIBs treated sets, variation in the intensity of isozyme fraction was noticed. In case of live PIBs inoculated sets the intensity of the band increased. In addition a new isozyme fraction with Rf 0.411 was appeared on last day of larval life only in live PIBs inoculated sets. This clearly indicated that the activity of superoxide dismutase might be triggered by the immunization as well as immunization followed by live PIBs treatment.

Esterases are a group of enzymes which catalyze the hydrolysis of various types of acetyl esters. The zymogram of beeta esterases are presented in figures 3 and 4. In the initial stages of pathogenesis the isozymes of esterase in live PIBs inoculated set showed more prominent; however, at the later stages it was significantly reduced. Esterase A appears to be closely related to the stimulation of embryogenesis in *Bombyx mori*[30]. Probably, during pathogenesis this might try to maintain healthy status of the host. In addition, on 9th day the isozyme of Rf. 0.208 was prominent in live PIBs inoculated set only when compared to remaining experimental sets. As the disease progressed, the intensity of bands were pale when compared to control, because of non availability of sufficient precursors to synthesize enzyme protein, as they were diverted to viral metabolism.

Hence, the present investigation clearly indicated that the information obtained from the biochemical experiments may be used during breeding of disease resistant silkworm strains. In addition, the information gathered from this research work contributes to basic virology in general.

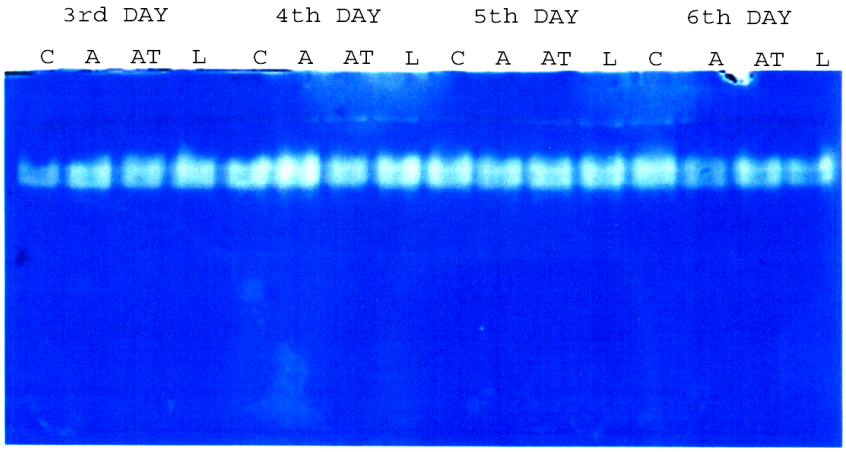

Figure 1: Zymogram of amylase from haemolymph of PM X CSR2 silkworms. Lanes: C-Control, A-Induced, AT-Induced followed by Live PIBs inoculated and L-Live PIBs inoculated batches.

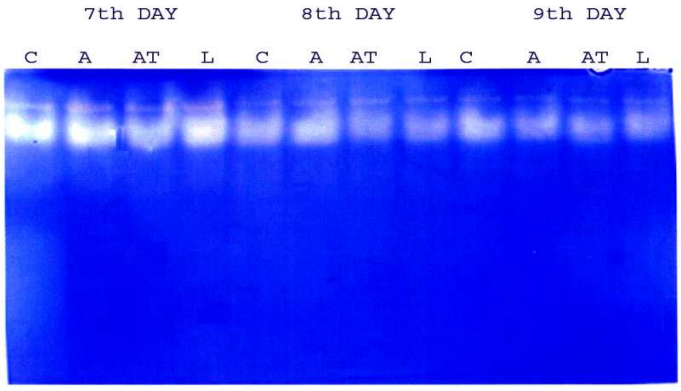

Figure 2: Zymogram of amylase from haemolymph of PM X CSR2 silkworms. Lanes: C- Control, A- Induced, AT-Induced followed by live PIBs inoculated and L- Live PIBs inoculated batches.

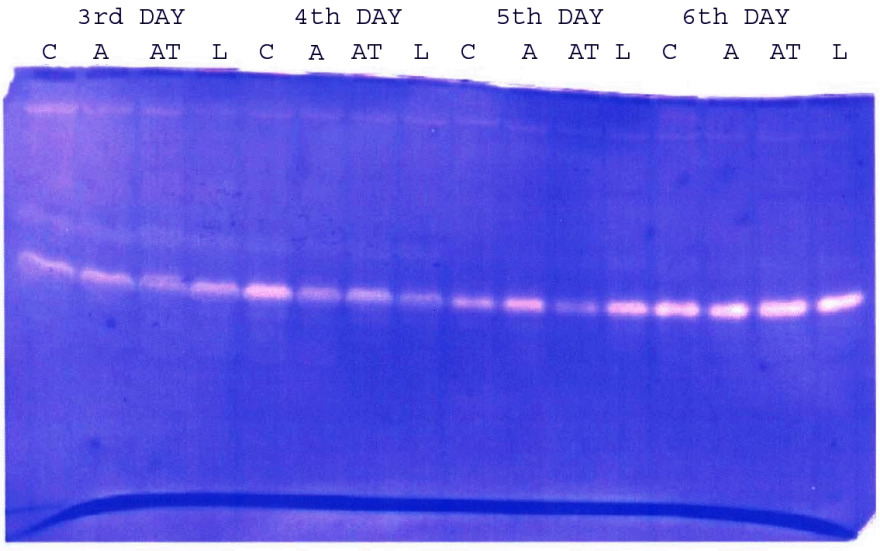

Figure 3:Zymogram of superoxide dismutase from haemolymph of PM X CSR2 silkworms. Lanes: C-control, A-Induced, AT-Induced followed by live PIBs inoculated and L-live PIBs inoculated silkwormm batches

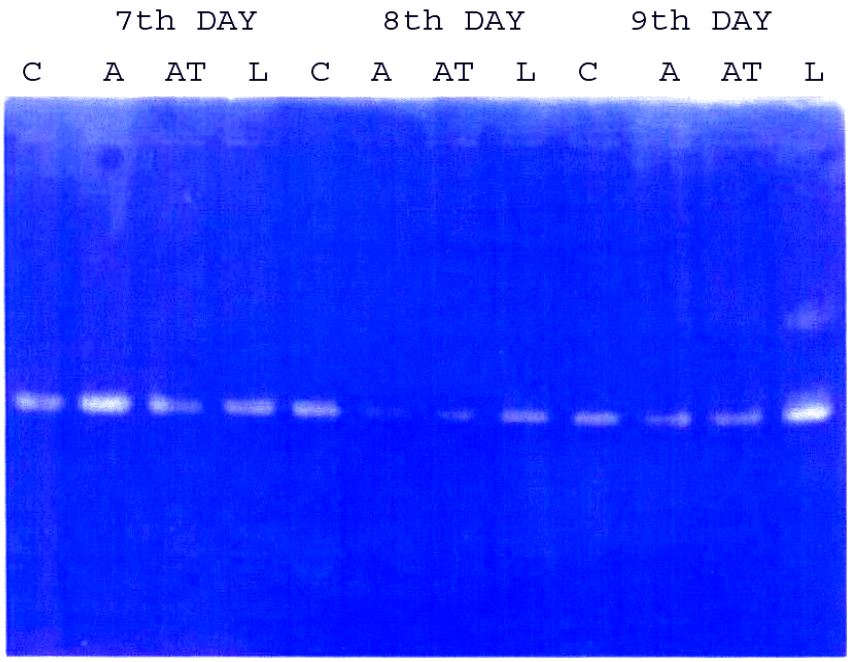

Figure 4: Zymograms of superoxide dismutase from haemolympg of PM X CSR2 silkworms. Lanes: C-Control, A- Induced, AT- Induced followed by live PIBs inoculated and L- live PIBs inoculated batches

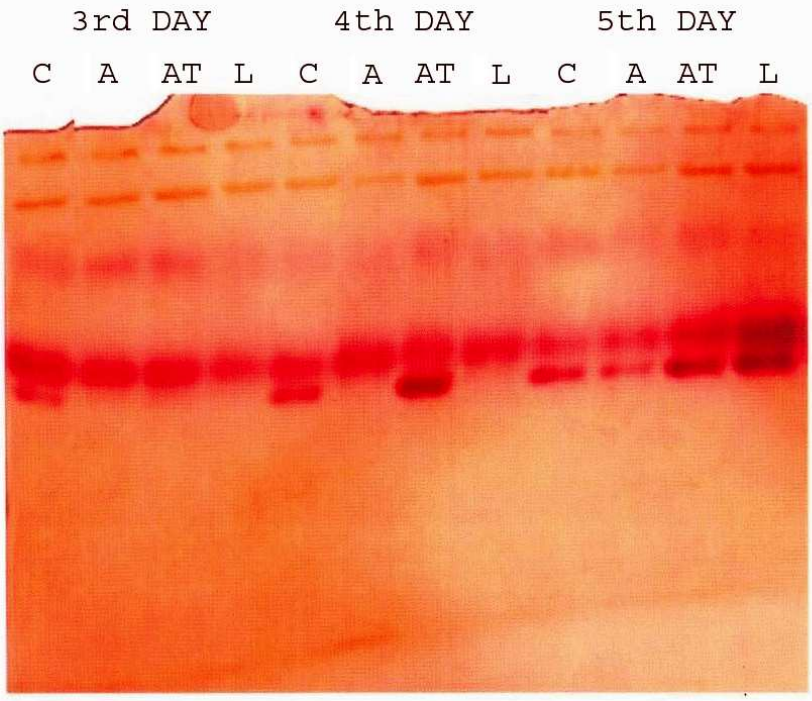

Figure 5: Zymograms of beeta esterase from haemolymph of PM X CSR2 silkworms.Lanes: C-Control, A-Induced, AT- Induced followed by live PIBs inoculated and L- Live PIBS inoculated bactches.

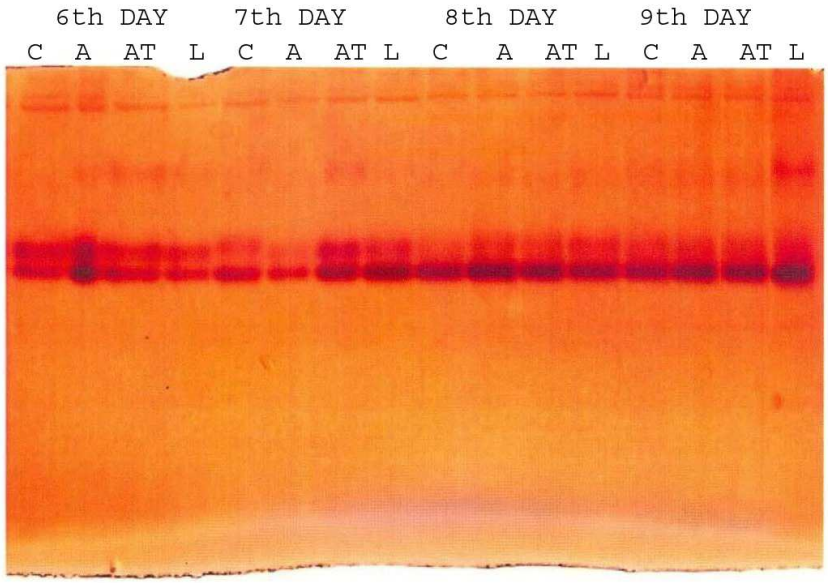

Figure 6: Zymogram of beeta esterase from haemolymph of PM X CSR2 silkworms. Lanes: C-Control, A- Induced, AT- Induced followed by live PIBs inoculated and L-Live PIBs inoculated batches.

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