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The Effect of Chemical Mutagen on Haemolymph Proteins of Silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) in F_1 Stage

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ABSTRACT: The chemical mutagen Diethylsulphate (DES) was treated to the mulberry silkworm, NB_4D_2 breed at the age, fifth instar fifth day with different concentrations (8mM and 10mM) by injection and spray methods. The F_1 progeny was obtained from DES treated and control sets by selfing the moths. The quantitative analysis of haemolymph proteins revealed that 8mM injection set exhibit higher levels when compared with control and treated sets. The quantitative analysis also clearly indicate the variations in the number as well as intensity of the protein bands. @JASEM

The various aspects of protein metabolism including quantitative changes in haemolymph protein synthesis and metabolic activity of specific enzymes have attracted the interest of many insect biochemists. The available results from these biochemical studies indicate that protein metabolism is of considerable importance in characterizing different stages of insect development (Chen, 1966). Haemolymph proteins play an important role in insects for transport functions, as well as for their enzyme action. The synthesis and utilization of haemolymph proteins are controlled by genetic and hormonal factors (Hurliman and Chen, 1974). Although our knowledge of insect haemolymph proteins has greatly advanced during the last decade, still only the origin and function of few major proteins are known (Riddiford and Law 1983). There is general agreement that the fat body is a main source of haemolymph proteins (Dean *et al.,* 1985 and Keeley, 1985) and others may come from haemocytes (Hughes and Price, 1976, Katagiri, 1977). Extensive studies of blood during development in *Hyalophora cecropia* and *Samia Cynthia* have been made using starch gel electrophoresis (Lauffer, 1960). It has been reported that blood proteins fluctuate during the development of *Bombyx mori* (Heller, 1927). The protein concentration was found to increase seven folds during the larval life of *Bombyx mori* (Bito, 1927 and Florkin, 1936). Recently number of researchers worked on silkworm protein variations, qualitatively and qualitatively occurs during mutagens treatment in general and chemical mutagens in particular during larval development (Mahesh, 1997; Bashamohideen and Ameen, 1998; Mahesha *et al*., 2000). However studies on chemical mutagens particularly effect on haemolymph is meagre. Hence, the present investigation was attempted to study the effect of chemical mutagen on haemolymph proteins of silkworm, *Bombyx mori* L. during larval fifth instar stage.

MATERIALS AND METHODS

The silkworm race, NB_4D_2 a bi-voltine breed and chemical mutagen DES (Diethyl sulphate) were used for the study. The eggs were procured from private agency and incubated at temperature 25±1° C and 70-80% relative humidity in an incubator. Block box was carried out on 8th day to achieve uniformity in hatching. The hatched larvae were reared in environmental chamber by providing with suitable quality mulberry leaves of *Morus alba* (variety V_1). The rearing was conducted by following the method discribed by Raja Ram (2000).

The silkworm at the age of fifth instar, fifth day larvae were taken for treatment. Two different concentrations of DES like 8mM and 10mM were selected and treated by spray and injection. For spray the solutions of 8mM and 10mM were prepared in distilled water. 20ml of the DES solution was sprayed on 20g of mulberry leaves and fed orally to the larvae for one day (4 feeds only) at the age of fifth instar fifth day. After the treatment of four feedings, next day onwards-normal feed was given. Similarly, one of the batch was fed with the leaves sprayed with distilled water and was kept as a control spray. At end of the instar, these larvae were allowed to spin the cocoons. Similarly for injections of 8mM and 10mM other batches were made. Forty µl of final concentration of DES freshly prepared in 0.75% sodium chloride (NaCl) solution and injected at the lateral side of the inter segmental region between 7th and 8th abdominal segments using micro syringe. Each larva was injected with 0.04ml of solution. One set of larvae was maintained as control (without any spray or injection). The three replications were maintained for each comprising of 100 larvae. Both the experimental batches were reared separately and the larvae were allowed to continue for development, metamorphosis and moth emergence. The F_1 progeny was obtained by selfing the moths emerged from

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batches treated with DES by spray and injection as well as controlled sets separately.

The rearing of F_1 progeny rearing was conducted in the environmental chamber by following standard method described by Raja Ram (2000). During fifth instar, a few larvae were collected daily at 11AM in each treatment and control batches from 1st day of 5th instar to spinning. The larvae were kept in refrigerator at 4-5 minutes to facilitate the free running of heamolymph. The caudal horn of the larvae was punctured and the haemolymph was collected in cleaned, sterilized and pre-cooled vials. A pinch of phenylthiourea was added to prevent the oxidation of heamolymph. From the collected haemolymph samples the total soluble protein was estimated by following standard procedure (Lowery *et al*., 1951). Finally the collected data were subjected to statistical analysis of ANOVA - test (Khan and Khanum, 1994) and discussed.

The qualitative analysis of total soluble protein was done in haemolymph by using the Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) as described by Ignacimuthu (2001). A uniform quantity of protein (100µg) from each batch was loaded to each slot of the gel. Molecular weight marker (broad range) was used in a slot to compare the molecular weight of the proteins separated from that of the samples. After electrophoresis, fixation of the gels was carried out in 12% trichloroacetic acid for 30 minutes, and then in 10% acetic acid for another 30 min. The gels were stained in 0.3% Coomassie brilliant blue R-250 in mixture of distilled water, methanol and acetic acid (9:6:3) for over night. After appropriate destaining with mixture of distilled water, methanol and acetic acid (9:6:1 v/v), the gels were photographed on Transilluminator.

RESULTS

The quantitative estimation and qualitative analysis of haemolymph total proteins in fifth instar larvae of various treatments, controls and untreated sets is presented in table –1 and Fig: 1-3.


Fig-I.gif


Fig-II.gif


Fig-III.gif

Fig-I, II and III: Polyacrylamide gel electrophoretic pattern of haemolymph protein in untreated, control and treated samples of V instar (1st day to 6th day) NB4D2 silkworm breed.

The total soluble protein content in the haemolymph increased with an increase in the age from first day to spinning day of fifth instar (table-1). The treatments 8mM spray, 10mM spray and 8mM injection sets show significantly (P>0.01) increased from 1st day to sixth day of fifth instar except in second and fourth day in 8mM and 10mM spray sets and 4th day in 8mM injection set, whereas the control spray set also showed a significant (P>0.01) increase in protein concentration in first and fifth day. However, the treatment with 8mM injection recorded highest value on final day when compared with the other injected, sprayed and controlled batches. On the other hand, the set with treatment 10mM injection recorded the least value on first day of fifth instar followed by untreated set, 10mM spray, control spray, 8mM spray and 8mM injection.

On first day of fifth instar, the protein bands of higher molecular weight were found in between 205kDa and 97kDa were found in all studied samples except in 8mM spray treatment and also there was disappearance of protein bands of molecular weight between 67kDa and 43kDa (fig-1). The protein bands were found to disappearance between 205kDa and 97kDa in the samples treated with 10mM injection when compared with other samples in second day (fig-1). In third day the staining/quantity of 68kDa protein showed a variations between treatment and control (fig-2). Thickly stained bands were absorbed in 10mM spray and in 10mM injection when compared with other samples. The number of protein bands was more absorbed in 4th day of treated 8mM injection and 10mM spray samples when compared with other samples, where as in all the treatments 28kDa protein band show a deep staining with thick bands. It was also absorbed that the 48kDa protein was disappeared in the sample treated with 8mM spray. However, the number of bands increased in all treated sets (fig-2). The 10mM spray batch showed more number of bands on fifth day when compared with other batches and there were variations with regard to the presence of 48kDa protein over other samples (fig-3). Before spinning day of fifth instar 8mM spray set showed thinly stained 68kDa protein band and had very less number of bands when compared with other samples. Also the number of bands was coming down in all sets when compared with early days (fig-3). The five protein bands such as 6kDa, 28kDa, 35kDa, 45kDa and 67kDa were found common throughout fifth instar and the bands of 18kDa and 48kda were found from second day to till spinning.

Table-I: Effect of chemical mutagen DES on total soluble proteins content in haemolymph of silkworm, *Bombyx mori* L., NB_4D_2 during 5th instar.

Treatments	5th Instar larval days					
	1	2	3	4	5	6
Control	11.36 ±0.288	16.40 ±0.115	44.06 ±0.061	58.67 ±0.13	67.82 ±0.058	78.32 ±0.055
Normal control spray	14.42 ±0.460**	15.15 ±0.061	41.67 ±0.050	51.94 ±0.047	69.32 ±0.050**	75.84 ±0.055
8mM Spray	15.94 ±0.072**	15.89 ±0.66	45.43 ±0.104**	57.65 ±0.065	69.95 ±0.061**	79.73** ±0.045
10mM Spray	13.37 ±0.051**	16.25 ±0.07	44.35 ±0.035**	50.43 ±0.045	68.44 ±0.060**	78.74 ±0.065**
8mM Injection	18.54 ±0.145**	17.89 ±0.025**	49.18 ±0.025**	58.40 ±0.076	73.38 ±0.065**	81.26 ±0.031**
10mM Injection	10.83 ±0.187	13.17 ±0.037	38.73 ±0.061	47.38 ±0.035	65.74 ±0.060	71.14 ±0.040
F-Value	363.39	1542.3	9999.1	1216.1	5332	9924
CD-Value at 5%	0.5708	0.1532	0.1395	0.1724	0.1292	0.1334
1%	0.8932	0.2397	0.2183	0.2679	0.2021	0.2087

Note: Values present in the table are Mean± Standard Deviation
** - Significant at 1% level

DISCUSSION

The proteins play an important role in the haemolymph of insects not only in specific transport functions, but also in their enzyme action. Hurlimann and Chen (1974) asserted that the synthesis and utilization of haemolymph proteins are conditioned by genetic and hormonal control. It also reported that, the concentration of protein in haemolymph increases progressively during larval development and reaches maximum in the late fifth instar larvae. Increase in the protein content is attributed to the development of reproductive organs (Sinha and Sinha, 1994).

The variations in the concentration of protein in the haemolymph of various treated sets of the study could be attributed to the differential concentration of mutagen also difference in the physiological activities of these batches under treatment. The treatment 10mM injection set recorded least concentration of protein when compared with control and other treatments. This clearly shows that, high concentration of chemical mutagen may be acts as toxic or alter the genetical characters and also might be due to physiological and biochemical disturbance in the metabolic activity of the silkworm. Meanwhile, the treatment 10mM spray set did not show to a large extent difference when compared with low concentration treated sets. This is may be due to the chemical mutagen during spray time may be wasted or sprayed mutagen may be evaporated during feeding period, for the reason that fixed quantity of chemical mutagen did not reached to the silkworm larvae.

The present results are agreement with the results of Mahesha *et al*., (2000) who reported, high protein concentration in 2.5mM (53.56mg/ml) and 10mM (41.50mg/ml) sets in EMS treated batches. Also Rao *et al*., (1981) they reported that an increase in total protein content of thoracic and mandibular museles on the third day of post irradiation and irradiation of high gamma rays affects circadian rhythm of tissue portion. Vaidya *et al*., (1974) and Prasad and Sethi (1980) also noticed an increase in the haemolymph protein content of four species of *Drosophilidae* and *Dacusdorsalis* when irradiated with 15k rad and 24k rad respectively.

A comparative study of proteins in all the samples drawn from different treatments revealed that the number of protein bands and specificity of bands vary from day to day. The presence and absence of few protein bands during the larval life was the feature, there by indicating probably the synthesis of particular protein to meet the need of the day.

In the present study, the presence of thick protein bands near the region 29kDa is assumed to be *Bombyx mori* serum protein (BmLSP), which was proposed by Fujiwara and Yamashita (1990). They demonstrated that BmLSP occurs as a major protein throughout the early instars until the initial day of last instar and gets decreased towards spinning stage. Sakurai (1984) reported that BmLSP was identified as a protein with a molecular weight 30,000 the amino acid composition was similar to that of 30kDA protein of silkworm (Izumi *et al*., 1981). Further, BmLSP was shown to be a different protein by immunological studies (Fujiwara and Yamashita,

1990). The variation in the number of protein bands and specificity of protein bands during various days of fifth instar development could be ascribed to the fact that the proteins in the haemolymph vary quantitatively during the development of silkworm. It has been reported that haemolymph proteins fluctuate during the development of *Bombyx mori* (Heller, 1924). Lakshmi kumari (1991) showed that the haemolymph proteins of silkworm fluctuates during fifth instar development and the number of protein bands showed highest in the middle of the instar in different doses of γ- radiation treated batches. The present study is in close conformation with the earlier studies of Lakshmi kumari (1991).

The appearance of the common bands throughout fifth instar observed in the present study is concomitant with earlier work of Shivenkappa (1991) who reported 28kDa, 35kDa, 42kDa and 82kDa protein bands were found throughout in fifth instar of NB_4D_2 breed. The number of protein bands was observed to be decreasing on final day of the instar. Disappearance of protein bands indicates either the non-production or utilization or the degradation of haemolymph proteins to maintain amino acid concentration in the haemolymph. 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. This hypothesis is in agreement with the results of Beadle and Shaw (1950) who reported the hydrolysis of proteins during the larval life of *Bombyx mori* for the maintenance of amino acid concentration in the haemolymph. Florkin (1937) has reported that the free amino acids in the blood of *Bombyx mori* are the efficient precursor for the production of silk. Cauterization of spinneret in the silkworm, leading to lyses of silk gland, causes a great increase in the amino acids of the haemolymph (Wigglesworth, 1965). Neisen and Mills (1968) have postulated a hypothesis for the appearance and disappearance of protein bands. According to them, causes the mid gut and fat body manufacture some of the haemolymph proteins. Such proteins could be reabsorbed or hydrolyzed to form amino acids during the life cycle, leading to disappearance of protein bands in the haemolymph; or they may be changed into one or more of the other protein components of the haemolymph, resulting in the appearance of new bands. However, any change in the protein pattern during the development can be considered as being directly determined by the gene function, which reflects the alteration in the metabolism of the developing organism (Posteur and Kastritsis, 1971).

In the present investigation, variations in the quality and quantity of proteins might be either due to one of the two possibilities. Firstly, the genotype of the silkworm larvae might be altered, as the DES is an effective chemical mutagen. Secondly, it might also be due to transmission of some influencing factor or factors generated by DES treatment, from parents to progeny. This alteration might enhance the levels of utilization of exogenous food material and efficient conversion at lower doses. Higher doses of DES might cause adverse effect on the silkworm and, eventually leads to lower levels of utilization and conversion of exogenous food material (Mahesha, 1997). This hypothesis is also supported by the work of Bashamohideen and Ameen (1998). They found rate of protein breakdown at lethal dose of *Dichlorovas* in haemolymph as well as fat body when compared to sub lethal dose. As the haemolymph composition of the insects reflects the nature and degree of metabolism of the tissue suffused in this fluid, changes in the proteins of the haemolymph may show the level of modification in the organism. Therefore, by studying the haemolymph protein, it is possible to have a clear picture of the protein metabolism of the insect after the treatment with a mutagen. The qualitative variations in the protein bands of different treatments and on different days during the larval life indicate both utilities of the specific proteins as well as the synthesis of new proteins by the insect.

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