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Protein Profiles in the Silkworm, Bombyx Mori (L) on Exposure to Trace Element and Nutrients

KEYWORDS: Fat body, Haemolymph, Methoprene, Muscle, Pyridoxine, Silk Gland

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ABSTRACT *The present study has been demonstrated severe perturbations in protein profiles of the silkworm Bombyx mori, in different tissues such as Silk Gland, Haemolymph, Fat body and Muscle, when fed on mulberry leaves fortified with selected trace element, Zinc, vitamin, Pyridoxine and hormone, Methoprene. The experimental worms were divided in to four groups and fed with mulberry leaves soaked in the selected compounds i.e. Zinc chloride, Pyridoxine, Methoprene and with Mixed dose (Zn+B6+H). The Control group of silkworm larvae was fed with normal mulberry leaves. All groups of silkworm larvae were fed four times in a day throughout the 5th instar larval period. Both Control and Experimental silkworm larvae were weighed daily and sacrificed on selected days viz. 1st, 3rd, 5th and 7th day. However protein utilization, especially for the silk fiber as well as the spinning activity probably remains more or less same at the selected dose of trace element, focusing on the possibility of variations in protein synthesis. The findings of the present study finally suggest that the Mixed dose induced active turnover of all profiles of protein metabolic events in the posterior silk gland, creating the conditions that are highly congenial for growth and silk production.*

1. INTRODUCTION:

The haemolymph of phytophagus insects have especially high potassium and low sodium levels, a balance that is characteristic of the mineral composition of plant foliage (Florkin and Jeuniaux, 1974). Because cofactor functions of iron (Nichol et al., 2002) and zinc, for example are considered as having almost universal importance to organisms, such trace elements can be assumed, tentatively, to be essential for insects in general. Few measurements on efficiencies of mineral and trace element utilization have been made in insects and, like vitamins their quantitative requirements are largely unknown. Mineral availabilities in plants and insects were reviewed by Muniandy et al., (2001). Zinc is involved in diverse cellular processes, including catalysis and gene expression, and has been implicated as an inhibitor of apoptosis and of oxidative stress.

The silkworm, *Bombyx mori* produces massive amount of silk proteins during the 5th stage of larval development. These proteins are stored in the middle silk gland and they are discharged out through the anterior duct and spinneret, at the end of the fifth instar. Two kinds of silk proteins have been distinguished as major components of silk cocoons, the first being **fibroin** and the second being **sericin,** a natural macromolecular protein, serving as an adhesive to unite fibroin for making silk cocoons of silkworm. Recently, silkworm is being used as bio factory for the production of useful protein using the silk gland, which has promoted the technological development in sericulture.

Earlier findings have reported that juvenile hormone (JH) and their derivative can be used as growth regulators of the commercial silk worm to increase silk yield. For example, Chowdhary, (1991) reported that 21% increase in silk yield and more than 40% increase in silk ratio on application of JH, SJ-42-F.

Silk production basically depends on the *B. mori* larval protein metabolism which in turn needs more energy generating events, spinning requires more muscular activity and silk is being produced by the silk gland. On these lines, the selection of enzymes involved in protein and energy metabolism as well as tissues like muscle and silk gland in the present study is justifiable.

2. MATERIALS AND METHODS:

Test species : Silkworm, *Bombyx mori* (Disease-free larvae from local grainages)

Mulberry : M$_5$ Variety

Larval Instar : 5th Instar

Test chemicals : 1. Zinc chloride (Fisher Inorganics & Aromatics Ltd,

2. Pyrol / Pyridoxine hydrochloride (vitamin B6) (FI & AL)

3. Methoprene Hormone (Seri-Agro market: Bangalore)

Duration of treatment : 7 Days

Dose Selected

Zinc chloride : 2 μg/ml

Pyridoxine hydrochloride : 2 μg/ml

Methoprene Hormone : 2 μg/ml

Tissues Selected : Silk Gland, Haemolymph, Fat body and Muscle

2.1. Test species:

The present investigation was carried out on the Pure Mysore x CSR2 hybrid variety of the silkworm, *Bombyx mori*. Since the experiments required continuous maintenance of the test species, silkworms were reared in the laboratory itself in accordance with the procedure (Krishnaswami, 1978).

2.2. Treatment of fifth instar larvae with Zinc, Pyridoxine and Methoprene:

Required concentration (2 μg/ml) of Zinc chloride, Pyridoxine and Methoprene solutions were prepared in distilled water as shown below.

2.3. Preparation of standard stock solutions

For the preparation of standard stock solution 1, 1g of zinc chloride was dissolved in 100ml of distilled water (1000 mg x 1000 μg /100 ml) which is equivalent to 10000 μg/ml. From this solution 1ml was taken and added to 99ml of distilled

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water (10000 µg /100 ml) which is equivalent to 100 µg/ml, known as standard stock solution 2. The same procedure was followed in the case of Pyridoxine , Methoprene and Mixed dose also.

For the preparation of 2 µg/ml concentration, 2ml of standard stock solution 2 was added to 98ml of distilled water. But in the preparation of Mixed dose, the above prepared standard stock solution 2, each 2ml i.e. Zinc +Pyridoxine + Methoprene (2ml+2ml+2ml) was added to 94ml of distilled water. 100 ml of each of these concentrations were prepared as per the above table and 25-75 mulberry leaves were soaked in these solutions, dried at room temperature till the wetness is removed & were used to feed four groups of experiment larvae of the 5th instar stage for 7 days.

2.4. Protein profiles:
Various protein profiles were estimated in selected tissues such as silk gland, haemolymph, fat body and muscle of control and experimental the silkworm by Lowry method (Lowry et al., 1951).

2.5. Isolation of Tissues:
Silk gland:
Larvae were dissected and the silk glands on both sides were extracted with the help of a forceps carefully. Weight of both silk glands from 5 silkworms i.e., 10 glands was recorded. The silk glands were cut into small pieces with the help of scissors and 0.05 g was taken and homogenated in 5ml of distilled water with the help of mortar and pestle. This homogenate (1% w/v) was used for Protein estimation.

Haemolymph:
Silkworm larvae held in between the index finger and thumb was punctured with a needle on the oral side and drops of haemolymph were collected in a veil up to 0.1 to 0.2 ml. From this, 0.1ml of haemolymph was taken and 0.9ml of distilled water was added and mixed well in a test tube to get a uniform mixture. This diluted haemolymph was used for Protein estimation.

Fat body:
After dissection of silkworms, fat bodies were collected from 6 to 8 worms and a uniform mixture was made using a needle. From this mixture, 0.05 g of fat body tissue was taken and homogenated in 5ml of distilled water with the help of mortar and pestle. This homogenate (1% w/v) was used for Protein estimation.

Muscle:
After dissection of silkworms, the muscle tissue was collected from 6-8 worms and a uniform mixture of the muscle tissue was made. From this mixture, 0.05 g of fat body tissue was taken and homogenated in 5ml of distilled water with the help of mortar and pestle. This homogenate (1% w/v) was used for Protein estimation.

STATISTICAL ANALYSIS:
Values of the measured parameters were expressed as Mean ± SEM. Repeated Measures of ANOVA was used to test the significance of difference among four different groups followed by Dunnet's Multiple Range Test (DMRT). Statistical analysis was performed by using Statistical Program of Social Sciences (SPSS) for windows (Version 16; SPSS Inc., Chicago, 1L, USA). The results were presented with the F-value and p-value. In all cases F-value was found to be significant with p-value less than 0.01**. This indicates that the effects of factors are significant.

3. RESULTS:
3.1. Total Proteins: (Tables: 1 to 4 and Figs: 1 to 4)
A common observation made in the present study was that the Protein Profiles in all four experimental groups of silkworms were significantly elevated in silk gland, haemolymph, fat body and muscle tissues on all selected days during the 5th instar larval stage. One significant observation was the effect of the **Mixed dose (E4)** was more pronounced on any given day, when compared to individual doses.

3.1.1. Control silkworms:
The total protein content of the silk gland, haemolymph, fat body and muscle of the control silkworm was 48.20, 11.46, 50.06 and 48.46 mg protein / g wet weight of tissue respectively and out of the four tissues, **haemolymph** had the lowest protein content while fat body recorded highest content.

3.1.2. Experimental silkworms Treated with 2µg/ml Zinc chloride:
After treatment with 2µg/ml Zinc chloride(E1), significant elevation in total protein content was observed in silk gland, haemolymph, fat body and muscle tissues from day 1 to day 7 and the increase was in the following order. On all days, the silk gland recorded highest protein content and haemolymph the least.

Selected days	Sg		Mc		Fb		Hl
Ist day							
Control	48.20	<	48.46	<	50.06	>	11.46
Experimental	65.52	>	58.44	>	56.05	>	14.86
7th day							
Control	180.20	>	70.50	>	55.63	>	27.69
Experimental	198.00	>	85.53	>	59.23	>	29.07

3.1.3. Experimental silkworms Treated with 2µg/ml Pyridoxine:
A similar trend in the elevation pattern of the protein content was also noticed in all the selected tissues of the silk worms treated with 2µg/ml of Pyridoxine on all selected days during the 5th instar larval stage against the respective controls. However, when compared with 2µg/ml Zinc chloride treatment, Pyridoxine caused slightly higher elevation in all selected tissues as given below. Here also, silk gland recorded more elevation than the other tissues.

Selected days	Sg		Mc		Fb		Hl
Ist day							
Control	48.20	<	48.46	<	50.06	>	11.46
Experimental	70.05	>	63.55	>	61.03	>	19.60
7th day							
Control	180.20	>	70.50	>	55.63	>	27.69
Experimental	202.00	>	90.20	>	65.48	>	34.10

3.1.4. Experimental silkworms Treated with 2µg/ml Methoprene:
Similar to Zinc and Pyridoxine, Methoprene treatment also caused a remarkable increase in total proteins in all selected tissues over their corresponding control tissues and silk gland once again recorded the peak elevation compared with other tissues.

Selected days	Sg		Mc		Fb		Hl
1st day							
Control	48.20	<	48.46	<	50.06	>	11.46
Experimental	74.40	>	68.28	>	65.25	>	24.08
7th day							
Control	180.20	>	70.50	>	55.63	>	27.69
Experimental	207.00	>	94.84	>	69.32	>	39.09

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3.1.5. Experimental silkworms Treated with Mixed dose:
In the Silk worm treated with Mixed dose of Zinc + Pyridoxine + Methoprene, there was a drastic hike in the total proteins content in all the selected tissues when compared with other experimental groups which received individual doses of Zinc, Pyridoxine and Methoprene separately.

Selected days	Sg		Mc		Fb		Hl
Ist day							
Control	48.20	<	48.46	<	50.06	>	11.46
Experimental	80.15	>	72.65	>	70.15	>	29.07
7th day							
Control	180.20	>	70.50	>	55.63	>	27.69
Experimental	211.20	>	97.75	>	74.44	>	44.08

One significant observation from the above results with respect to the total proteins was that the silk gland tissue showed highest protein content and also registered peak elevation with the Mixed dose treatment when compared with other tissues and other experimental groups from day 1 to day 7.

3.2. Soluble Proteins: (Tables: 1 to 4 and Figs: 1 to 4)
As a contrary to the total proteins, the soluble protein levels in silk gland, haemolymph, fat body and muscle tissues were decreased in the silkworms on treatment with selected compounds on all selected days during the 5th instar larval stage. The results of the present study further clearly demonstrated that the effect of Mixed dose has more profound effect throughout the experimental duration.

3.2.1. Control Silkworms:
The soluble protein content of the silk gland, haemolymph, fat body and muscle of the control silk worms was 41.43, 8.57, 37.54 and 32.37 mg protein / g wet weight of tissue respectively. As in the case of total proteins, the soluble proteins level was highest in the silk gland and least in haemolymph.

3.2.2. Experimental silkworms Treated with 2µg/ml Zinc chloride:
After treatment with 2µg/ml of Zinc chloride, the silk gland registered peak elevation in soluble protein content from day 1 to day 7 and the increasing trend in the soluble protein content in other tissues was in the following order.

Selected days	Sg		Mc		Fb		Hl
1st day							
Control	41.43	>	32.37	<	37.54	>	8.57
Experimental	59.10	>	41.36	<	46.07	>	12.23
7th day							
Control	132.30	>	4.27	<	13.12	<	16.10
Experimental	143.00	>	5.35	<	23.43	>	17.47

3.2.3. Experimental silkworms Treated with 2µg/ml Pyridoxine:
When compared with 2µg/ml Zinc chloride treatment, at Pyridoxine (2µg/ml) dosage, the soluble proteins showed slightly significant elevation in all selected tissues on all selected days during the 5th instar larval stage compared with control.

Selected days	Sg		Mc		Fb		Hl
Ist day							
Control	41.43	>	32.37	<	37.54	>	8.57
Experimental	65.11	>	46.39	<	51.16	>	17.30
7th day							
Control	132.30	>	4.27	<	13.12	>	16.10
Experimental	146.45	>	6.74	<	35.36	>	23.07

3.2.4. Experimental silkworms Treated with 2µg/ml Methoprene:
A similar trend as seen in the case of 2µg/ml Pyridoxine was observed at 2µg/ml methoprene treatment also and the silk gland showed more elevation compared with other tissues.

Selected days	Sg		Mc		Fb		Hl
Ist day							
Control	41.43	>	32.37	<	37.54	>	8.57
Experimental	69.12	>	52.05	<	56.01	>	22.11
7th day							
Control	132.30	>	4.27	<	13.12	<	16.10
Experimental	152.55	>	8.60	<	43.06	>	27.53

3.2.5. Experimental silkworms Treated with Mixed dose:
In silk worms treated with the mixed dose, even though the soluble proteins levels in all the tissues were elevated highest when compared to other experimental groups, they were far less than the levels observed at lower dose such as 2µg/ml.

Selected days	Sg		Mc		Fb		Hl
Ist day							
Control	41.43	>	32.37	<	37.54	>	8.57
Experimental	74.06	>	57.14	<	61.05	>	27.25
7th day							
Control	132.30	>	4.27	<	13.12	<	16.10
Experimental	157.28	>	10.04	<	45.84	>	33.66

One significant observation from the above results with respect to the soluble proteins was that the silk gland showed highest protein content and registered peak elevation with the Mixed dose treatment and muscle showed least soluble protein content when compared with other tissues and other experimental groups from day 1 to day 7. However, when compared to total proteins the soluble proteins elevation is less in all the experimental groups as well as control.

3.3. Structural Proteins: (Tables: 1 to 4 and Figs: 1 to 4)
Structural protein levels in the silk gland, haemolymph, fat body and muscle tissues of silk worms treated with different compounds as already mentioned showed an increasing trend from day 1 to day 7 in all selected tissues. It was also noted that the effect of mixed dose was more pronounced on 7th day when compared to other selected days and groups.

3.3.1. Control silkworms:
The structural protein content of the silk gland, haemolymph, fat body and muscle of the control silkworm was 6.74, 2.90, 12.58 and 16.10 as mg protein / g wet weight of tissue respectively.

3.3.2. Experimental silkworms Treated with 2µg/ml Zinc chloride:
After treatment with 2µg/ml l of Zinc chloride, more elevation in structural protein content was observed in silk gland, haemolymph, fat body and muscle tissues from day 1 to day 7 and the increase was in the following order.

Selected days	Sg		Mc		Fb		Hl
1st day							
Control	6.74	<	16.10	>	12.52	>	2.90
Experimental	6.45	<	17.14	>	9.98	>	2.63
7th day							
Control	48.20	<	66.23	>	42.50	>	11.53
Experimental	55.04	<	80.18	>	35.80	>	11.60

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3.3.3. Experimental silkworms Treated with Pyridoxine:
In the Pyridoxine treated group, structural proteins showed slight elevation in all selected tissues on all selected days during the 5th instar larval stage compared with control. When compared with 2µg/ml Zinc chloride treatment, structural proteins showed slightly more elevation in all selected tissues and the increase was in the following order.

Selected days	Sg		Mc		Fb		Hl
Ist day Control Experimental	 6.74 4.95	 < <	 16.10 17.16	 > >	 12.52 9.97	 > >	 2.90 2.30
7th day Control Experimental	 48.20 55.48	 < <	 66.23 83.46	 > >	 42.50 30.12	 > >	 11.53 11.03

3.3.4. Experimental silkworms Treated with Methoprene:
Similar to Pyridoxine treatment, with Methoprene treatment also, the silk gland showed less elevation compared with control tissues. Structural proteins showed less elevation in all selected tissues and the increase was observed in the following order.

Selected days	Sg		Mc		Fb		Hl
Ist day Control Experimental	 6.74 5.26	 < <	 16.10 16.23	 > >	 12.52 9.33	 > >	 2.90 1.97
7th day Control Experimental	 48.20 54.58	 < <	 66.23 86.24	 > >	 42.50 26.50	 > >	11.53 11.56

3.3.5. Experimental silkworms Treated with Mixed dose:
Silk worm, when treated with Mixed dose, the structural protein contents in the selected tissues were elevated slightly high when compared with control tissues and other experimental groups, and they were in the following manner.

Selected days	Sg		Mc		Fb		Hl
Ist day Control Experimental	 6.74 6.10	 < <	 16.10 15.54	 > >	 12.52 9.19	 > >	 2.90 1.82
7th day Control Experimental	 48.20 53.92	 < <	 66.23 87.71	 > >	 42.50 28.60	 > >	 11.53 10.42

Again, one significant observation from the above results with respect to the structural proteins was that muscle tissue showed highest protein content and registered peak elevation with the Mixed dose treatment and haemolymph showed least structural protein content when compared with other tissues and other experimental groups from day 1 to day 7.

Figure .1: Changes in Protein Profiles in the Silk gland of Control and different Experimental groups of 5th instar silkworms

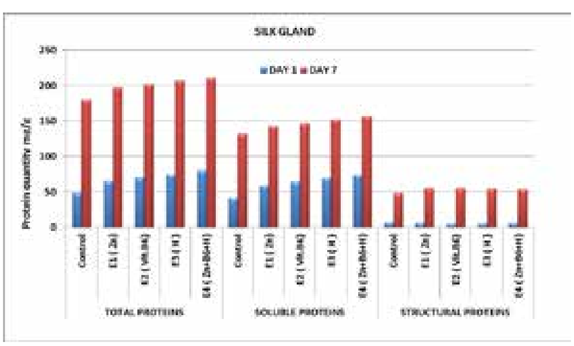


Figure 2: Changes in Protein Profiles in the Haemolymph of Control and different Experimental groups of 5th instar silkworms

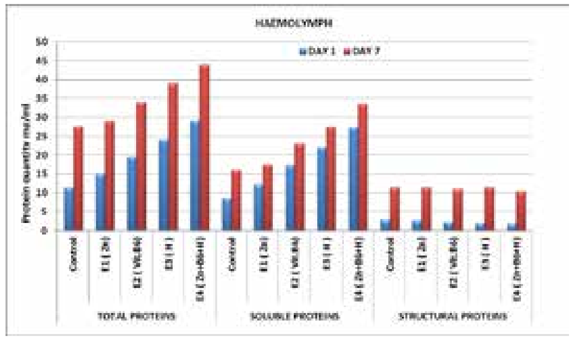


Figure 3: Changes in Protein Profiles in the Fat body of Control and different Experimental groups of 5th instar silkworms

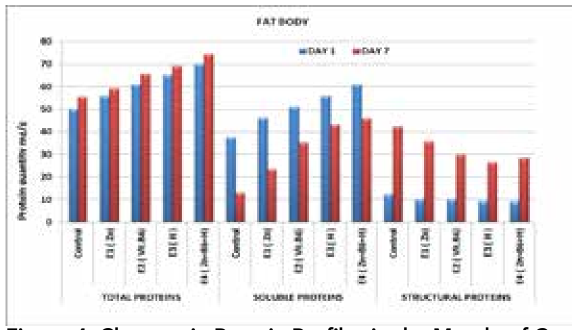
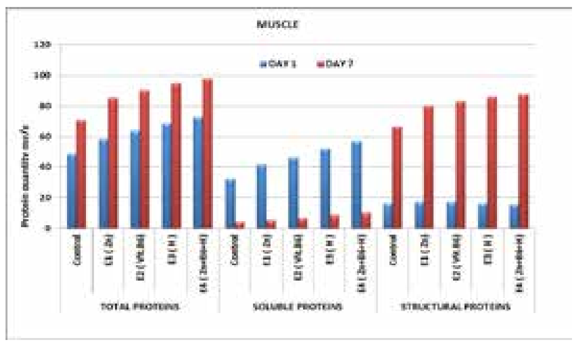


Figure 4: Changes in Protein Profiles in the Muscle of Control and different Experimental groups of 5th instar silkworms



4. DISCUSSION:
The results of the present study demonstrated severe perturbations in protein profiles of the silk worm (*B. mori*) in different tissues such as silk gland, haemolymph, fat body and muscle, when fed on mulberry leaves fortified with selected trace element, Zinc chloride, vitamin, Pyridoxine and hormone, Methoprene. However, protein utilization, especially for the silk fiber as well as the spinning activity probably remains more or less same at the selected dose of trace element, focusing on the possibility of variations in protein synthesis. Several researchers demonstrated phagostimulatory effect of vitamins and minerals in insects (Kanafi, 2007).

Feeding of mulberry leaves with supplements of potassium iodide, cobalt chloride and calcium chloride increase the quantity of proteins in the sericigene gland (Dasmahapatra et al., 1989). Administering potassium sulfate in the food determines protein decrease in the fat body and the haemolymph (Nirwani and Kaliwal, 1996), but Zinc chloride caused a significant decrease of fat body protein content and a significant increase in haemolymph protein content (Hugar, 1998). Oral supplements of potassium permanganate lead to increase of protein content in both the fat body and the haemolymph (Bhattacharya and Kaliwal, 2004).

According to the article "Application of Multi-vitamins as Supplementary Nutrients on Biological and Economical Characteristics of Silkworm *Bombyx mori* L." (By Kayvan Etebari and Leila Matindoost, 2005), multivitamin supplements had significantly increased or inhibited based on the concentration they were administered at.

The present findings clearly demonstrated the perturbations in protein profiles of the silk worm (*B.mori*) in different tissues like silk gland, haemolymph, fat body and muscle, when fed on mulberry leaves treated with selected compounds such as Zinc, Pyridoxine, Methoprene and Mixed dose. In Zinc treated group, the total and soluble proteins were elevated from day-1 to day-7. The results of the present study indicated that Zinc chloride at all selected doses significantly elevated the total protein levels in silk gland, haemolymph, fat body and muscle tissues of silk worm on all selected days during the 5th instar larval stage. It was also noted that the effect of Zinc was more pronounced on **7th day,** when compared to other selected days and other experimental groups. On all days, the **silk gland** recorded highest protein content and **haemolymph** the least.

As a corollary to the total proteins, the soluble protein levels in silk gland, haemolymph, fat body and muscle tissues were also elevated in the silkworms on treatment with Zinc chloride throughout the 5th instar larval stage. The results of the present study further clearly demonstrated that the effect of Zinc was more pronounced on 7th day when compared to other selected days. But when compared to total proteins the soluble proteins elevation was low from day-1 to day-7.

Similarly, the structural protein levels in the silk gland, haemolymph, fat body and muscle tissues of silk worms treated with Zinc chloride showed an increasing trend from day 1 to day 7 in all selected tissues. It was also noted that the effect of Zinc was more pronounced on 7th day. One common and most significant observation made in the case of structural proteins was they decrease in earlier days and elevated on 5th day as well as on 7th day.

In Pyridoxine treated group, elevation of total protein levels increased and the levels of soluble proteins were decreased when compared to total proteins. But the structural proteins were declined in earlier days and elevated on 5th day as well as on 7th day. As in the case of Pyridoxine treated group, the Methoprene treated group also recorded the total protein levels significantly and the levels of soluble proteins were decreased when compared to total proteins. The structural protein levels in the silk gland, haemolymph, fat body and muscle tissues of silk worms treated with Methoprene showed an increasing trend from day 1 to day 7. But the activity of structural proteins were declined in earlier days and elevated significantly on later days. Elevation in the levels of both total and soluble proteins in Mixed dose treated group showed high protein synthesis in all the selected tissues of *Bombyx mori,* when compared to the remaining experimental groups such as Zinc, Pyridoxine and Methoprene treated group. The increase in these levels at different experimental groups indicates an elevation of protein synthesis or a decrease in proteolysis at these selected experimental groups. However, protein utilization, especially for the silk fibre as well as the spinning activity probably remains more or less same at these groups, focusing on the possibility of variations in protein synthesis. Further, among the four experimental groups, Mixed dose treated group exhibited the overall level of protein synthesis remained high followed by Methoprene, Pyridoxine and Zinc.

Protein metabolism is an important process in building up of body i.e., in the development of muscles, tissues etc. Nutritive values of different proteins for the silk worm vary largely. These differences in the food value seem to depend on qualitative and quantitative amino acid composition of proteins (Crighton, 1984). Zinc ions entering into the silk gland cells, haemolymph, fat body, and into the muscle cells, initiate the protein elevations depending upon the tissue. Because of the changes in the protein elevations, the body size of the silkworm also showed variations. Several researchers demonstrated phagostimulatory effect of vitamins and minerals in insects.

In the present study, the **silk gland** registered more protein content when compared to other tissues in both control and the experimental worms during 5th instar larval stage. The **silk gland** is a reservoir for two important silk proteins such as fibroin and sericin (Zhang *et al.*, 2006) and other proteins such as chaperones, metabolic enzymes, heat shock proteins, immuno proteins, serpins, transport proteins, cytoskeleton proteins and those involved in gene expression (Kyung *et al.,* 2006). Though, in all experimental groups, various protein profiles were elevated, the maximum impact was observed in the experimental groups treated with Mixed dose. From this, it is inferred that a mixture of nutrients like a trace element, vitamin and a hormone seems to favor silk gland growth by stimulating protein synthesis during fifth instar development. Obviously, the silk production in *B. mori* could be modulated profitably for the sericultural industry by feeding the larvae with the mulberry leaves dipped in $ZnCl_2$, Pyridoxine and Methoprene at 2 µg / 100 ml / 100 worms, dosage.

Similarly, the **haemolymph** of ***B. mori*** is the chief circulating fluid and flowing reservoir of about 241 to 298 proteins (Lix *et al.,* 2006) that promote larval growth, metamorphosis, silk production, apoptosis, chitin and haemocyte formation, growth of salivary glands, reproductive organs and ecdysis (Naga Jyothi *et al*., 2010). Though, the impact of higher doses of minerals and vitamins on the haemolymph proteins is negative, it exerted significantly positive role at lower dose viz.2µg. Besides these functions, haemolymph serves as a transitory storage organ for various proteins and enzymes, until they are taken up and utilized by other tissues of which the silk gland requires special mention. Zinc could exert its modulatory role on the proteins in the circulating medium of haemolymph. In case of Pyridoxine, increase in elevation was noticed. When compared to these groups, Methoprene treated group exerted high elevation. Among the four experimental groups, peak elevation was recorded in the Mixed dose treated group.

Haemolymph proteins are synthesized by fat body cells and then secreted into the haemolymph in a time-dependent situation during post embryonic development and metamorphosis (Kishimoto et al., 1999). Several classes of abundant insect haemolymph proteins have been identified of which productions are regulated by hormones (Cole et al., 1990). Therefore, in this study, silkworms were topically treated a juvenile hormone analogue (JHA), Methoprene in 5th instar larval stage after fourth larval ecdysis and it was observed that haemolymph exerted peak levels on 7th day of fifth instar larvae of silkworm, *B. mori*.

In insects, JH exhibited a regulatory effect on protein synthesis. JH can suppress or induce protein synthesis independently or antagonistically (Fang et al., 2005). However, these hormones can also interact synergistically (Fang et al., 2005) and can be more effective to induce protein synthesis (Flatt et al., 2006). At the fifth instar larvae of *Bombyx mori*, JH induces the release haemolymph from the prothoracic gland on day 3, day 6, and day 9 (Sakurai and Imokowa, 1988). JH is secreted during the fourth ecdysis and then disappears from the haemolymph during early days of the fifth instar. Following this, JH titer increases gradually from day 5 until pupation (Niimi and Sakurai, 1997).

B. mori haemolymph contains many different proteins. They are usually classified according to their functions but their functions have not been completely clarified yet. Total protein concentrations in *Drosophila* and *Samia cynthia* were low at early stages but increased just before puparium formation. In our study, control groups showed similar results. The protein concentrations in the control haemolymph remained low at the beginning of the final instar and then increased slowly until day 7 in the silkworm larvae. A peak occurred on last day, just before pupation, and following this peak, the protein content of haemolymph decreased gradually. Endocrine conditions during the fifth larval stage differ from those of other larval instars and are important for larval-pupal metamorphosis. The ecdysone levels during the first 3 days of the last instar are undetectable, but juvenile hormone is still found in the haemolymph (Sakurai et al., 1998). Disappearance of juvenile hormone on day 3 is required for recovery of the prothoracic gland activity for secretion ecdysone. Both ecdysone and juvenile hormone can be detected in the haemolymph (Sakurai et al., 1989) after day 5 of the fifth instar. It has been shown that a juvenile hormone analogue on *B. mori* causes increase of the haemolymph protein concentrations and this result arises due to preventing sequestration of the storage proteins by the fat body (Kajiura and Yamashita, 1992). Previous investigation on the influence of the juvenile hormone analogue fenoxycarb on major haemolymph proteins in the silkworm, *Bombyx mori* during the last larval instar revealed that lipid transport in insects occurs via reusable transport proteins called lipophorins (Chino et al., 1981). They were also involved in lipid resorption in the intestine and transport of sterols, hydrocarbons and carotenoids. It has been reported that juvenile hormone also binds to lipophorins (Urich, 1994). Investigations on the influence of the juvenile hormone analogue Methoprene on major haemolymph proteins of the silkworm *Bombyx mori* during the last larval instar, all of these results suggest that major haemolymph proteins have different sensitivities to the juvenile hormone analogue Methoprene. The day of application and dose are more important for the response and these results may also be related to developmental progression of all experimental groups.

Infact, the insect **fat body** plays a key role in the metabolism similar to that of the mammalian liver and adipose tissues. In ***B. mori,*** it synthesizes and stores over 177 proteins that constitute nine glycolysis related proteins, several metabolically related proteins like diacylglycerol binding protein, triacylglycerol lipase, putative hydrolases, defense proteins etc. (Hou et al., 2007b). From the detailed analysis on the protein profiles of the fat body, it was obvious that the impact of $ZnCl_2$ was opposite to that of silk gland and haemolymph (Nirvani and Kaliwal, 1996). Of course, Pyridoxine is important in protein metabolism, without this the third instar significantly reduced the fecundity (Faruki, 2005). The impact of Zinc, Pyridoxine and Methoprene on fat body protein profiles is opposite to that of silk gland and haemolymph.

The silkworm **muscle** is known to contain many metabolically important proteins that are transported to it from other tissues during metamorphosis and includes those of the cytoskeleton, muscle contraction, feeding, locomotion, respiration and cocoon spinning (Naga Jyothi *et al.*, 2010) apart from those involved in oxidative metabolism and ATP production (David and Ananthakrishnan, 2006).

The overall effect of $ZnCl_2$, Pyridoxine and Methoprene, on muscle proteins was similar to that of the fat body, with lower doses eliciting a more positive effect. From these observations, it was suggested that the metabolism in this tissue could be positively modulated in all the selected experimental groups. The muscle of silk worm is known to synthesize and store about 258 proteins (Zhang et al., 2007).

Pyridoxine is necessary for the proper functioning of over 60 enzymes that participate in amino acid metabolism. It is also involved in carbohydrate and fat metabolism. Without Pyridoxine or its derivatives no larva reached the third instar under aseptic condition. In support of the present study, Pyridoxine is important in protein metabolism (Ziegler et al., 2000) and its deficiency results in decrease in phosphorylases (National Research Council, U.S. 1987).

The preliminary studies with juvenile hormone analogues in *Bombyx mori* were accomplished through topical applications on the insect. Akai et al., (1985) reported 30% increase on silk - ratio over the control, after the application of C18JH synthetic hormone. Chowdhary et al., (1986) noticed a 21% increment of silk production by the use of SJ-42-F juvenile hormone.

Silk proteins like **sericin and fibroin** will immensely contribute to the cocoon size and ultimately to the productivity of sericulture industry. The proteins of intestine, haemolymph and fat body may provide the raw material for the synthesis of silk protein in the silk gland. Contractile proteins of muscle in this economic insect would be important for feeding and for coordinated muscular movements during spinning activity. Thus higher the efficiency of the muscle, greater would be the efficiency of the spinning because during the 5^{th} instar stage, massive development of body musculature vis-à-vis its innervations by segmental nerves was observed. There are also reports on the improvement of economic characters of the silkworm, *B. mori* after administration of PMSG (Pregnant Mare's Serum Gonadotropin) and thyroxin (Thyagaraja et al., 1991).

Total, Soluble, Structural protein levels in the silk gland, haemolymph, fat body and muscle tissues of silk worms treated with Zinc, Pyridoxine and Methoprene showed an increasing trend from day 1 to day 7 in all selected tissues. It was also noted that the effect of Mixed dose was more pronounced on 7^{th} day. From the above results, it was observed that all fractions of proteins were elevated significantly in the silk gland, haemolymph, fat body and the muscle of the silkworms on treatment with selected doses of the above mentioned compounds on all selected days during the 5^{th} instar larval stage and the effect of Mixed dose (Zn+B6+H) was more pronounced due to their cumulative effects.

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