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Effect of copper stress on biochemical changes in *Philosamia ricini's* gland

P Bhaskar, SK Srivastava and Pakkala Abhiram

Abstract
The purpose of the study was to analyze the effect of Copper sulphate stress on total protein, protease, and level of free amino acids in silkworm. Enzymatic activity of alanine amino transferase and aspartate amino transferase in silkworm were also assed. For this study collected eri silkworm eggs from Regional Sericulture Research Station, Hyderabad, Andhra Pradesh (Central Silk Board). These eggs were hatched after seven days at 26°C. The larvae at early stage were fed with the chopped green variety of castor leaves (*Ricinus communis*). After the larvae enter to the second instar, it fed with Copper sulphate treated castor leaves at different concentration of 5, 10, and 15ppm with control. During the time of experiments also analyze the length and weight of the larvae. At 15ppm Copper sulphate treatment both third and fourth instars maximum length (2.7 and 3.8cm), weight (0.29 and 0.79gm), total protein (65.11±0.08 and 58.86±0.09 mg/gm), free amino acid third instar 15ppm (13.48±0.08µg/ml), In fourth instar highest on 5ppm (14.97±0.05 µg/ml) of the larvae observed and minimum was observed at Copper sulphate of 10ppm concentration. In this study also analyzed Protease activity, AAT and ALAT was highest at 10 ppm concentration.

Keywords: Free amino acid, protease, alanine aminotransferase, aspertate aminotransferase, *Philosamia ricini*, total protein

Introduction
Sericulture is an agro-based, labour intensive and foreign exchange earning commercial activity. It is an important cottage industry in India. Sericulture has been successful in eradication of rural poverty, resulting in social as well as economic development, mainly in rural areas. This industry is capable of generating employment and earning income continuously with low investment. Sericulture includes two sectors namely farm and industry. The farm sector involves growing food plants of silkworm, rearing silkworm to produce cocoons and laying production. Reeling, twisting, dyeing, printing, finishing, knitting and felting form the industry sector. Sericulture is much valued in creating mainly rural and marginally urban employment.

Silk is the queen of textiles; synthesis of silk is started after the 4th moult of the silkworm (Borgohain, 2015) [7]. Silk was a functional term used to describe natural protein fibres that are secreted by arthropods (Chowdhary, 2006) [11]. India is the only country in the world producing all the varieties of natural silk, *viz*. Mulberry, Eri, Tasar, Oak tasar, and Muga. Among these commercially exploited silkworms, eri silkworm is completely domesticated multi voltine, poly-phagous species under non-mulberry sector which is reared throughout the year (Subramanianan *et al*. 2013) [36].

The eri silkworm, *Philosamia ricini* is raised in India and parts of the orient for its silk. India continues to the second largest producer of silk in the world after China. Mulberry accounts for 92%, eri 5.5%, tasar 2% and muga 0.5% of the total raw silk production of the country. The silk produced by *Philosamia ricini* is not as fine or delicate as that of *Bombyx mori*, the mulberry silkworm, but it is more durable (Srivastava and Gupta, 2015) [35]. Ericulture is an agro-based traditional activity that has played an important role in generating income and employment for people living in rural areas in some parts of the world. It is ideal for rural areas as it requires low capital, is labor intensive thus creates jobs and it is commercially attractive (Oduor *et al.*, 2016) [23].

The environmental factors play a major role in Eri silk production. Since eri worms are quite delicate and sensitive to environmental conditions the prospect of obtaining silk production depends more on the food or host plant nutrition (Renuka and Shamitha, 2014) [27]. Eri fabric is called "Poor man's Silk" because it is much cheaper than muga and mulberry silk. So, it can appeal to a wide range of population. It is a domesticated silkworm and can be reared indoors and outdoors. It feeds on over 29 species of host plants (Reddy *et al.*, 2002) [26].

Proteins are the most important and characteristic constituents of living matter. Proteins must be constantly supplied to the organisms for growth and they are maintained at constant levels because of the dynamic state of constant turn over (Dunlap *et al*., 1978 and Hershku and Ciechanover, 1982) [14, 17]. Minor losses of proteins may result in several neurological disorders. Studies were made to analyse haemolymph proteins in insects such as *Philosamia ricini* (Poonia, 1978) [24], *Bombyx mori* L. (Venkata Reddy, 1984 and Seong *et al*., 1985) [38, 31] and *Calliphora* (Anderson, 1984) [2]. Reported the total and soluble proteins during metamorphosis of the silkworm. The protein levels in the fifth instar larvae of silkworm in different environmental conditions were reported (Maheswaramma, 1994) [21].

Proteases are the most commonly found digestive enzymes in insects (Ann Sorensen *et al*., 1983) [3]. Several factors responsible for the secretion of the proteolytic enzymes have been investigated (Briegel and Lea 1975) [8]. A strong protease activity has been demonstrated in the digestive fluid of the silkworm *Bombyx mori* L. (Eguchi and Iwamoto, 1976 and Sasaki and Sazuki, 1982) [15, 29]. Protease activity was reported in the fifth instar larvae of *Bombyx mori* L. (Maheswaramma, 1994) [21].

Amino acids play an important role in the osmotic homeostasis of blood (Beadle and Shaw, 1950) [6]. Insects, in addition to sugars and lipids, use amino acids as the readily available source of respiratory fuels (Bursell, 1963) [9]. The occurrence of free amino acids in high concentration in insect haemolymph was first observed (Nazari, 1902) [22]. Free amino acids in insect haemolymph was in general much higher than in vertebrate blood (Duchateau and Florkin, 1958 and Auclair, 1959) [13, 5]. Observed that a very high titre of free amino acids in the haemolymph is characteristic of winged insects. The amino acids in the silk gland of *Bombyx mori* L. were determined (Chitra and Sridhara, 1972) [10]. Effect of dietary amino acids in the haemolymph of the larvae was studied (Inokuchi, 1970) [18].

The aminotransferases (ALAT and AAT) mediate the transfer of amino groups of the amino acids to α- oxoglutarate, oxaloacetate and pyruvate to form glutamate, aspartate and alanine respectively (Lehninger, 1978) [19]. Amino transferasses have been detected in the tissues and eggs of silkworm. The activity levels of aspartate and alanine amino transferases showed an increase in silk gland, while they showed decreased levels in the central nervous system, muscle and haemolymph (Sivaprasad and Murali Mohan, 1990) [33].

Materials and Methods

The experiments were carried out in the laboratory at Department of Biochemistry and Biochemical Engineering, Jacob Institute of biotechnology and Bioengineering, Sam Higginbottom University of Agriculture, Technology and Science, Allahabad. The Eri silkworm (*Philosamia ricini*) was selected as the test organism for the investigation. Healthy, disease free seeds of eri silkworm were collected from "Regional Sericulture Research Station, Hyderabad, Andhra Pradesh (Central Silk Board)" and reared up to the pupation stage. The larvae were fed with the green variety of castor leaves (*Ricinus communis*). Fresh castor leaves were supplied three to four times a day with a care so that no larvae suffer from starvation. Proper cleanliness and hygiene were maintained during the time of rearing to prevent the occurrence of any diseases in larvae. After hatching hierarchy of the instar i.e. (1st insatr, 2nd instar, 3rd instar, 4th instar, 5th instar) for this experiment 3rdand4th instar will be selected.

Rearing of Eri silkworm *Philosamia ricini*

The rearing of eri silkworm was done by the method described by (Lowry *et al*., 1951) [20].

Eri silkworm *Philosamia ricini* mainly feed the castor (*Ricinus communis*) plant leaves. This species of silkworm reared easily in the laboratory. The rearing house of eri silkworm should be well ventilated and free from dust. The optimum temperature and humidity required are 26°C and 85 to 90 percent respectively. The different method of eri silkworm rearing are (a) Bunch rearing (b) Tray rearing and (c) Low cast bamboo platform rearing. In present study the "TRAY rearing method" was fallowed in the laboratory.

Treatments

In the present study silkworm (*Philosamia ricini)* feeded castor leaf treated with different concentrations of copper sulphate *i.e* 5ppm, 10ppm, 15ppm, 20ppm, along with control.

Sample preparation of silk gland

The silk gland of silkworm dissected out from the larvae of 3rd and 4thstage were washed in chilled bodenstein insect ringer solution and collected on a chilled dish kept over ice. The pooled glands were homogenized using chilled distilled water and acid washed sand in a homogenizer, kept immersed in crushed ice, and made into a solution of 10% silk gland tissue concentration (W/V). The homogenate was strained through muslin cloth and the filtrate used for the biochemical assay.

Biochemical analysis

Effect of copper Stress on total protein in silkworm

Protein was estimated by the method of (Lowry *et al*., 1951) [20] by using BSA as standard protein.

Effect of copper stress on the level of free amino acid in silkworm

Free amino acid was estimated by the method of

Estimation of Protease activity

Protease activity in the tissues was estimated using the ninhydrin method as described by Davis and Smith (1955) [12].

Effect of copper stress on enzymatic activity of AAT and ALAT in silkworm

Aspartate and alanine aminotransferase were assayed by the method of (Harper, 1985) [16].

Results and Discussion

Present studies entitled "Effect of copper stress on biochemical changes in *philosamia ricini*'s gland" were investigated and data obtained are hereby discussed in the following sub headings. The larval period in silkworm shows profound changes in the levels of biochemical parameters *viz.* total protein, free amino acid, protease activity, alanine aminotransferase and aspartate aminotransferase on 3rd and 4th in stars in the silk gland treated with different concentration of copper sulphate.

Effect of Copper sulphate on length of Eri silkworm (*Philasomia ricini*) on 3rd and 4th instar

In 3rd instar the length is decreased at 5ppm concentration @ 10 days while in the 4th instar length is increased at 5ppm concentration 16 days due to the duration of days. And the 3rd

instar @ 5ppm (19.23 %) decreased and the length of 4th instar increased @ 5ppm (26.31 %) when compared to control.

In 3rd instar the length is decreased at 15ppm concentration @ 10 days while in the 4th instar length is increased at 15ppm concentration 16 days due to the duration of days. And the 3rd instar @ 15ppm (42.10 %) decreased and the length of 4th instar increased @ 15ppm (46.15 %) when compared to control (Figure 1).

Similarly, Ali (2009) [1] Ashfaq *et al*. (2010) [4]. Tucker *et al*. (2004) [37] reported that Length was significantly increased by Chromium concentration in larval body.

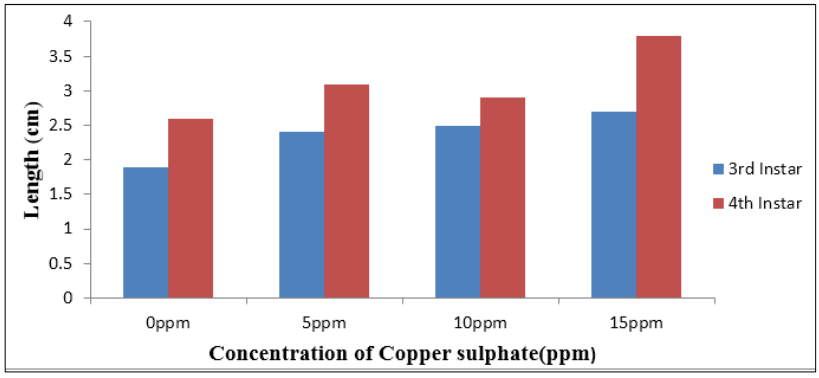

Fig 1: Effect of Copper sulphate on length of Eri silkworm *(Philasomia ricini*) on 3rd and 4th instar

Effect of Copper sulphate on weight (gm) of Eri silkworm (*Philasomia ricini)* on 3rd and 4th instar

In the present study larvae were feed with copper sulphate treated castor leaves of various concentrations 0ppm, 5ppm, 10ppm, and 15ppm. Increasing weight of larvae in 3rd instar 0.18, 0.29, 0.32, 0.25gm and 4th instar 0.41, 0.79, 0.5, 0.68 gm, respectively**.**

In 3rd instar the weight is decreased at 5ppm concentration @ 10 days while in the 4th instar weight is increased at 5ppm concentration 16 days due to the duration of days. And the 3rd instar @ 5ppm (61.11 %) decreased and the weight of 4th instar increased @ 5ppm (92.68 %) when compared to control.

In 3rd instar the weight is decreased at 15ppm concentration @ 10 days while in the 4th instar weight is increased at 15ppm concentration 16 days due to the duration of days. And the 3rd instar @ 15ppm (38.88 %) decreased and the weight of 4th instar increased @ 15ppm (65.85 %) when compared to control (Figure 2). And also increased body weight due to accumulation of various biochemical constituents like proteins, amino acids and enzymes like proteases, glutamate dehydrogenase and amino transferases (Venkata Reddy, S. (1984) [38].

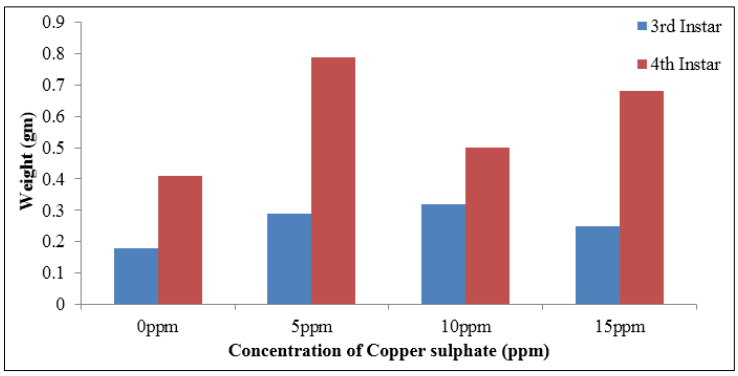

Fig 2: Effect of Copper sulphate on weight (gm) of Eri silkworm (*Philasomia ricini*) on 3rd and 4th instar

Effect of Copper sulphate on protein content (mg/gm) in gland of Eri silkworm (*Philasomia ricini*) on 3rd and 4th instar

Total protein showed significant increase (P<0.005) after copper sulphate treatment in various concentration 0ppm, 5ppm, 10ppm and 15ppm. Increased protein content were observed in silk gland of 3rd instar were 28.76±0.10, 44.42±0.05, 65.11±0.08, 50.91±0.18 mg/g, respectively. On 4th instar protein content were increased to 34.57±0.04, 49.40±0.04, 58.86±0.09, 51.92±0.04 mg/g, respectively**.** Highest protein content were observed in silk gland of third instar treated with 10ppm (65.11±0.08 mg/gm), minimum in 5ppm treatment (44.42±0.05 mg/gm) and in fourth instar highest protein observed on 10ppm (58.86±0.09mg/gm) and minimum (49.40±0.04 mg/gm) at 5ppm concentration (Figure 3).

In 3rd instar at 5ppm and 10ppm in silk gland may be due to either increased or decreased proteolysis which might lead to accumulated protein content. The protein profiles of the cell are indicative of the physiological status of the animal and there exists a dynamic equilibrium between the synthetic and degradative pathways associated with these molecules. In 4th instar 5ppm and 10ppm either increased or decreased due to the stimulate protein synthesis in fat body leading to an increase in its protein content and consequently more protein is released into the haemolymph from this organ. Thus the present result of enhancement of proteins in silk gland may be supported by the mobilization of more proteins from the haemolymph and fat bodies of copper sulphate treated silkworms. Similar results are found by (Harper, 1985) [16].

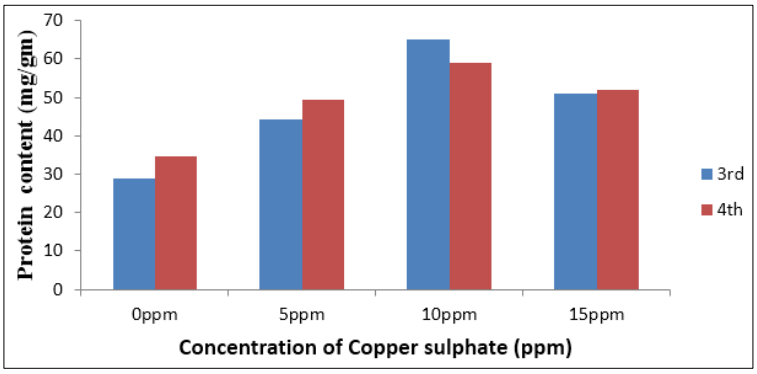

Fig 3: Effect of Copper sulphate on protein content (mg/gm) in gland of Eri silkworm *(Philasomia ricini*) on 3rd and 4th instar

Effect of Copper sulphate on free amino acid in gland of Eri silkworm (*Philasomia ricini*) on 3rd and 4th instar

The free amino acid showed significant increase (P<0.005) after copper sulphate treatment in various concentration 0ppm, 5ppm, 10ppm, and 15ppm. Increased free amino acid content were observed in silk gland of 3rd instar were 0.77±0.002, 0.78±0.021, 0.80±0.001, 0.83±0.001µg/ml, respectively. On 4th instar protein content were increased to 4th instar 0.65±0.002, 0.67±0.003, 0.73±0.002, 0.79±0.001µg/ml, respectively**.** Highest free amino acid content was observed in silk gland of third instar treated with 15ppm (13.48±0.08µg/ml), minimum in 5ppm treatment (9.14±0.59µg/ml).In fourth instar highest protein observed on 5ppm (14.97±0.05 µg/ml) and minimum (11.51±0.03 µg/ml) at 10ppm concentration (Figure 4).

After copper sulphate treatment free amino acid levels showed a decrease in the silk gland which indicates the faster mobilization of free amino acids into oxidative metabolism in the presence of copper sulphate. In 3rd instar free amino acid the 5ppm is decreased when compared to the 3rd instar of protein at 5ppm is increased and 4th instar free amino acid the 15ppm decreased when compared to the 4th instar of protein at 15ppm is increased. Free amino acid levels showed a decreased in the silk gland when indicates the faster mobilization of free amino acids into oxidative metabolism in the presence copper sulphate. TCA cycle through transamination. Amino acids in the silk gland serve as the pool for silk protein synthesis**.** Similar results are found by Siva Rami Reddy *et al*., (1984) [34], Prudhommo *et al*., (1985) [25], Sehnal and Akai, (1990) [30], Prasad and Murali Mohan (1990) [33]. The decreased free amino acid content may be due to decreased proteolysis Venkata Rami Reddy *et al*., (1992) [39].

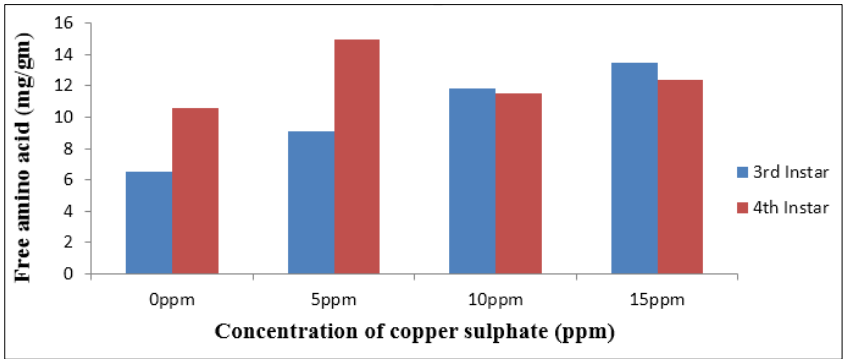

Fig 4: Effect of Copper sulphate on free amino acid in gland of Eri silkworm *(Philasomia ricini*) on 3rd and 4th instar

The effect of Copper sulphate on Protease activity (µmol tyrosine equivalents/mg protein/ hr) in gland of Eri silkworm on 3rd and 4th instar

The protease activity showed significant decrease (P<0.005) after copper sulphate treatment in various concentration 0ppm, 5ppm, 10ppm, and 15ppm, and show decreased protease activity in silk gland of 3rd instar were 0.90±0.00, 0.69±0.00, 0.68±0.00, 0.70±0.00 µmol tyrosine equivalents/mg protein/ hr. respectively. On 4th instar 0.61±0.06, 0.32±0.02, 0.32±0.00, 0.12±0.00 µmol tyrosine equivalents/mg protein/ hr, respectively**.**

In 3rd instar of protease the 10ppm is decreased when compared to the 3rd instar of free amino acid the 10ppm is increased due to the proteases are the enzymes responsible for the hydrolysis of proteins into amino acids. In 4rd instar of protease the 15ppm is decreased when compared to the 4rd instar of free amino acid the 15ppm is increased due to the proteases are the enzymes responsible for the hydrolysis of proteins into amino acids (Figure 5). due to participate in protein digestion in intestine and histolysis in other tissues. The decrease of protease activity level may be due to lower rate of histolysis. Similar results are found by (Sailaja and Bharathi 2015) [28].

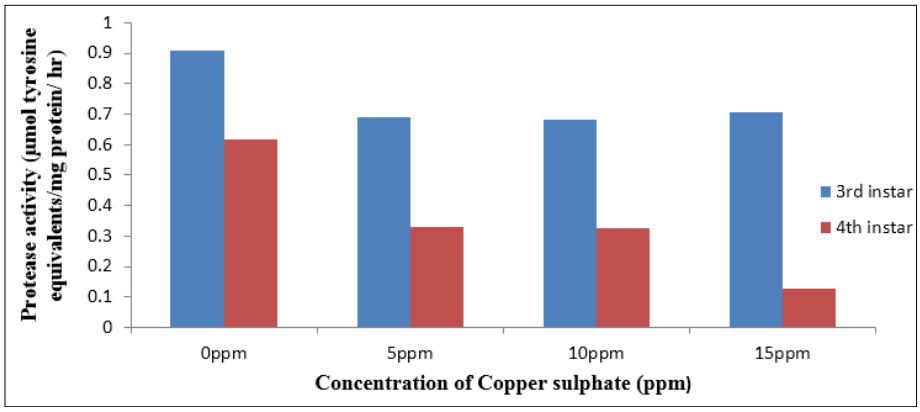

Fig 5: The effect of Copper sulphate on Protease activity (µmol tyrosine equivalents/mg protein/ hr) in gland of Eri silkworm on 3rd and 4th instar

Alanine aminotransferase (ALAT) activity

Significant result was noticed in ALAT activity (p<0.005) after copper sulphate treatment in various concentration 0ppm, 5ppm, 10ppm, and 15ppm and showed increase alanine amino transferase in silk gland in 3rd instar are 1.34±0.01, 0.49±0.01, 0.69±0.00, 0.37±0.00 mg/g respectively and 4th instar 1.16±0.01, 0.54±0.00, 0.85±0.01, 0.57±0.00 mg/g respectively**.** The highest level of alanine amino transferase activity was observed in silk gland of third instar treated with 10ppm (0.69±0.00 µmol pyruvate equivalents/mg protein/ hr), minimum in 15ppm (0.37±0.00 µmol pyruvate equivalents/mg protein/ hr). In fourth instar highest protease activity observed on 10ppm (0.85±0.01 µmol pyruvate equivalents/mg protein/ hr) and minimum (0.54±0.00 µmol pyruvate equivalents/mg protein/ hr) at 5ppm concentration.

Alanine aminotransferase activity is decreased when compared to the protein and free amino acid are increased with the copper sulphate treatment indicating the active involvement in the protein synthesis.

Higher amino transferase activities are the indicative of higher conversion of amino acid pool and subsequently greater protein synthesis. The enhanced activity of alanine aminotransferase reflected the general index of mobilization of free amino acids into gluconeogenesis and oxidation of amino acids respectively. Similar results are found by Venkatarami Reddy *et al*., (1992) [39] and Sinha *et al*., (1996). Thus it can be concluded that cobalt chloride enhanced the amino acid metabolism which effects the growth production of silkworm. Sailaja and Bharathi (2015) [28].

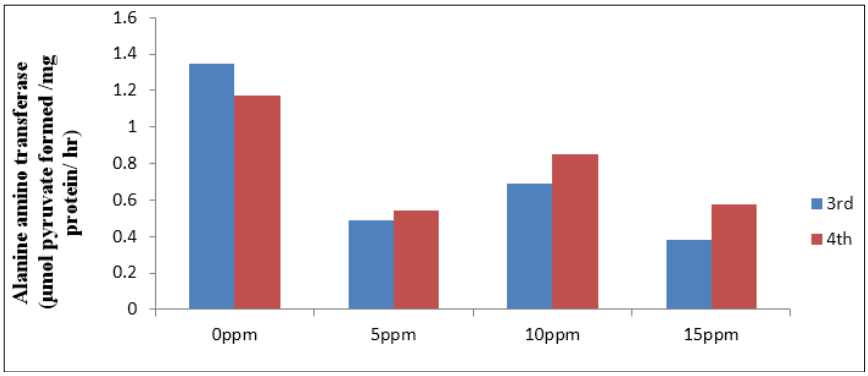

Fig 6: Effect of Copper sulphate on alanine amino transferase (µmol pyruvate formed /mg protein/ hr) in gland of Eri silkworm *(philasomia ricini*) on 3rd and 4th instar

Aspartate amino transferase (AAT) activity

Significant result was noticed in ALAT activity (p<0.005) after copper sulphate treatmentin various concentration 0ppm, 5ppm, 10ppm, and 15ppm and showed increased aspartate amino transferase in silk gland in 3rd instar are 0.45±0.18, 0.21±0.04, 0.35±0.21, 0.17±0.00 and in 4th instar 0.62±0.00, 0.26±0.09, 0.28±0.02, 0.10±0.05 respectively**.**

The highest level of aspartate amino transferase activity was observed in silk gland of third instar treated with 10ppm (0.35±0.21µmol pyruvate equivalents/mg protein/ hr), minimum in 15ppm (0.17±0.00µmol pyruvate equivalents/mg protein/ hr). In fourth instar highest activity observed on 10ppm (0.28±0.02µmol pyruvate equivalents/mg protein/ hr) and minimum (0.10±0.05 µmol pyruvate equivalents/mg protein/ hr) at 15ppm concentration. Aapartate aminotransferase activity is decreased when compared to the protein and free amino acid are increased with the copper sulphate treatment indicating the active involvement in the protein synthesis.

Higher amino transferase activities are the indicative of higher conversion of amino acid pool and subsequently greater protein synthesis. The enhanced activity of aspartate aminotransferase reflected the general index of mobilization of free amino acids into gluconeogenesis and oxidation of amino acids respectively. Similar results are found by Venkatarami Reddy *et al*., (1992) [39] and Sinha *et al*., (1996). Thus it can be concluded that cobalt chloride enhanced the amino acid metabolism which effects the growth production of silkworm.

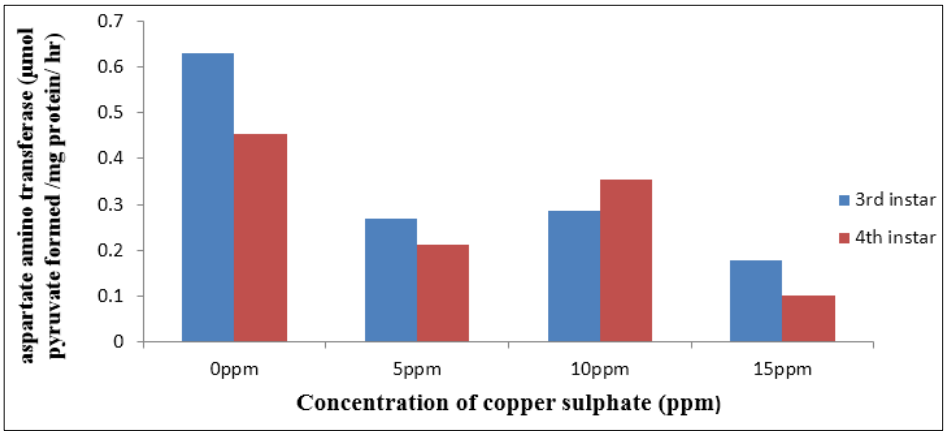

Fig 7: Effect of Copper sulphate on aspartate amino transferase (µmol pyruvate formed /mg protein/ hr) in gland of Eri silkworm *(philasomia ricini*) on 3rd and 4th instar

Conclusion

On the basis of above investigation it can be concluded that the eri silk worm can easily able to grew inside the lab. Current studies proved that eri silkworm were sensitive to environmental factors. In present study silkworm feed the heavy metal copper sulphate treated castors leaves and it resist to copper sulphate up to 15ppm, so eri silkworm can able to culture using castor leaves grown in heavy metal (copper sulphate) up to maximum 15ppm concentration accumulated soil area. Amino acids were the major precursor of silk synthesis in present study proved that silkworm feed with 15ppm treated copper sulphate leaves increases the amino acids contents in the silk gland of the eri silkworm, which increases the more silk production. Current study proved that Copper sulphate stress increased the protein content and decreased the enzyme activities due to heavy metal stress.

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