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Effect of Feeding Mulberry Leaves Supplemented with $CaCl_2$ on Biochemical Contents of the Silkworm, *Bombyx mori* L.

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ABSTRACT

Oral Supplementation with 50, 100 and 150 µg/ml calcium chloride to the fifth stadium larvae of CSR2xCSR4 biovoltine hybrid silkworm, *B. mori* on fat body glycogen, protein and total lipids and haemolymph trehalose and protein have been studied. One of the four normal feeds per day was substituted with treated leaves fed to silkworms at fifth instar larvae. The controls were fed with the leaves dipped in distilled water and normal leaves. The fat body glycogen with 50 and 100 µg/ml, haemolymph trehalose with 100 and 150 µg/ml and fat body protein with all the calcium chloride treated groups were increased significantly when compared over respective carrier controls. There was significant increase in the haemolymph protein with 150 µg/ml calcium chloride treated group. However, there was no significant change in the haemolymph protein with 50 and 100 µg/ml calcium chloride treated groups when compared over the respective carrier controls. The fat body total lipids decreased significantly in all the groups treated with calcium chloride when compared over the respective carrier controls. These results indicated that the content of glycogen and protein of the fartbody and trehalose and protein content of the haemolymph to calcium chloride showed good response in CSR2xCSR4 bivoltine hybrid silkworm, *B. mori*.

Key words: Biochemical Contents, *Bombyx mori*, Calcium Chloride, Silkworm

INTRODUCTION

The silkworm, *Bombyx mori* L. derives almost all the nutrients required for its growth from the mulberry leaf. The requirement of different minerals in various insects has been investigated (House, 1974). It has been reported that silkworm larvae require essentially potassium, phosphorus, magnesium and zinc for their growth and survival (Horie *et al.*, 1967). It is interesting to note that there are similarities in dose requirements by each elements among insect species, and that mulberry leaves contain the adequate amounts of minerals to maintain good growth (Ito and Niminura, 1966).

The carbohydrates, proteins and lipids are essential for the development of insects and are derived from the food material. The metabolic fuels are stored in the fat body and haemolymph during fifth instar. Fat body in insects performs many functions like that of mammalian liver. Thakare *et al.*, (1980) have reported that trehalose is a major haemolymph sugar in the last nymphal stage of the dragonfly, *Orthetram chrysis* and haemolymph serves as a storage tissue (Jungreis, 1980; Mullins, 1985). It has also been reported that minerals influences on the biochemical contents of the silkworm, (Nirwani and Kaliwal, 1996; Hugar *et al.*, 1998; Goudar and Kaliwal, 2001; Bhattacharya and Kaliwal, 2004). It has been showed that magnesium is essential for complete activity of trechalose synthesis (Murphy and Wyatt, 1965). It has been reported that potassium iodide, cobalt chloride, calcium chloride and potassium nitrate influences the protein, DNA and RNA content of the silkgland of the silkworm, *B. mori* Nistari race (Dasmahapatra *et al.*, 1989).

Etebari *et al.*, (2004) have reported on different aspects of mulberry leaves supple-mentation with various nutritional com-pounds in sericulture. The metals and their salts acting as a catalyst important to biologi-cal system. The late age larval stage is the most active feeding stage during which the larva accumulates large quantity of fuel reserves in various tissues and is endowed with unique biochemical adaptations to conserve nutritional resources available dur-ing active larval stage of the silkworm.

The calcium is another important element that influences on the metabolism of the silkworm. Therefore, the present investtiga-tion was undertaken to study the effect of feeding mulberry leaves supplemented with calcium chloride on the fat body protein, glycogen, haemolymph trehalose, protein and fat body total lipids of the silkworm.

MATERIALS AND METHODS

Rearing technique and treatment

The disease free layings (DFLs) of the bivoltine hybrid (CSR2 x CSR4) silkworm, *Bombyx mori* L. used in the present investing-ation were obtained from the grainage centre Rayapur, Dharwad district, Karnataka State. The hatched larvae were reared in the rearing room and fed with fresh mulberry leaves (S36 variety) four times a day by maintaining optimum humidity (85-95%) and tempera-ture (27° C) providing optimum spacing and mulberry leaves ad libitum raised by improved methods of rearing techniques (Krishnaswami, 1978). The fifth instar larvae were selected randomly and grouped into different batches for the experiment. Each group consisted of five replications each with 20 larvae. The calcium chloride was procured from Fisher Inorganics and Aromatics Limited, Madras.

It was dissolved in small quantity of distilled water and diluted to 50,100 and 150 µg/ml by adding distilled water. The mulberry leaves were dipped for 30 min in calcium chloride of known quantity and then the leaves were dried under the shade and fed to the silkworm larvae. In each supplementation, 1 kg of mulberry leaves were dipped in 500 ml of solution. Amongst the four feeding per day, feeding of treated leaves was given one time at 8.00 am with the feeding of untreated leaves. Treatment was given alternate day. The carrier controls were fed with mulberry leaves dipped in distilled water while the normal controls were fed with untreated leaves.

Tissue preparation

The silkworm larvae were dissected in 0.9% saline at pH 6.5 on the 6th day of the fifth stadium. The fat body was immediately collected and used for estimation of glycogen, protein and lipids.

The haemolymph was collected from 2-3 larvae by amputing one of the thorasic legs in a pre chilled centrifuge tube and was used for the estimation of trehalose and total protein.

Glycogen estimation

Anthrone method of Sciefter *et al.* (1950) was used to determine the fat body glycogen. A known quantity of fat body was homogenized with 2ml of 20% potassium hydroxide. The glycogen was precipitated by adding equal volume of 80% ethanol and the mixture was kept overnight at room temperature for digestion. It was then centrifuged at 3000 rpm for 15 min and the supernatant was discarded. The precipitate was dissolved in a known volume of distilled water. Glycogen content was estimated with known aliquots in triplicate by the anthrone method. Glucose-D was used as the reference standard and the intensity of the colour was read on the spectrophotometer at 620 nm.

Trehalose estimation

The estimation of haemolymph trehalose was carried out according to the method of Roe (1955). Known quantity of haemolymph was collected in each test tube, and added 0.5 ml of 2% of sodium hydroxide to each test tube. After shaking, the tubes were kept in boiling water for 10 min and then the tubes were cooled in an ice box. Then 5 ml of anthrone reagent (0.05% anthrone in 70% sulphuric acid) was added to the tubes, and they were again kept in boiling water for 15 min for the development of color. Then the tubes were cooled to room temperature. Then the color intensity was read on the spectrophotometer at 620 nm. For the reference standard the trehalose (Sigma, USA) was used. Anthrone positive carbo-hydrate in the haemolynmph is considered as trehalose.

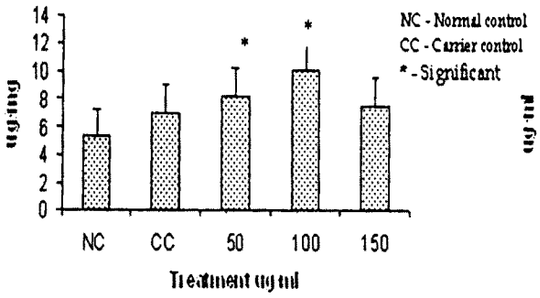

Fig 1. Effect of CaCl2 on the fat body glycogen of the silkworm

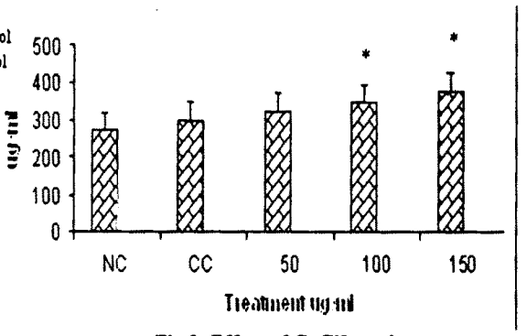

Fig 2. Effect of CaCl2 on the haemolymph trehalose of the silkworm.

Total protein estimation

The method of Lowry *et al.* (1951) was used for the estimation of total protein in the fat body and haemolymph. The tissue protein was precipitated by the addition of 1 ml of 30% trichloroacetic acid (TCA) solution followed by centrifugation at 3000 rpm for 30 min. It was repeated twice, then the precipitate was dissolved in 1ml of 0.1 N sodium hydroxide. A known aliquot of this solution was then mixed with 5 ml of alkaline copper reagent (20% sodium carbonate prepared in 0.1 N sodium hydroxide containing sodium potassium tartarate and 1% copper sulphate). After 10 min 0.5 ml of Folin Ciocalteu's reagent was added to the tubes and the tubes were shaken thoroughly. Then the tubes were kept for 20 min for color development. The readings were taken on the spectrophotometer at 650 nm.

The estimation of total haemolymph protein was also carried out. A known quantity of (1 ml)haemolymph was diluted with 0.5 ml of distilled water. A known aliquot of this solution was added with 5 ml of alkaline copper reagent. After 10 min 0.5 ml of folin Ciocalteu's reagent was added and were mixed thoroughly and kept for 20 min until color develops. The readings were taken on the spectrophotometer at 650 nm. For the reference standard Bovine Serum Albumen (BSA) (Fatty acid free) was used.

Extraction and estimation of total lipids

The method of Folch *et al.* (1957) was used for the lipid estimation, using chloroform: methanol mixture (2: 1 V/V). First, all the fat body was homogenized with appropriate volume of chloroform: methanol mixture (1:10). Then homogenate was then quantitatively transferred to a 50 ml separating funnel and then similar volume of chloroform was added. The two solvents were partitioned by the addition of 0.2 volume of water. After the funnel was shaken, the mixture was allowed to stand overnight. The lower chloroform layer containing lipids was drawn off. The lipids sample was kept in a vacuum desiccators until constant weight was obtained.

Statistical analysis

The experiments were designed by the complete randomized block design (CRBD) method and the data collected were subjected to the statistical analysis of variance (ANOVA) test to determine the significant difference between the treatment and control groups (Raghava Rao, 1983).

RESULTS

The results on the effect of oral supplementation with three different concentrations 50, 100 and 150 µg/ml of calcium chloride on glycogen, protein and total lipids content of the fat body and trehalose and protein content of the haemolymph in the bivoltine hybrid of the silkworm, *Bombyx mori* are presented in Figures 1 to 5.

Carbohydrates

Oral supplementation with 50 µg/ml calcium chloride to fifth stadium silkworm larvae resulted in an increase of 17% fat body glycogen (Fig 1) and 8% haemolymph trehalose (Fig 2). The oral supplementation with 100 µg/ml calcium chloride to silkworm larvae resulted in an increase of 44% fat body glycogen and 16% haemolymph trehalose. The oral supplementation with 150 µg/ml calcium chloride to silkworm larvae showed in an increase of 4% fat body glycogen and 26% haemolymph trehalose The present

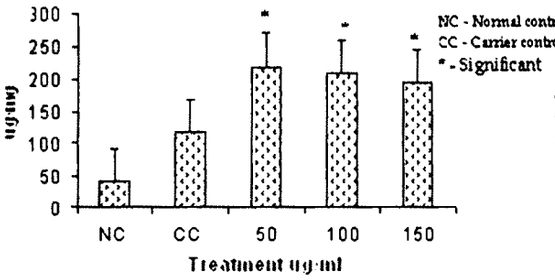

Fig 3. Effect of CaCl2 on the fat body protein of the silkworm

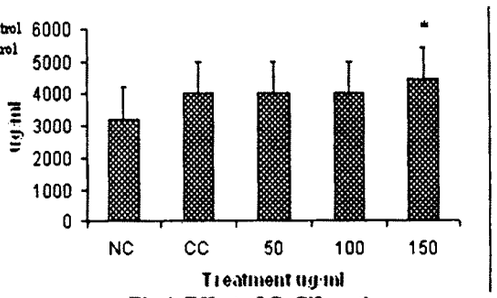

Fig 4. Effect of CaCl2 on the haemolymph protein of the silkworm.

results suggested that the dietary supplementation with calcium chloride significantly increased the fat body glycogen with 50 and 100 µg/ml, haemolymph trehalose with 100 and 150 µg/ml treated groups when compared over the respective carrier controls.

Total Protein

The dietary supplementation with 50 µg/ml calcium chloride to fifth instar silkworm larvae showed an increase of 86% fat body protein (Fig 3) and 2% haemolymph protein (Fig 4). The dietary supplementation with 100 µg/ml calcium chloride to silkworm larvae resulted in an increase of 76% fat body protein and 1% haemolymph protein when compared over the respective carrier controls. The oral supplementation with 150 µg/ml calcium chloride to silkworm larvae showed an increase of 64% fat body protein and 10% haemolymph protein (Figs. 3 and 4). The above result also suggested that the oral supplementation with calcium chloride significantly increased the fat body protein with all the calcium chloride treated groups where as haemolymph protein was significantly increased only in 150 µg/ml calcium chloride treated group when compare over the respective carrier control.

Total lipids

The dietary supplementation with 50, 100 and 150 µg/ml calcium chloride to silkworm larvae resulted in decrease of 8%, 10% and 20% fat body total lipids respectively (Fig 5). The decreases in the content of fat body total lipids were found to be statistically significant when compared over the respective carrier controls.

In the present results it was interesting to note that there were an increase in glycogen, protein and total lipids contents in the fat body and trechalose and protein contents in the haemolymph in carrier controls when compared over the respective normal controls. This indicated that even the distilled water influences the biochemical contents of the silkworms.

DISCUSSIONS

The results of the present study showed that the fat body glycogen significantly increased in the groups treated with 50 and 100 µg/ml calcium chloride. Similar increase in the fat body glycogen content has been reported after supplementing the feed with potassium and magnesium sulphates and potassium permanganate in the bivoltine silkworm, *B. mori* (Nirwani and Kaliwal, 1995; Bhattacharya and Kaliwal, 2004). Recently it has been reported that after supplementing the feed with minerals increases the glycogen and trechalose contents of the fat body and haemolymph in the silkworm (Bhattacharya and Kaliwal,

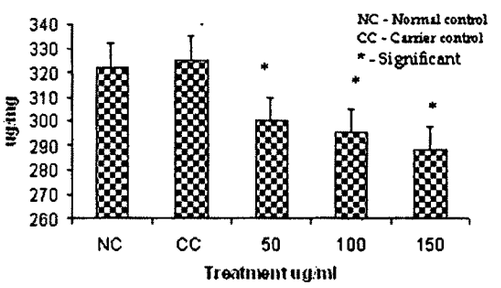

Fig. 5. Effect of calcium chloride on the Total lipids of the silkworm *B. mori* L.

2005a, b, c). Accumulation of glycogen during the feeding period in bivoltine silkworm is shown to be due to the increased amylase activity and glycogenesis (Pant and Morris, 1969). The increase in the amylase activity of the midgut and the increased production of carbohydrates has been reported after supplementing the feed with mineral samples in the beetle, *Leptinotarsa decemlineata* (Izhevaskiy, 1976).

However, in the present study the increased fat body glycogen after supplementing the feed with calcium chloride may possibly be due to the stimulatory effect on the amylase activity of the midgut resulting in an increased production of glycogen as suggested by earlier workers (Pant and Morris 1969; Izhevaskiy, 1976). The results of the present study also indicate that the trehalose content of the haemolymph was significantly increased in high doses of calcium chloride feed groups. Morever, the amount of trehalose present in the haemolymph is directly related to the glycogen content of the fat body, which is influenced by a number of endogenous organic and inorganic factors and also has been reported that calcium ions enhance the production of trehalose by the fat body in the insect *Periplaneta americana* as suggested by Downer (1979). The increase in the haemolymph trehalose content has also been revealed after feeding with magnesium and potassium sulphates and potassium perman-ganate in the bivoltine NB4D2, CSR2 x CSR4 bivoltine hybrid of the silkworm, (Nirwani and Kaliwal, 1995; Bhattacharya and Kaliwal, 2004). The significant increase in the haemolymph trehalose might possibly be due to the conversion of glycogen into trehalose and its subsequent release into the haemolymph by the fat body.

Wigglesworth (1972) has stated that the fat body in insects is the main site of protein synthesis as well as the intermediating metabolisms of amino acids. In the present study, treatment with calcium chloride has resulted in increase of the fat body protein in all the treated groups and haemolymph protein in 150 µg/ml calcium chloride treated group. The increased protein content of the fat body and haemolymph might possible be due to the stimulatory effect of the eral salt on the synthetic activity of the at body and the increased haemolymph protein content might be due to the release of excess of protein by the fat body into the haemolymph which also influences on the silkgland, cocoon weight and its shell weight of the silkworm, It has also been reported in an increase in the protein content of the silkgland after supplementing the feed with minerals in the nistari race of, *B. mori* (Dasmahapatra *et al.*, 1989). Recently, Similar results have been reported in the silkworm treated with minerals (Goudar and Kaliwal, 2000; Bhattacharya and Kaliwal, 2004, 2005 a, b, c).

The lipids are the energy reserves and important component of the fat body which can be mobilized rapidly during starvation, oogensis, embryogenesis, moulting and is used to sustain continuous muscular activity (Wyatt, 1967; Gilbert and Chino, 1974). In the present study, treatment with calcium chloride has resulted in a significant decrease in the fat body total lipids in all the groups. In contrast to our findings it has been reported that the oral supplementation with potassium nitrate significantly increased the total lipids, phospholipids and neutral lipids of the fat body in the silkworm, (Goudar and Kaliwal, 2001). Recently it has also been reported that oral supplementation with minerals significantly increased the total lipids of the fat body in the silkworm, (Bhattacharya and Kaliwal, 2004, 2005 a,b,c). The decreased total lipids of the fat body might possibly be due to the inhibitory effect of the calcium chloride at a given concentration on the synthetic activity of the fat body. It was also interesting to note that there was an increase in the biochemical contents of the fat body and haemolymph in distilled water treated carrier controls indicated that the distilled water may influence the synthetic activity of glycogen and protein of the fat body and trehalose and protein of the haemolymph may be due to increase in moisture content of the silkworm.

The possible mechanism of action of calcium chloride on the biochemical contents of the fat body and haemolymph of the silkworm, was due to their influence on nervous system or hormones, or the stimulation or inhibition of enzyme activity, or the induction or suppression of enzyme synthesis is not known. Hence, further investigation is essential to known the exact mechanism of action of calcium chloride on

the biochemical contents in the fat body and haemolymph of the silkworm, *B. mori.*

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