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*Research Paper*

# INFLUENCE OF THE STEVIA INULIN TREATED LEAVES OF MULBERRY, Morusalba (L) ON THE MID GUT ENZYMES IN FIFTH INSTAR LARVAE OF SILKWORM, Bombyxmori (L) (RACE: PM X $CSR_2$)

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The inulin is a heterogeneous collection of fructose polymers anda soluble dietary fiber. Four different concentrations of aqueous solution of herbal formulation: Stevia inulin powder (5.0 ppm; 10.0 ppm; 20.0 ppm and 50.0 ppm) were used to treat the leaves of mulberry and fed to the fifth instar larvae of polyvoltine, crossbreed silkworm, *Bombyxmori* (L) for first three days, second day, third day and third day (only). The larvae fed with untreated and water treated leaves were also maintained. Bioassays of proteins (S.P. and T.P.) and enzymes (protease and amylase) were carried out on fifth day through the use of mid gut homogenate. Treating the mulberry leaves with herbal formulation: Stevia inulin powder and feeding them to fifth instar larvae was found reflected into significant improvement in the levels of proteins (S.P. and T.P.) and velocities of biochemical reactions catalyzed by protease and amylase. The pattern of increase in soluble proteins and total proteins in the mid gut tissue were 32.147 to 90.074% and 5.657 to 39.052% respectively. The activities of mid gut protease and amylase were increased by 21.444 to 83.706% and 14.54 to 52.257% respectively. The nutrient contents of herbal formulation: Stevia inulin powder serve to improve the digestibility and exert the influence on efficient metabolism in the fifth instar larvae of silkworm, *Bombyxmori* (L). The herbal Stevia inulin powder treatment may gear overall biochemical constituency of silkworm larvae, through mid gut enzymes. Use of herbal Stevia inulin powder to treat mulberry leaves and feeding the fifth instar larvae of silk worm, *Bombyxmori* (L) may be introduced in the rearing schedule to fortify the digestibility and qualitative silk production.

Keywords: Silkworm, Bombyxmori, Stevia, Inulin, Midgut enzymes

## INTRODUCTION

The life of insect herbivores is in the orchestrate progression, which closely interlinked with plant metabolites. The biochemical constituents of

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plants could have been the factors of growth and metamorphosis for insects (Bowers *et al*., 1966). The phytophagous insects are able to avoid poor quality food or able to select a high quanlitative food from variety available to them. The silkworm, *Bombyxmori* (L) is a monophagous insect, feeding exclusively on the leaves of mulberry *Morusalba* (L). It is therefore, essential to improve either food quality or appetite (or both) of larval instars of silkworm for better performance in silk production. The factors responsible to influence the growth, development and subsequent physiology of insect body include: nutritional qualities of food, biochemical status of nutrients in the food, hormonal level in the body and environmental conditions (Murugan and Georgr, 1992). Elements of the insect body are primarily derived from the food source. For silkworm, the leaves of mulberry contain many stimulants (Ito, 1960 and 1961; Nayar and Fraenkel, 1962; Ito *et al*., 1964; and Ito and Hyashiya, 1965). Nutrition quality in silkworm, *Bombyxmori* (L) serve to accelerate the growth, metamorphosis and forms the physiological foundation for sericulture. The leaves of mulberry are the sole source of food for larval instars of silkworm, *Bombyxmori* (L), biochemically constituted with proteins, lipids, carbohydrates (Murali, 1992) and minerals (Subramanyam Reddy, 1992). Therefore, corresponding diversity of enzymes capable of hydrolyzing the biocompounds of mulberry is exhibited by mid gut of larval instars of silkworm, *Bombyxmori* (L). The body tissues of larval instars of silkworm, *Bombyxmori* (L) especially, the fat bodies accumulates large quantity of proteins, lipids and glycogen during the development, which is nothing but the reflection of efficient consumption and utilization of nutrient biocompounds of mulberry leaves. The variation in the food consumption in phytophagous insects may be for varied biochemical processes, ultimately for successful adaptations (Slansky, 1982). It has been suggested that, there is a functional difference between the activity of digestion by the digestive fluid in mid gut and tissue of mid gut. It has been reported by Horie, *et al.* (1963) that, molecular proteins are hydrolyzed into peptides by digestive fluid content and into amino acids with peptidases in the mid gut tissue. Likewise, the polysaccharides, are digested in the insect gut lumen by digestive fluid and disaccharides and/or trisaccharides get hydrolysed into their constituent monasaccharide sugars mainly in the gut tissue (Horie, 1967).

Lipase, the lipid digesting enzyme of the insect mid gut has been reported to have analogy with pancreatic lipase of vertebrates (Yamafugi and Yonezawa, 1935). The efforts towards the qualitative silk production through the improvement in the efficiency of consumption and utilization of food by larval instars of silkworm, *Bombyxmori* (L) include: improvement in the quality of mulberry leaves and supplementation of nutrient biocompounds like soya protein; potassium iodide, copper sulphate, other mineral salts, herbal products (or drugs) like digoxin (Vitthalrao and Kulkarni, 2011) and kho-go (Desai *et al.*, 2011).

Steviol glycosides belongs to the stevia plant (*Steviareboudiana* Bertoni). They are responsible for sweet taste and are heat stable, pH stable and do not ferment (Brandle, 2004). Structurally, Steviol is a diterpene, aglycone unit of the sweet glycoside (Stevoside) of *Stevia reboudiana* Bertoni (Bridel and Lavieille, 1931; and Dolder *et al.*, 1960). The European Food Safety Authority evaluated the safety of steviol glycosides, extracted from the leaves of *Stevia reboudiana* Bertoni plant, as sweetener and expressed the

units to be consumed for human health (Commission Regulation/EU/No. 1131/2011). Medicinally important status of Steviol; it's glycoside nature (glycoside treated mulberry leaves may accelerate the rate of midgut enzyme catalyzed biochemical reactions in the larval instars of silkworm). Most of the Juvenoids used for topical application to the larval instars of silkworm are terpenoids. Glycoside nature of steviol; it's diterpene structure and it's pharmaceutical status made to plan to screen aqueous solution this herbal formulation through treating the leaves of mulberry, *Morus alba* (L) and feeding them to the fifth instar larvae of silkworm, *Bombyxmori* (L) (Race: PM x $CSR_2$).

## MATERIAL AND METHODS

The Disease Free Layings (DFL) of multivoltine, crossbreed race: PM x $CSR_2$ of silkworm, *Bombyxmori* (L) were procured through the sericulture unit of Agriculture Development Trust, Malegaon (Baramati). They were processed for incubation (through black boxing); transfer of hatched larvae on the rearing bed of mulberry leaves and reared through the methods prescribed by Krishnaswami *et al.* (1978) and explained by Khyade (2004). Soon after the fourth moult, the fifth instar larvae were divided into various groups like untreated control (1), water treated control (3) and the treated (12), each with 100 individuals. Four feedings were followed (6 am, 11 am, 4 pm and 10 pm). 100 grams of fresh mulberry leaves were used to feed the group of 100 larvae, for each feeding. The *herbal formulation:* Stevia inulin powder was procured from local medical store and utilized for treating the mulberry leaves. Known quantity of herbal powder was mixed in known volume of distilled water to prepare aqueous solution of desired strength. The stock solutions of herbal powder were of strength: 05 ppm; 10 ppm; 20 ppm and 50 ppm. The stock solutions of herbal drug were prepared freshly before the feeding. 400 ml of aqueous solution of herbal powder was used to soak 100 grams of fresh mulberry leaves. The soaking was carried out for half an hour before feeding. The soaked/treated mulberry leaves were drained off completely and then fed to the fifth instar larvae of silkworm, *Bombyxmori* (L). Hundred grams of mulberry leaves were utilized for feeding each time, for the group of hundred larvae. The untreated control group of larvae were supplied with untreated leaves of mulberry. Water treated group of larvae were supplied with water treated leaves of mulberry. For each concentration (ppm) of herbal powder, three groups of larvae were made, out of which the first group was fed with treated leaves of mulberry for the first three days; second group for second day and third day. Third group of larvae for each concentration was supplied with treated leaves for only third day (Table 1). For remaining days, the larvae were fed with untreated leaves of mulberry. The bioassay of activity of mid gut protease and amylase was carried out on fifth day of fifth instar. Twenty larvae from each group were selected randomly; anaesthetized with chloroform soaked cotton pads and dissected for mid gut in chilled saline (0.9% NaCl). The larvae were opened from dorsal side; the entire alimentary canal was removed from each larva; flushed with ice cold saline so as to remove the debris of mulberry leaf and washed with ice cold saline. The mid gut from alimentary canal was separated; washed with saline; blotted and weighed accurately on electronic balance. The mid gut tissue was fragmented and then homogenized in chilled saline. Homogenate was centrifuged at 40 °C for 15 min at 10000 rpm. The supernatant was equalized to the volume,

Table 1: Schedule of Treating the Mulberry Leaves with Aqueous Solution of Stevia Inulin Powder Herbal Formulation and Feeding to the Fifth Instar Larvae of Silkworm, Bombyxmori (L) (Race: PM x $CSR_2$)

| Group | Day for Feeding Concentration of Stevia Inulin (ppm) | 1 | 2 | 3 |
|---|---|---|---|---|
| 0-0 | Untreated control | - | - | - |
| 0-I | Water treated control | □ | □ | □ |
| 0-II | Water treated control | - | □ | □ |
| 0-III | Water treated control | - | - | □ |
| A-1 | 5 ppm | + | + | + |
| A-2 | 5 ppm | - | + | + |
| A-3 | 5 ppm | - | - | + |
| B-1 | 10 ppm | + | + | + |
| B-2 | 10 ppm | - | + | + |
| B-3 | 10 ppm | - | - | + |
| C-1 | 20 ppm | + | + | + |
| C-2 | 20 ppm | - | + | + |
| C-3 | 20 ppm | - | - | + |
| D-1 | 50 ppm | + | + | + |
| D-2 | 50 ppm | - | + | + |
| D-3 | 50 ppm | - | - | + |

Note: - = Untreated mulberry leaves; □ = Water treated mulberry leaves; + = Steviol inulin powder treated mulberry leaves.

aliquots of which contain 10 mg per ml and used as assay sample. Half the volume of assay sample was utilized for bioassay of soluble proteins and another half for mid gut enzymes (protease and amylase).

Bioassay of soluble proteins was carried out through the methods of Lowery *et al*. (1951). For each assay sample (of each group), bioassay was carried in the triplicate set. One ml of assay sample was added in each test tube. The blank test tube was also prepared simultaneously, in which the assay sample was replaced with distilled water. Addition of 5 ml Lowery's "C" solution was made in each test tube, mixed well and kept for 15 minutes for the purpose to form the copper-protein complex. After fifteen minutes; 0.5 ml Folin's phenol reagent was added in each test tube and mixed well. The content in each test tube was allowed to develop colour. Then the optical density of content of each test tube was recorded at 660 nm on spectrophotometer. The concentration of soluble proteins of each assay sample was calculated through the reference of optical density assay sample and standard proteins (BSA) (the plot of optical density against conc. of Bovine Serum Albumen). The experimentations were repeated for thrice to obtain consistent results.

The content of soluble proteins in each assay sample was expressed in the unit as microgram protein per mg tissue.

For the purpose to determine total protein contents of tissue, another set of twenty larvae was selected randomly from each group. They were anaesthetized with chloroform soaked cotton pads and dissected for mid gut tissue.

The mid gut tissue was homogenized in chilled distilled water by using clean and sterilized morter and pestle in one normal (1.0 N) solution of sodium hydroxide and kept at 37 °C for 24 hours. Then it was precipitated with equal volume of ten percent solution of TCA and centrifuged at 10000 rpm for 10 minutes. The precipitate was dissolved in 1.0 NaOH and used as assay sample for total proteins. Further methods of determination of contents of total proteins are similar as described for soluble proteins.

The activity of mid gut protease was carried out according to the method of Brik *et al*. (1962)

with modifications suggested by Isshaya *et al*. (1971), outlined by Chougale (1992) and Khyade (2004). The mid gut protease activity was determined in triplicate setalong with the blank. The mixture of incubation consisted of substrate (one ml of ten percent caein solution); source of enzyme (0.5 ml assay sample) and 0.5 ml of 0.2 M Trisbuffer (pH = 8.4). For the blank, assay sample was replaced by distilled water. The incubation was carried out in water bath at 30 °C for 20 minutes with constant shaking. Addition of 6 ml of 2% trichloroacitic acid was made. The content was centrifuged at 8000 rpm for 15 minutes. The supernatant was used to read the optical density at 280 nm on spectrophotometer. Amount of tyrosine liberated from the casein due to action of mid gut protease was calculated through the use of optical density readings for assay sample; tyrosine (from standard graph) and predetermined soluble protein contents of each assay sample. The activity of mid gut protease was expressed in terms of specific activity: microgram tyrosine liberated per mg protein per minute.

The activity of mid gut amylase was determined according to the methods of Bernfeld (1955); explained by Ishaaya and Swirski (1970), with modifications suggested by Gaikwad (1998), outlined by Khyade (2004) and Desai *et al*. (2011).

For the purpose to determine the activity of mid gut amylase, 20 larvae were selected randomly and processed for assay sample preparation as described for soluble proteins. Mid gut amylase was determined in triplicate set along with blank. The incubation mixture consisted of one ml of one percent starch solution (as substrate), phosphate buffer (pH = 9.2) and 0.5 ml of assay sample. For the blank, assay sample was replaced by distilled water. The process of incubation was carried out in water bath at 30 °C for 20 minutes. After incubation the termination of activity of enzyme was made by addition of 2 ml DNSA and 2 ml distilled water. The contents were heated in boiling water bath exactly for five minutes, cooled immediately and the optical density of content was read at 540 nm on spectrophotometer.

For the purpose to calculate the mid gut amylase activity; the optical density readings for each assay sample; standard solution of maltase (from graph) and soluble proteins were utilized. The enzyme activity was expressed in specific activity: micrograms of maltose liberated per mg protein per minute.

The experimentations were repeated for thrice for the purpose to obtain consistency in the results. The collected data was subjected for statistical analysis (mean, standard deviation, percent change and significance through student t-test) by the methods of Norman and Baily (1955).

## RESULTS AND DISCUSSION

The results on the biochemical response of the mid gut tissue in the fifth instar larvae of polyvoltine, crossbreed, silkworm, *Bombyxmori* (L) to the Stevia inulin powder herbal formulation treated leaves of mulberry *Morus alba* (L) (M-5: variety) are summarized in Table 2.

Treating the mulberry leaves with various concentrations of Stevia inulin powder herbal formulation and feeding them to the fifth instar larvae of silkworm, *Bombyxmori* (L) for first three days; second and third days and for third day (only) was found variously reflected in the levels of contents of proteins (soluble and total) and activity of enzymes (protease and amylase) in the mid gut tissue homogenate. The soluble

Table 2: Contents of Proteins and Activity of Enzymes in the Mid Gut Tissue of the Fifth Instar Larvae of Silkworm, Bombyxmori (L) (Race: PM x $CSR_2$) Fed with the Aqueous Solution of Stevia Inulin Powder Herbal Formulation Treated Leaves of Mulberry, Morus alba (L) (M-5: Variety)

| S. No. | Moiety | Soluble Proteins | Total Proteins | Protease Activity | Amylase Activity |
|---|---|---|---|---|---|
| Group | | | | | |
| 1 | Untreated control (0-0) | 138.83 | 579.43 | 1.786 | 3.817 |
| | | (± 49.851) | (± 126.51) | (± 0.154) | (± 0.229) |
| 2 | Water treated control (0-I) | 134.11 | 566.14 | 1.797 | 3.839 |
| | | (± 61.486) | (± 159.38) | (± 0.468) | (± 0.897) |
| 3 | Water treated control (0-II) | 136.07 | 567.89 | 1.786 | 3.839 |
| | | (± 55.668) | (± 143.23) | (± 0.521) | (± 0.897) |
| 4 | Water treated control (0-III) | 136.69 | 571.51 | 1.791 | 3.851 |
| | | (± 52.579) | (+ 139.28) | (± 0.815) | (± 0.914) |
| 5 | A-1 | 183.46** | 612.21* | 2.169*** | 4.372* |
| | | (± 52.409) | (± 166.35) | (± 0.043) | (± 0.631) |
| | | 32.147 | 5.657 | 21.444 | 14.54 |
| 6 | A-2 | 185.29** | 619.16* | 2.236** | 4.465* |
| | | (± 59.931) | (± 143.78) | (± 0.147) | (± 0.871) |
| | | 33.465 | 6.856 | 25.195 | 16.976 |
| 7 | A-3 | 185.47** | 621.09* | 2.274** | 4.478* |
| | | (± 59.126) | (± 151.71) | (± 0.263) | (± 0.889) |
| | | 33.595 | 7.185 | 27.323 | 17.317 |
| 8 | B-1 | 219.78** | 683.27* | 2.313*** | 4.559* |
| | | (± 98.213) | (± 298.68) | (± 0.279) | (± 0.914) |
| | | 98.306 | 17.921 | 29.507 | 19.439 |
| 9 | B-2 | 238.01** | 758.58* | 2.889*** | 4.842* |
| | | (± 77.324) | (± 121.29) | (± 0.348) | (± 0.929) |
| | | 71.439 | 30.918 | 61.758 | 26.853 |
| 10 | B-3 | 241.79** | 758.61** | 2.989*** | 4.913** |
| | | (± 68.763) | (± 189.73) | (± 0.348) | (± 0.782) |
| | | 74.162 | 30.923 | 67.357 | 28.713 |
| 11 | C-1 | 242.47** | 789.43** | 3.026*** | 5.483** |
| | | (± 76.296) | (± 134.18) | (± 0.312) | (± 0.859) |
| | | 74.652 | 36.242 | 69.428 | 43.646 |
| 12 | C-2 | 242.51** | 789.59 | 3.083*** | 5.489** |
| | | (±79.012) | (±247.26) | (±0.617) | (±1.012) |
| | | 74.681 | 36.27 | 72.62 | 43.804 |
| 13 | C-3 | 242.57** | 789.63** | 3.087*** | 5.588** |
| | | (± 88.136) | (± 313.13) | (± 0.983) | (± 1.132) |
| | | 74.724 | 36.277 | 72.844 | 46.397 |

Note: Each figure is the mean & three replications. Figure in parenthesis with ± sign is the standard deviation. Figure below parenthesis is percent change. * : p<0.05; ** : p<0.01; *** : p<0.001.

proteins of untreated control (0-0) and water treated (for first three days) (0-I) control were found measured 138.83 (±49.851) and 134.11 (±61.486) units respectively. The water treated control groups for second day and third day (0-II) and for only third day were exhibited 136.07 (±55.668) and 136.69 (±52.579) units of soluble proteins in their mid gut homogenate. The contents of total proteins in the mid gut tissue of fifth instar larvae fed with untreated mulberry leaves were measured 579.43 (±126.51) units. The group of larvae fed with water treated mulberry leaves for first three days; second day and third day, only for third day were found 566.14 (±159.38); 567.89 (±143.19) and 571.51 (±139.28) units of total proteins respectively in their mid gut tissue homogenate. Treating the mulberry leaves with water may affect either digestibility in the mid gut lumen or absorption of digested matter by epithelial surface of mid gut in fifth instar larvae of silkworm, *Bombyxmori* (L) (Vitthalrao Khyade and Jyoti Kulkarni, 2011; and Desai *et al*., 2011). To allow the water treated mulberry leaves for complete draining or shade drying may help for the larval efficiency.

The contents of soluble and total proteins in the mid gut tissue homogenate of larvae fed with 5 ppm steviol herbal formulation treated mulberry leaves for first three days; second day and third day and third day (only) were found increased by 32.147 to 33.595 and 5.657 to 7.185% respectively. The mid gut protease activity and

amylase activity in these groups were found elevated from 21.444 to 27.323 and from 14.54 to 17.317% respectively. Increase in the concentration of herbal drug for treating mulberry leaves and feeding as per the schedule was found reflected into significant improvement in the levels of proteins (soluble and total) and the activity of enzymes in the mid gut tissue homogenate. Soluble proteins seems to increase significantly in their level (up to 90%) and total proteins (up to 39%).

The mid gut protease activity in all the groups of herbal drug treatment was found increased significantly. The levels of significance for the improvement in the mid gut amylase activity were found similar for the groups: 5, 6, 7, 8 and 9. The mid gut amylase activity in the groups: 10-16 was found with higher level of significance.

Increase in the levels of proteins (S.P. and T.P.) in the fifth instar larvae of silkworm, *Bombyxmori* (L) fed with mulberry leaves treated with various concentrations of steviolherbal formulation may be explained away as due to enhanced break down of contents of mulberry leaves through the elevated action of mid gut protease and amylase. The glycoside terpenoids through mulberry leaves are known to increase the capability of consumption and utilization of food by insects like silkworm. In phytophagous insects, the exogeneous compounds through herbal feed mimic the action of natural juvenile hormone, which enhance the synthesis of poly (A) RNA for major silk protein (Sen, 1988). Most significant response for herbal drug treatment in the study seems to be the levels of soluble proteins and activities of mid gut protease and mid gut amylase. The soluble proteins contribute in the tissue metabolism through enzymes. According to Applebaum (1985), continuous feeding in insects get reflect into advancement of production of mid gut enzymes, which improve the enzyme efficiencies. Most significant improvement in the protease activity in the treated group of study may be concerned with contents of specific plants. Individual plant extractive treatment may screen out the plant responsible for improved protease activity. Likewise the amylase enhancing herbal constituents of drug should be screened.

The final larval instars of lepidopteran insects have four phases of growth which include: preparatory (first two days); Accumulation phase (third and fifth days); Regression phase (sixth day) and Degeneration phase (day of spinning). The initial preparatory phase is characterized by high rate of DNA synthesis, high rate of digestion, moderate RNA synthesis and low protein synthesis. This phase seems to be juvenile hormone dependent. Accumulation phase, regression phase and degeneration phase are concerned mainly with silk glands. Improvement in the levels of mid gut proteins (S.P. and T.P.); efficiency of mid gut protease and amylase in the group of larvae fed with mulberry leaves treated with steviol herbal formulation in the present study seems to be affecting the growth phases of larva. Treating the mulberry leaves with herbal drug: khogo and feeding them to the fifth instar larvae of silkworm, *Bombyxmori* (L) for first three days, seems to be significant in comparison with others (2). Feeding treated leaves for first three days possibly availing the herbal nutrients, which affect digestibility of larvae and may contribute phyto-juvenoids or other compounds of growth and development. The study should be extended for screening juvenoid activity of herbal drug. Treating the mulberry leaves with herbal drug and feeding the fifth instar larvae of silk worm may be introduced in the schedule of rearing (Bridel and Lavieille, 1931; and Dolder *et al.*, 1960).

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