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Original Article

Studies on the changes in the activities of digestive enzymes in the midgut of silkworm *Bombyx mori.(L).(Lepidoptera: Bombycidae)* fed with mulberry leaves supplemented with Indian bean *(Dolichos lablab)*

Saravanan Manjula[a], Selvi Sabhanayakam [a], Veeranarayanan Mathivanan [a], Nadanam Saravanan [b]*.

[a]*Department of Zoology, Faculty of Science, Annamalai University, Annamalainagar - 608 002,Tamilnadu, India*
[b]*Division of Biochemistry, Rani Meyyammai College of Nursing, Faculty of Medicine, Annamalai University, Annamalainagar - 608 002, Tamilnadu, India.*

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ABSTRACT

To evaluate the changes in the activities of digestive enzymes activities of silkworm supplemented with Indian bean *(Dolichos lablab)*. Finely powdered *Dolichos lablab* was dissolved in distilled water and diluted to 2.5 %, 5 %, 7.5 % and 10 % concentrations. Fresh mulberry leaves *(Morus alba L.)* were sprayed by each concentration and were fed to silkworms, from 3rd to 5th instar, five feedings/day. Group 1 larvae received mulberry leaves sprayed with distilled water and served as control, group 2 larvae received 2.5% *Dolichos lablab* sprayed mulberry leaves, group 3 larvae received 5 % *Dolichos lablab* sprayed mulberry leaves, group 4 larvae received 7.5 % *Dolichos lablab* sprayed mulberry leaves and group 5 larvae received 10 % *Dolichos lablab* sprayed mulberry leaves. Silkworm larvae fed on *Morus alba. L.* (mulberry) leaves enriched with 7.5 % concentrations of *Dolichos lablab,* significantly gained more pupa weight, silk length and silk weight as compared to those fed on normal MR2 mulberry leaves. Hence, 7.5% dose was fixed as an effective dose. Further, same study was conducted to find out the changes in the digestive enzymes activities in the midgut occurred in the fourth day of IVth instar larvae. There was a significant increase in the midgut urease, amylase, sucrase and protease activities. But midgut trehalase activity was significantly decreased. The results suggest that coadministration of *Dolichos lablab* with mulberry leaves at a concentration of 7.5% has enhanced the digestion of ingested food which in turn reflects in the quantity of silk produced.

1. Introduction

The nutrition, particularly as it relates to the physiology of digestion, is the most fundamental and important challenges in the sericulture. Effective culture cannot occur unless a species can be grown quickly and economically. The *Bombyx mori L* (silkworm) is a phytophagous lepidopteran insect that is monophagous feeder on *Morus alba.L* (mulberry leaves). According to Kellner, [1] the silkworm digests albumin, fat and carbohydrates except cellulose. The ability of silkworm to produce and secrete digestive enzymes is to a great extent influenced by the nutrient composition of the meal. Scientists have tried alternative food for the rearing of silkworm, but they were not cost effective. So they used some nutrients, minerals and vitamins as food supplements. Mulberry leaves have been supplemented with various nutrients for silkworm feeding to promote silk quality and quantity. Mahmood *et al.* [2] found that silkworm larvae, when fed on mulberry leaves treated with farm yard manure and ammonia solution significantly consumed more food, gained more larval weight and produced heavier cocoons as compared with those fed on untreated leaves. Ravikumar has [3] emphasized that the quality and the nutritional status of mulberry has a great influence on the silkworm growth, silk yield and disease resistance. Silkworm requires specific essential sugars, amino acids, proteins and vitamins for its normal growth [4]. Javed and Gondal [5] have also reported that silkworm fed with nitrogen and ascorbic acid supplemented mulberry leaves showed higher growth and lower mortality. Silkworm midgut digestive enzymes have been studied in detail by various scientists. [6-10]. Rationalization of some of these enzymes is a feature of the silkworm [11]. Midgut enzyme activity is also a

* Corresponding Author : Dr. N. Saravanan
Lecturer, Division of Biochemistry,
Rani Meyyammai College of Nursing, Faculty of Medicine,
Annamalai University, Annamalainagar - 608 002,
Tamilnadu, India. Telephone: (91) 4144-237225
Fax:(91) 4144-237225
E-mail: biosaravanan@gmail.com.

developmental stage dependent [12,13], and the diapause nature has relevance to enzymatic activities in the midgut of silkworm [14]. An understanding of the change in the digestive physiology when supplemented with the Indian bean *Dolichos lablab (D.lablab)* may help to maximize the commercial production of silkworm.

D. lablab is a leguminous plant, found in India, is a seasonal dicotyledonous legume. It is commonly called as Indian bean. For the fulfillment of need of dietary proteins, the population of the subtropics, being predominantly vegetarian, looks to legumes like *D. lablab* as it is having more protein content. It is also called as poor man's bean as it is cheap when compared to other beans. Extracts of *D. lablab* seeds were found to be having mitogenic properties [15]. Although the effects of nitrogen, vitamin and salts supplementation on the growth of silkworm have been investigated by many researchers, the effect of mulberry leaves enriched with *D. lablab* was not investigated. So, the present study was aimed to find out the effective dose of *D. lablab* application to mulberry leaves on pupa weight, silk length and silk weight. By using the effective dose, further analysis of the activities of the digestive enzymes were done in the midgut of fourth day of fouth instar larvae of silkworm and an ultimate aim to find out whether the change in activities of the enzymes have impact on the growth and silk production of silkworm.

2. Materials and methods

The eggs of silkworm L NB4, D2 (local Bivoltine) race were collected from farmers' training centre at Jayankodapattiam, Tamilnadu, India. The eggs were placed at ambient temperature of 25±2^0 C and relative humidity of 70 to 80 % in an incubator for hatching. After hatching, larvae were isolated from stock culture. The larvae were divided into 5 experimental groups including controls (distilled water control), each group consisting of 10 larvae. The larvae were reared in card board boxes measuring 22 X 15X 5 cm3 covered with polythene sheet and placed in an iron stand with ant wells. The larvae were subjected to the following treatments. *D. lablab* was purchased from the local market surrounding Chidambaram, Tamil Nadu, India, identified and authenticated from the Department of Botany, Annamalai University. It was shade dried and powdered using mortar. Finely powdered *D. lablab* was dissolved in distilled water and diluted to 2.5 %, 5 %, 7.5 % and 10 % concentrations. Fresh mulberry leaves were sprayed by each concentration and then dried in air for 10 min. The supplementary leaves were fed to silkworms, five feedings/day. Group 1 larvae received mulberry leaves sprayed with distilled water and served as control, group 2 larvae received 2.5% *D. lablab* sprayed mulberry leaves, group 3 larvae received 5 % *D. lablab* sprayed mulberry leaves, group 4 larvae received 7.5 % *D. lablab* sprayed mulberry leaves and group 5 larvae received 10 % *D. lablab* sprayed mulberry leaves, respectively. And they were maintained up to cocoon. Pupa weight, silk length and silk weight were determined for all groups.

The same protocol was repeated only with 7.5% *D. lablab* sprayed mulberry leaves and control larvae received mulberry leaves sprayed with distilled water for the estimation of digestive enzymes. On fourth day of fourth instar, the larval midgets were isolated and homogeised.

2.1. Preparation of tissue extracts for enzyme assays

The whole midgut was isolated from prefrozen larvae kept at -20°C for 12 h and a 10% homogenate was prepared (after separating the Malpighian tubules, fat bodies, and other tissue fragments adhering to the gut) in ice-cold buffer solution. The homogenate was centrifuged at 3000 rpm and the supernatant was used as the enzyme source with appropriate dilution.

2.2. Assay of enzyme activities

Assay of urease was done by the following procedure. 0.5 ml of enzyme solution was incubated with the assay buffer consisted of 0.1 M KH_2PO_4 (pH 7.5) containing 120 mM urea, 5 mM EDTA, 0.1% (v/v) 2-mercaptoethanol and 0.5% (w/v) ascorbic acid for 3 h at 30°C. After incubation, the reaction was terminated by adding 1/24 volume of 1 N H_2SO_4. Ammonia released from urea was assayed by Nessler's method. One unit of the enzyme was defined as the amount that hydrolyzed 1 µmol of urea per min under the assay condition. Amylase activity was evaluated by the Bernfeld [16] method using glucose as standard. The reaction mixture contained 50 mM phosphate buffer (pH 6.5), 1% starch (freshly prepared), and appropriately diluted enzyme. Sucrase activity was measured according to the method of Ishaaya and Swirski [17] with glucose as standard. The incubation mixture contained 50 mM phosphate buffer (pH 6.5), 3.42 M sucrose, and appropriately diluted enzyme. Trehalase activity was determined by the Dahlman [18] method with slight modification of pH from 5.6 to 6.0. The assay mixture contained 50 mM phosphate buffer (pH 6.0), 3.78M trehalose, and appropriately diluted enzyme. The incubation period and temperature of incubation were 30 min and 24 ± 1°C for amylase, and 60 min and 37°C for sucrase and trehalase. The amount of glucose liberated was measured at 540 nm after inhibition of the reaction with dinitrosalicylic acid (DNS) reagent in the cases of amylase and sucrase and with concentrated H_2SO_4 in the trehalase assay. The mixture was boiled over a boiling water bath for 10 min and diluted with distilled water. Activity is expressed as milligrams of glucose liberated per minute per milligram of protein in all three estimations. The protease enzyme assay was carried out with the method of Eguchi and Iwamoto [19] with slight modification of the pH of borate buffer (pH 11.0) as outlined by Sarangi [20] using tyrosine as standard. The reaction mixture contained 1% casein, 0.1M borate buffer (pH 11.0), and appropriately diluted enzyme. The incubation was carried out for 30 min at 30°C. The reaction was inhibited by adding 8.2M trichloroacetic acid (TCA) and centrifuged. The supernatant was used with 0.5 N NaOH and Folin's reagent to measure the tyrosine liberated at 660 nm. Protein content in all assays was estimated with the Folin phenol reagent [21] using bovine serum albumin as standard.

2.3. Satistical analysis

Data were analyzed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using a commercially available statistics software package (SPSS® for Windows, V. 16.0, Chicago, USA). Results were presented as means ±SD. p values < 0.05 were regarded as statistically significant.

3. Results and discussion

Table 1 shows the effects of various concentrations of *D. lablab* supplementation with mulberry on the pupa weight, silk length and silk weight. There is a significant raise in the pupa weight, silk length and silk weight of the larvae fed with *D. lablab* supplemented mulberry when compared with control groups. This may be due to the increased protein content of the mulberry supplemented with *D. lablab.* This is in agreement with the work done by Hiware [22] regarding the increased pupa weight, silk length and silk weight when silkworm treated with homeopathic drug *Nux vomica*. There is a significant increase in the all parameters while there is no significance between the 7.5% and 10% dose of *D. lablab* with respect to pupa weight, silk length and silk weight. So, 7.5% was fixed as the effective dose.

Table 1. Effects of various concentrations of *Dolichos lablab* supplementation with mulberry leaves on the pupa weight, silk length and silk weight of *Bombyx mori*.

Group	Pupa weight (grams)	Silk length (meters)	Silk weight (grams)
Control	1.55 ± 0.03[a]	717.48 ± 13.53[a]	0.209 ± 0.004[a]
Mulburry + 2.5% *Dolichos lablab*	1.63 ± 0.04[b]	724.26 ± 16.78[a]	0.229 ± 0.005[b]
Mulburry + 5% *Dolichos lablab*	1.92 ± 0.01[c]	768.99 ± 5.39[b]	0.241 ± 0.002[c]
Mulburry + 7.5% *Dolichos lablab*	2.09 ± 0.02[d]	794.44 ± 8.75[c]	0.260 ± 0.003[d]
Mulburry + 10% *Dolichos lablab*	2.11 ± 0.05[d]	800.10 ± 20.25[c]	0.262 ± 0.007[d]

Values are means ± s.d. for 10 larvae in each group. [a-d] Values not sharing a common superscript letter within each column differ significantly at P < 0.05 (DMRT).

Table 2. Effects of 7.5% concentrations of *Dolichos lablab* supplementation with mulberry leaves on the midgut urease, amylase and sucrase activities of silkworm *Bombyx mori.*

Group	Urease (mU/g)	Amylase (mg glucose/ min/mg protein X 10^{-3})	Sucrase (mg glucose /min/mg protein X 10^{-3})
Control	16.14 ± 0.80[a]	10.69 ± 0.58[a]	11.91 ± 0.68[a]
Mulburry + 7.5% *Dolichos lablab*	18.62 ± 0.99[b]	11.27 ± 0.80[b]	13.39 ± 0.72[b]

U- amount that hydrolyses 1 µ mol of urea /min at 30°C Values are means ± s.d. for 10 larvae in each group. [a, b]Values not sharing a common superscript letter within each column differ significantly at P < 0.05.

Table 3. Effects of 7.5% concentrations of *Dolichos lablab* supplementation with mulberry leaves on the midgut protease and trehalase activities of silkworm *Bombyx mori.*

Group	Protease (µ mol tyrosine/min/ mg protein X 10^{-2})	Trehalase (mg glucose/min/ mg protein X 10^{-2})
Control	33.29 ± 1.58[a]	12.91 ± 0.64[a]
Mulburry + 7.5% *Dolichos lablab*	37.26 ± 2.0[b]	10.77 ± 0.58[b]

Values are means ± s.d. for 10 larvae in each group. [a, b]Values not sharing a common superscript letter within each column differ significantly at P < 0.05.

Table 2 and 3 show the effects of 7.5% D. lablab supplementation with mulberry on the midgut urease, amylase, sucrase, protease and trehalase activities of silkworm. There is a significant increase in the midgut urease, amylase, sucrase, protease in the *D. lablab* supplemented group when compared with control group supplemented with distilled water.

Ammonia produced from urea by the action of mulberry leaf urease is assimilated into amino acids via the glutamine synthetase/glutamate synthase pathway in the same way as plants and micro organisms [23]. Rosenthal et al. [24] also explained about the utilization of urea in insects. Larvae of the bruchid beetle *Caryedes brasiliensis* feeds on a neotropical legume *Dioclea megacarpa*. It possess high urease activity, and are capable of utilizing urea generated from canavanine, a toxic amino acid stored in the seeds of the host plant. However, the origin of the urease activity detected in the insect has not been clarified. The beetle's urease might originate from the legume seeds rather than from the insect itself as observed in the silkworm. Generally, legume seeds have high urease activity [25]. So in the present study the *D.lablab*, the leguminous plant, may also have more urease activity. Urea utilization as a nitrogen source for protein synthesis has been well studied in mammals [26-30] and chicks [31]. It has been shown that intestinal flora was indispensable for utilizing urea nitrogen for protein synthesis [32, 33]. The urea recycling system found in the silkworm more or less resembles that of mammals, but it is noteworthy that silkworm utilizes an enzyme of the host plant for the insect and that urea metabolism in silkworm is completely dependent on the diets that the insect is given. Mulberry leaf urease and the supplemented *D.lablab* urease may make a significant contribution to silk production in the silkworm by converting useless urea into ammonia available as a nitrogen source of silk-protein. As the pupa weight, silk length and silk weight are significantly increased upon supplementation of *D.lablab*, it is in agreement with Hirayama [34] who found that the silk production of the larvae reared on mulberry leaves was larger than that of the larvae reared on the artificial diet.

Poor nutrition and low-nutrient diets have direct effects on primary biochemical and physiological systems, and thus may decrease the performance of insects by effecting changes in the detoxification system that can alter the susceptibility of the insect [35], the poor feeding behavior may be correlated with the alteration in digestive enzyme activity on insecticide treatment [36]. In the present study activity of the enzymes amylase, sucrase, and protease were increased, which may be due to the sufficient amount of substrate resulting from high food intake. Sumida *et al.* [13] have reported that midgut sucrase is activated by sucrose at a higher concentration (<100 mM) derived from the ingested food in the midgut lumen. The rational food consumption by a lepidotpteran larva was correlated directly with the activities of amylase and invertase by Christopher and Mathavan [37], with the larva receiving 100% food found to have the highest amylase and invertase activites, which declined as the percentage of food offered was reduced. Similarly, the heavier pupa weight on *D.lablab* supplementation could have resulted in the increased activity of amylase and sucrase.

Digestion of leaf proteins is aided by the proteolytic enzymes, proteases. Late silkworms are generally eat coarse leafs, and are suppose to have a highly specific protease enzyme system that hydrolyzes the fibrous protein found in abundance in coarse mulberry leaves [38]. The proteolytic activity of the alimentary canal in relation to feeding of proteins has been studied in many insects [39, 40]. In the present study, protease activity has been increased on *D.lablab* supplementation and it is presumed that the bean may activate the enzyme molecules to act on their substrates, or the enzyme molecules may be have sufficient amount of substrate. Protease activity is influenced by the age, sex and feeding behaviour of silkworms and decreases significantly on starvation during late fifth instar [41]. The observations of the present investigation can thus be correlated with increased feeding behavior and increased quantity of food ingested by the silkworm for the active participation of these enzymes in the process of digestion, which in turn reflects in the high pupa weight, silk length and silk weight.

Trehalase activity to the contrary, was inhibited in the midgut of silkworms supplemented with *D.lablab*. Azuma and Yamashita [42] reported an increase in midgut trehalase activity serves for the utilization of hemolymph trehalose for metabolic energy to maintain active processes in various situations like starvation. The decreased trehalase activity may also be due to decreased hydrolysis of

trehalose to release glucose molecules in drastic conditions and in high energy demand [43]. The energy demand, which might have been supplied in the form of glucose molecules by the hydrolysis of trehalose in the midgut by trehalase [44]. In the present study as the trehalase activity was significantly decreased it is presumed that there is no such disturbance in the carbohydrate metabolism and also no drastic situations for the silkworm.

4. Conclusion

So it is concluded that the supplementation of *D.lablab* at the level of 7.5% may have beneficial effects on the growth of the silkworm and also increase the quantity of the silk production by enhancing the digestibility of the mulberry leaves. So this supplementation could be prescribed to the farmers to get more quantity of silk.

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