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Research Article

EFFECT OF FOOD ADDITIVES ON THE PROTEIN CONTENT AND MIDGUT ENZYME ACTIVITIES IN THE 5TH INSTAR LARVAE OF BOMBYX MORI (L)"

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ABSTRACT

Sericulture activities are agro-based and also industrial based. The Agro-based part involves mulberry cultivation and silkworm rearing. India is the second largest producer of raw silk after China. Fortification of mulberry leaves with supplementary nutrient (Muniandy *et al*., 2001) and feeding silkworm is a useful modern technique to increase economic value of cocoon. Mulberry leaf quality is one of the major factor which have significant role in silkworm development. Keeping in view the above fact, an attempt was made by using V1 mulberry variety and food additives such as Corn, Soya, horse gram flour as supplementary nutrients. The purpose of this study is to know whether the food additives used in the present study have any effect on the total protein content of the silk gland and the activity of midgut enzymes such as Amylase and Protease. The present investigation clearly depicts that foliar application of horse gram flour as well as corn flour, increased protein content in the silk gland and protease as well as amylase enzyme activity in the midgut of FC1XFC2 hybrid. Hence, it is concluded that the supplementation of horse gram flour at a concentration of 10g/100g of leaves for 100 larvae may have beneficial effect in increasing the quantity of the silk production by enhancing the digestibility of the mulberry leaves and subsequent increase in the protein content of silk glands.

INTRODUCTION

Sericulture activities are agro-based and also industrial based. The Agro-based part involves mulberry cultivation and silkworm rearing. Sericulture in India is an important cottage industry based on Agro forestry. India is the second largest producer of raw silk after China.

The silkworm, *Bombyx mori* is a monophagous insect, feeding exclusively on the leaves of mulberry *Morus alba* (L). It is therefore, essential to improve either food quality or appetite (or both) of larval instars of silk worm for better performance in silk production. Legay (1958) stated that silk production is dependent on the larval nutrition and nutritive value of mulberry leaves which plays a very effective role in producing good quality of cocoons.

Mulberry leaf quality is one of the major factor which have significant role in silkworm development, different types of mulberry plants are seen, among them V1 variety have their own significant role in silkworm growth. Few studies such as Bohidar *et al*. (2007) reported effect of different mulberry genotypes on the economic parameters of silkworm in Orissa climate and made suggestion for use of mulberry variety (V1, DD) for more silk production and it was observed that the larval protein content was highest in V1 varieties, least in local varieties (Ruth LalfePuii *et al*., 2014). Because of the above facts, in the present study V1 mulberry variety was used.

Supplementary nutrients or fortification agents when added to the normal diet enriched the nutritional value of the diet, making it more useful from the nutritional point of view (Bajpeyi *et al*., 1991). Keeping in view the above fact, an attempt was made by using V1 mulberry variety and food additives such as Corn, Soya and horse gram flour as supplementary nutrients.

Enzymes

Enzymes are large biological molecules responsible for the thousands of biochemical interconversion that sustain life. Without Enzymes, metabolism would neither progress through the same steps, nor be fast enough to serve the needs of the cell. Silkworm midgut digestive enzymes have been studied in

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detail by various scientists (Kanekatsu, 1972; Eguchi *et al*., 1976; Sumida *et al*., 1990).

Protease Enzyme

Among many digestive enzymes, protease enzyme in Silkworm plays a key role in converting the mulberry protein to silk protein. Eguchi *et al.* (1976) showed that the protease activity changed according to age, sex and feeding behaviour in midgut of fifth instar larvae. Protease are in higher rate during Silkworm larval stage (Kumari *et al.,* 1997).

Amylase Enzyme

Amylase is one of the key enzyme involved in digestion and carbohydrate metabolism in insects (Daone *et al*., 1975; Buonocore *et al*., 1976; Horie and Watanabe, 1980). Amylase activity was measured with DNSA procedure using soluble starch as substrate (Bernfeld, 1955; Baker, 1991).

In view of all the above, present study aims at investigating protein and enzyme activities in silkworm larvae fed with different food additives (corn, soya, horse gram flour).

MATERIALS AND METHODS

Test animal

In the present study FC1×FC2 bivoltine double hybrid silkworm were used as experimental animals in order to study the effect of food additives on the enzymatic and protein profile. Silkworm species as model animals because of their availability, cost which is much lower than with mice, low ethical problems and no biohazards.

Fig 1 FC1xFC2 double hybrid silkworm larvae

Collection and maintenance of test animal

The FC1×FC2 double hybrid silkworm bivoltine variety were collected from the farmers who are involved in sericulture activity in Kachinkatte village, Shivamogga thaluk. 5th instar larvae were collected soon after the fourth moult and grouped into four experimental groups and maintained separately in silkworm rearing house on the shelf made by wood wrapped with thread and covered with newspaper without any gap.

Larvae were fed with fresh mulberry leaves (V1) harvested from the garden and selected healthy leaves were chopped into suitable size and treated proper dosage of flour as food additives as shown in the Table 1.

Table 1 Treatment groups

Group	Concentration of mulberry leaves (gm)	Concentration of flour (gm)	days for feeding					
			1	2	3	4	5	6
Control	100	-	-	-	-	-	-	-
Corn flour	100	10	+	+	+	+	+	+
Soya bean flour	100	10	+	+	+	+	+	+
Horse gram flour	100	10	+	+	+	+	+	+

+:Fed with mulberry leaves and flour for 3 times/day
- :Fed with mulberry leaves only for 3times/day

Each group had 100 larvae. Group-1 were treated with 100g of mulberry leaves along with 10g corn flour for 3 times/day i.e. morning 7:30-8:30 AM, afternoon 2-3 PM and evening 6-7 PM. The treatment schedule was continued for about 6 days for every group with different flour, horse gram flour (Group-2), soya flour (Group-3) and Group-4 considered as control group.

Determination of enzyme activity and estimation of total protein content of silk gland was done using standard procedures.

Determination of enzyme activity

Preparation of Enzyme extract

The enzyme extract was prepared according to the method of Ishaaya and Swiriski (1970). In each group larvae were selected randomly and midgut part were dissected and cleaned it properly and weighed accurately in electronic balance (3gm). The midgut tissue was homogenized in a prechilled mortar and pestle along with 0.7% NaCl. The homogenate was centrifuged at 3000 rpm for 15 minutes. Supernatant was used as enzyme extract.

The reaction mixture consists of 0.5ml of enzyme extract, 1ml of substrate (starch for Amylase and Casein for Protease) and 1ml of buffer of various pH levels, reaction mixture was heated on water bath for 30min, after heating, the mixture was kept for incubation for about 30min and 2.5ml of DNSA reagent was added (The DNSA was prepared as suggested by Noelting and Benfeld (1948). The reaction was stopped and the mixture was heated for 5min on boiling water bath followed by immediate cooling in ice cold water, 2.5ml of distilled water was added to the same test tube, mixed thoroughly and reading was taken at 540nm on Spectrophotometer. The specific activity was expressed in terms of µg tyrosine/ml/g tissue for protease and mg maltose/ml/g tissue for amylase respectively. The pH was determined by appropriate buffers, 3-6 for acetate buffer, 7-11 for phosphate buffer, substrate concentration was determined in the range 0.5-2.5 each enzyme shows its own pH, Substrate concentration.

Estimation of protein in silk gland

Protein estimation was done by Lowry *et al.* (1951) method.

Statistical analysis

The experiment was repeated thrice to obtain consistency in the results. The collected data was subjected for statistical analysis by the methods of ANOVA (One-way ANOVA)

RESULTS

The effect of food additives on the silkworm physiology corresponding to the important digestive enzymes Amylase and Protease in the midgut and protein content in the silk gland were recorded as follows:

Total protein content in the silk gland (Table 2)

Fifth instar larval Protein content was analyzed. Larval protein content ranges from 0.1607-0.1913mg BSA/ml/mg tissue. Maximum Protein content observed in Horse gram flour and corn flour treated groups were 1.051 and 1.022 fold higher than Control group, whereas Soya flour shows 1.132 fold lower activity than that of Control group. The result of one way ANOVA revealed that the variation among the experimental groups are all found to be Significant at $p<0.001$ and $p<0.05$.

Table 2 Protein content in the Silk gland of 5th instar larvae of Silkworm *Bombyx mori* (L.) fed with the leaves of Mulberry, (V1 variety) treated with different food additives

		Treated		
Protein (mg BSA/ ml/g tissue)	**Control**	**Corn flour**	**Soya flour**	**Horse gram flour**
	0.1820±0.0001	0.1861±0.0003* (1.022↑)	0.1607±0.0002* (1.132↓)	0.1913±0.001* (1.051↑)

Each value represented as Mean± SEM, n=3
*P < 0.05.

Protease activity in the midgut of silkworm larvae at different pH (Table 3)

Among all the treatment groups Horse gram flour group (10gm/100gm of leaves) recorded the highest Protease activity compared with control group i.e., 1.303, 1.670, 2.135, 3.85, fold higher than control group at different pH 7, 8, 9, 10 respectively.

Table 3 Quantitative analysis of Protease enzyme in the midgut of Silkworm at different Ph

			Treated		
Enzyme	**pH**	**Control**	**Corn Flour**	**Soya Flour**	**Horse Flour**
Protease (µg tyrosine/ ml/g tissue)	7	18.93±0.26	18.81±0.19 (1.006↓)	13.99±0.13* (1.353↓)	24.67±0.11* (1.303↑)
	8	20.74±0.24	18.62±0.05* (1.113↓)	13.67±0.07* (1.517↓)	34.64±0.15* (1.670↑)
	9	16.70±0.077	16.70±0.097	16.41±0.149 (1.017↓)	35.67±0.077*** (2.135↑)
	10	8.173±0.09	15.30±0.149* (1.87↑)	16.52±0.07* (2.02↑)	31.52±0.04* (3.85↑)

Each value represented as Mean± SEM, n=3
*P < 0.05.

Protease activity in the midgut of silkworm larvae at different substrate (Table 4)

The highest enzyme activity was observed in Horse gram flour treated Silkworms group followed by Corn flour treated group. Horse gram flour treated group showed 1.315 fold higher than Control group at 1% Substrate concentration and in case of Corn flour treated group at 1.5% Substrate concentration showed 1.60 fold higher than control group. The result of one way ANOVA revealed that the variation among the experimental groups are all found to be significant at $p<0.001$ and $p<0.01$.

Table 4 Quantitative analysis of Protease enzyme in the midgut of Silkworm at different substrate concentration

			Treated		
Enzyme	**Substrate (%)**	**Control**	**Corn flour**	**Soya flour**	**Horse gram flour**
Protease(µg tyrosine/ml/g tissue)	0.5	18.94±0.07	26.42±0.12* (1.394↑)	12.25±0.12* (1.546↓)	22.95±0.10* (1.211↑)
	1	23.84±0.10	27.56±0.06* (1.156↑)	12.99±0.13* (1.835↓)	31.35±0.05* (1.315↑)
	1.5	22.37±0.08	35.98±0.044* (1.60↑)	12.41±0.056* (1.80↓)	23.22±0.15* (1.03↑)
	2	24.38±0.06	30.09±0.10* (1.23↑)	13.80±0.08* (1.76↓)	27.86±0.16* (1.14↑)
	2.5	24.56±0.13	38.26±0.38* (1.557↑)	12.14±0.13* (2.02↓)	46.04±3.77* (1.87↑)

Each value represented as Mean± SEM, n=3
*P < 0.05.

Amylase activity in the midgut of silkworm larvae at different pH (Table 5)

Amylase activity for the liberation of maltose at different pH in treated groups (Corn, Soya, Horse gram flour) showed decreasing activity of Amylase enzyme when compared with control group.

Table 5 Quantitative analysis of amylase enzyme in the midgut of Silkworm at different pH

			TREATED		
Enzyme	**pH**	**Control**	**Corn Flour**	**Soya Flour**	**Horse Flour**
Amylase (mg maltose/ ml/g tissue)	3	6.529±0.165	2.412±0.020* (2.70↓)	4.157±0.020* (1.57↓)	5.797±0.031* (1.12↓)
	4	8.510±0.036	2.868±0.020* (2.967↓)	5.865±0.047* (1450↓)	7.784±0.010* (1.093↓)
	5	8.745±0.1045	2.918±0.021* (2.996↓)	5.196±0.012* (1.683↓)	6.212±0.013* (1.407↓)
	6	9.260±0.111	2.718±0.009* (3.40↓)	5.850±0.014* (1.58↓)	5.529±0.014* (1.674↓)

Each value represented as Mean± SEM, n=3
*P < 0.05.

Amylase activity in the midgut of silkworm larvae at different substrate (Table 6)

The data showed that the highest Amylase activity was recorded on supplementation of Horse gram flour 10g/100g of leaves followed by Corn flour 10g/100g of leaves in respect to the different Substrate concentration. Amylase activity was higher in Horse gram flour treated group i.e., 1.982, 2.216, 1.369, 1.282, 1.832 folds higher than that of control group at different Substrate concentration of 0.5%, 1%, 1.5%, 2%, 2.5% and in case of Corn flour treated group was 1.579, 1.726, 1.687, 1.662, 1.301 folds higher than that of control group. The result of One-way ANOVA revealed that the variation among the experimental group were all found to be Significant at $p<0.001$.

Table 6 Quantitative analysis of amylase enzyme in the midgut of Silkworm at different substrate concentration

			Treated		
Enzyme	**Substrate (%)**	**Control**	**Corn flour**	**Soya flour**	**Horse gram flour**
Amylase (mg maltose/ ml/g tissue)	0.5	2.734±0.010	4.317±0.014* (1.579↑)	1.891±0.018* (1.44↓)	5.419±0.009* (1.982↑)
	1	2.624±0.012	4.531±0.010* (1.726↑)	1.622±0.012* (1.617↓)	5.816±0.018* (2.216↑)
	1.5	2.809±0.031	4.740±0.022* (1.687↑)	1.930±0.017* (1.455↓)	3.848±0.010* (1.369↑)
	2	3.520±0.009	5.852±0.020* (1.662↑)	2.964±0.012* (1.187↓)	4.513±0.040* (1.282↑)
	2.5	3.545±0.018	4.613±0.017* (1.301↑)	4.570±0.039* (1.289↑)	6.496±0.017* (1.832↑)

DISCUSSION

Protein

The quantitative estimation of total protein in silk gland of bivoltine hybrid showed significant increase in horse gram flour treated groups followed by corn flour treated group. In the present study, the higher protein content in the silk gland of the silkworm is due to the supplementation of horse gram flour and corn flour enriched leaves to silkworm, this clearly indicate the influence of dietary protein on increasing protein content in silkworm during fifth instar which is considered as prime feeding stage of the silkworm larvae wherein about 80-85% of the total leaves are consumed.

Krishna Swamy *et al*. (1978) observed that the increase in the protein concentration in the silkworm body after the fourth moult is due to the regular feeding activity. Sodani *et al.* (2004) observed that horse gram is an inexpensive source of protein. Our data also revealed that major protein activity was observed in horse gram flour treated group.

Enzymes

The enzyme system in the silkworm plays a vital role in determining the performance of the larvae in terms of effective transformation of organic food molecules of the leaf into useful biomolecules. The consumption of mulberry leaf during final instar accounts for more than 80% of the total consumption during its larval life. Food consumed in this stage is effectively utilized for the production of silk proteins as well as to support its metabolism (Lokesh *et al.,* 2006).

Protease enzyme

Midgut protease enzyme is one of the important enzyme that helps in converting the mulberry protein to silk protein in *Bombyx mori* (L.) protease are important digestive enzyme which is synthesized in higher rate during silkworm larval stage (Kumari *et al.,* 1997) highly responsible for dietary protein metabolism (Abudabos, 2012) in the digestive system of the silkworms.

Present study revealed that protease activity in midgut was optimum at pH 10 and substrate concentration 2.5% in FC1XFC2 hybrid. According to Eguchi *et al.,* (1972) the optimum pH of larval protease is 11, while in adult about 9. The protease activity changed according to age, sex and feeding in midgut of fifth instar.

Amylase

Amylase enzyme activity in silkworm larvae (FC1XFC2) was increased when treated with optimum concentration of horse gram flour followed by corn flour at optimum substrate concentration 2% and 2.5% respectively. These findings are confirmative with Ishaya *et al.,* (1971). Their findings indicate the larvae of *Spodoptera littoralis* when fed on leaves with additional factor acts as a stimulant for digestive enzymes probably through hormonal mechanism. From the above, it may be inferred that the decrease in protein and enzyme activities in soya flour treated silkworm group may be due to the insufficient consumption of soya flour treated mulberry leaves.

The present investigation clearly depicts that foliar application of horse gram flour as well as corn flour, increased protein content in the silk gland and protease as well as amylase enzyme activity in the midgut of FC1XFC2 hybrid. Among these groups horse gram flour treated group has given better results indicating its involvement in the expression of physiological activities when compared with corn flour and soya flour treated group. Hence, it is concluded that the supplementation of horse gram flour at a concentration of 10g/100g of leaves for 100 larvae may have beneficial effect in increasing the quantity of the silk production by enhancing the digestibility of the mulberry leaves and subsequent increase in the protein content of silk glands.

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