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RESEARCH ARTICLE

Estimation of protein concentration in different tissues of popular silkworm (*Bombyx mori* L.) races

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Abstract

Three silkworm races viz., pure Mysore (PM), bivoltine (CSR_2) and crossbreed ($PMxCSR_2$) were analyzed for their protein concentrations. Quantitative protein estimation was carried out in different body tissues of silkworms. Data obtained are statistically analyzed with one way ANOVA at $p<0.05$ significance level and presented in the result section. Results revealed that, significantly higher (at $p<0.05$) proteins were recorded in bivoltines compared to crossbreeds and multivoltine silkworms. Bivoltine silkworms were comparatively superior in protein concentrations tested in different body tissues and the experiment can be adopted for screening and characterization of different silkworm breeds.

INTRODUCTION

Generally silkworm *Bombyx mori* L. is monophagous in character and feeds solely on mulberry leaves. Silkworm larva obtains different amino acids from the mulberry leaves and uses to synthesize silk proteins secreted during spinning. Proteins play an important physiological role in growth and development of silkworm and silk proteins synthesis. Necessary amino acids are derived from the amino acids present in body fluid in a free state and in the posterior silk gland cells. Silk worm requires all the ten essential amino acids for growth and development (Ito, 1978). Silk is made up of two proteins such as fibroin and sericin. Fibroin forms the core and is surrounded by sericin. These two proteins differ in their characteristics and secreted from different parts of silk gland. Fibroin is secreted from the posterior part and sericin is secreted from middle part of silk gland. Fibroin is formed from the amino acids of posterior silk gland cells. Sericin quality is one of the important features of cocoon. Sericin is classified in various ways, but generally as α-sericin and β-sericin. α-sericin being presents in the inner layer of cocoon and differs from β-sericin present in the outer layer. Amount of sericin in cocoon varies in different strains of *Bombyx mori* L. By knowing the economic importance and convenience, silkworm has almost become an important tool for several biochemical, physiological and genetic studies in insects. Physiological and biochemical studies includes general metabolism and morphogenesis in insects, digestion and digestive enzyme, protein synthesis and their metabolism, hormones and their mechanisms of action, structure and function of chromosomes etc., for better productivity. Major biomolecules such as carbohydrates, lipids, proteins, hormones and chromosomes etc., play an important role in biochemical process underlying growth and development of insects (Ito and Horie, 1959; Wyatt, 1967). Metabolism and accumulation of these biomolecules in insect tissues during their development in different stages of life cycle was studied by many workers (Murphy and Wyatt, 1965; Wiens and Gilbert, 1967; Horie *et al*., 1968; Yamashita *et al*., 1972; Keeley, 1978; Tanaka and Kusano, 1980; Friedman, 1985; Bhattacharya and Kaliwal, 2004). The concentrations of these biomolecules mainly depend on mulberry leaf quality. Proteins in haemolymph are at higher concentration during development and are useful in silk proteins synthesis. Keeping this in view, in the present experiment an attempt has been made to study the protein concentration during silkworm larval development in different silkworm races.

Materials and Methods

In the present study, three different silkworm races namely multivoltine (pure Mysore PM), crossbreed ($PMxCSR_2$) and bivoltine (CSR_2) were selected. Disease-free silkworm layings were procured from National Silkworm Seed Project (NSSP), Madiwala, Bengaluru. Eggs were incubated at 25±1 °C temperature and relative humidity of 75% on eighth day. Then eggs were black boxed for 48 h to obtain uniform hatching. Brushing of the eggs was done at 10 am on 10^{th} day. Appropriate cellular rearing techniques were adopted and separate rearing trails were conducted for different silkworm races (Krishnaswami *et al*., 1970; Krishnaswami, 1978; 1990; Benchamin *et al*., 1983; Benchamin and Nagaraj, 1987). Rearing house and other appliances were thoroughly disinfected with 2% formalin 2 days before the commencement of rearing. Adding lime (500 mg to 1 L of 2% formalin) to formalin is formed to be more effective. Necessary precautions were taken during rearing to avoid diseases and contamination. Three different silkworm races reared under standard rearing conditions on V_1 mulberry variety leaves. During chawki rearing (1^{st}, 2^{nd} and 3^{rd} instar) optimum temperature (25-27 °C) and relative humidity (80-90%) and for late age rearing (4^{th} and 5^{th} instar) optimum temperature (23-25 °C) and relative humidity (70-75%) were maintained for the vigorous growth and development of silkworm larvae. Leaves were harvested during cooler hours of the day and preserved in wet gunny cloth till the feeding time. Larvae were fed three times daily (7.00 am, 3.00 pm and 11.00 pm) with healthy, fresh mulberry leaves from hatching to spinning except during moulting. Young age larvae fed with tender, succulent and nutritious leaves required to favour the growth and development of chawki silkworms. While mature and coarse leaves fed to late age silkworms as they grow till ripening. Bed cleaning was done once in 1^{st} instar, twice in 2^{nd} and 3^{rd} instars and every day in 4^{th} and 5^{th} instars during silkworm rearing. Lime powder was dusted during moulting in order to keep the rearing bed dry.

Collection and preparation of tissue sample

Protein estimation was carried out in each instar with three replications. Young age silkworms body size was very small and it was difficult to handle different tissues. Therefore during first four instars, entire larval body was considered. Larvae were selected on second day of each instar for the analysis. During 5^{th} instar different tissues such as haemolymph and midgut were collected at 24 h interval on all the days after fourth moult to spinning.

Haemolymph

Silkworm larvae were randomly collected daily at 11 am during 5^{th} instar and kept at 5 °C for 5-10 min to facilitate the free running of blood. Larval caudal horn was cut open and haemolymph was collected into a clean pre-cooled 5 ml glass vials containing few crystals of phenylthiourea to prevent oxidation. Vials containing samples were preserved in refrigerator at 4 °C as stock for estimation of proteins. Haemolymph was diluted appropriately with demineralised water. After dilution, samples were centrifuged at 3000 rpm for 5-10 min and supernatant was collected and used as sample for protein estimation.

Midgut Tissue

Silkworm larvae were randomly collected daily at 11 am during 5^{th} instar. They were kept separately in petridishes at 4 °C. Midgut was excised by cutting larval skin dorsally in cold condition. 10% (W/V) homogenate of the midgut tissue was prepared in ice-cold distilled water using a glass homogeniser. Homogenate was centrifuged at 3000 rpm for 10 min, supernatant was collected, diluted and used as sample for protein estimation.

Silkworm Excreta

Silkworm excreta were collected every day immediately after bed cleaning from hatching to spinning from all the three silkworm races reared. Collected excreta was cleaned off with leaf bits and dried in a hot air oven at 50 °C for 2 h. Cleaned and dried excreta used for the preparation of sample. One percent homogenate of the excreta was prepared in distilled water using mortar and pestle. Homogenate was centrifuged at 3000 rpm for 10 min and supernatant was collected and used as sample for protein analysis.

Protein Estimation

Quantitative estimation of protein was done spectrophotometrically in different body tissue samples by Lowry *et al*., (1951) method using bovine serum albumin (BSA) as standard.

Statistical Analysis

One-way analysis of variance *ANOVA* was used to test the significance of differences between mean values of independent observations of biochemical parameters in entire body, midgut, haemolymph and excreta of silkworm races. Comparisons were performed to find significant differences between the silkworm races. Differences were significant at $p < 0.05$.

Results

Protein concentration in silkworm entire body.

In multivoltine silkworms protein concentration in the entire body during 1st instar was found 28.18 mg/g and gradually increased during 2nd instar (31.02 mg/g) and 3rd instar (32.42 mg/g) and reaches maximum during 4th instar (34.86 mg/g). In bivoltine silkworms protein concentration was 31.46 mg/g in 1st instar and increased during 2nd instar (31.95 mg/g) followed by 3rd instar (36.45 mg/g) and 4th instar (39.27 mg/g). In cross breed silkworms, protein concentration revealed that, in 1st instar (30.78 mg/g) followed by 2nd instar (31.10 mg/g), 3rd instar (34.68 mg/g) and maximum in 4th instar (37.42 mg/g) (Table-1). Bivoltine silkworms recorded maximum protein concentration followed by crossbreed and multivoltine silkworms in all the instars.

Protein concentration in 5th instar silkworm

Midgut

In multivoltine silkworms, protein concentration was minimum on 1st day (10.31 mg/g) and gradually increased with the advancement of instar and reached peak on 7th day (28.89 mg/g). Protein concentration in bivoltine silkworms was 20.28 mg/g on 1st day and gradually increased till 5th day (21.36 mg/g, 26.12 mg/g, 33.82 mg/g and 37.86 mg/g on 2nd day, 3rd day, 4th day and 5th day respectively). Then decreased gradually on 6th day (37.22 mg/g) and 7th day (36.46 mg/g). In crossbreed silkworms, it increased with the advancement of larval development from 1st day to 7th day. On the 1st day, it was minimum 17.12 mg/g followed by 18.62 mg/g, 20.56 mg/g, 26.18 mg/g, 32.50 mg/g, 34.86 mg/g and 35.66 mg/g on 2nd day, 3rd day, 4th day, 5th day, 6th day and 7th day respectively (Table-2).

Haemolymph

Protein concentration varied significantly in multivoltine silkworms from beginning to end of 5th instar. It was 13.18 mg/g on 1st day that increased to 14.14 mg/g on 2nd day, 17.68 mg/g on 3rd day, 19.89 mg/g on 4th day. On 5th day it was increased significantly to 30.02 mg/g and on 6th day 35.23 mg/g and decreased slightly on 7th day (34.01 mg/g). In bivoltine silkworms there is a significant increase from 1st day to 7th day of larval growth. Protein concentration was 20.10 mg/g on 1st day, 22.18 mg/g, 25.26 mg/g, 30.40 mg/g, 36.71 mg/g, 39.48 mg/g and 43.30 mg/g on 2nd, 3rd, 4th, 5th, 6th & 7th day respectively. In cross breed silkworms, protein concentration on 1st day is 16.82 mg/g and increases as the larva grows and reached maximum on 7th day (37.02 mg/g). Protein concentration was recorded highest in bivoltine silkworms (43.30 mg/g) followed by crossbreed silkworms (33.02 mg/g) on 7th day and 35.23 mg/g in multivoltine silkworms on 6th day. Lowest protein concentration was also found in the same hierarchy that, 20.10 mg/g in bivoltines followed by crossbreeds (16.82 mg/g) and multivoltine silkworms (13.18 mg/g) (Table-3). Haemolymph proteins have always been an interesting tool for insect biochemists because of their pertinent role in development, morphogenesis and in almost all intermediary metabolic pathways in insects.

Silkworm excreta

Silkworm excreta was subjected to protein analysis once in 1st instar to 4th instar and all the 7 days during 5th instar and results showed the variation in protein concentration in all the days.

Multivoltine silkworm excreta

Protein concentration during 1st instar was 8.01% and gradually increases as larva develops. During 2nd instar it was 10.80% followed by 11.76% and 12.20% in 3rd instar and 4th instar respectively (Table-4). Protein concentration on the 1st day of 5th instar was 9.63% followed by an increase on 2nd day 10.76%. Again it decreases on 3rd day 9.86%, further decreased on 4th day 8.02%. From 5th day onwards protein concentration gradually increased to 10.29% followed by 10.78% and 11.28% on 6th day and 7th day respectively (Table-5).

Bivoltine silkworm excreta

Protein concentration gradually increases from 1st instar (11.19%), 2nd instar (11.86%) to 3rd instar (12.69%) and then decreases significantly during 4th instar (10.26%) (Table-4). During 5th instar, in the beginning it increases and then decreases as larval development proceeds. Protein concentration on 1st day was 13.26% followed by 14.12% on 2nd day. On 3rd day, there was a significant decrease in protein concentration (12.86%) that further decreased on the penultimate days. On 4th day it was 12.20% followed by 11.86%, 11.62% and 11.30% on 5th, 6th and 7th day respectively (Table-5).

Crossbreed silkworm excreta

Protein concentration was 10.28% during 1st instar followed by 2nd instar (11.54%), 3rd instar (12.06%) and decreased on 4th instar (10.11%) (Table-4). During 5th instar, on 1st day it was 11.36% followed by 11.89% on 2nd day and decreased on 3rd day (11.03%). It was significantly increased on 4th day (12.62%) followed by decrease on 5th day (11.89%) and 6th day (10.27%) and on 7th day protein concentration increased significantly to 12.26% (Table-5).

Table 1: Protein concentration in silkworm entire body from 1st to 4th instar.

Stage	Multivoltine (PM)	Bivoltine (CSR_2)	Crossbreed ($PMxCSR_2$)
	Mean ± SD	Mean ± SD	Mean ± SD
1st instar	28.18 ± 0.29	31.46 ± 0.51	30.78 ± 0.46
2nd instar	31.02 ± 0.50	31.95 ± 0.49	31.10 ± 0.50
3rd instar	32.42 ± 0.38	36.45 ± 0.30	34.68 ± 0.48
4th instar	34.86 ± 1.02	39.27 ± 0.31	37.42 ± 0.30

Table 2: Protein concentration in silkworm midgut during 5th instar.

Days	Multivoltine (PM)	Bivoltine (CSR_2)	Crossbreed ($PMxCSR_2$)
	Mean ± SD	Mean ± SD	Mean ± SD
1st day	10.31 ± 0.28	20.28 ± 0.60	17.12 ± 0.30
2nd day	12.94 ± 0.48	21.36 ± 0.41	18.62 ± 0.32
3rd day	16.32 ± 0.52	26.12 ± 0.62	20.56 ± 1.26
4th day	21.76 ± 0.37	33.82 ± 0.38	26.18 ± 0.40
5th day	24.38 ± 0.47	37.86 ± 1.06	32.50 ± 0.52
6th day	27.70 ± 0.43	37.22 ± 0.52	34.86 ± 0.42
7th day	28.89 ± 0.56	36.46 ± 0.08	35.66 ± 1.04

Table 3: Protein concentration in silkworm haemolymph during 5th instar.

Days	Multivoltine (PM)	Bivoltine (CSR_2)	Crossbreed ($PMxCSR_2$)
	Mean ± SD	Mean ± SD	Mean ± SD
1st day	13.18 ± 0.51	20.10 ± 0.51	16.82 ± 0.86
2nd day	14.14 ± 0.09	22.18 ± 0.46	18.27 ± 0.51
3rd day	17.68 ± 0.54	25.26 ± 0.38	19.86 ± 0.30
4th day	19.89 ± 0.34	30.40 ± 0.60	22.76 ± 1.27
5th day	30.02 ± 0.56	36.71 ± 0.56	35.16 ± 1.06
6th day	35.23 ± 0.51	39.48 ± 0.30	38.72 ± 0.68
7th day	34.01 ± 0.40	43.30 ± 0.28	37.02 ± 0.30

Table 4: Protein concentration in silkworm excreta from 1st to 4th instar.

Stage	Multivoltine (PM)	Bivoltine (CSR_2)	Crossbreed ($PMxCSR_2$)
	Mean ± SD	Mean ± SD	Mean ± SD
1st instar	08.01 ± 0.11	11.19 ± 0.19	10.28 ± 0.32
2nd instar	10.80 ± 0.56	11.86 ± 0.21	11.54 ± 0.30
3rd instar	11.76 ± 0.48	12.69 ± 0.30	12.06 ± 0.24
4th instar	12.20 ± 0.37	10.26 ± 0.19	10.11 ± 0.19

Table 5: Protein concentration in silkworm excreta during 5th instar.

Days	Multivoltine (PM)	Bivoltine (CSR_2)	Crossbreed ($PMxCSR_2$)
	Mean ± SD	Mean ± SD	Mean ± SD
1st day	09.63 ± 0.17	13.26 ± 0.42	11.36 ± 0.31
2nd day	10.76 ± 0.28	14.12 ± 0.22	11.89 ± 0.27
3rd day	09.86 ± 0.31	12.86 ± 0.18	11.03 ± 0.18
4th day	08.02 ± 0.07	12.20 ± 0.23	12.62 ± 0.23
5th day	10.29 ± 0.18	11.86 ± 0.52	11.89 ± 0.36
6th day	10.78 ± 0.09	12.30 ± 0.21	10.27 ± 0.32
7th day	11.28 ± 0.27	11.62 ± 0.51	12.26 ± 0.19

Discussion

Data revealed that, protein concentration in selected silkworms varied significantly such as entire body during 1st to 4th instar, in 5th instar different tissues like midgut, haemolymph and silkworm excreta. Growth and development of silkworm larvae and economic characters such as cocoon yield, cocoon weight, shell weight, silk percentage and renditta are greatly influenced by the nutritional level of mulberry leaf (Krishnaswami *et al*., 1970; 1971). Proteins are biomolecules plays a fundamental and physiological role in the growth and development of silkworm *Bombyx mori* L. and synthesis of silk proteins in silk gland during larval development (Seo *et al*., 1985). It is known fact that, nearly 70% of proteins directly derived from mulberry leaf and is the source of silk protein production by silkworm (Kawase, 1975). It is observed that supplementation of proteins through mulberry leaves improve the cocoon quality and reduces larval mortality (Ito, 1978). Supplementation of glycol and alanine with mulberry leaves enhances the cocoon layer and weight. It also increases the glycine content of silk protein and reduces nitrogen excretion (Kim *et al*., 1983). If the leaf is given with additional tyrosine and phenylalanine, silk quality and quantity is increased, particularly the fibroin (Anonymous, 1970). Protein concentration increased rapidly from 1st instar and reaches maximum level at the end of 4th instar. Similar findings were also observed by Banno *et al*., (1993). Higher body protein concentration in bivoltine silkworm larvae is perhaps due to the increased consumption of mulberry leaves. It results in high rate of conversion and accumulation of proteins in silkworm larval body compared to crossbreed and multivoltine silkworm larvae.

In midgut tissue, protein concentration was found high in bivoltines followed by crossbreed and multivoltine silkworms. Midgut protein content in all the silkworms races increases from 1st day to 7th day of 5th

instar is in conformity with the earlier findings of Seo *et al*., (1985). This is due to active absorption of food constituents by the midgut and increase in assimilation and conversion rates during 5th instar larval development. From the present findings it is observed that, efficiency of conversion of food in to proteins is higher in bivoltine silkworms compared to crossbreeds and multivoltines as a result midgut protein content is significantly highest in bivoltine silkworms followed by crossbreed and multivoltines. It is in confirmation with the earlier findings of Ito and Arai, (1965) that, they observed higher protease activity in bivoltine silkworms might contribute for the increased accumulation of proteins. Maso and Narumi, (1989) observed total proteins in midgut and silk gland decreased in early stages and increased in later stages of silkworm larval development. Haemolymph acts as storage reservoir for many materials essential for insects and its composition tends to vary in response to various activities (Hirano and Yamashita, 1983). As haemolymph is transport tissue, it transports more proteins to and from the different tissues. This may be required to meet the higher physiological activities in silkworm such as increased body growth as well as silk synthesis. Synthesis of proteins in fat body will be increased during moulting period (Yashitake and Nagata, 1989). Variation in protein concentration in haemolymph is due to differential rate of metabolism and synthesis. Results are in confirmation with the earlier works of Sathish, (1998) that, haemolymph protein in sericigenous insects is responsible for the formation of silk proteins in silk glands. Shimura, (1978) reported that, haemolymph acts an amino acid reservoir between midgut and silk gland, supplied amino acids to silk gland for silk synthesis. Lauffer, (1960) was the first ever observed haemolymph proteins in Silkworm *B. mori* L. Haemolymph protein content increases throughout 5th instar and reaches maximum at the end of 5th instar in all the three silkworm races. Nagata and Kobayashi, (1990) reported a quantitative change in storage proteins during silkworm larval development.

Dietary proteins are valuable nutritive components of the food required for the growth and development of silkworm larva and to carry out several physiological functions. Gowda, (1982) reported that, silkworm excreta contain more proteins compared to wheat bran and paddy husk and percentage of crude proteins on the dry matter basis ranges from 13-14%. It is observed that, consumption, digestion and assimilation rates are less in case of multivoltine compared to bivoltine and crossbreed silkworms (Benchamin and Nagaraj, 1987). Silkworm litter contains 3.06% nitrogen that is roughly 19% of the crude protein (Mathur and Mukhopadhyaya, 1988). Further, bivoltine and crossbreed silkworm excreta recorded corresponding increase in protein content in comparison to multivoltine. This clearly revealed that, conversion of leaf materials to body materials is higher in both bivoltine and crossbreed races. During 4th instar, interestingly, crossbreed silkworms excreta recorded lower protein content (10.11%) and highest in pure mysore (12.20%) that supports the earlier findings of Banno *et al*., (1993). At the same time an increase in excretal protein in multivoltine reveals a decline in the conversion efficiency where in low digestion, assimilation and conversion rates are associated with high protein excretion. Present results are in favour of the earlier findings of Nagata and Kobayashi, (1990). They opinioned that, concentration of storage proteins in silkworm larva is shown to increase during larval growth. In case of crossbreed, it follows the same trend as that of bivoltine. Body protein concentration is high in relation to excretal protein concentration during I instar (30.78%) that gradually increases to 31.10% during 2nd instar, 34.68% during 3rd instar and 37.42% in 4th instar. This clearly shows that, the conversion efficiency of crossbreed race is higher than the multivoltine. Yogananda Murthy *et al*., (2014) reported that, bivoltine silkworms are superior in all the biochemical parameters examined compared to crossbreed and multivoltine silkworms.

Conclusion

The foregoing results clearly indicate that, three silkworm races examined differ significantly in protein concentration. During early instar, whole body was used (1st to 4th instar) and in 5th instar, different tissues such as haemolymph and midgut were analysed for protein analysis. Larval excreta was also analysed for the protein concentration. Results confirmed that, bivoltine silkworms are superior over crossbreed and multivoltine silkworms in protein concentration in different body tissues analysed. Differences between silkworms races are due to genetic endowment. Quantity and quality of proteins in silkworms attributes the robustness and healthiness that reliably considered being better in rearing performance and cocoon yield. Screening of silkworm genetic resources using protein analysis can be more useful and dependable for the silkworm selection in breeding programme as well as silkworm races exploitation for commercial purpose.

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