Critical Biochemical Analysis in Different Body Tissues in Three Commercial Silkworm (*Bombyx mori* L.) Races

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ABSTRACT

Three silkworm races of Bombyx mori L viz., Pure Mysore (PM), Bivoltine (CSR_2) and Crossbreed ($PMxCSR_2$) were analyzed for their biochemical compositions. Quantitative assay of proteins, reducing sugars and nucleic acids were carried out in different body tissues of silkworm. Data obtained are statistically analyzed with one-way ANOVA at $p<0.05$ significance level and presented in the results. Significantly higher (at $p<0.05$) proteins were recorded in bivoltine silkworms compared to crossbreeds and multivoltine silkworms. Higher reducing sugar was recorded in midgut and silk glands of bivoltines followed by crossbreed and pure Mysore silkworms. DNA and RNA concentrations were recorded comparatively higher in haemolymph and midgut of bivoltine silkworms. Results revealed that, bivoltine silkworms are comparatively superior in all the biochemical parameters examined and these studies can be expediently adopt for screening and characterization of different silkworm breeds.

Keywords: *Bombyx mori*, midgut, hemolymph, silk gland, biochemical analysis

INTRODUCTION

Mulberry silkworm *Bombyx mori* L. is a monophagous lepidopteron insect derives the required nutrients for its growth from mulberry leaves. Success of silkworm development, cocoon crop and silk yield is mainly depending on the environmental conditions and mulberry leaf quality during rearing (Benchamin and Anantharaman, 1990).Silkworm breeds differ in their nutritional requirements depending on the variety, rearing environment, season and quantum of nutrition upon physiological and metabolic activities (Ramesha et al., 2010). Nutritional studies in silkworm with respect to moulting, cocoon productivity, economic characters, silk production, silk protein and body weight gain have elucidated their dependency on quantitative and qualitative variations of mulberry leaves used as feed (Sumoika et al., 1982). Major biomolecules such as proteins, carbohydrates and lipids play an important role in biochemical process underlying growth and development of insects (Ito and Horie, 1959). Proteins are known to have remarkable influence on various developmental stages. Protein is necessary for various biological activities during development, metamorphosis and maintenance of various physiological functions in different tissues (Kumar et al., 2011). Carbohydrates are protecting organisms during adverse conditions and are essential components of energy source for different biological activities in silkworms. Biomolecules such as glycerol, sorbitol acts as thermo protectants synthesize in the tissues and released into haemolymph (Shamitha and Purushotham Rao, 2008). Reducing sugars account 5% of total blood sugars in insects. Lipids in the fat body are energy reservoirs and can be mobilised rapidly during moulting, starvation, oogenesis and embryo genesis and used

to sustain continues muscular activity (Kochi and Kaliwal, 2005). It is a fact that, silkworm requires essential proteins, amino acids, sugars, fatty acids, vitamins, enzymes and micronutrients for its growth and production of silk protein (Bhattacharya *et al*., 2011). Though a number of biochemical works in respect of silkworms qualitative and quantitative nature have been carried out, information on basic biochemical constituents and their variations during larval development in silkworms are scanty. Keeping this in view, an attempt has been made to analyze some biomolecules in different tissues of commercial silkworm races.

MATERIAL AND METHODS

In the present experiment, multivoltine (Pure Mysore), bivoltine (CSR_2) and crossbreed ($PMxCSR_2$) silkworm races were selected. Disease free layings obtained from National Silkworm Seed Production Centre (NSSPC), Madiwala, Bangalore. Different races were reared separately under standard laboratory conditions using cellular rearing methods and replicates were maintained separately (Krishnaswami et al., 1970; Krishnaswami, 1990; Benchamin and Nagaraj, 1987). Larvae fed three times daily (7.00am, 3.00pm, 11.00pm). Young age larvae were fed with healthy fresh S_{36} mulberry variety leaves known to favour growth and development of chawki silkworms and late age silkworms fed with healthy fresh M_5 mulberry variety leaves.

Collection and Preparation of Tissue Samples

Tissue samples for biochemical assays were collected during each instar. As the body size of young silkworms is very small and it is difficult to handle different tissues during first four instars, entire larval body was considered. Larvae were selected on second day of each instar for analysis. During 5^{th} instar different tissues such as hemolymph, midgut and silk gland were collected at 24 hours interval on all the days.

Haemolymph

Haemolymph was collected from 5^{th} instar silkworm larvae in a pre-chilled test tube containing a few crystals of phenyl thiourea by cutting the caudal horn. Haemolymph was centrifuged at 3000 rpm for 10 minutes at 4^oC and the supernatant was collected and stored at -20^oC until further use.

Midgut

Midgut tissue was excised by cutting larval skin dorsally in a dissection tray containing ice cold ringer solution with Tris-HCL buffer (pH 7). Midgut was collected by separating anterior and posterior part of the gut and transferred to a pre-cooled plastic vials. 10% (W/V) homogenate was prepared in ice-cold PBS using a glass homogeniser. Homogenate was centrifuged at 3000 rpm for 10 minutes and supernatant was collected, diluted appropriately and used as sample for the assay.

Silk Glands

Silk glands were collected and allowed for 5-7 minutes in the buffer, then remove excess moisture on glands surface by thin blotting paper. Transferred silk glands to a sterilized glass homogenizer and homogenized with 20% (W/V) 50mM Tris-HCl buffer (pH 7). Homogenate was transferred to a clean centrifuge tube and centrifuged at 10000 rpm for 30 minutes in cooling condition. Supernatant was collected in a clean glass test tube for the analysis.

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Biochemical Analysis

Quantitative estimation of proteins was done spectrophotometrically by Lowry *et al*., method (1951) using bovine serum albumin (BSA) as standard. Reducing sugar assay was carried out according to Burton (1956) using glucose as standard. DNA and RNA quantification done according to Schneider (1957) method using calf thymus DNA and yeast RNA as standards.

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 midgut, haemolymph and silk gland of different silkworm races. Comparisons were performed to find significant differences between the silkworm races. Differences were significant at $p < 0.05$.

RESULTS AND DISCUSSION

Total Proteins

Nutritional efficiency in larval stages significantly influences the resulting pupae and adult particularly in lepidopteron insects where in adult is a non-feeding stage (Srivastava et al., 1982). Seo et al., (1985) opined that, proteins are biomolecules plays a fundamental and physiological role in growth and development of silkworms and synthesis of silk proteins in silk gland during larval development. In the present experiment, total proteins in whole body showed a rapid increase from 1st instar and reaches maximum at the end of 4th instar in all the three silkworm races analysed. Varied protein concentrations were observed among whole body tissues of different races and significantly higher proteins recorded at $p<0.05$ in bivoltine silkworms during 1st to 4th instar (Table-1). Comparatively, bivoltine silkworms recorded highest proteins during 4th instar (93.62 mg g^{-1}) followed by crossbreed (89 mg g^{-1}) and pure Mysore silkworms (80.72 mg g^{-1}). A linear increase in protein concentration was recorded in the haemolymph and silk gland from 1st day (40.12 mg ml^{-1} and 98.64 mg g^{-1}) to day before spinning (98.80 mg ml^{-1} and 178 mg g^{-1}) during 5th instar larva. In contrast to this, midgut protein concentration was higher (61.34 mg g^{-1}) during 5th instar and gradually reduced before spinning. Apparently significant differences (at $p<0.05$) in protein concentrations in different tissues were recorded and between different silkworm races reported (Table-2). Hurlimann and Chen (1974) asserted that, synthesis and utilization of haemolymph proteins are conditioned by genetic and hormonal control. In silkworms, haemolymph protein fluctuates during its developmental stages and concentration was found to increase positively correlated with silkworm growth. High protein concentration is an indication of a greater metabolic activity of the tissue. Higher protein concentration was recorded in bivoltine silkworms. Similar findings were observed by Banno et al., (1993), is due to increased consumption and assimilation of mulberry leaves and subsequent high rate of conversion and accumulation of proteins in bivoltine silkworms compared to crossbreed and multivoltine silkworms. Higher protein content in whole body of silkworm larva (1st-4th instar) may be due to tender leaves fed than medium and coarse leaves (HisaoAruga, 1994). Results revealed that, quantitatively total proteins of haemolymph and silk gland showed variations during larval development and maximum was on last day of 5th instar and minimum on 1st day after fourth moult. This indicates the influence of dietary protein on the increase in haemolymph and silk gland protein in 5th instar, since 5th instar is considered as prime feeding stage of silkworms where in 80%-85% of total leaves consumed. Ito and Arai (1963) observed that, accumulation of biomolecules is largely depending on mulberry leaf quality and amount of leaf consumed by silkworms. It is reported that, physiological and biochemical activities in silkworm body are affected by the availability of nutrients such as

carbohydrates, proteins, amino acids, lipids, minerals and vitamins (Sridhara and Bhat; 1966; Ohmura et al., 1989).

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). Haemolymph transports more proteins to and from the different tissues. Synthesis of proteins in fat body will be increased during moulting period (Yashitake and Nagata, 1989; Nagata and Kobayashi, 1990). Variation in protein concentration in haemolymph and silk glands 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. Performance in silkworm races studied in the present study may be due to differential physiological activities and attributed to genetic differences among these races. Since, bivoltine silkworms are characterised by high silk yielding nature, conversion of haemolymph proteins into silk substances is obvious. In crossbreed and pure Mysore silkworms a significant difference is observed in haemolymph proteins throughout 5^{th} instar. Total proteins in silk gland showed a gradual increase in all the three silkworm races (Shimada et al., 1985). In midgut, protein concentration showed an increasing trend from 1^{st} day to 4^{th} day and then gradually decreases till 8^{th} day in all the three races. Midgut tissue of silkworms plays an important role in active absorption of food constituents viz., proteins and amino acids from digested mulberry leaves. In turn midgut proteins help in growth and development of silk gland, reproductive organs in silkworms and increase the assimilation and conversion rates during 5^{th} instar larval development (Seo et al., 1985).

Reducing Sugars

Reducing sugars in the whole body was significantly increasing from 1^{st} to 4^{th} instar in all the three silkworm races. An increasing reducing sugar concentration was observed as the larval growth increases from 1^{st} to 4^{th} instar in all the silkworm races studied. Higher reducing sugar was recorded in bivoltine silkworms compared to crossbreed and pure Mysore silkworms (Table-1). Reducing sugars recorded daily during 5^{th} instar showed similar pattern, linear increase in the concentration from 1^{st} day up to spinning was observed in all the silkworm races. Higher concentration was recorded in bivoltine silkworm haemolymph (1.914 mg ml^{-1}) day before spinning. Comparatively lower reducing sugars observed in crossbreed and pure Mysore silkworms respectively (Table-3).Carbohydrates like glycogen and trehalose, other nitrogenous compounds are the main haemolymph constituents reported to be crucial during growth, development and in maintenance of diapause in insect(Hyun-Mi Jo and Yonggyun Kim, 2001). Many diapausing species are reported to utilize stores of glycogen to generate glycerol, sorbitol and trehalose. Silkworms conserve sufficient quantity of energy reserves during larval stage to be utilized during pupal and adult stages (Mishra et al., 2010). Simex and Kodrik (1986) have reported that, glycogen content in fat body, body wall and silk gland and free carbohydrates in haemolymph changed significantly during final larval instar and metamorphosis in silkworms. Carbohydrates are the major components in living organisms' food that either directly or indirectly used as energy source for all vital activities. Reducing sugars are food reserves for immediate energy demand of tissue. Reducing sugars concentration in midgut and haemolymph showed significant variations between days that support dynamic nature of haemolymph. In midgut and haemolymph of all the three silkworm races, there was a gradual increase in reducing sugar concentration from 1^{st} day to 8^{th} day. Reducing sugar level was significantly lower in pure Mysore silkworms compare to

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cross breed and bivoltine silkworms. This can be explained through productiveness of the race and food intake that is more in bivoltines than crossbreed and pure Mysore silkworms.

Nucleic Acids (DNA & RNA)

Nucleic acids recorded higher concentration in whole body during 4th instar (Table-1). DNA concentration in different races recorded as follows BV: 4.98 mg g-1, CB: 4.82 mg g-1 and MV: 4.16 mg g-1. Concomitantly, RNA content in the whole body was also recorded highest during 4th instar in all the races (BV: 17.46 mg g-1, CB: 13.6 mg g-1 and MV: 10.44 mg g-1). In 5th instar DNA and RNA revealed higher in midgut during middle part of the instar and subsequently reduced as the larvae initiates spinning. In contrast, DNA and RNA in haemolymph revealed a linear increase from 1st day of 5th instar up to cocoon spinning. There was a significant variation in DNA and RNA concentration in different races studied (Table-4 & 5). Couble et al., (1983) reported that, DNA is known as informational molecule contains genetic information responsible for propagation of specific proteins and remains constant in a cell and any increase would reflect the growth is accomplished partly by an increase in cell number. Couble et al., (1987) suggested that, synthesis of DNA, RNA, fibroin and sericin in silk glands are developmentally regulated and these events are synchronized with the specific events of metamorphosis. DNA content in silkworms' whole body showed significant difference between the races analysed. DNA was gradually increasing from 1st to 4th instar indicating that, it is required for synthetic activity like protein synthesis. Further DNA content observed high in bivoltine followed by crossbreed and pure Mysore silkworms. DNA concentration increases gradually from 1st day and maximum on 5th day, corresponds to the active silk synthesis period (Tazima, 1978) and maximum food intake period (Gururaj, 1995), then gradually decreases as larva grows in all the three silkworm races till 8th day. Variations in DNA concentration in haemolymph is due to the fact that, haemolymph being a circulatory fluid and a metabolic reservoir for various physiological processes tends to alter its biochemical constituents with food intake and daily fluctuations in environmental conditions (Yokoyama, 1962a). RNA content in silkworms' whole body showed significant difference among three races and it gradually increases from 1st to 4th instar. Bivoltines showed high RNA content followed by crossbreed and pure Mysore silkworms. Similar results were observed by Srivastava et al., (1979) that, RNA content increased during aphid larval growth. RNA concentration in both midgut and haemolymph is peak on 8th day in all the silkworm races supports the earlier findings that, RNA synthesis precedes protein synthesis as it is known that DNA makes RNA and RNA makes proteins.

CONCLUSION

Results confirmed that, bivoltine silkworms are superior over crossbreed and multivoltine silkworms in biochemical contents in different body tissues analysed. Difference between silkworms is due to genetic endowment of the races. Quantity and quality of biomolecules in silkworms attributes the robustness and healthiness that reliably considered being better in rearing performance and cocoon yield. Screening of silkworm genetic resources using biochemical analysis as a tool may be more dependable for the selection in silkworm breeding programmes as well as for commercial exploitation of silkworm races.

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APPENDIX

Table 1. Biochemical constituents concentration in silkworms (whole body)

#	*Silkworm Races*	*Silkworm Instars*	*Total Proteins (mg/g)*	*Reducing Sugars (mg/g)*	*DNA (mg/g)*	*RNA (mg/g)*
1.	BV (CSR_2)	1st	70.23	2.84	2.18	11.08
		2nd	78.96	3.02	3.10	12.28
		3rd	85.10	4.30	3.46	15.03
		4th	93.62	5.24	4.98	17.46
2.	CB (PM x CSR_2)	1st	63.78	2.36	2.16	10.21
		2nd	70.60	2.81	2.88	10.36
		3rd	78.53	3.92	3.16	11.74
		4th	89.45	4.80	4.82	13.86
3.	PM (Pure Mysore)	1st	58.63	2.02	1.96	07.64
		2nd	62.00	2.74	2.10	07.95
		3rd	71.19	3.10	2.81	09.36
		4th	80.72	3.80	4.16	10.44
SEM ±			0.982	0.062	0.102	0.572
CD @5%			02.58	00.18	0.264	1.660

Table 2. Total proteins concentration in 5th instar silkworm tissues

#	*Silkworm Races*	*Silkworm Tissues*	*Days*							
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	BV (CSR_2)	Haemolymph (mg/ml)	40.12	41.80	42.96	58.60	74.80	80.16	85.80	98.80
		Silk gland (mg/g)	98.64	103.18	117.22	124.56	128.60	134.16	148.65	178.00
		Mid gut (mg/g)	42.12	51.65	54.57	61.34	58.02	56.40	47.38	45.17
2	CB (PM x CSR_2)	Haemolymph (mg/ml)	38.20	40.12	41.03	46.06	50.08	56.48	61.88	66.84
		Silk gland (mg/g)	93.55	95.81	97.92	98.53	101.64	109.44	116.04	125.44
		Mid gut (mg/g)	40.94	47.18	49.33	59.41	56.16	52.25	44.00	42.13
3	PM (Pure Mysore)	Haemolymph (mg/ml)	34.23	35.60	36.18	40.03	44.22	48.92	54.60	58.43
		Silk gland (mg/g)	82.64	84.40	86.13	87.84	90.18	94.24	97.96	102.25
		Mid gut (mg/g)	34.63	41.55	44.83	52.24	49.63	46.44	37.93	36.19
SEM ±			1.926	3.368	3.156	3.612	2.746	1.880	2.604	4.012
CD @5%			5.348	9.109	8.304	9.843	6.944	4.945	6.916	7.456

Table 3. Reducing sugars concentration in 5th instar silkworm tissues

#	*Silkworm Races*	*Silkworm Tissues*	*Days*							
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	BV (CSR_2)	Mid gut (mg/g)	0.670	0.815	0.906	0.976	1.213	1.435	1.857	1.914
		Haemolymph (mg/ml)	0.546	0.613	0.682	0.694	0.746	0.794	0.812	0.843
2	CB (PM x CSR_2)	Mid gut (mg/g)	0.546	0.724	0.852	0.860	0.925	1.102	1.356	1.632
		Haemolymph (mg/ml)	0.438	0.574	0.623	0.659	0.705	0.732	0.783	0.838
3	PM (Pure Mysore)	Mid gut (mg/g)	0.421	0.496	0.532	0.695	0.869	0.943	1.094	1.286
		Haemolymph (mg/ml)	0.279	0.422	0.450	0.540	0.598	0.634	0.695	0.725
SEM ±			0.023	0.019	0.026	0.027	0.020	0.021	0.017	0.019
CD @5%			0.062	0.050	0.078	0.074	0.058	0.058	0.051	0.053

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Table 4. DNA concentration in 5th instar silkworm tissues

#	*Silkworm Races*	*Silkworm Tissues*	*Days*							
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	BV (CSR_2)	Mid gut (mg/g)	1.486	1.546	1.744	1.842	2.128	1.346	1.257	1.139
		Haemolymph (mg/ml)	0.768	0.960	0.964	1.253	1.436	1.462	1.610	1.885
2	CB (PM x CSR_2)	Mid gut (mg/g)	1.385	1.410	1.516	1.814	1.986	1.261	1.104	1.046
		Haemolymph (mg/ml)	0.746	0.896	0.914	1.135	1.223	1.312	1.486	1.698
3	PM (Pure Mysore)	Mid gut (mg/g)	1.120	1.234	1.374	1.748	1.786	0.984	0.923	0.846
		Haemolymph (mg/ml)	0.550	0.689	0.746	0.890	0.965	1.126	1.214	1.406
SEM ±			0.100	0.121	0.152	0.126	0.130	0.128	0.120	0.116
CD @5%			0.312	0.308	0.416	0.948	0.356	0.264	0.382	0.312

Table 5. RNA concentration in 5th instar silkworm tissues

#	*Silkworm Races*	*Silkworm Tissues*	*Days*							
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	BV (CSR_2)	Mid gut (mg/g)	3.042	3.289	3.630	3.945	4.126	4.548	4.846	5.643
		Haemolymph (mg/ml)	0.944	1.230	1.300	1.377	1.384	1.460	1.782	2.516
2	CB (PM x CSR_2)	Mid gut (mg/g)	2.680	3.426	4.120	4.484	4.580	4.867	5.168	5.641
		Haemolymph (mg/ml)	0.322	0.546	0.580	0.674	0.683	0.691	0.784	0.942
3	PM (Pure Mysore)	Mid gut (mg/g)	1.466	1.640	1.760	1.800	1.982	2.104	2.586	3.188
		Haemolymph (mg/ml)	0.165	0.224	0.316	0.325	0.342	0.386	0.480	0.537
SEM ±			0.160	0.212	0.194	0.204	0.264	0.260	0.219	0.233
CD @5%			0.482	0.611	0.526	0.610	0.648	0.665	0.602	0.631