

STUDIES ON LARVAL HAEMOLYMPH PROTEIN LEVELS OF SELECTED RACES OF THE SILKWORM, *BOMBYX MORI* L. UNDER TEMPERATE CLIMATES OF KASHMIR

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ABSTRACT

Changes in the levels of protein in the haemolymph of eleven selected races of the silkworm, *Bombyx mori* L. viz., SH-6, SKAU-R-6, SKUAST-31, Dun6, Yakwei and CSR-5 (oval races) and NB4D2, SKAU-R-1, SKUAST-13, SKUAST-28, CSR-4 (constricted races), were investigated during 5th instar larval development in spring and summer season. The levels of total protein in the haemolymph, increased from first day of 5th instar till sixth day. From seventh day, the protein levels decreased significantly in all the races. Racial differences in the changes in the levels of protein in the haemolymph were observed in the larvae when reared during spring and summer season. The haemolymph proteins in spring season increased significantly from 41.34 to 66.79 mg/mL in SKAU-R-6 followed by SH-6 (38.88-61.13 mg/mL), SKUAST-31 (41.07-60.17 mg/mL), Dun6 (39.66-58.13 mg/mL), CSR-5 (38.29-55.85 mg/mL) and Yakwei (35.80-54.93 mg/mL). However, during summer season, there was a significant decrease in the haemolymph protein levels in all the races. But, the order of decrease was significantly higher in Yakwei > CSR-5 > Dun6 > SKAU-R-6 > SH-6 > SKUAST-31. The present study revealed that the high temperature during summer induces specific changes in the metabolism (reversible thermal stress) that have different adaptive value in different races of the silkworm. Relatively higher increase in the protein levels in the haemolymph of SKAU-R-6, SKAU-R-1 and NB4D2 presumably provides protective cover to tissues against high temperature during summer. The absence of such a mechanism may be responsible for temperature susceptibility of the bivoltine races like Yakwei, CSR-5, SKUAST-13 and CSR-4.

INTRODUCTION

Unlike other terrestrial insects, the mulberry silkworm is domesticated and reared in enclosed spaces and thus not exposed directly to external environment, though is exposed to fluctuating conditions of ambient temperatures and leaf quality during different seasons of the year. The long domestication and monophagous food habit of *Bombyx mori* limits the acquisition of the nutrients required for optimal growth and development of the silkworm to mulberry leaf. Seasonal variation plays a major role in biochemical profiles of different tissues of the silkworm larva during metamorphosis. Proteins are important biological macromolecules that are required for growth and development of the silkworm as well as biosynthesis of silk. They are major source of nitrogen and essential amino acids, which are to be obtained only through mulberry leaves in the monophagous silkworm *Bombyx mori*. Species specific variations in the ontogenic pattern of haemolymph free amino acids and haemolymph proteins have been found during the larval development of a number of insect species (Chen, 1971; Ranjini and Mohamed, 2004; Mishra *et al.*, 2005). The haemolymph protein concentration in *Bombyx mori* decreases normally by about one-fourth during pharate adult development (Doira and Kawaguchi, 1972). Such variations probably reflect the balance between the synthesis, storage, transport and degradation of structural and functional proteins

during ontogeny as well as a response to particular ecological and physiological conditions (Florkin and Jenniaux, 1974). Haemolymph proteins undergo radical changes both in quality and quantity during the development in lepidopteron insects (Srivastava and Pareek, 1976). Nagata and Kobayashi (1990) reported that the increase in the protein content of haemolymph and silk gland from the beginning to the end of the fifth instar may be due to active secretion of proteins by other tissues like fat bodies.

During insect metamorphosis, profound biochemical changes occur in the haemolymph, in particular, the concentrations of certain pools of proteins and amino acids undergo changes. Most importantly, special classes of haemolymph proteins known as storage proteins undergo significant changes (Levenbook, 1985). These storage proteins function as the main reservoirs for the supply of amino acids both at the time of larval moults and during metamorphosis (Nagata and Kobayashi, 1990). It has been reported that these larval storage proteins accumulate in the organism at the time of development. The fat body synthesizes a number of proteins and releases them into the haemolymph during active feeding larval period (Kumar *et al.*, 1998).

In the light of the above considerations, an attempt was made to analyze the haemolymph protein levels of oval (SH6, SKAU-R-6, SKUAST-31, Dun6, Yakwei and CSR5) and constricted races (NB4D2, SK1, SKUAST-13, SKUAST-28, CSR4) of the

silkworm during spring and summer seasons.

MATERIALS AND METHODS

Eleven popular pure races of silkworm, *Bombyx mori* L. namely SH-6, SKAU-R-6, SKUAST-31, Dun6, Yakwei and CSR-5 (oval races) and NB4D2, SKAU-R-1, SKUAST-13, SKUAST-28, CSR-4 (constricted races) were selected for the present study. NB4D2 and SH-6 a constricted and an oval race included in this study have been designated as control. Two disease free layings (DFL's) of each of the ten races of the silkworm were obtained from Germplasm Bank, Division of Sericulture, Mirgund, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir and one race namely Dun6 was obtained from CSR and TI Pampore. The larvae were reared cellularly under standard rearing conditions (Krishnaswami, 1978) during spring and summer season.

Haemolymph collection

Haemolymph was collected in a pre-chilled test tube containing a few crystals of thiourea by cutting the first proleg of larva. The haemolymph was collected from the 1st to 7th day of 5th instar larval development and centrifuged at 3000g for 10 min at 4°C, and the supernatant was used in the haemolymph protein estimation.

Estimation of total protein

Total protein was estimated by following the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard. To 0.1mL of the sample, 0.9mL of distilled water was added. This was followed by the addition of 5mL of the protein reagent. The tubes were left for 15 minutes at room temperature. Then 0.5mL of Folin reagent was added and the tubes were allowed to stand for 30 minutes. The reading was taken at 660 nm using spectrophotometer. The concentration of protein in the sample was calculated with the standard curve and results were expressed in mg of protein per mL of haemolymph.

Statistical analysis

Analysis of variance (ANOVA) was done test the significance of differences between the mean values of the haemolymph protein levels of the silkworm races reared during different seasons. Least Significant Difference (LSD) was used to find significance of differences between the means in races, seasons and days. Differences were considered significant at $p \leq 0.05$.

RESULTS

The levels of total protein in the haemolymph were significantly different at different stages of 5th instar larval development in both oval and constricted races of the silkworm during spring and summer season (Tables 1 to 4).

Haemolymph protein levels in oval races

The mean haemolymph protein levels were found to be significantly higher in SKAU-R-6 (51.87 mg/mL) followed by SH-6 (49.12 mg/mL), SKUAST-31 (48.57 mg/mL), Dun6 (45.51 mg/mL), CSR-5 (45.05 mg/mL) and Yakwei (42.73 mg/mL) (Table 1). Among the seasons, there was a significant increase in haemolymph protein levels during spring season in all the races. The Haemolymph protein levels increased on different days of larval growth period in 5th instar larvae of all the races. The protein levels in haemolymph showed a consistent increase from 1st to 6th day, and a significant fall on 7th day of 5th instar larval development.

During spring season, the haemolymph proteins of SKAU-R-6 increased significantly from 41.34 to 66.79 mg/mL followed by SH-6 (38.88-61.13 mg/mL), SKUAST-31 (41.07-60.17 mg/mL), Dun6 (39.66-58.13 mg/mL), CSR-5 (38.29-55.85 mg/mL) and Yakwei (35.80-54.93 mg/mL). When the oval races were reared during summer season, there was a significant decrease in the haemolymph protein levels in all the races. But, the order of decrease was significantly higher in Yakwei > CSR-5 > Dun6 > SKAU-R-6 > SH-6 > SKUAST-31. The protein levels in SKAU-R-6 during summer season ranged from 38.85 to

Table 1: Protein levels in the larval haemolymph of oval races of the silkworm, *Bombyx mori* L. reared during different seasons (Data pooled over same seasons of two years, 2009-2010)

Season	Days	Oval races					
		SH6 (Control)	SKAU-R-6	SKUAST-31	Dun6	Yakwei	CSR5
Spring	1-day	38.88 ± 1.15	41.34 ± 1.37	41.07 ± 1.24	39.66 ± 1.51	35.80 ± 0.99	38.29 ± 0.99
	2-day	42.52 ± 0.95	45.85 ± 0.88	45.12 ± 1.35	42.18 ± 1.00	39.65 ± 1.14	42.14 ± 1.14
	3-day	46.01 ± 1.16	50.24 ± 1.15	46.16 ± 0.83	45.30 ± 0.79	42.10 ± 0.73	44.94 ± 0.51
	4-day	53.65 ± 1.11	57.91 ± 2.52	53.29 ± 0.99	49.21 ± 0.76	46.68 ± 0.97	49.18 ± 0.97
	5-day	58.34 ± 0.69	62.41 ± 1.00	55.79 ± 0.99	52.46 ± 0.99	49.92 ± 1.31	52.43 ± 1.31
	6-day	61.13 ± 0.95	66.79 ± 0.85	60.17 ± 0.85	58.13 ± 0.92	54.93 ± 0.82	55.85 ± 0.82
	7-day	52.62 ± 1.33	53.82 ± 1.97	50.46 ± 3.00	47.64 ± 2.07	46.76 ± 1.22	47.88 ± 1.00
Summer	1-day	36.09 ± 0.76	38.85 ± 0.94	36.82 ± 1.53	35.30 ± 2.03	31.52 ± 1.51	34.03 ± 1.51
	2-day	39.73 ± 1.21	43.30 ± 0.62	40.67 ± 1.17	37.82 ± 1.47	35.38 ± 0.13	37.88 ± 1.14
	3-day	43.10 ± 0.99	44.84 ± 1.23	43.17 ± 0.77	40.66 ± 1.20	37.88 ± 0.72	40.38 ± 0.73
	4-day	51.75 ± 0.76	53.17 ± 0.92	49.97 ± 0.58	44.57 ± 0.99	42.33 ± 1.69	44.63 ± 1.45
	5-day	56.10 ± 0.80	57.00 ± 0.16	53.14 ± 1.16	47.82 ± 0.58	45.16 ± 1.61	47.87 ± 1.36
	6-day	59.89 ± 0.48	61.39 ± 0.90	57.52 ± 0.90	51.91 ± 0.76	48.79 ± 0.72	51.30 ± 0.73
	7-day	47.97 ± 0.93	49.35 ± 1.01	46.74 ± 1.21	44.56 ± 1.20	41.45 ± 1.17	43.95 ± 1.18
Mean of races irrespective of seasons		49.12 ± 8.05 ^b	51.87 ± 8.33 ^a	48.57 ± 6.81 ^c	45.51 ± 6.15 ^d	42.73 ± 6.32 ^f	45.05 ± 6.05 ^e
LSD ($p \leq 0.05$)		0.276					

Mean values with different letters in rows are significantly different from each other (as indicated by Tukey's HSD) Each value is the mean ± SE of 6 separate replications; Values expressed as mg of protein/mL of haemolymph

Table 2: Analysis of variance (ANOVA) showing the effect of seasons on haemolymph protein levels of oval races of the silkworm, *Bombyx mori* L. during 5th instar larval development

Source	Degree of freedom	Mean sum of squares	F-value
Environment	1	2038.42	1456.78**
Genotype	5	920.29	657.69**
Days	6	3516.52	2513.13**
Genotype × Environment	5	13.93	9.95*
Environment × Days	6	0.91	0.65NS
Genotype × Days	30	21.14	15.10**
Environment × Days	30	2.69	1.93NS
Error	420	1.39	

** Significant ($p \leq 0.01$); * Significant ($p \leq 0.05$); NS- Non significant

61.39 mg/mL followed by SH-6 (36.09-59.89 mg/mL), SKUAST-31 (36.82-57.52 mg/mL), Dun6 (35.30-51.91 mg/mL), CSR-5 (34.03-51.30 mg/mL) and Yakwei (31.52-48.79 mg/mL).

Haemolymph protein levels in constricted races

Among the constricted races, the larvae of SKAU-R-1 showed relatively higher mean levels of protein (51.66 mg/mL) followed by NB4D2 (50.96 mg/mL), SKUAST-28 (49.38 mg/mL), CSR-4 (47.35 mg/mL) and SKUAST-13 (46.46 mg/mL) (Table 2). The haemolymph protein levels in all the constricted races also showed a significant linear increase from 1st to 6th day during the 5th instar larval development. However, a significant increase in the haemolymph protein levels was observed in SKAU-R-1 from 42.00 to 65.91 followed by NB4D2 (41.31-64.22 mg/mL), SKUAST-28 (40.03-62.01 mg/mL), CSR-4 (42.24-58.71 mg/mL) and SKUAST-13 (39.93-56.62 mg/mL) during spring season. Whereas, during summer season a significant decrease in protein levels was observed in all the races, but, the decrease was found to be significantly higher in CSR-4 and SKUAST-13 races. The protein levels in SKAU-R-1, NB4D2, SKUAST-28, SKUAST-13 and CSR-4 during summer season ranged from 38.78 to 61.55, 37.75 to 60.92, 36.51 to 59.38, 34.56 to 54.00 and 37.92 to 52.52 respectively.

Table 4: Analysis of variance (ANOVA) showing the effect of seasons on haemolymph protein levels of constricted races of the silkworm, *Bombyx mori* L. during 5th instar larval development

Source	Degree of freedom	Mean sum of squares	F-value
Environment	1	1677.28	1478.95**
Genotype	4	422.24	372.31**
Days	6	3212.58	2832.71**
Genotype × Environment	4	7.62	6.72*
Environment × Days	7	0.84	0.74NS
Genotype × Days	24	24.45	21.56**
Environment × Days	24	3.31	2.92*
Error	350	1.13	

** Significant ($p \leq 0.01$); * Significant ($p \leq 0.05$); NS- Non significant

DISCUSSION

The results of the present investigation demonstrate changes in the levels of protein in haemolymph of oval (SH-6, SKAU-R-6, SKUAST-31, Dun6, Yakwei and CSR-5) and constricted races (NB4D2, SKAU-R-1, SKUAST-13, SKUAST-28 and CSR-4) of silkworm from 1st day to the end of the 5th instar with reference to spring and summer season. The major biochemical process underlying morphogenesis is protein synthesis. The storage proteins are mostly synthesized by the larval fat body and secreted into the haemolymph (Seong *et al.*, 1985). An enormous accumulation of storage proteins occur in the haemolymph at the final instar and the proteins are sequestered by the fat body during the larval-pupal transformation (Nagata and Kobayashi, 1990). The principal function of storage proteins are considered to be those of amino acid reservoirs during metamorphosis, with arylphorin being involved in cuticle formation because of its high content of tyrosine and phenylalanine (Levenbook, 1985). Storage proteins, however, may have another role in larval development.

The silkworm growth and development are extremely variable depending upon the environmental conditions and quality of leaf used as the feed (Krishnaswami, 1978; Tazima 1978;

Table 3: Protein levels in the larval haemolymph of constricted races of the silkworm, *Bombyx mori* L. reared during different seasons (Data pooled over same seasons of two years, 2009-2010)

Season	Days	Constricted races				
		NB4D2 (Control)	SKAU-R-1	SKUAST-13	SKUAST-28	CSR4
Spring	1-day	41.31 ± 0.61	42.00 ± 1.08	39.93 ± 0.62	40.03 ± 0.64	42.24 ± 0.72
	2-day	44.95 ± 0.78	45.64 ± 1.60	43.72 ± 0.78	43.82 ± 0.80	44.43 ± 0.80
	3-day	47.43 ± 0.82	49.71 ± 1.20	46.26 ± 1.08	46.80 ± 1.42	47.79 ± 1.09
	4-day	56.08 ± 0.61	56.21 ± 0.79	49.95 ± 0.98	55.13 ± 1.18	51.04 ± 0.98
	5-day	61.10 ± 1.18	61.79 ± 0.79	53.20 ± 1.32	58.63 ± 1.18	55.28 ± 0.81
	6-day	64.22 ± 0.88	65.91 ± 0.90	56.62 ± 0.83	62.01 ± 0.77	58.71 ± 0.83
	7-day	53.52 ± 0.74	54.25 ± 0.80	48.91 ± 1.12	52.72 ± 1.13	49.50 ± 0.76
Summer	1-day	37.75 ± 0.76	38.78 ± 1.05	34.56 ± 0.54	36.51 ± 0.55	37.92 ± 1.21
	2-day	41.38 ± 1.21	42.42 ± 1.11	38.35 ± 1.29	40.29 ± 1.30	40.11 ± 1.26
	3-day	44.76 ± 0.99	45.46 ± 0.93	40.88 ± 1.19	42.83 ± 1.20	42.61 ± 0.71
	4-day	52.41 ± 1.54	53.11 ± 1.12	48.18 ± 1.96	50.98 ± 2.44	45.86 ± 1.45
	5-day	57.42 ± 0.73	56.46 ± 1.24	50.57 ± 1.35	54.99 ± 1.13	49.77 ± 1.11
	6-day	60.92 ± 1.43	61.55 ± 0.47	54.00 ± 0.72	59.38 ± 0.86	52.52 ± 0.71
	7-day	49.62 ± 0.93	50.70 ± 0.65	45.39 ± 0.97	47.34 ± 0.97	45.18 ± 1.17
Mean of races irrespective of seasons		50.96 ± 8.17 ^b	51.66 ± 7.98 ^a	46.46 ± 6.29 ^e	49.38 ± 7.88 ^d	47.35 ± 5.76 ^c
LSD ($p \leq 0.05$)			0.245			

Mean values with different letters in rows are significantly different from each other (as indicated by Tukey's HSD) Each value is the mean ± SE of 6 separate replications; Values expressed as mg of protein/mL of haemolymph

Takamiya *et al.*, 1982; Kobayashi *et al.*, 1986). The changes in the protein levels of haemolymph reflect tissue specific changes in 5th instar larval development and in response to seasons in the oval and constricted races of the silkworm, *Bombyx mori*. Among the seasons, there was a significant increase in haemolymph protein levels during spring season in all the races. The relative increase in protein levels among the oval races were significantly higher in SKAU-R-6 followed by SKUAST-31, SH-6, Dun6, CSR-5 and Yakwei (Table 1) during spring and summer seasons. Also, among the constricted races, the larvae of SKAU-R-1 showed relatively higher mean levels of protein followed by NB4D2, SKUAST-28, CSR-4 and SKUAST-13 (Table 2). The protein levels in the haemolymph increased significantly during the 5th instar larval development. The relative increases in protein levels during both seasons were more in SKAU-R-6 followed by SKAU-R-1, NB4D2 and SKUAST-28. The increases in protein levels of haemolymph were due to synthesis of new proteins by the tissues and release into haemolymph. Martin (1969) observed that the increase in soluble proteins during early stages of last instar larvae in *Calliphora stygia* was due to high rate of protein synthesis by the fat body. Nagata and Kobayashi (1990) also reported an increase in protein synthesis during feeding stage in *Bombyx mori*. The protein levels showed a significant drop on the last day of spinning (7th day) in all the oval and constricted races which could be correlated to their sequestration and retention in the larval fat body or due to reduced rate of synthesis as observed by Kinnear *et al.* (1971).

During summer season, the haemolymph protein levels decreased significantly in all the races but the decrease was found to be significantly higher in Yakwei followed by CSR-5, SKUAST-13 and CSR-4. Several tissues including the fat body synthesize haemolymph proteins and the rates of synthesis and their export are impaired at higher ambient temperature during summer season. Thus, it could be concluded that temperate and tropical bivoltine races like SKAU-R-6, SKAU-R-1 and NB4D2 relatively have the ability to utilize the available nitrogen and carbon resources even under nutrition stress conditions during summer season towards biomass production and this tendency may be one of the reasons responsible for the temperature tolerance observed in these races.

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