RESEARCH PAPER | Asian Journal of Bio Science, Volume **8** | Issue 1 | April, 2013 | 47-51
Received : 07.12.2012; **Revised :** 03.02.2013; **Accepted :** 05.03.2013

Effect of mulberry nutrition on protein and free amino acid levels of haemolymph of selected races of the silkworm, *Bombyx mori* L. under temperate climates of Kashmir

AWQUIB SABHAT[1], M.A. MALIK[1], A.S. KAMILI[2], G.N. MALIK[1], S.A. MIR[3] **AND** FIRDOSE AHMAD MALIK[1]

[1]Temperate Sericulture Research Institute (SKUAST-K), MIRGUND (J&K) INDIA
[2]Directrate of Extension, Sher-e-Kashmir University of Agricultural Sciences and Technology (K), Shalimar, SRINAGAR (J&K) INDIA
[3]Division of Agricultural Statistics, Sher-e-Kashmir University of Agricultural Sciences and Technology (K), Shalimar, SRINAGAR (J&K) INDIA

Changes in the levels of protein and free amino acids in the haemolymph of three selected silkworm breeds *viz*., SKAU-R-1, SH-6 and NB4D2, fed with selected mulberry varieties like Ichinose (control), Goshoerami (uniformly the best), and Kokuso-20 (least productive) were investigated in 4th moult, and 5th instar larval development during spring and summer seasons. The protein levels in haemolymph were lower at moult in all the three races but the levels showed a sharp increase upon resumption of feeding. The mean levels of protein irrespective of seasons were relatively higher in larvae of SKAU-R-1 (44.97 mg/ml) followed by NB4D2 (44.35 mg/ml) and SH6 (42.48 mg/ml). Haemolymph free amino acid levels decreased during 5th instar from the level observed at 4th moult. The mean free amino acid level for the entire 5th instar larval stage in both seasons was relatively higher in NB4D2 (4.55 mg/ml) followed by SKAU-R-1 (4.25 mg/ml) and SH6 (4.17 mg/ml). Free amino acid levels dropped through the 5th instar larval development in all the three races. The changes in the haemolymph protein and free amino acid levels were observed from different base levels in the larvae feeding on different mulberry varieties. Racial differences in the haemolymph protein and free amino acid contents were observed during both seasons in relation to mulberry nutrition being higher in larvae feeding on the foliage of Goshoerami than Ichinose and Kokuso-20.

Key words : Mulberry, *Bombyx mori*, Haemolymph, Protein, Free amino acid

How to cite this paper : Sabhat, Awquib, Malik, M.A., Kamili, A.S., Malik, G.N., MIR, S.A. and Malik, Firdose Ahmad (2013). Effect of mulberry nutrition on protein and free amino acid levels of haemolymph of selected races of the silkworm, *Bombyx mori* L. under temperate climates of Kashmir. *Asian J. Bio. Sci.*, **8** (1) : 47-51.

INTRODUCTION

Silkworm (*Bombyx mori* L.) is a monophagous insect. The silkworm, *Bombyx mori* feeds voraciously on mulberry foliage and increases in mass over five thousand times in about four weeks of larval life. Normal silk production and weight of the matured larvae during 5th instar are directly related to protein content of the leaves. Growth rates and maximal larval weights are significantly different in silkworm races like SK1, SH6 and NB4D2 (Sabhat *et al.*, 2011) and accordingly their protein requirements vary. Miyashita (1986) reported that the productivity of the silkworm is controlled by mulberry leaf (38.20%), climate (37.00%), silkworm rearing techniques (9.30%), silkworm race (4.20%), silkworm egg (3.10%) and other factors (8.20%). The two factors that affect the successful cocoon crop production most are, therefore, environment and leaf quality. The growth rates also vary at different stages of larval development and largely depend on the leaf ingestion level. About 80 per cent of leaf is ingested in 5th instar which increases by each day in the instar. Silk protein biosynthesis begins at the end of larval growth phase and lasts a couple of days. A major portion (about 70%) of the silk protein produced by the silkworm is directly derived from the proteins of the mulberry leaves (Fukuda, 1960) and therefore, the utility of a mulberry protein depends on its solubility and leaf moisture content. Insects have very high free amino acid content in the haemolymph which is considered as a biochemical characteristic of insects (Florkin, 1959). Silkworms obtain 72-

86 per cent of their amino-acids from mulberry leaves and more than 60 per cent of the absorbed amino acids are used for silk production. The best known case of utilization of haemolymph free amino acids in *Bombyx mori* is silk protein synthesis (Corrigan, 1970). The utilization of free amino acids by silk glands of *Bombyx mori* for silk production could be easily observed by direct measurements of amino acid levels in the haemolymph during silk biosynthesis. The synthesis of the larval proteins and their conversion into silk proteins depends on various physiological aspects like maintenance of amino acid pool for protein synthesis, supply of metabolites for energy metabolism, catalysis of interactions between proteins and carbohydrate and protein/amino acid metabolism is brought about by group of enzymes known as transaminases (Mullins, 1985).

Although, the nutritional value of leaf varies in different mulberry genotypes and in different leaf positions within a genotype, how the differences are reflected in the silkworm requires more investigation. It is with this background, that the relative influence of race, mulberry variety and day of 5th instar on haemolymph protein and free amino acids were studied.

RESEARCH METHODOLOGY

Silkworm rearing:

Disease free layings (DFLs) of the three selected silkworm breeds *viz.*, SKAU-R-1, SH-6 and NB4D2 were brushed and reared separately as per the standard rearing technique of Dandin *et al*. (2003) on fresh leaves of the mulberry varieties of Ichinose, Kokuso-20 and Goshoerrami. The experiment was conducted during spring and summer seasons of 2009 and 2010. The total protein and free amino acid levels in the haemolymph of the silkworm races, reared on the leaves of different mulberry varieties, were estimated.

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 4th moult to 5th instar larvae and centrifuged at 3000g for 10 min at 4^{0}C, and the supernatant was collected and used in total protein and free amino acid estimations.

Estimation of total protein:

Protein content was estimated by the method of Lowry *et al*. (1951) using crystalline Bovine Serum Albumin (BSA) as standard and the values were expressed in terms of mg of protein/ml of haemolymph.

Estimation of total free amino acid:

The total free amino acid was estimated by using the method of Moore and Stein (1968) using tyrosine as standard. The total free amino acid content was expressed as mg of free amino acid /ml of haemolymph.

Statistical analysis:

Duncan's Multiple Range Test (DMRT) using a commercially available statistics software package (SPSS for Windows, V. 16.0, Chicago, USA) were used to find significance of differences between the mean levels of protein and free amino acids in the haemolymph of silkworm races and mulberry varieties. Differences were considered significant at $P< 0.05$.

RESEARCH FINDINGS AND ANALYSIS

The levels of total protein and free amino acids in the haemolymph were significantly different at different stages of larval development in all the three races of the silkworm (Fig. 1 and 2 and Table 1 and 2).

Table 1: ANOVA showing the summary of the effect of mulberry varieties on haemolymph protein levels of the selected silkworm races

Treatments	Spring	Summer	Pooled mean of seasons
Varieties			
Ichinose (Control)	45.50±9.82	41.33±8.58	43.41±9.44
Kokuso-20	43.28±9.21	38.84±8.22	41.06±9.00
Goshoerami	49.45±11.2	45.20±10.4	47.32±11.0
CD/LSD @ (P=0.05)	0.227	0.202	0.163
Races			
SKAU-R-1	47.25±10.8	42.68±9.77	44.97±10.53
SH-6	44.83±10.1	40.13±9.13	42.48±9.88
NB4D2	46.14±10.3	42.56±9.33	44.35±10.0
CD/LSD @ (P=0.05)	0.717	0.203	0.163

Means with different superscripts are significantly different from each other for races and mulberry varieties separately

Table 2: ANOVA showing the summary of the effect of mulberry varieties on haemolymph free amino acid levels of the selected silkworm races

Treatments	Spring	Summer	Pooled mean of seasons
Varieties			
Ichinose (Control)	4.80±1.39	3.81±1.02	4.31±1.32
Kokuso-20	4.25±1.05	3.23±0.95	3.74±1.12
Goshoerami	5.64±1.24	4.22±1.09	4.93±1.37
CD/LSD @ (P=0.05)	0.047	0.373	0.032
Races			
SKAU-R-1	4.61±1.49	3.90±1.15	4.25±1.38
SH-6	4.81±1.08	3.53±0.98	4.17±1.21
NB4D2	5.27±1.40	3.84±1.12	4.55±1.45
CD/LSD @ (P=0.05)	0.047	0.037	0.032

Means with different superscripts are significantly different from each other for races and mulberry varieties separately

Haemolymph protein concentration:

The haemolymph protein levels showed a significant linear increase from 0-day to 6th day of 5th instar but dropped on the last day of larval life in all the three races (Fig.1). The mean levels of protein irrespective of seasons were relatively higher in larvae of SKAU-R-1 (44.97 mg/ml) followed by NB4D2 (44.35 mg/ml) and SH-6 (42.48 mg/ml) (Table 1). Among the varieties, the mean haemolymph protein levels of all the races were significantly higher on Goshoerami (47.32 mg/ml) followed by Ichinose (43.41 mg/ml) and Kokuso-20 (41.06 mg/ml). The mean haemolymph protein levels in spring larvae were significantly higher than summer larvae. During spring season, the mean haemolymph protein levels among the varieties were significantly higher on Goshoerami (49.45 mg/ml) followed by Ichinose (45.50 mg/ml) and Kokuso-20 (43.28 mg/ml). However, during summer season, the mean haemolymph protein levels were relatively higher on Goshoerami (45.20 mg/ml) followed by Ichinose (41.33 mg/ml) and Kokuso-20 (38.84 mg/ml). Among the races, the mean haemolymph protein levels during spring season were significantly higher in SKAU-R-1 (47.25 mg/ml) followed by NB4D2 (46.14 mg/ml) and SH-6 (44.83 mg/ml). However, during summer season, the larvae of SKAU-R-1 showed relatively higher mean levels of protein (42.68 mg/ml) than NB4D2 (42.56 mg/ml) and SH-6 (40.13 mg/ml).

Haemolymph free amino acid concentration:

The free amino acid levels at 4th moult in all the three races were found to be significantly higher than other days of 5th instar larval development. The free amino acid levels showed a significant linear decrease from 4th moult to 7th day in the 5th instar in all the three races (Fig. 2). Haemolymph free amino acid levels were found to be lowest on the last day of larval development. The mean free amino acid level for the entire 5th instar larval stage in both seasons was relatively higher in NB4D2 (4.55 mg/ml) followed by SKAU-R-1 (4.25

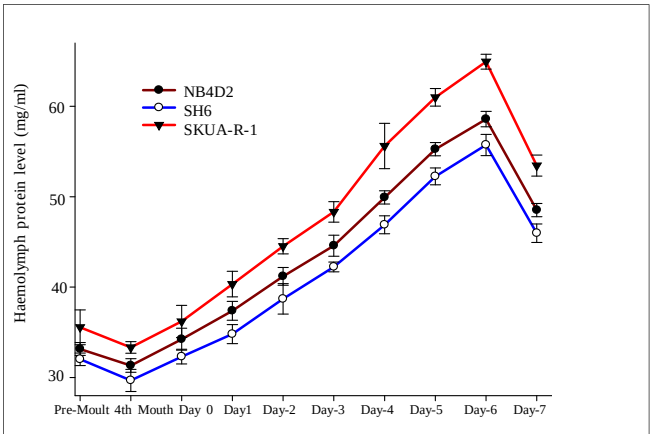

Fig. 1: Mean haemolymph protein levels of SKAU-R-1, SH-6 and NB4D2 on different days of 5th instar larval development

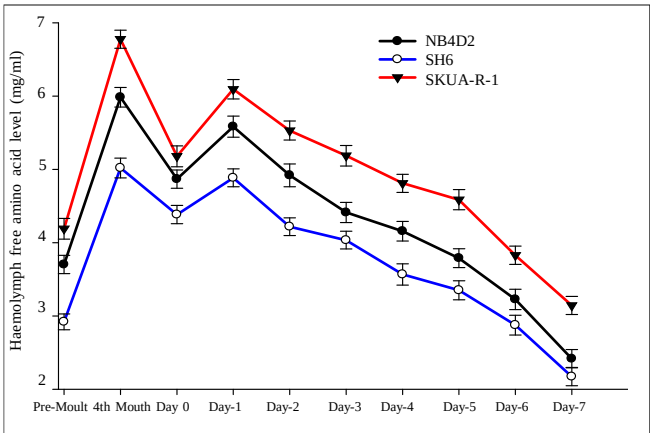

Fig. 2: Mean haemolymph free amino acid levels of SKAU-R-1, SH-6 and NB4D2 on different days of 5th instar larval development

mg/ml) and SH-6 (4.17 mg/ml) (Table 2). Among the varieties, the mean haemolymph free amino acid levels of all the races were significantly higher on Goshoerami (4.93 mg/ml) followed by Ichinose (4.31 mg/ml) and Kokuso-20 (3.74 mg/ml). During spring season, the haemolymph free amino acid levels of all the races were significantly higher on Goshoerami from 4.93 mg/ml followed by Ichinose (4.80 mg/ml) and Kokuso-20 (4.25 mg/ml) (Table 2). However, during summer season, the free amino acid levels showed a general decrease through out the 5th instar larval development and the rate of decrease was found to be greater in larvae reared on Kokuso-20 and Ichinose mulberry variety than on Goshoerami.

The levels of the protein and free amino acid in haemolymph varied significantly when the three selected silkworm races *viz*., SKAU-R-1, SH6 and NB4D2 were reared on different varieties of mulberry *viz.*, Ichinose (control), Goshoerami (uniformly the best), and Kokuso-20 (least productive) mulberry varieties. The results obtained from the above study showed significantly higher increase in haemolymph protein content during spring and summer season in Goshoerami (49.45, 45.20 mg/g) followed by Ichinose (45.50, 41.33 mg/g) and Kokuso-20 (43.28, 38.84 mg/g). Accordingly, a progressive increase in the protein content of the larval haemolymph observed in batches fed with Goshoerami and Ichinose and can be attributed to the nutritional superiority of the leaf and increased consumption by the larvae. The high moisture content with optimal levels of protein fulfilled the nutritional requirements of the larvae, thereby providing essential materials for the synthesis of new membranes and other sub-cellular structures required for cell architecture and are in conformity with the studies of Chen and Levenbook (1966). Ito (1978) reported that utilization of exogenous proteins is an important factor for growth and development of the larva in insects and about 60 per cent of the total nitrogen content of the mulberry leaf is used for silk biosynthesis.

Haemolymph protein levels are in accordance with the growth patterns of the silkworm races. The larvae of SKAU-R-1, SH6 and NB4D2 fed with Goshoerami mulberry variety showed a significantly higher increase from '0' day to 6th day followed by Ichinose and Kokuso-20. But, all the three races responded to mulberry nutrition by an upward change in the haemolymph protein in larvae feeding on leaf of higher protein content. SKAU-R-1 and NB4D2 responded more significantly than SH6 by increasing haemolymph protein content in relation to leaf protein content. Mulberry leaf protein is the dominant source of amino acids though silkworm can synthesize some nonessential amino acids through transamination reactions. Storage proteins synthesized in the fat body, and stored in the fat body and haemolymph serve as rich and continuous sources of free amino acids during periods of high demand or food scarcity (Levenbook, 1985; Nagata and Kobayashi, 1990). Haemolymph protein levels increased during active feeding period from '0' day to the last day of 5th instar. The two factors which controlled haemolymph protein levels were leaf ingestion levels and its protein content. The leaf ingestion level depended on the silkworm race and day of larval development in 5th instar. Gokulamma and Srinivasa Reddy (2003) reported that PM, NB_4D_2 and CSR5 consume 3.16, 7.4 and 6.5 g of mulberry leaf, respectively during 5th instar (Gokulamma and Srinivasa Reddy, 2003). The leaf ingested increased every day and highest consumption levels were observed towards the end of the instar. The drop in the haemolymph protein on the last day of 5th instar resulted from lower levels of leaf consumption and lower protein content of the coarse leaf which is fed during the period and utilization of haemolymph proteins for silk biosynthesis.

The composition of free amino acids in the haemolymph and the influence of dietary amino acids on the haemolymph amino acids is well documented (Inokuchi, 1971; Watanabe, 1980). The content of free amino acids in mulberry leaves affects the cocoon shell of the silkworm (Yamada *et al.*, 1976). It is reported that omission of any of the essential amino acids from the artificial diet leads to block the protein synthesis and enhance the uric acid excretion (Horie *et al.*, 1970). In the present study, the free amino acid levels at 4th moult in all the three races (SKAU-R-1, SH6 and NB4D2) were significantly higher than other days of 5th instar larval development. The free amino acid levels showed a significant linear decrease from 4th moult to 7th day in the 5th instar in all the three races. High haemolymph free amino acid level in silkworm serves many functions like increasing haemolymph osmotic pressure and supporting protein synthesis in tissues during moulting, metamorphosis and silk protein biosynthesis (Mullins, 1985). Haemolymph free amino acid levels respond more significantly to mulberry nutrition than the silkworm race (Table 2). Leaf protein contents which differed more significantly than their free amino acid levels in the three mulberry varieties could have greater role in the response of the haemolymph free amino acid levels to mulberry nutrition. Haemolymph free amino acid levels showed a steady and significant decline during development and were lowest on the last day of 5th instar (Fig. 2). The importance of free amino acid levels appears to be reduced by a gradual increase in haemolymph protein which provide more complete source of all the amino acids required for new proteins. The significant drop in haemolymph protein along with free amino acids on the last day of 5th instar is an evidence of its supportive role in silk protein biosynthesis.

Acknowledgement:

The authors express their gratitude to the authorities of Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir for providing laboratory facilities for the study.

LITERATURE CITED

Chen, P.S. and Levenbook, L. (1966). Studies on the haemolymph proteins of the blow fly *Phormia regina*. I. Changes in ontogenic patterns. *J. Insect. Physiol*., **12**: 1595-1609.

Corrigan, J.J. (1970). Nitrogen metabolism in insects. In: *Comparative biochemistry of nitrogen metabolism*. *The Invertebrates* (Ed. J.W. Campbell, Academic Press, LONDON (U.S.). pp. 387-488.

Dandin, S.B., Jayaswal, Jayant and Giridhar, K. (2003). *Handbook of sericulture technologies*. Central Silk Board, BENGALURU (KARNATAKA) INDIA.

Florkin, M. (1959). The free amino acids of insect haemolymph. In: *Biochemistry of insects* (Ed. L. Levenbook). Pergamon Press, OXFORD (U.S.A.) pp.63-77.

Fukuda, T. (1960). Biochemical studies on the formation of the silk protein. XI. The kinds of free amino acids concerned in the biosynthesis of the silk protein. *J. Seric. Sci., Japan,* **29**: 120.

Gokulama, K. and Srinivasa Reddy, Y. (2003). Role of nutrition and environment on the consumption, growth and utilization indices of selected silkworm breeds of *Bombyx mori* L., *Adv. Tropical Sericul.*, pp. 249-252.

Horie, Y., Inokuchi, T. and Watanabe, K. (1970). Effects of dietary amino acid balances on growth and cocoon quality in the silkworm, *Bombyx mori* L. *Bull. Seric. Exp. Stn*., **24**: 335.

Inokuchi, T. (1971). Effect of dietary condition of free amino acid patterns in haemolymph of the silkworm, *Bombyx mori* L. *J. Seric. Sci. Japan,* **40**: 281.

Ito, T. (1978). Silkworm nutrition, In The Silkworm: *An important laboratory tool*. (Ed: Tazima, Y.) Kodansha Ltd., Japan. pp. 121-157.

Levenbook, L. (1985). Insect storage proteins. In: *Comprehensive insect physiology biochemistry and pharmocology*. Kerkut, G.A. and Gilbert, L.I. (Eds.) Pergmon Press: Oxford, **10**: 307-346.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.S. (1951). Protein measurement with the folin phenol reagent. *J.Biol. Chem*., **93**: 265-275.

Miyashita, Y. (1986). A report on mulberry cultivation and training methods suitable to bivoltine rearing in Karnataka. pp. 1-7.

Moore, S. and Stein, W.H. (1968). A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J. Biol. Chem*., **211**: 907-913.

Mullins, D.E. (1985). Chemistry and physiology of the haemolymph. Comprehensive insects physiology. (Eds. G.A. Kerkut and L.I. Gilbert). Pergamon Press, Oxford, **3**: 355-400.

Nagata, M. and Kobayashi, M. (1990). Effects of nutrition on storage protein concentration in the larval haemolymph of silkworm, *Bombyx mori* L. *J. Sericul. Sci. Japan,* **59**: 469-474.

Sabhat, A., Malik, M.A., Malik, F.A., Sofi, A.M. and Mir, M.R. (2011). Nutritional efficiency of selected silkworm breeds of *Bombyx mori* L. reared on different varieties of mulberry under temperate climate of Kashmir. *African J. agric. Res.,* **6**(1): 120-126.

Watanabe, K. and Horie, Y. (1980). Effects of dietary levels of essential amino acids on the growth on silk glands, components of haemolymph and uric acid excretion in the silk worm, *Bombyx mori. J. Seric. Sci. Japan,* **49**: 177-185.

Yamada, M. (1976). Acid hydrolytic conditions of the silkworm, *Bombyx mori* fibroin for automatic amino acid analysis, *Acta Sericologica*, **98**: 74-83.