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ASPECTS OF PROTEIN METABOLISM IN THE SILKWORM, *BOMBYX MORI* (L), DURING LARVAL-PUPAL METAMORPHOSIS

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ABSTRACT : Changes in the levels of proteins and total free amino acids and the activities of aminotransferase enzymes were assayed in the tissues of the hybrid of LR and NB_4D_2 varieties of the silkworm, *Bombyx mori*, during the larval-pupal transition period. The total protein levels recorded a continuous increase in the larval tissues, viz., the central nervous system (CNS), muscle, silk-gland, fat-body and hemolymph throughout the 5th instar development, but dropped in the pupal tissues. In contrast, the levels of soluble proteins declined in all the larval tissues except in the silk-gland. Significantly, the levels of total free amino acids recorded a fall throughout the 5th instar development, but increased during the larval-pupal transition period. The turnover in the levels of proteins and free amino acids in the larval tissues reflects continuous protein synthesis, particularly the silk protein fibroin, in the silk-gland during the larval growth and development. The activity levels of aspartate (AAT) and alanine (AlAT) aminotransferases registered a continuous decline in the larval tissues, but were significantly elevated during the larval-pupal transition period, apparently suggesting a limited role for transamination process during larval development. The changes in the levels of the biochemical parameters were discussed with reference to histogenesis and histolysis during silkworm metamorphosis.

KEYWORDS : *Bombyx mori*, Metamorphosis, Proteins, Amino acids, Aminotransferases

INTRODUCTION

Silkworm metamorphosis is a dynamic activity, involving many biochemical events, mostly related to protein metabolism. The report of Chen[1] highlighted the role of biochemical constituents in insect metamorphosis. Both qualitative and quantitative aspects of protein metabolism of silkworm have attracted the attention of several investigators[2,3,4,5]. It has been reported that the growth of silkworm during metamorphosis is accompanied by an increase in the body weight and accumulation of various biochemical constituents like proteins, amino acids and enzymes like proteases, glutamate dehydrogenase and aminotransferases[2,6,7,8]. Since silkworm is an economically important insect, physiologists have attempted to elucidate the role of biochemical constituents in silk protein synthesis and egg formation[9]. More importantly, the parameters of protein metabolism have been examined because of their role in development, morphogenesis and in the intermediary metabolism[10]. As a corollary to such studies, the present investigation has been taken up to investigate the changes in some aspects of protein metabolism during the development of the 5th instar larvae and during the transformation of the silkworm larva to the pupa.

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MATERIALS AND METHOD

The present investigation was carried out

on the hybrid of LR and NB_4D_2 varieties of the silkworm, *Bombyx mori*. Day-to-day changes in parameters of protein metabolism, such as the levels of proteins and free amino acids and activity levels of aminotransferase enzymes were examined in the 5th instar larvae and in early pupa. Tissues such as the central nervous system (CNS), muscle, silk-gland, fat-body and hemolymph, isolated by dissecting the larvae in ice-cold silkworm Ringer's solution[11] were used for the biochemical assays. The protein content was estimated by the method of Lowry *et al.*[12] and the free amino acid content by the method of Moore and Stein[13]. The activity levels of aspartate (AAT) and alanine (AlAT) aminotransferases were estimated by the method of Reitman and Frankel[14].

Statistical treatment of data

The difference in levels of different parameters examined between day1 and the remaining days of the instar and early pupa in different tissues was statistically tested. All the assays were carried out with six separate replicates from each group. Values were expressed as mean ± standard deviation (SD) from six replicates. The mean and SD were worked out using INSTAT statistical software and Dunnett's multiple comparison test followed by one-way Analysis of Variance (ANOVA) using the SPSS statistical tool (SPSS for windows, release 17.0.1, 2008, SPSS Inc., Chicago, IL) to assess the differences. P values of <0.05 were considered as statistically significant. Per cent changes were calculated from day1 to each of the remaining days of the instar and early pupa and presented along with the statistical test.

RESULTS AND DISCUSSION

The larval-pupal transition period in silkworm shows profound changes in the levels of biochemical parameters. The present investigation demonstrates that daily changes occur in all parameters of protein metabolism during the larval-pupal transition period.

The data presented in Tables 1 to 6 highlight the day-to-day changes in the levels of proteins, free amino acids and aminotransferases in the 5th instar larva and pupa of the silkworm.

The levels of total and soluble proteins recorded an increasing trend in all the tissues from day 1 to day 6 during the development of 5th instar larvae and then a declining trend in the last day of the instar and in early pupa. The extent of increase varied from tissue to tissue. Contrary to this trend, from the 6th day the levels of total proteins declined on the last day of the instar (i.e. 7th day) and in the early pupa in all the tissues (Table-1).

The soluble proteins witnessed a similar increasing trend in all the tissues except silk-gland, in which their levels dropped continuously up to the early pupa. In contrast, the soluble protein content of silk-gland recorded a drop on the 7th day of the instar and in the early pupa. [The levels of soluble proteins on the 7th day of the instar and in the early pupa were by and large similar to those of total proteins in CNS, muscle fat-body and hemolymph, wherein from the 6th day they decreased on the 7th day and early pupa (Table-2)].

The structural protein levels were elevated in all the tissues up to the 6th day of the instar with minor variations. However, their levels witnessed an overall decreasing trend on the last day of the instar and in the early pupa in all the tissues (Table-3).

The increase in protein levels during the first six days of the 5th instar indicates high rate of protein synthesis during the larval growth and development. The rate of protein synthesis is maximal in the silk-gland followed

by the muscle, fat-body, CNS and hemolymph. The rate of protein synthesis seems to decline during the conclusion of 5th instar and start of pupal stage, as evidenced by the lower levels of proteins in all the tissues of the silkworm (Tables 1 & 2).

In the CNS many proteins are used for the growth and development of the brain and ventral ganglia, while some proteins are used as cholinergic enzymes[15,16] and some others as biological clock proteins[17]. In muscle, most of the proteins are contractile in nature and facilitate feeding and spinning behaviors of silkworm[2]. Obviously, the intensification of these two behaviors is of paramount importance for the 5th instar larvae. The feeding behaviour is more pronounced in early stages and is responsible for the uptake of nutrients, while the spinning behavior is initiated at the end of the 5th instar and is responsible for the spinning of the cocoon. If this is so, the accumulation of higher levels of structural proteins from the beginning of the 5th instar through the 6th day (Table-3) is indicative of consolidation of muscle tissue for increased efficiency of feeding and spinning behaviors during the larval development. In silk-gland, the proteins are used for the synthesis of silk proteins, viz. fibroin and sericin[18]. The great increase (about 317%) in the levels of structural proteins coupled with a decline in the levels of soluble proteins in silk-gland is indicative of continuous synthesis and accumulation of silk proteins. A higher rate of protein synthesis was reported in the fat-body by Martin *et al.*[19] and it seems to act as the storage organ for a variety of proteins and facilitate their migration to other tissues during metabolism. The hemolymph proteins are

Table-1 : Total protein levels (mg protein/g wet wt of tissue or 1 ml of hemolymph) during the 5th instar development of the silkworm *Bombyx mori*

		Fifth Instar							
Tissue	Indices	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Early Pupa
CNS	Mean ± SD % Change 't' test	47.3±4.5	51.9±5.5 +10 NS	59.2±5.8 +25 P<0.05	62.3±6.1 +31.7 P<0.001	70.7±6.3 +49.5 P<0.001	77.4±6.6 +64 P<0.001	73.8±6.3 +56 P<0.001	52.5±5.6 +11.0 NS
Muscle	Mean ± SD % Change 't' test	143.2±13.1	157.6±14.3 +10 NS	165.5±15.5 +16 NS	240.5±13.0 +68 P<0.001	357.4±14.1 +150 P<0.001	379.1±15.9 +165 P<0.001	238.1±12.3 +66 P<0.001	107.0±10.0 -25 P<0.001
Silk-gland	Mean ± SD % Change 't' test	278.8±11.9	310.4±13.9 +11 NS	329.6±15.9 +18 P<0.05	461.0±14.9 +65 P<0.001	589.3±16.8 +111 P<0.001	683.3±16.1 +145 P<0.001	450.0±13.0 +61 P<0.001	212.5±12.4 -24 P<0.001
Fat-body	Mean ± SD % Change 't' test	96.7±8.4	102.1± 9.8 +6 NS	107.6±9.8 +11 NS	108.7±9.9 +12 NS	108.8±10.0 +12 NS	109.9±9.4 +14 NS	66.6±5.0 -31 P<0.001	45.8±4.0 -53 P<0.001
Hemo-lymph	Mean ± SD % Change 't' test	27.3±2.9	32.2±3.2 +18 P<0.01	38.5±3.3 +41 P<0.001	47.4±4.7 +74 P<0.001	93.0±8.3 +241 P<0.001	112.0±10.4 +310 P<0.001	79.5±6.7 +191 P<0.001	75.0±6.5 +175 P<0.001

Note: Each value is the mean ± standard deviation (SD) of six separate observations. For each observation tissue from 20 to 30 animals was pooled. The per cent changes (PC) for the means for each day were calculated against the mean of the first day as the control. Values in parentheses indicate the total per cent changes up to the respective days, taking the first day of the instar as the control. Changes are significant at least at 5% level. NS: Not Significant.

Table-2 : Soluble protein levels (mg protein/g wet wt of tissue or 1 ml of hemolymph) during the 5th instar development of the silkworm *Bombyx mori*

Fifth Instar									
Tissue	Indices	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Early Pupa
CNS	Mean ± SD % Change 't' test	32.9±2.0	35.6±2.2 +8 NS	39.6±3.4 +20 P<0.01	43.0±3.5 +31 P<0.001	45.6±3.6 +37 P<0.001	49.0±3.9 +49 P<0.001	43.9±3.5 +33 P<0.001	38.9±3.4 +18 P<0.05
Muscle	Mean ± SD % Change 't' test	32.3±2.0	39.4±3.1 +22 P<0.01	48.3±3.9 +22 P<0.01	53.3±4.4 +50 P<0.001	55.3±4.6 +71 P<0.001	59.4±4.7 +84 P<0.001	48.0±3.6 +49 P<0.001	37.1±2.5 +15 NS
Silk-gland	Mean ± SD % Change 't' test	127.3±6.6	101.2±5.9 -20 P<0.001	92.1±4.0 -28 P<0.001	71.8±3.5 -44 P<0.001	66.4±3.1 -48 P<0.001	51.5±4.2 -59 P<0.001	44.4±3.4 -64 P<0.001	16.8±1.2 -87 P<0.001
Fat-body	Mean ± SD % Change 't' test	33.8±2.1	35.8±2.9 +6 NS	38.6±3.4 +14 NS	39.5±3.5 +17 NS	41.8±3.9 +24 P<0.05	41.9±3.9 +24 P<0.05	38.1±3.4 +13 NS	5.1±2.8 +4 NS
Hemo-lymph	Mean ± SD % Change 't' test	11.6±2.0	13.5±1.9 +16 NS	19.0±2.0 +64 P<0.001	21.6±2.1 +86 P<0.001	29.1±2.9 +151 P<0.001	33.8±3.2 +191 P<0.001	32.3±3.3 +178 P<0.001	25.5±2.8 +120 P<0.001

Note: Footnote is the same as in Table 1. NS – Not significant.

Table-3 : Structural protein levels (mg protein/g wet wt of tissue or 1 ml of hemolymph) during the 5th instars development of the silkworm *Bombyx mori*

Fifth Instar									
Tissue	Indices	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Early Pupa
CNS	Mean PC	14.4	16.3+13	19.6+36	19.3+34	25.1+74	28.4+97	29.9+108	13.6-6
Muscle	Mean PC	110.9	118.2+7	117.2+6	187.2+69	302.1+172	319.7+188	190.1+71	69.9-37
Silk-gland	Mean PC	151.5	209.2+38	237.5+57	389.2+157	522.9+245	631.8+317	405.6+168	195.7+29
Fat-body	Mean PC	62.9	66.3+5	69.0+10	69.2+10	67.0+6	68.0+8	28.5-55	10.7-83
Hemo-lymph	Mean PC	15.7	18.7+19	19.5+24	25.8+64	63.9+307	78.2+398	47.2+201	49.5+215

Note**:** Each value, obtained by subtracting the corresponding soluble protein value of Table 2 from the corresponding total protein value of Table 1, is the mean of six separate observations. The other details are the same as in Table 1. The data was not given statistical treatment as it represents derived data.

Table-4 : Total free amino acid levels (m moles of tyrosine/g wet wt of tissues or 1 ml of hemolymph) during the 5th instar development of the silkworm *Bombyx mori*

Fifth Instar									
Tissue	Indices	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Early Pupa
CNS	Mean ± SD % Change 't' test	41.4±5.7	31.4±4.7 -24 P<0.05	17.5±2.4 -58 P<0.001	12.8±2.2 -69 P<0.001	10.3±1.4 -75 P<0.001	6.5±1.0 -84 P<0.001	33.4±3.7 -19 NS	9.9±1.4 -77 P<0.001
Muscle	Mean ± SD % Change 't' test	80.5±7.0	68.0±5.6 -15 NS	43.2±5.5 -46 P<0.001	28.7±3.4 -64 P<0.001	25.2±3.0 -69 P<0.001	18.9±2.3 -76 P<0.001	36.2±3.1 -55 P<0.001	20.6±2.9 -74 P<0.001
Silk-gland	Mean ± SD % Change 't' test	83.8±5.5	59.8±5.3 -29 P<0.001	36.7±3.3 -56 P<0.001	15.4±2.4 -82 P<0.001	8.3±2.0 -90 P<0.001	7.6±1.4 -91 P<0.001	19.6±3.8 -77 P<0.001	11.8±2.9 -86 P<0.001
Fat-body	Mean ± SD % Change 't' test	49.4±3.8	47.2±2.9 -4 NS	35.1±3.4 -29 P<0.001	19.8±2.1 -60 P<0.001	9.8±1.4 -80 P<0.001	8.5±1.3 -83 P<0.001	11.9±1.7 -76 P<0.001	10.7±2.2 -78 NS
Hemo-lymph	Mean ± SD % Change 't' test	82.0±6.6	65.7±4.7 -20 P<0.001	36.6±2.0 -55 P<0.001	16.2±2.0 -80 P<0.001	12.5±2.0 -85 P<0.001	6.8±1.6 -92 P<0.001	18.5±2.8 -77 P<0.001	14.0±2.1 -82 P<0.001

Footnote is the same as in Table 1.

Table-5 : Activity levels of aspartate aminotransferase (AAT) (mmoles of pyruvate formed/mg protein/h) during the 5th instar development of the silkworm *Bombyx mori*

Fifth Instar									
Tissue	Indices	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Early Pupa
CNS	Mean ± SD % Change 't' test	1.3±0.07	1.0±0.14 -23 P<0.01	0.9±0.05 -31 P<0.001	0.7±0.04 -46 P<0.001	0.5±0.04 -61 P<0.001	0.3±0.02 -77 P<0.001	1.0±0.07 -23 P<0.01	1.4±0.16 +8 NS
Muscle	Mean ± SD % Change 't' test	1.5±0.08	1.2±0.07 -20 P<0.001	1.0±0.06 -33 P<0.001	0.9±0.04 -40 P<0.001	0.6±0.04 -60 P<0.001	0.5±0.02 -67 P<0.001	1.1±0.06 -27 P<0.001	2.4±0.09 +60 P<0.001
Silk-gland	Mean ± SD % Change 't' test	1.4±0.1	1.1±0.15 -21 P<0.01	0.8±0.03 -43 P<0.001	0.6±0.02 -57 P<0.001	0.4±0.02 -71 P<0.001	0.3±0.04 -79 P<0.001	0.9±0.05 -36 P<0.001	1.1±0.01 -21 P<0.001
Fat-body	Mean ± SD % Change 't' test	2.4±0.06	2.07±0.09 -14 P<0.01	1.9±0.08 -21 P<0.001	1.8±0.08 -25 P<0.001	1.7±0.06 -29 P<0.001	1.5±0.07 -37 P<0.001	2.1±0.1 -12 P<0.05	3.2±0.1 +33 P<0.001
Hemo-lymph	Mean ± SD % Change 't' test	4.0±0.19	2.9±0.1 -27 P<0.001	1.7±0.17 -57 P<0.001	1.4±0.08 -65 P<0.001	1.2±0.07 -70 P<0.001	0.9±0.05 -77 P<0.001	1.2±0.04 -70 P<0.001	1.6±0.11 -60 P<0.001

Footnote is the same as in Table 1.

Table-5 : Activity levels of aspartate aminotransferase (AAT) (mmoles of pyruvate formed/mg protein/h) during the 5th instar development of the silkworm *Bombyx mori*

Fifth Instar									
Tissue	Indices	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Early Pupa
CNS	Mean ± SD % Change 't' test	2.5±0.09	2.1±0.07 -16 P<0.01	1.6±0.1 -36 P<0.001	1.3±0.06 -48 P<0.01	1.0±0.05 -60 P<0.001	0.6±0.05 -76 P<0.001	1.8±0.08 -28 P<0.001	2.3±0.07 -8 NS
Muscle	Mean ± SD % Change 't' test	2.0±0.06	1.7±0.06 -15 P<0.05	1.3±0.05 -35 P<0.001	1.1±0.05 -45 P<0.001	0.8±0.04 -60 P<0.001	0.7±0.02 -65 P<0.001	1.5±0.02 -25 P<0.001	2.8±0.11 +40 P<0.001
Silk-gland	Mean ± SD % Change 't' test	2.4±0.07	1.8±0.05 -25 P<0.001	1.3±0.08 -46 P<0.001	0.9±0.04 -62 P<0.001	0.6±0.04 -75 P<0.001	0.4±0.06 -83 P<0.001	0.6±0.01 -75 P<0.001	1.4±0.07 -42 P<0.001
Fat-body	Mean ± SD % Change 't' test	3.4±0.21	3.1±0.1 -9 NS	2.7±0.09 -21 P<0.001	2.6±0.09 -23 P<0.001	2.5±0.06 -26 P<0.001	2.2±0.05 -35 P<0.001	3.2±0.12 -6 NS	4.3±0.14 +26 P<0.001
Hemo-lymph	Mean ± SD % Change 't' test	5.1±0.27	3.9±0.19 -23 P<0.001	2.4±0.11 -53 P<0.001	1.9±0.07 -62 P<0.001	1.1±0.08 -78 P<0.001	0.7±0.05 -86 P<0.001	1.3±0.05 -74 P<0.001	1.7±0.06 -67 P<0.001

Footnote is the same as in Table 1. NS – Not significant.

implicated in ecdysis, growth of reproductive organs and salivary glands, formation of haemocytes and chitin[20].

Insect metamorphosis is a dynamic process involving both histogenesis and histolysis[21]. The increase in the levels of total, soluble and structural proteins up to the end of sixth day of the 5th instar (Tables 1, 2 & 3) is indicative of histogenesis during larval growth. On the other hand, the declining levels of proteins in all tissues and the corresponding increase in the levels of free amino acids at the end of the 5th instar and in the early pupa reflect the commencement of histolytic activity on the last day of the 5th instar which continues throughout the pupal stage.

Contrary to the proteins, the free amino acids showed a reverse trend in the silkworm tissues, wherein their levels decreased up to the 7th day of 5th instar and dramatically increased on the last day of larva, but once again dropped in the pupa. Their levels dropped until the silkworm reached the 6th day of the 5th instar, and then a partial recovery was recorded on the last day of 5th instar. In the early pupa also their levels recorded a partial recovery in different tissues examined in tune with the trend on the 7th day (Table-4).

The amino acid pool in silkworm is derived both from proteins through histolysis and from non-protein sources like carbohydrates and lipids through *de novo* synthesis[8]. It is likely that amino acids are mobilized from other tissues into the silk-gland and fat-body via the hemolymph, as suggested by Noguchi *et al.*[22]. The larval-pupal transition seems to trigger some assigned metabolic events[6], such as transamination, lipogenesis, maintenance of homeostasis, energy metabolism, formation of hemocytes etc, by actively mobilizing the amino acid pool from the hemolymph and fat-body during metamorphosis. In the whole process,

the fat-body seems to act as the storage organ similar to the liver in vertebrates[23]. Further, hemolymph seems to act as a transitory repository of biochemical constituents, from which tissues retrieve them depending on their need. Thus, a dynamic biochemical exchange mechanism like that of liver and plasma seems to operate in silkworm and other insects that could facilitate the exchange of substances between the fat-body and hemolymph[10,24].

The activity levels of aminotransferase enzymes, viz., aspartate aminotransferase (AAT) and alanine aminotransferase (AlAT) showed a declining trend up to the 6th day of 5th instar and a recovery trend on the 7th day and early pupa. Following this, the enzyme activity recorded a recovery trend in all the tissues On the 7th day and in the early pupa its levels presented a recovery trend (Tables 5 & 6).

Aminotransferases enable the transfer of amino groups of all amino acids except lysine and threonine to 2-oxo-glutarate, oxaloacetate and pyruvate to form glutamate and alanine respectively[25]. The presence of aspartate (AAT) and alanine (AlAT) aminotransferase activity was detected in the silkworm tissues as reported in earlier investigations[2,6,26]. The declining levels of these two aminotransferases from the 1st day to the 6th day of the 5th instar indicate a minimal role for transamination reaction during larval growth and development. This trend seems to be heading for a reversal during the larval-pupal transition period, which indicates increased turnover of amino acids and glutamate formation. The higher levels of free amino acids observed in the present investigation during the early pupal stage *vis-à-vis* the trend towards restoration of activity levels of AAT and AlAT (Tables 4, 5 & 6), support this assumption. This indicates the role for aminotransferases in protein synthesis in silkworm tissues during the pupal growth and development. Through active deamination of amino acids, facilitated by AAT and AlAT, some of the energy requirements of the silkworm are presumably met by way of enhanced intermediary metabolism. The role of aminotransferases in manipulation of various metabolic events like glucogenesis, gluconeogenesis, biological oxidation, histolysis and histogenesis in silkworm[6,26] needs to be ascertained. Such an approach could bring down the duration of life cycle of the silkworm by triggering appropriate metamorphic changes.

The present study demonstrates two important facets of protein metabolism in *Bombyx mori* during larval-pupal metamorphosis. Firstly, the preponderance of protein biosynthesis during larval development and the mobilisation of amino acid pool for enhancing this metabolic step in all the tissues. Secondly, a shift in the amino acid turnover during the larval-pupal transition period in which the transamination reaction takes precedence over protein synthesis. Further, the larval-pupal transition period is characterized by the prevalence of both constructive (histogenesis) and destructive (histolysis) changes. The protein metabolism is stimulated to achieve greater turnover of silk proteins, greater spinning activity, and consequently greater silk output.

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