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Proteolysis-Triggered Muscular Atrophy in The Abdominal Segments of The Silkworm, *Bombyx Mori* During Pupal-Adult Metamorphosis

KEYWORDS: Bombyx mori, Abdominal muscles, Muscle degeneration, Proteolysis

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ABSTRACT *The metamorphic changes in the musculature of the third (AS-3) and seventh (AS-7) abdominal segments were studied in Bombyx mori. During pupal development the abdominal musculature undergoes degeneration to the extent of ~95% in AS-3 and ~77% in AS-7. The persisting pupal musculature includes 9 dorsal internal median muscles (DIM), 4 dorsal lateral internal muscles (DIL), 8 ventral internal median muscles (VIM), 8 ventral internal lateral muscles (VIL), 4 ventral external median muscles (VEM), 6 ventral external lateral muscles (VEL), 21 sternocoxal muscles (SCM) 6 spiracular muscles (SM), 18 tergopleural muscles (TPM) in AS-3 and 7 DIM, 3 DIL, 3 VIM, 5 VEM, 5 VIL, 3 SM and 3 pleurosternal muscles (PSM) in AS-7. Similarly, the persisting adult musculature includes 4 VIM, 4 VIL and 3 DIL in AS-3 and 6 DIM, 6 DIL, 2 VIM, 2 VIL and 3 TPM in AS-7. The muscular atrophy is discussed with reference to the ongoing proteolytic activity.*

INTRODUCTION

The silkworm, *Bombyx mori* is a holometabolous insect. It undergoes a period of postembryonic development and metamorphosis in which larval, pupal and adult stages are produced with distinct form and function. The larva forms shows unique set of behavioural activities such as feeding, crawling, moulting or ecdysis and cocoon spinning, which are markedly different from those of adult activities such as walking, flight, mating and egg-laying. During moulting, it forms a new cuticle underneath the old cuticle, sheds it at regular intervals and finally escapes from the latter by a series of patterned muscular movements that are coordinated by its segmental muscles. Soon after the completion of larval life, it spins the silk cocoon and remains in it in a quiescent but metabolically dynamic state, a process called pupation. The pupal stage represents a transitory stage between the voraciously feeding larval stage and actively mating adult stage. In this stage, the larval tissues of insects are transformed to adult tissues which acquire new characters and functions in tune with the changed roles (Wigglesworth, 1972). During pupal stage too, it forms a new cuticle and sheds the older one and displays a pupal-specific behaviour called gin trap behaviour that is accompanied by the formation of a gin trap circuit involving the posterior abdominal segments (Levine, 1984). Most of the larval muscles, except those involved in gin trap behaviour are retained in the larval abdominal segments. Keeping in view the unique behaviour of pupal and adult stages, enormous constraints are placed on the muscular system. It has been reported that some larval muscles, which have no role in the pupal and adult stages undergo degeneration and new muscles are formed in the adult (Truman and Levine, 1983; Kent *et al.*, 1995). Thus, the silkworm muscle undergoes dramatic transformations in its form and function during metamorphosis, which is of considerable interest for investigators in the field.

Snodgrass (1935 and 1958) delineated a clear roadmap for future investigations in the neuromuscular systems of insects. Since then, the insect muscle has been studied in terms of its morphology, physiology and anatomy (Eaton, 1982, 1987; Burrows, 2007; Kondoh and Obara, 1982). Of late, attempts were made to trace the gross organization of neuromuscular systems in *B. mori* (Sivaprasad, 2009a and 2009 b). Needless to say the larval musculature undergoes dramatic metamorphic changes such as atrophy and reorganization in accordance with the demands of pupal and adult stages. The goal of the present investigation is to trace the extent of muscle transformation in the selected abdominal segments of silkworm, *Bombyx mori* and to correlate muscular atrophy with the ongoing proteolytic activity during pupal-adult metamorphosis.

MATERIALS AND METHODS

The present investigation was carried out on Pure Mysore x CSR_2 hybrid strain of *Bombyx mori*, reared under standard environmental conditions of 28°C, 85% RH as per Krishnaswami, 1986. After hatching, the worms were fed with M_5 variety of mulberry leaves, five times a day at 6 AM, 10 AM, 2 PM, 6 PM and10 PM, under normal 12 hr light and 12 hr dark conditions. The study attempted to trace the pattern of muscle degeneration in the selected abdominal segments vis-à-vis the proteolytic activity in the abdominal musculature during pupal-adult metamorphosis. The experimental design included the study of the organisation of abdominal musculature during pupal and adult stages together with the assay of muscle proteins, protease activity and free amino acid levels.

1. Mapping of abdominal musculature:

The pupal and adult stages were fixed for 24 h in a fixative consisting of 25 ml of 40% formalin, 1.25 ml of acetic acid and 10 g of chloral hydrate in 100 ml of distilled water (Chauthani and Callahan, 1966). They were pinned dorsal side up on a wax block and dissected out in the mid-dorsal region from the last abdominal segment to the head. The reminiscent of gut, fat body and associated trachea were carefully removed along with the fat body and attached tracheae. The gross organization of the abdominal musculature of third (AS-3) and seventh (AS-7) abdominal segments was studied under a stereo-binocular microscope, by applying 1% methylene blue stain in distilled water. Occasionally alcoholic Bouin's fluid was added to the preparation in order to stain the muscle preparation blue-green and to distinguish its lines of demarcation clearly. From such preparations, sketches of musculature were made directly from the dissections. At least five to ten larvae were used to draw the sketches from each segment.

2. Assay of proteolytic activity:

The ongoing proteolysis was determined by assaying the levels of total proteins, protease activity and free amino acid in the abdominal during the pupal and adult stages. The muscle tissue was isolated carefully by mid-dorsal dissection of the silkworm pupae and adults in ice cold *Bombyx* Ringer (Yamaoka *et al.*, 1971) and stored it in ice-cold conditions. The protein profiles of the muscle were estimated on 3rd, 6th and 9th day of pupal development and on 1st day in the adult stage by the method of Lowry *et al* (1951) in 1% homogenate of the tissue in ice cold

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distilled water. The protein levels computed using standard bovine serum albumin, were expressed as mg/g wet weight of tissue. Protease activity was estimated by the method of Davis and Smith (1955) in 5% homogenate of the muscle in ice cold distilled water. The enzyme activity was computed using an amino acid standard and expressed in μ moles of tyrosine/ mg protein/ hour. The free amino acid content of the muscle tissue was estimated by the method of Moore and Stein (1954) as described by Colowick and Kaplan (1957) in 10% homogenate in trichloroacetic acid and expressed as mg/g wet weight of the tissue.

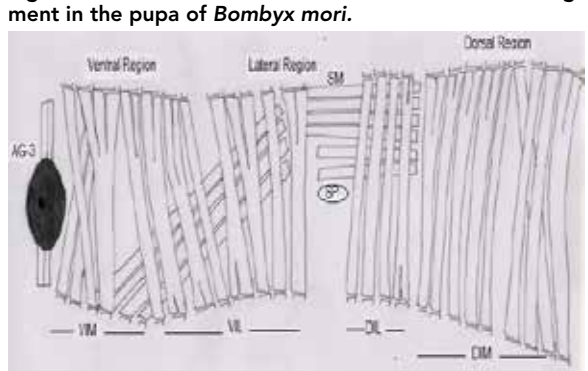
Statistical analysis:
The data were analyzed statistically in terms of mean, standard deviation (SD), percent changes and test of significance. While the mean and SD were computed using M.S Excel platform, the test of significance and percent changes was calculated online by using the Graph Pad (*www.graphpad.com/quick calcs/ index cfm/*)and Percent Change (*www.percent – change com/index php*) tools respectively. The extent of muscle damage during metamorphosis was analyzed by a new statistical parameter called compound periodical growth rate (CPGR), which highlights the extent of daily deterioration of muscle as given by Sivaprasad (2012).

RESULTS AND DISCUSSION
Metamorphic changes in the abdominal musculature in the third and seventh abdominal segments (AS-3 and AS-7) of the pupal and adult stages of *B. mori* have been mapped and its proteolytic activity analyzed with reference to muscle degeneration and the relevant data are presented in figures 1 to 6 and tables 1 and 2.

Metamorphic changes in the abdominal musculature
The fundamental pattern of abdominal musculature in *B. mori* is in consonance with the segmentation of the body (Snodgrass, 1935, 1958; Deshpande and Pathan,1982). Comprehensive pictures of musculatures in the thoracic and abdominal segments of silkworm larva have been drawn-up (Sivaprasad, 2009a, 2009b). The silkworm musculature comprises dorsal, ventral and lateral groups. The dorsal group includes four sets of muscle bands, namely, the musculi dorsales interni mediales or dorsal internal median muscles (DIM), *musculi dorsales externi mediales* or dorsal median external muscles (DEM), *musculi dorsales interni laterales* or dorsal lateral internal muscles (DIL), and *musculi dorsales externi laterales* or dorsal lateral external muscles (DEL). Similarly, the ventral group comprises *musculi ventrales interni mediales* or ventral internal median muscles (VIM), *musculi ventrales externi mediales* or ventral external median muscles (VEM), *musculi ventrales interni laterales* or ventral internal lateral muscles (VIL) and *musculi ventrales externi laterals* or ventral external lateral muscles (VEL). The lateral group encompasses the musculi laterales interni or lateral internal muscles (LIM) and musculi laterals externi or lateral external muscles (LEM). Principally, the former includes tergosternal (TSM), sternopleural (SPM) muscles and spiracular muscles (SM), while the latter is represented by tergopleural (TPM), pleurosternal (PSM) and pleurocoxal (PCM) muscles. In addition, the proleg-bearing AS-3 comprises a group of intrasegmental muscles called sternocoxal muscles (SCM) that connect the sternum with the coxal base of the proleg. Most of these muscles in the third and seventh abdominal segments are either partly represented or seen in the form of remnants of larval muscles or absent altogether in both the stages under study.

Pupal musculature:
In the pupa, the musculature of the third abdominal segment (AS-3) consists of all the three groups of muscles; dorsal, ventral and lateral. These include 9 DIM and 4 DIL in dorsal group, 8VIM 8 VIL, 4 VEM, 6 VEL and 21 SCM in ventral group and 6 SM and 18 TPM in lateral group (Figs. 1 and 2). In the seventh abdominal segment (AS-7), the musculature is simple and includes 7 DIM, 3 DIL, 3 VIM, 5 VEM, 5 VIL, 3LEM and 3 PSM in all the three groups (Table 3). The DIM, DIL, VIM and VIL are represented in the form of longitudinal intersegmental bands, while the SM, SCM, TPM and PSM are represented in the form of either horizontal or oblique intrasegmental strips. Topologically, the DIM are of segmental length and are arranged in intersegmental area of the abdomen in two sub-groups of 4+5 in AS-3 and in one sub-group of 7 in AS-7. Likewise, DIL are arranged in single sub-group, in both the abdominal segments. Both the groups of dorsal muscles (DIM and DIL) are attached to the phragma at either end of AS-3. The topological arrangement of ventral muscles is similar to that of dorsal muscles. They are connected to the furca of intersegmental wall at both the ends. In this arrangement, the VIM is organized in the form of three sub-groups of 3+1+4 in AS-3 and in one sub-group of 3 in AS-7 (Tables 1, 2 and 3). The VIL are organized as a single group of 8 in AS-3 and 5 in AS-7, while the VEM are arranged in a single sub-group of 4 in AS-3 and 5 in AS-7. The lateral group of muscles is disposed either horizontally or obliquely in the abdominal segment. The spiracular muscles (SM) are arranged in the form of two horizontal sub-groups of 4+2 in AS-3, while they are absent altogether in AS-7 (Figs. 1 and 2). They originate on the posterolateral part of the sternal area below the VIL and are inserted on the pleural wall at the spiracle. In AS-3, the TPM are represented as two groups (6+12) of thin flaps on the body wall (shown in dotted lines in figure 2). They arise on the extreme lateral portions of the tergum and are inserted on the pleural portions of the segment. Their appearance and disposition in the folds of abdominal body wall indicate that these two groups of muscles are subjected to degeneration during pupal development. The PSM arise on the sternum at the coxal base and extended horizontally up to the lateral region and connected to the pleura at the other end. The SCM are typically intrasegmental muscles present in the leg-bearing AS-3. They arise on the sterna wall around the leg and extend towards the coxal base from all the directions either horizontally or vertically or obliquely and are attached to the rim of coxa or in its vicinity. Surprisingly, most of them are represented in a state of degeneration on the sterna body wall (shown in dotted lines around coxa in Fig.2). These muscles are not represented in AS-7, due to absence of prologs. It is likely that the degenerating abdominal muscles (mostly external) are finally absorbed into the new cuticle secreted by the epidermal cells, which later becomes brownish and forms the hard exoskeleton of the silkworm pupa.

Fig.1: Internal Musculature of the third abdominal segment in the pupa of *Bombyx mori.*



AG-3: Third abdominal ganglion; DIL: musculi dorsales interni laterale; DIM: musculi dorsales interni mediales; SM: Spiracular muscles; SP: Spiravle; VIL: musculi ventrales interni mediales; VIM: musculi ventrales interni laterals.

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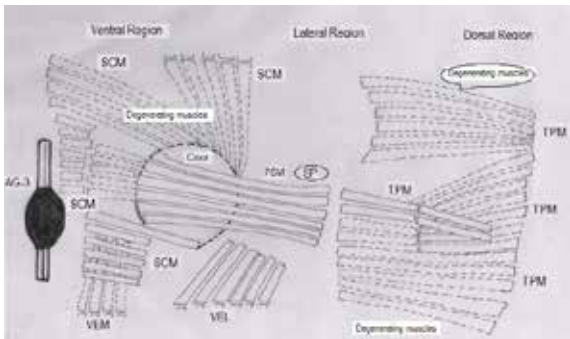
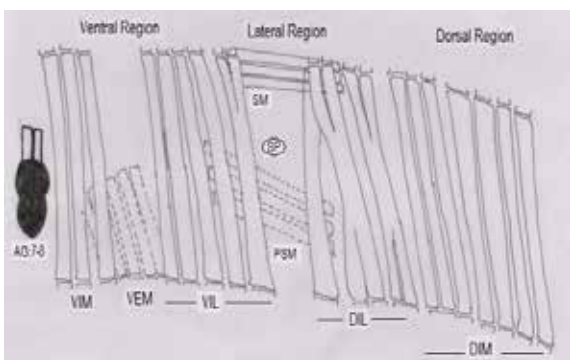
Fig.2: External Musculature of the third abdominal segment in the pupa of Bombyx mori.



AG-3: Third abdominal ganglion; SCM: Sternocoxal muscles; SP: Spiravle; PSM:Pleurosternal muscles; TPM: Tergopleural muscles; VEL: musculi ventrales externi laterales; VEM: musculi ventrales externi mediates.

Fig.3: External and internal musculature of the seventh abdominal segment in the pupa of *Bombyx mori.*

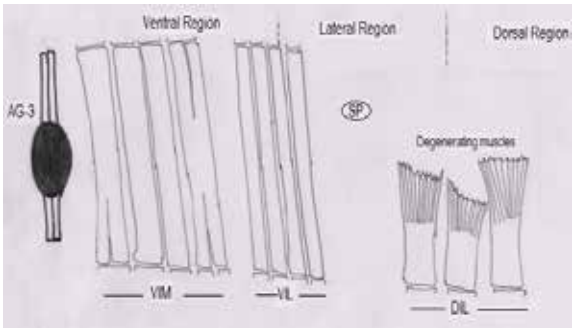


AG 7-8: Seventh and eighth abdominal ganglia; DIL: musculi dorsales interni laterale; DIM: musculi dorsales interni mediales; PSM: Pleurosternal muscles; SM: Spiracular muscles; SP: Spiravle; VEM: Musculi ventrales externi mediales; VIL: musculi ventrales interni mediales; VIM: musculi ventrales interni laterals.

Adult musculature:

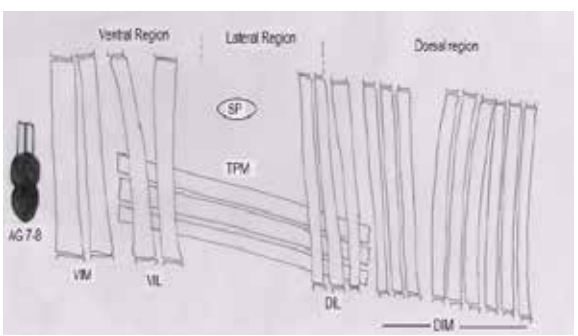
The abdominal musculature in the adult segments is much simpler than that of the pupa and appears to have undergone degeneration during the pupal-adult transition period. In AS-3, the entire musculature is represented in the form of three sub-groups, comprising 4 VIM, 4 VIL and 3 DIL (Fig. 4). Interestingly, the DIL are seen as a degeneration group and are restricted to the posterolateral region of the seventh abdominal segment, where they are seen as short fragmented pieces, with broken anterior ends. The other pupal complements of both internal (DIM, DIL, VIM, VIL, SM) and external (VEM, TPM, PSM) muscles are absent in this adult segment (Fig. 5). However, in AS-3, the musculature is more elaborate and consists of 6 DIM, 6 DIL, 2 VIM, 2 VIL and 3 TPM. The persistence of certain dorsal and ventral groups of muscles in the silkworm pupal and adult stages substantiates the observation that the body movements are confined to dorso-ventral plane due to the formation of rigid cuticle during pupal stage (Levine, 1984).

Fig.4: Musculature of the third abdominal segment in the adult *Bombyx mori.*



AG-3: Third abdominal ganglion; DIL: musculi dorsales interni laterale; SM: Spiracular muscles; SP: Spiravle; VIL: musculi ventrales interni mediales; VIM: musculi ventrales interni laterals.

Fig.5: Musculature of the seventh abdominal segment in the adult *Bombyx mori.*



(AG 7-8: Seventh and eighth abdominal ganglia; DIL: musculi dorsales interni laterale; DIM: musculi dorsales interni mediales; SP: Spiravle; VIL: musculi ventrales interni mediales; VIM: musculi ventrales interni laterals)

Metamorphosis involves muscle degeneration and muscle fusion

A comparative analysis of the muscle maps of the larval, pupal and adult stages indicate that the abdominal musculature is subjected to two metamorphic events; muscle fusion and muscle degeneration. These two events continue throughout pupal stage, either singularly or together. It seems, the external muscles undergo muscle degeneration, while their internal complements are subjected to both muscle fusion and muscle degeneration. A close look at the larval musculature, drawn-up earlier from this laboratory (Sivaprasad, 2009 a, 2009 b) and at the musculature of two abdominal segments recorded in the present study, confirms this fact (Table 1). The rate of muscle degeneration is analyzed in terms of two percentile parameters, namely overall degeneration rate and compound periodical growth rate (CPGR) in order to draw meaningful conclusion. While the former describes the total percentage of damage caused to the muscle at the end of pupal development, the latter provides the extent of damage caused in a day (average daily degeneration rate) during metamorphosis (Sivaprasad, 2012). For instance, during pupal-adult metamorphosis the number of dorsal muscles in AS-3, gets reduced from 38 to 3, showing an overall degeneration rate (ODR) of ~ 92% and a negative compound periodical growth rate (CPGR) of 22.42%. At the same time, its ventral muscles are reduced from 45 to 8 with an ODR of ~82% and a CPGR of -15.86 and the number of lateral muscles declined from 35 to zero, reflecting 100% decline in its ODR and CPGR. The layer-wise analysis of musculature in this

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segment indicate that the muscle degeneration is complete in the external layer, wherein both the parameters; ODR and CPGR stood at 100% level, while those of internal musculature showed an ODR of ~83% and a CPGR of -16.15 during metamorphosis. But in the posterior abdominal segment (AS-7), the muscle degeneration is not comprehensive in any region or part thereof. For instance, the number of dorsal, ventral and lateral muscles is reduced from 35 to 12, 28 to 4 and 30 to 3 respectively with a range of about 52 to 93% in ODR and about 7 to 24% in CPGR (Table 1). Contrary to that of AS-3, the external musculature in this segment is not completely reduced. Its ODR is limited to 93% with a CPGR of -23.72%, while the internal musculature recorded an ODR of ~58% and a CPGR of 8.29% pupal-adult metamorphosis. In all the AS-3 of the fifth instar larva consists of 148 muscles in hemi-segment and 296 in the whole segment and that of AS-7 comprises 83 in hemi-segment and 166 in the whole segment. Obviously, when the larval form transforms into adult through the intervening pupal stage, as many as 280 muscles (296 – 16) disintegrated in the whole AS-3, depicting an ODR of ~95% and a CPGR of 25.31% during silkworm metamorphosis. At the same time, in AS-7, as many as 128 muscles disintegrated (166 – 38) with an ODR of ~77% and a CPGR of 13.71% during the same period (Table 1). Thus, the muscle disintegration is more extensive in the AS-3, while it is not so in AS-3, in which some muscles involved in are retained. The loss of intersegmental muscles (DIM, DIL, VIM , VIL) largely affects the pupal movements, but only such muscles that are essential for pupal defense reflex mechanism, gin trap behaviour, eclosion (adult emergence from cocoon) persist in the abdominal segments (Levine, 1984) . The event of muscle degeneration is associated with another metamorphic event called muscle fusion, especially in the internal musculature. This is clearly observed in the organization of external muscular layers in both the abdominal segments. Morphologically, all the dorsal and ventral muscles in the pupal segments are larger than their counterparts in the larval segments and show vertical partition lines in their middle regions, a point that demonstrates the occurrence of muscle fusion during metamorphosis.

Table 1: Number of muscles present in the third (AS-3) and seventh (AS-7) abdominal segments of *Bombyx mori during metamorphosis.*

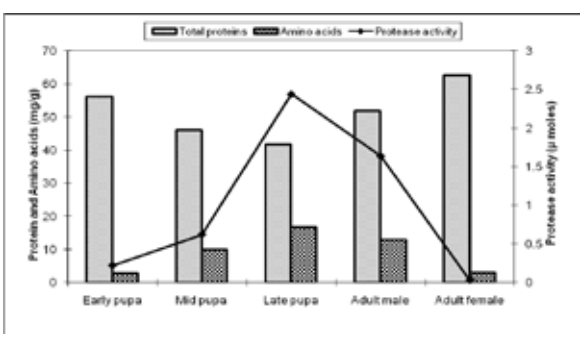
Segment	Stage	No. of muscles (Region-wise)			No. of muscles (Layer-wise)		Total No. of muscles	
		Dorsal	Ventral	lateral	External	Internal	Hemi-segment	Whole segment
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
AS - 1	Larva*	38	45	35	54	64	148	296
	Pupa	13	16	06	29	06	35	70
	Adult	03	08	-	00	11	08	16
	CPGR (%)	-22.42	-15.86	-100	-100	-16.15	-25.31	-25.31
	ODR (%)	-92.1	-82.2	-100	-100	-82.8	-94.6	-94.6
AS - 2	Larva*	25	28	30	45	38	83	166
	Pupa	10	13	06	06	23	21	42
	Adult	12	04	03	03	16	19	38
	CPGR (%)	-7.08	-17.68	-20.57	-23.72	-8.29	-13.71	-13.71
	ODR (%)	-52.0	-85.7	90.0	-93.3	57.9	-77.1	-77.1

***Source: Sivaprasad, 2009 b.(CPGR: Compound periodical growth rate; ODR: Overall degeneration rate).**

Muscle degeneration involves proteolysis

In order to ascertain as to what extent the proteolysis is responsible for muscle degeneration, the levels of total proteins, free amino acids and protease activity has been estimated in the abdominal muscle tissue (Table 2; Fig. 6). The protein profiles of muscle tissue recoded about 18% decline in the mid pupa and ~9% decline in the late pupa. At the same time, the protease activity recorded an increase of ~192% in mid pupa and ~294% in the late pupa. The increase in proteolysis is accompanied by accumulation of free amino acids to the extent of ~267% in the mid pupa and ~68% in the late pupa (Table 2). However, the levels of all the three biochemical constituents in the adult muscle tissue showed trends contrary to those in the pupa. The total protein levels of adult male recorded an increase ~24% and in the adult female it recorded an elevation of 21% during pupal-adult transformation. At the same time the protease activity recorded a decline of ~33% in male and ~82% in female. In tune with declining trends in protease activity, the free amino acid levels recorded a decrease of ~23% in males and ~77% in females (Table 2; Fig. 6). Notwithstanding the prevalence of low proteolytic activity in the adult silkworm, the same in pupal stage demonstrates that the muscle degeneration is accompanied to a large extent by another metamorphic biochemical event called proteolysis. Needless to say, the decline in the levels of total proteins during pupal development with concomitant increase in the activity levels of protease and free amino acid pool amply demonstrates this assumption (Sivaprasad and Sailaja, 2010). It is likely that the degraded muscle proteins are effectively utilized by the silkworm for its energy needs during metamorphosis. Female flies seem to have higher energy requirements than males due to demands from the developing ovaries.

Fig. 6: Levels of total proteins, protease activity and free amino acids in the abdominal muscle of *Bombyx mori* during pupal-adult transformation.



Source: Table 2.

Table 2: Levels of total proteins, free amino acids and protease activity in the abdominal musculature of Bombyx mori during pupal-adult metamorphosis.(Each value represents the mean ± standard deviation (SD) of four separate observations. For each observation tissue from 10 to 15 larvae was pooled).

Stage	Statistical Parameter	Total proteins (mg / g wet wt.)	Protease activity (µ m of tyrosine/ mg protein / hr)	Free amino acids (mg / g wet wt.)
Early pupa	Mean S.D	56.1 1.74	0.22 0.04	2.70 0.3
Mid pupa	Mean P.C S.D	45.9 -18.2 0.57*	0.62 181.8 0.06*	9.90 266.7 0.3*
Late Pupa	Mean P.C S.D	41.7 -9.2 2.0*	2.44 293.5 0.27*	16.7 68.7 0.70*

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Adult Male	Mean	51.8	1.64	12.8
	P.C	24.2	-32.8	-23.4
	S.D	1.73*	0.11*	1.59*
Adult Female	Mean	62.5	0.30	2.90
	P.C	20.7	-81.7	-77.3
	S.D	2.39*	0.03*	0.21*

***Statistically significant (P values: <0.001).**

Thus, the proteolytic activity during plays a vital role in mobilizing proteins during metamorphosis in the silkworm as demonstrated by Yaginuma and Ushizima (2005). The opposing trends in the levels of proteolytic activity in the pupal and adult stages indicate that the muscle degeneration vis-à-vis proteolytic activity is arrested in the adult tissue, obviously due to revamp of abdominal musculature in the genital segments, a fact to be demonstrated in future investigations.

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