

## CHANGES IN THE LEVELS OF PROTEOLYTIC PARAMETERS IN THE FAT BODY AND HAEMOLYMPH OF *BOMBYX MORI* DURING PUPAL-ADULT METAMORPHOSIS

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### ABSTRACT

The parameters of proteolytic activity (total proteins, free amino acids and protease activity) were analyzed in the silkworm, *Bombyx mori* in the thoracic (TFB) and abdominal (AFB) fat bodies and in the haemolymph during pupal-adult metamorphosis. The fat body exists in two different functional forms in the thorax and abdomen and plays regionally distinct roles in the segregation and synthesis of the parameters examined. Findings indicate that the AFB is more resistant to protease degradation and accordingly maintains higher protein-retention capacity and lower protease activity than those of TFB. It is likely that the proteolytic products of one tissue are used-up in other tissues for their growth and development during the ongoing metamorphic events of histogenesis and histolysis. The study also highlights that the growth and differentiation of the thoracic musculature is coupled with degeneration of TFB in the thoracic segments. Conversely, the degeneration of abdominal musculature is coupled with the growth and development of AFB and genital organs in the abdominal segments. In this process, the haemolymph acts as a transport vehicle for proteolytically-derived nutrients and energy reserves and shuttles them among the tissues depending on metamorphic needs and demands.

**Keywords:** *Bombyx mori*, Free amino acids, Protease activity, Proteins, Proteolysis,

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## INTRODUCTION

The pupal-adult metamorphosis of the mulberry silkworm, *Bombyx mori* is characterized by distinct changes in its form and behavior. The metamorphic events result from concomitant changes in the body biochemistry and physiology (Archana, 2006; Hou et al., 2010). The structural changes in its morphology and anatomy are accompanied by two metamorphic events called histolysis and histogenesis (Argell, 1953; Wag et al., 2008). Biochemically these two dynamic events are measurable by analyzing the relative levels of total proteins vis-à-vis free amino acids on one hand and by comparing them with the contemporary changes in the protease activity on the other (Sivaprasad and Sailaja, 2010; Hemalatha et al., 2013; 2014). The protein and free amino acid reserves not only play a vital role in the formation of tissue structures, but also provide the much needed alternative energy base for metamorphosis (Archana et al., 2006; Ramesh Babu et al., 2009). The protease activity maintains homeostasis in the levels of proteins and free amino acids and thus indirectly controls the events of histolysis and histogenesis during pupal and adult metamorphosis in insects (Chen and You, 2004; Wang et al., 2008). The current study takes stock of the metamorphosis-induced changes in the proteolytic activity in the fat body and haemolymph of *B. mori* by analyzing the growth trends in the levels of proteolytic parameters such as the total proteins, free amino acids (FAA) and protease activity.

## MATERIAL AND METHODS

Biochemical investigations on the proteolytic parameters (proteins, free amino acids and protease activity) were carried out on the mulberry silkworm, *Bombyx mori* during pupal and adult stages. Earlier the silkworm larvae were fed with M<sub>5</sub> variety of mulberry leaves, five times a day (at 6 AM, 10 AM, 2PM, 6PM, 10 PM) and reared under standard environmental conditions of 28°C, 85% RH as per Krishnaswamy (1986). Two tissues (i.e., fat body and haemolymph) and four stages (i.e., early, mid and late pupal and adult stages) were chosen for the study. While the haemolymph was collected from pupa and adult by puncturing the dorsal side of the abdomen with a fine needle, the fat body was collected separately from the thorax and abdominal regions by mid dorsal dissection of the pupal and adult bodies in the *Bombyx* Ringer (Yamaoka et al., 1971). The total protein levels were estimated by the method of Lowry et al., (1951), in the 1% homogenate of fat body and 1:19 diluted haemolymph in distilled water. Protease activity was estimated by the method of Davis and Smith (1955) in 5% homogenate of fat body and in the 1:4 diluted haemolymph in distilled water. The free amino acid (FAA) levels were estimated by the method of Moore and Stein (1954) in the 5% homogenate of the fat body and in the 1:19 diluted haemolymph in 10% TCA. The experimental data was statistically analyzed, using an online software package ([www.graphpad.com/quick](http://www.graphpad.com/quick)

calcs/ index cfm/) / (www.percent – change com/index php) and MS Excel platforms. The growth trends in proteolytic parameters were interpreted in terms of compound periodical growth rates (CPGR's) as given by Sivaprasad (2012).

## RESULTS AND DISCUSSION

In insects, the fat body and haemolymph maintain a dynamic metabolic relationship with each other like that of the liver and plasma in vertebrates by coordinating their storage and transport functions (Attardo *et al.*, 2005; Hanson *et al.*, 2005; Arrase and Soulages, 2010). Their pupal-adult metamorphosis is accompanied by constructive and destructive changes in form and function involving histolysis and histogenesis (Yazimura and Ushizima, 2005; Sailaja *et al.*, 2011). In *B. mori*, the constructive metamorphic changes include the formation head, sense organs, thoracic dorsum, wings, wing musculature and the genitalia, while the destructive changes include the termination of moulting cycle, disintegration of gut, salivary glands and abdominal appendages and the selective degeneration of neuro-muscular systems (Singh *et al.*, 2001; Mitra, 2013; Sivaprasad, 2014).

### Proteolytic activity in the fat body

The insect fat body exists in two different functional forms in the thorax and abdomen and performs a myriad of metabolic functions and maintains

biochemical homeostasis (Dean *et al.*, 1985; Haunerland and Shirk, 1995). This function becomes more imperative during pupal-adult metamorphosis due to remarkable changes in physiology and biochemistry in a regionally distinctive manner in the insect body. Such metabolic demands impose enormous constrains on the fat body and make it alter its metabolism in tune with the regionally-distinct metamorphic demands.

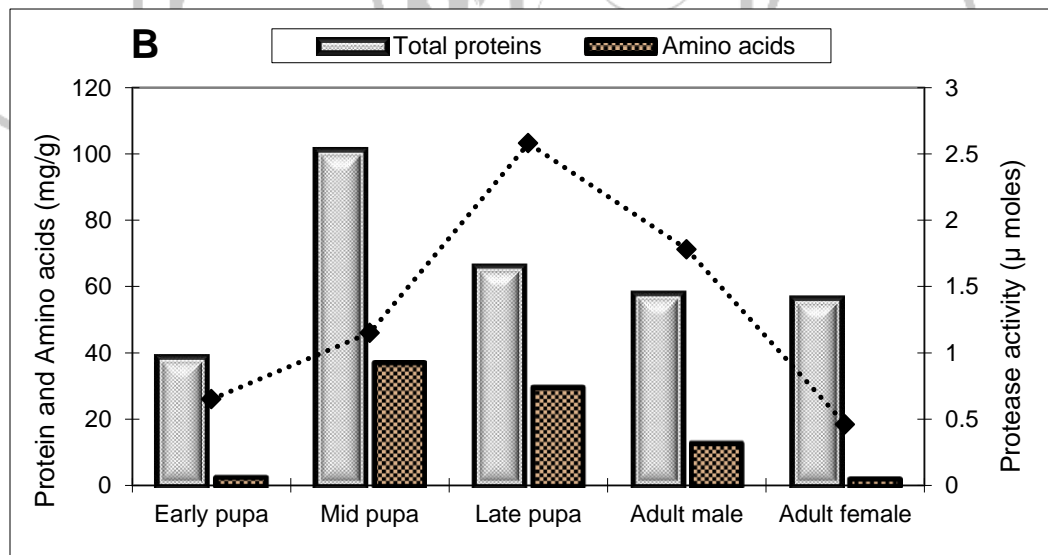
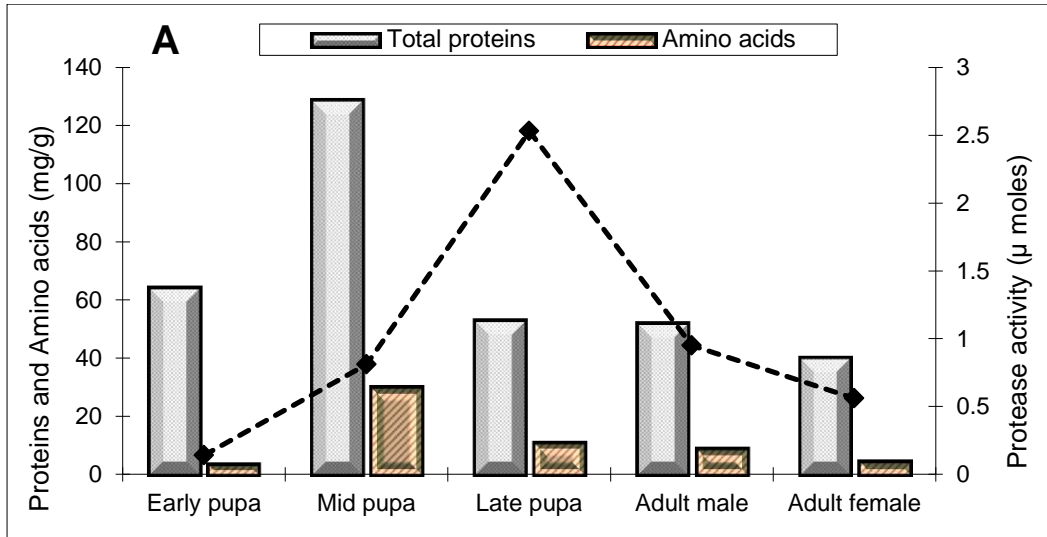
**Thoracic fat body:** The fat body present in the first three thoracic segments (pro, meso and meta-thoracic) was considered as the thoracic fat body (TFB). All the proteolytic parameters of this part of the fat body recorded upward growth trends from the early (Day 1) to mid-pupal (Day 5) stage. During this period, the CPGRs grew @18.83% per day in protein levels, @53.09% per day in protease activity and @66.4% per day in FAA levels. However, during the next four days of pupal development (i.e., from mid pupa to late pupa) the growth rates of proteins and FAA declined @25.48% and 27.70% per day respectively, while those of the protease activity were elevated @46.18% per day (Table 1). During the pupal-adult transition period, their levels recorded significant falls in a sex-specific manner. The levels of total proteins declined marginally (- 0.63% per day) in male moths but moderately (- 8.75% per day) in females. At the same time the protease activity declined @62.45% per day in males and 77.87% in females and FAA levels @18.58% in males and @56.64% per day in females (Table 1; Fig 1A).

**Table 1:** Changes in the levels of proteolytic parameters (Total proteins, protease activity and free amino acids) in the fat body and haemolymph of *Bombyx mori* during pupal adult metamorphosis.

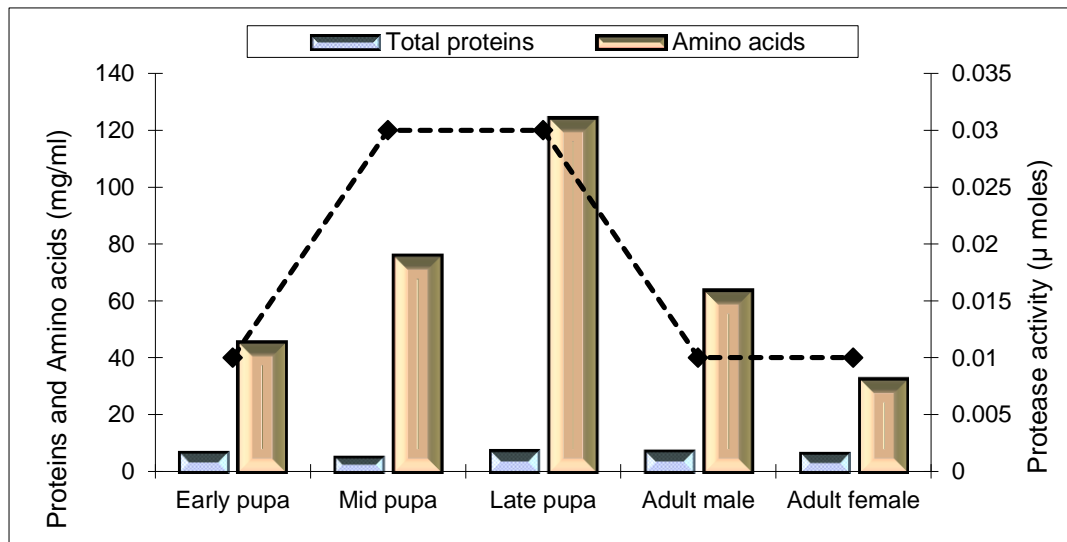
Stage	Statistical tool	Thoracic fat body			Abdominal fat body			Haemolymph		
		Total proteins	Protease activity	FAA	Total proteins	Protease activity	FAA	Total proteins	Protease activity	FAA
Early Pupa (Day-1)	Mean	64.6	0.14	3.9	39.0	0.65	2.8	6.9	0.01	45.9
	S.D	2.21	0.08	0.30	3.04	0.12	0.27	0.24	0.001	0.88
Mid Pupa (Day-5)	Mean	128.8	0.81	29.9	101.2	1.15	36.8	5.4	0.03	76.3
	S.D	±3.49*	±0.09*	±0.42*	±3.90*	±0.06*	±2.54*	±0.22*	±0.005*	±1.37*
	CPGR (%)	18.83	55.09	66.40	26.92	15.33	90.40	-5.94%	31.61%	13.55%
Late Pupa (Day-9)	Mean	53.3	2.53	11.3	66.2	2.58	29.4	7.53	0.03	124.5
	S.D	±2.04*	±0.09*	±0.94*	±2.98*	±0.08*	±1.35*	±0.10*	±0.001**	±3.71*
	CPGR (%)	-25.48	46.18	-27.70	-13.19	30.91	-7.21	11.72%	0.00%	17.73%
Adult Male	Mean	52.3	0.95	9.2	58.0	1.78	12.9	7.4	0.01	64.0
	S.D	±2.77**	±0.10*	±0.32*	±0.70*	±0.15*	±1.08*	±0.05**	±0.001*	±0.45*
	CPGR (%)	-0.63	-62.45	-18.58	-12.39	-31.01	-56.12	-1.73	-66.67	-48.59
Adult Female	Mean	40.5	0.56	4.9	56.6	0.46	2.3	6.6	0.01	32.9
	S.D	±2.89*	±0.05*	±0.66*	±1.07**	±0.02*	±0.05*	±0.05*	±0.001**	±0.95*
	CPGR (%)	-8.75	-77.87	-56.64	-14.50	-82.17	-92.18	-12.35	-66.67	-73.59

The protein values are expressed in terms of mg / gm wet of fat body or mg / ml of haemolymph and the protease activity in terms of  $\mu$  moles of tyrosine / mg protein / hour. Each value represents the mean  $\pm$  standard deviation (SD) of four separate observations, each representing tissues from 10 to 15 larvae. The compound periodical growth rates (CPGR) were computed for different periods on the basis of initial and final values, taken separately as day1- day 5, day 5- day 9 and day 9 - adult stages respectively. \*Statistically significant (P value < 0.001); \*\* Statistically not significant.

**Fig 1:** Changes in the levels of total proteins, protease activity and free amino acids in the thoracic (A) and abdominal (B) fat body of *Bombyx mori* during pupal adult metamorphosis.



**Fig.2:** Changes in the levels of total proteins, protease activity and free amino acids in the haemolymph of *Bombyx mori* during pupal adult metamorphosis.



### Abdominal Fat body

The part of the fat body that extends throughout the eight abdominal segments of the body constitutes the abdominal fat body (AFB). The levels of its proteolytic parameters recorded significant positive CPGRs up to mid pupal stage (i.e., from day 1 to day 5) during which, the proteins grew @26.92%, protease activity @15.33% and FAA @90.40% per day. However, in the second half of the pupal life (i.e., from day 6 to day 9), the CPGR of total proteins declined @13.19% and those of FAA @7.21% per day. But, the protease activity recorded an elevated growth rate of 30.91% per

day during this period (Table 1; Fig 1B). Similar to those of the TFB, the CPGRs of all proteolytic parameters in AFB declined considerably during the pupal-adult transition in a sex-specific fashion. Accordingly, the levels of proteins declined moderately in both males (-12.39% per day) and females (14.50% per day). At the same time the protease activity declined @31.01% in males and @82.17% in females, while the FAA levels were downed @56.12% in males and @-92.18% per day in females (Table 1; Fig 2A).

The current study highlights that AFB is more resistant to protease degradation and consistently



maintains relatively higher profiles of proteins compared to that of TFB. While the rate of accumulation of proteins during the first half of the pupal development (i.e., from day 1 to day 5) grew by 18.83% in TFB, it did so by 26.92% in AFB, representing an additional mobilization of proteins by 8.09 (26.92 - 18.83%) percentile points per day in the abdomen. Interestingly, the day-to-day levels in the protease activity recorded a low growth rate in the abdomen (15.33%) compared to that of the thorax (55.09%). The AFB seems to maintain higher protein-retention capacity than that of TFB during the second phase of pupal-adult metamorphosis. This is evidenced by the prevalence of relatively lower negative growth trends, wherein the protein levels declined @13.19% in the AFB and @25.48% per day in the TFB. The growth trends in the protease activity indicate that the rate of protein degradation is more rapid (CPGR: + 46.18%) in the TFB, compared to that of AFB (CPGR: + 30.91%). The declining trends in the growth rates of total proteins and protease activity were extended into the adult stage with significant difference in male and females. While the TFB in males incurred an additional loss of 11.76 percentile

points (12.39 – 0.63%) in its protein levels, the females suffered a loss of just 5.75 percentile points (14.50 – 8.75%) per day. In consonance with changes in protein levels, the protease activity of TFB declined by 31.44 additional percentile points (62.45 – 31.01%) in males, and by minus 4.3 percentile points (77.87 – 82.17%) per day in females (Fig. 1A, 1B).

Some vital proteins are synthesized, either exclusively or predominantly, in the fat body and other growing tissues of thorax and abdomen in *B. mori* (Pascini et al., 2011). The ups and downs in the proteolytic activity of TFB and AFB are probably caused due to concurrent counter trends in the proteolytic activity in the muscle and other tissues. It has been reported that some larval muscles, which have no role in adults, become defunct and degenerate, while some new muscles develop and assume new roles in the adult moth (Truman and Levine, 1983). Probably, the histogenesis in thoracic musculature and proteolysis in the adjacent TFB are coupled in which the proteolytic products of the latter are mobilized and channelized for the formation of wing and limb musculatures in the growing thoracic dorsum. Thus, greater demands for

organogenesis in the thorax of *B. mori* would have triggered higher proteolytic activity in the TFB, a fact that has amply been demonstrated in the present investigation. Conversely, a high degree (82 to 92% per day) of muscular atrophy has been recorded in the abdomen of *B. mori* during metamorphosis due to increased levels of proteolytic activity in the abdominal intersegmental muscles (Sivaprasad, 2014). Presumably, the products of muscle degeneration (proteins and amino acids) are mobilized to the AFB and contributed to its reserve pool during pupal-adult metamorphosis. Probably because of this reason, the latter is able to sustain its protein levels relatively at higher levels by actively withstanding the onslaught of the persistent protease activity. It is likely that the raw materials required for histogenesis are met from their mobilization from the other tissue and the same has been facilitated by the event of proteolysis (Sivaprasad and Sailaja, 2010).

### **Proteolytic activity in the haemolymph**

The insect haemolymph, being the chief transport system in the body, extends as a continuous channel

between the thorax and abdomen without regional distinction. The early growth trends in the levels total proteins of haemolymph are contrary to that of the fat body. Its protein levels declined from early pupa to mid pupa and increased during the late pupal stage and maintained marginal growth trends thereafter till the adult stage. During pupal development, the protein levels declined @ 5.94% per day from day 1 to day 5 and elevated @11.72% per day from day 5 to day 9. During pupal-adult transition, their levels recorded a marginal decline of 1.73% in males and maximal decline of 12.35% in females (Table 1). The protease activity grew @31.61% per day from day 1 to day 5 of pupal life and remained at the same level during the remaining period of the pupal development. However, the enzyme activity declined by 66.67% in both the sexes examined. On the other hand, the FAA levels were elevated @13.55% per day during the first five days and @17.73% per day during the remaining phase of pupal development. However, during pupal-adult transition, their levels declined by 48.59% in males and by 73.59% in females (Table 1; Fig.2). It is known that the chemical composition of haemolymph varies



as a function of the rate of histolysis and organogenesis (Seong *et al.*, 2005; Sailaja *et al.*, 2011; Sivaprasad, 2014). In a dynamic exchange process, the haemolymph transports and distributes over 298 proteins among the tissues. Because of this reason, the CPGRs of proteolytic parameters rise and fall during metamorphosis. In this mechanism, the fat body alone contributes about 90% of haemolymph proteins (Hoshizaki, 2005). However, most of the haemolymph proteins are mobilized for the growth and development of gonads in adult moths, notwithstanding the fact that some of them are degraded during transportation by the protease activity and provide the energy reserves for eclosion and mating (Sun *et al.*, 2007; Malik and Malik, 2009; Hemalatha *et al.*, 2013). The falls and elevations in the growth rates of proteins, protease activity and FAA in haemolymph, together with their counter trends in the TFB and AFB, amply substantiates this fact. The dramatic disappearance of haemolymph amino acids in the adult males and females is indicative of their mobilization as energy sources through transdemidation during pupal-adult transformation (Hemalatha

*et al.*, 2014). Further, maintenance of sustained levels of proteins in the haemolymph, coupled with slump in protease activity, observed in the present investigation indicates that they play a vital role in ensuring reproductive competence in adult moths of *B. mori* as reported earlier (Maki and Yamashita, 2001).

### CONCLUSION

Broadly, the silkworm metamorphosis is accompanied by histogenesis and histolysis, which involve proteogenesis and proteolysis respectively. Obviously, histolysis in one tissue contributes to histogenesis in the other tissue in during metamorphosis in silkworm. The study demonstrates that the growth and differentiation of the thoracic musculature is coupled with degeneration of TFB in the thoracic segments. Conversely, the degeneration of abdominal musculature is coupled with the growth and development of AFB and gonads in the abdominal segments. In this process, the haemolymph acts as a transport vehicle for proteolytically-derived nutrients and energy reserves among tissues depending on metamorphic needs and demands. Obviously, the nutrients and energy reserves shuttle predominantly among the three

tissues; muscle, fat body and genitals and this shuttling process is facilitated by the haemolymph without regional bias.

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