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Metamorphosis-Triggered Trans-Deamination of Amino Acids in the Silkworm, *Bombyx mori*

KEYWORDS: *Aminotranferases, Bombyx mori, Glutamate dehydrogenase, Metamorphosis,* Transdeamination,

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ABSTRACT *The biochemical process of trans-deamination has been studied during pupal-adult metamorphosis in Bombyx mori with reference to the levels of free amino acids (FAA) and activity levels of aspartate amino transferase (AAT), alanine amino transferase (AlAT) and glutamate dehydrogenase (GDH). The activity levels of AAT, AlAT and GDH in the fat body and haemolymph and the transamination process thereof, appears to be on a raising path in the first half of pupal life and on a declining path in its second half, but raises again during pupal-adult transition. The FAA levels sink and sail with those of the three enzyme activities examined in the present investigation, but notably on a low magnitude. The overall growth trends in the biochemical parameters indicate that trans-deamination process meets the energy demands of metabolism and reproduction and powers the pupal-adult metamorphosis in B. mori, in tissue-specific and gender-specific manner.*

INTRODUCTION

The silkworm, *Bombyx mori* is a holometabolus insect that undergoes remarkable transition from the feeding larval stage to quiescent pupal stage and finally to reproductive adult stage. Being a voracious feeder of mulberry leaves, its larval form obtains all nutrients required for the entire period of its life and the larval-derived energy resources provide energy reserves for metamorphosis and reproduction (Li *et al*., 2001). Its metamorphosis, being an energy-intensive process, involves histolysis, histogenesis, differentiation and morphogenesis which ultimately re-architecture the larval and pupal structures to those of an adult. The so called, pupal-adult metamorphosis is accompanied by a multitude of biochemical events in the fat body, haemolymph, gut and muscle (Sivaprasad and Sailaja 2010; Hemalatha *et al*., 2013). A dynamic metabolic relationship among the tissues is brought out by changing levels of proteins, carbohydrates, lipids, amino acids, enzymes and many other biochemical constituents (Seong *et al*., 2005; Yaginuma and Ushizima, 2005). The fat body and haemolymph are the two important metabolically active tissues which complements each other functions during metamorphosis. In this relationship, the fat body diverts the metabolic activities and produces, stores and releases components for the metamorphic events (Haunerland and Shirk, 1995).

Amino acids are the precursors of proteins and are essential to all living organisms. Their quantitative requirement varies from tissue to tissue and accordingly, they shuttle between the fat body and haemolymph during pupal-adult transformation (Nair and Kumar, 2004; Hou *et al*., 2010). Though the silkworm does not have enzymes required for the synthesis of all amino acids, the nutritionally derived amino acids are reserved in the fat body in the form of storage proteins (Trivedy *et al*., 2009). As the larva prepares to pupate, the protein synthesis is stopped and most of the haemolymph proteins are removed and stored in the fat body (Hoshizaki, 2005). Most of the energy and nutritional requirement of pupal and adult stages are likely to be maintained through trans-deamination, a dynamic biochemical event facilitated by the activity levels of aminotransferases and glutamate dehydrogenase (Bharathi and Sucharitha, 2006). The present study aims at correlating this biochemical process with activity levels of aspartate amino transferase (AAT), alanine amino transferase (AlAT) and glutamate dehydrogenase (GDH) during pupal-adult metamorphosis in *B. mori*.

MATERIAL AND METHODS

The Pure Mysore x CSR_2 hybrid strain of silkworm *Bombyx mori* was selected as test species for the present study. The silkworm larvae were reared under standard environmental conditions of 28° C, 85% RH as per Krishnaswamy, 1986. After hatching, the worms were fed with M_5 variety of mulberry leaves, 5 times a day at 6 A.M, 10 A.M, 2 P.M, 6 P.M and 10 P.M throughout the larval period. Haemolymph was collected from the pupae by making puncture on their dorsal surface with fine needle and the fat body was collected by dissecting them mid – dorsally in *Bombyx* Ringer (Yamaoka *et al.,* 1971). The Free amino acid levels were estimated by the method of Moore and Stein (1954) in 5% homogenate of fat body and 1:20 diluted haemolymph in 10% trichloroacetic acid (TCA). AAT and AlAT activities were estimated by the method of Reitman and Frankel (1957) in 5% homogenate of fat body and 1:19 diluted haemolymph in distilled water. The GDH activity was estimated by the method of Lee and Lardy (1965) in the homogenates of same concentrations prepared in ice-cold 0.25 M sucrose solution. The experimental data was statistically analyzed using online software (www.graphpad.com/quick calcs/ index cfm/) / (www.percent – change com/index php) and MS Excel platforms. In order to draw meaningful conclusions, the growth trend in all biochemical con-

Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

stituents were interpreted in terms of compound periodical growth rates (CPGR's) as given by Sivaprasad (2012).

RESULTS

The trans-deamination process and changes thereof are determined by analyzing the levels of free amino acids, vis-à-vis, the activity levels of AAT, AlAT and GDH and presented in tables 1, 2 and figures1 and 2.

Trans-deamination in fat body

Free amino acids: The results showed that the free amino acids levels of fat body undergo significant quantitative changes during pupal-adult transition. Their levels showed an increasing trend during the first 3 days of pupal development, but subsequently declined till the pupa reaches the adult stage. The elevation in their levels was ~51% during the 3 days of pupal life, but their levels declined by ~20% on day-5, ~26% on day-7, and maximally by ~58% on day-9. But surprisingly, they were elevated by ~223% in male flies and by ~35% in female flies (Table.1and Fig.1).

AAT and AlAT activity: In the fat body both AAT and AlAT activities showed fluctuating trends. The activity levels of these two aminotransferases recorded increasing trends till mid-pupal life. The activity levels of AAT and AlAT, respectively recorded an increase of ~51% and 657% on day-3, in the fat body on day-5, though the AAT activity declined by ~20%, while the AlAT activity rose by ~44% in this tissue . At the end of the pupal life (that is day-9), while the AAT activity declined by ~58%, the AlAT activity increased by ~107% in the fat body. During pupal-adult transition, the AAT activity shot up maximally by ~ 223% in males and marginally by ~35% in females. At the same time, the AlAT activity of fat body was elevated in males by ~65%, but declined in females by ~53% (Table.1 and Fig.1).

GDH activity: In the fat body GDH activity recorded a 3-fold increase (~300%) on day-3, ~21% increase on day-5 and ~85% increase on day-7 of pupal stage. At the end of pupal life (that is on day-9), its activity was slashed by 50%. During pupal-adult transformation, its activity recorded120% increase in males and 60% decrease in females (Table 1 and Fig.1).

Table.1. The levels of free amino acids, AAT, AlAT and GDH activity in the fat body of the silkworm, *Bombyx mori***, during pupal- adult metamorphosis.**

Stage	Statistical parameter	Amino acids	AAT	AlAT	GDH
Pupa day-1	Mean	37.4	0.202	0.17	0.014
	S.D	±1.26	±0.016*	±0.005	±0.004
Pupa day-3	Mean	56.4	1.53	1.12	0.056
	P.C	(50.81)	(657)	(538)	(300)
	S.D	±2.06*	±0.075*	±0.05*	±0.035**
	CPGR	22.8%	-	-	-
Pupa day-5	Mean	45.17	2.20	1.50	0.068
	P.C	(-19.85)	(43.8)	(33.9)	(21.42)
	S.D	±1.46*	±0.081*	±0.04*	±0.017**
	CPGR	-	81.61%	72.35%	48.46%
Pupa day-7	Mean	33.39	0.30	0.21	0.010
	P.C	(-26.07)	(-86.36)	(-86)	(85.29)
	S.D	±2.46	±0.01*	±0.007*	±0.001*
	CPGR	-	-63.07%	-62.58%	-
Pupa day-9	Mean	13.86	0.62	0.47	0.005
	P.C	(-58.49)	(106.6)	(123.8)	(-50)
	S.D	±1.18*	±0.03*	±0.016*	±0.0002*
	CPGR	-20.86%	43.75%	49.60%	-47.93%
Adult Male	Mean	44.75	1.02	0.79	0.011
	S.D	±3.88*	±0.05*	±0.042*	±0.006**
	CPGR	222.87%	64.5%	68.07%	120%
Adult Female	Mean	18.7	0.29	0.26	0.002
	S.D	±1.85*	±0.02*	±0.011*	±0.0002*
	CPGR	34.92%	-53.22%	-44.68%	-60%

***Statistically significant (P values: <0.001). **Statistically not significant.**

Each value in the above table, expressed as mg / g wet weight of tissue in case of amino acids, µ moles of pyruvate formed / mg protein / hour in case of aspartate amino transferase (AAT), alanine amino transferase (AlAT) and glutamate dehydrogenase (GDH) , *represents the mean ± standard deviation (SD) of four separate observations. For each observation tissue from 10 to 15 larvae was pooled. The percent change (PC) for each day was calculated taking its previous value as the control. The compound periodical growth rates (CPGR) were computed for different periods on the basis of initial and final values, taken separately as day1-day 3, day1- day 5, day 5- day 9 and day 9 - adult stages respectively.*

Trans-deamination in haemolymph

Free amino acids: In the haemolymph the trends in the levels of FAA were slightly different from those of the fat body (Table 2 and Fig.2). During pupal development, their levels recorded increasing trends up to the 5th day, but declined marginally on day-3 (-1%) and shot-up again by ~48% on day-5. But, thereafter their levels declined by ~10% on day-7 and by ~31% on day-9. However, during the pupal-adult transition period, their levels declined maximally in males (~39%) and minimally in females (~2%).

AAT and AlAT activity: In the circulating fluid of haemolymph, the AAT and AlAT activities were marked by significant ups and downs (Table 2 and Fig.2). The AAT activity declined by ~77% on day-3, but dramatically rose on day-5 by 281%, only to fall by ~34%, on day-7 and rose again by ~162% on day-9. But, during pupal-adult transition, its activity declined significantly in both males (-69%) and females (94%). At the same time, the AlAT activity declined by ~27% on day-3, increased by ~258% on day-5, again decreased by ~25% on day-7 and increased by ~ 145% on day-9 of pupal life. During transition to adult life, the AlAT activity of haemolymph showed contrasting trends in the two sexes examined. While its activity declined by ~63% in males, dramatically increased by ~302% in females.

GDH activity: The trends of GDH enzyme in haemolymph are by and large contradictory to those of the fat body. Its activity declined by 65% on day-3, but rose to unbelievable heights (~757%) on day-5, only to come down subsequently by 95% on day-7 and by 33% on day-9 during pupal development. During pupal-adult transformation, its activity remained unchanged in males but doubled (100% increase) in females (Fig.2 and Table.2).

Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

Table. 2. The levels of free amino acids, AAT, AlAT and GDH activity in the haemolymph of the silkworm, *Bombyx mori***, during pupal- adult metamorphosis.**

Stage	Statistical parameter	Amino acids	AAT	AlAT	GDH
Pupa day-1	Mean	101.97	0.091	0.026	0.002
	S.D	±3.7	±0.13**	±0.001	±0.0003
Pupa day-3	Mean	100.86	0.021	0.019	0.0007
	P.C	(-1.08)	(-76.9)	(-26.9)	(-65)
	S.D	±1.8**	±0.002*	±0.0005*	±0.0002*
	CPGR	-0.55%	-51.96%	-14.51%	-40.84%
Pupa day-5	Mean	149.13	0.080	0.068	0.006
	P.C	(-47.85)	(281)	(257.8)	(757)
	S.D	±2.25*	±0.001*	±0.005*	±0.002*
	CPGR	9.97%	95.18%	89.18%	192.77%
Pupa day-7	Mean	134.31	0.053	0.051	0.0003
	P.C	(-9.93)	(-34)	(-25)	(-95)
	S.D	±0.99*	±0.004*	±0.003*	±0.00005*
	CPGR	-	-18.6%	-13.40%	-
Pupa day-9	Mean	93.14	0.139	0.125	0.0002
	P.C	(-30.65)	(162.26)	(145)	(-33)
	S.D	±0.98*	±0.009*	±0.003*	±0.0001**
	CPGR	-11.1%	61.94%	56.56%	57.27%
Adult Male	Mean	57.0	0.043	0.046	0.0002
	S.D	±2.98*	±0.004*	±0.021*	±0.0003**
	CPGR	-38.8%	-69.06%	-63.20%	0%
Adult Female	Mean	91.06	0.197	0.185	0.0004
	S.D	±3.8*	±0.011*	±0.005*	±0.0002**
	CPGR	2.23%	-94.41%	302.17%	100%

***Statistically significant (P values: <0.001). **Statistically not significant.**

Each value in the above table, expressed as mg / ml of tissue in case of amino acids, µ moles of pyruvate formed / mg protein / hour in case of aspartate amino transferase (AAT), alanine amino transferase (AlAT) and glutamate dehydrogenase (GDH), *represents the mean ± standard deviation (SD) of four separate observations. For each observation tissue from 10 to 15 larvae was pooled. The percent change (PC) for each day was calculated taking its previous value as the control. The compound periodical growth rates (CPGR) were computed for different periods on the basis of initial and final values, taken separately as day1- day 3, day1- day 5, day 5- day 9 and day 9 - adult stages respectively.*

DISCUSSION

When glucose is not available in adequate amounts from the dietary sources, its requirements are met by synthesis from non-carbohydrate sources, through gluconeogenesis. The silkworm faces this situation during pupal adult metamorphosis. When it's feeding larval form transforms into pupa, it becomes quiescent till it metamorphosis into an adult. Needless to say, the metamorphosis of silkworm involves great many changes in its morphology, anatomy, physiology and biochemistry. The morphological and anatomical events do not fall under the preview of present investigation. But, nonetheless, the physiological and biochemical processes are directed towards the single objective of generating energy resources (i.e. glucose) during metamorphosis. One such mechanism is transdeamination in which three enzymes, viz., AAT, AlAT and GDH metabolize selective free amino acids and provides the much needed carbon skeleton for the generation of glucose that drives the metabolic machinery during silkworm metamorphosis (Li and Xu, 1982).

Amino acids act not only as precursors for proteins but also participate in a variety of functions. More importantly, they are converted to carbohydrates and serve as glucogenic amino acids and give rise to hormones, vitamins and bile salts. The fat body of insects plays an important role in amino acid metabolism principally, because it is the major site of protein storage. Aminotransferases are the enzymes, which catalyze the transfer of the amino group of one amino acid to a keto acid, so as to form another amino acid. During transamination reaction amino group from many different amino acids are collected in the form of L-glutamate, which then acts as an amino group donor for biosynthetic pathways or for excretion pathways that lead to the elimination of nitrogenous wastes. Glutamate dehydrogenase (GDH) is the enzyme present in the mitochondrial matrix, which causes the conversion of glutamate to alpha-keto glutarate, during which energy is released. The combined reaction, catalyzed by transaminases (AAT, AlAT) and GDH is called trans-deamination (Harper, 1963). The alpha –keto glutarate during this process enters the citric acid cycle for glucose synthesis. The fat body, being a dynamic metabolic organ, monitors these physiological functions and coordinates the growth, metamorphosis and reproduction in insects (Scott *et al.*, 2004; Hoshizaki, 2005;Arrese and Soulages, 2010).

Fig. 1: Changes in the levels of FAA and AAT and AlAT and GDH activity in the fat body of metamorphosing silkworm *Bombyx mori***. The amino acid values are expressed in mg /g wet weight of tissue and AAT, AlAT and GDH levels were expressed as pyruvate formed / mg protein / hour. (P values: <0.001)**

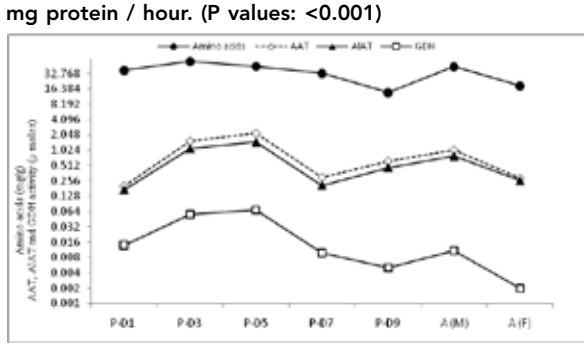
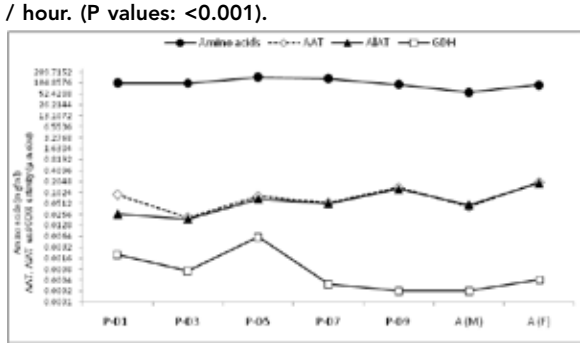


Fig. 2: Changes in the levels of FAA and AAT, AlAT and GDH activity in the haemolymph of metamorphosing silkworm *Bombyx mori***. The amino acid values are expressed in mg /ml of tissue and AAT, AlAT and GDH levels were expressed as pyruvate formed / mg protein / hour. (P values: <0.001).**



Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

The present study reveals that the amino acids of fat body raised up to third day of pupal development with a positive compound periodical growth rate of 22.8%, and declined continuously during the remaining phase of pupal life depicting a negative CPGR of 20.86%. During pupal-adult transition period its free amino acid levels were elevated by ~223% in the male flies and by ~35% in female flies (Table 1 and Fig.1). It is known that the fat body synthesizes and accumulates a variety of proteins during metamorphosis (Haemalatha *et al.*, 2013). The free amino acids required for protein synthesis, are probably supplied through the circulating fluid of haemolymph. The elevation in their levels in males is higher than that in females, mainly due to the difference in the rate of degradation of sulphur amino acids between male and females, which are involved in egg shell formation (Inokuchi, 1981; Trivedy *et al.*, 2009; Hou *et al.,* 2010). Contrary to those of the fat body, the haemolymph amino acids showed declining CPGR of 0. 55% during the first 3 days of pupal development, but their levels recorded a positive CPGR of 9.97% by mid pupal life. Thereafter, they declined continuously till the end of pupal life. Further decline in their levels were noticed in adults, which recorded a negative CPGR of 38.8% in males and 2.23% in the females (Table 2 and Fig.2). Thus, the haemolymph acts as a transient reservoir for amino acids and shuttles them between histolytic and histogenic organs (Hemavathi, 2001; Sivaprasad and Sailaja, 2011).

Our study confirms the prevalence of an exchange mechanism between fat body and haemolymph – an analogous transitory mechanism that operates in between the liver and blood in higher vertebrates. This of course, follows a specific trend in *Bombyx mori, in which* the elevation in the levels of free amino acids are accompanied by raise in the activity levels of AAT, AlAT and GDH until day-5 of pupal life. Later on, except for AAT, the activity levels of AlAT and GDH showed declining trends up to the last day of pupal life (Fig. 1 and 2). This suggests that the trans-deamination process continues actively till mid pupal life (day-5 of pupa) and meets the energy requirements all through the period of pupal development either through enhanced gluconeogenesis or other processes as cited in earlier reports (Venkataramireddy *et al.,* 1992a; Sinha *et al.,* 1996; Ramakrishna and Jayaprakash, 2007). The decline in the activity levels of AAT, AlAT and GDH vis-à-vis free amino acids levels in the second half of pupal life (i.e from day-5 to day-7) indicates that the energy reserves, so created are used-up during the later half of the pupal development. It is significant to note that all the enzymes involved in transamination in the fat body increase during pupal-adult transformation and reach peaks in male flies. This amply demonstrates that the trans-deamination process meets the energy demands of the reproductive functions such as the sperm motility, copulation and fertilization in males and egg-laying behavior in females in *B. mori* (Bharathi, 1984; Osanail *et al.*, 1986; Venkataramireddy *et al.*,1992b; Pushparani, 1997; Pushparani and Bharathi, 1999). Interestingly, the current study further indicates that the tissue-specific transamination reaction takes place in the silkworm in a sex-specific pattern. While, the trans-deamination process takes precedence in the fat body of males, it does so in the haemolymph of females. Notwithstanding the fluctuations in the rate of transamination, the free amino acid levels are mobilized at a constant rate through the haemolymph, both in males and females, probably with a view to maintain nitrogen balance and homeostasis during metamorphosis (Bhaskar, 1982; Bharathi and Govindappa, 1987; Hemavathi, 2001).

Though the present investigation is not exhaustive, nevertheless it highlights the occurrence of heavy turnover of amino acids during pupal-adult metamorphosis in the silkworms. This turnover, probably involves high protein synthesis in newly emerging organs like gonads and amino acid catabolism through trans-deamination in the remodeling tissues like the fat body, which apparently represents the main source for energy generation during metamorphosis.

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