



**Ultrasound induced enhancement of Protein Metabolism  
and Enzyme Activities in the Fat body of Fifth instar  
Silkworm, *Bombyx mori* L.**

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**Abstract**

The parameters of protein metabolism, such as levels of total, soluble proteins free amino acids, activity levels of protease, glutamate dehydrogenase, aspartate amino transferase and alanine aminotransferase were assayed in the fat body of the fifth instar larva of silkworm, *Bombyx mori*, under the impact of ultrasound. In general ultrasound has an elevatory effect on these parameters. Changes in the levels of these biochemical constituents are correlated with the events of histogenesis and histolysis associated with the silkworm metamorphosis. Under the influence of ultrasound protein metabolism is stimulated to achieve greater turnover of silk proteins, greater spinning activity, and consequently greater sericultural output.

Key-Words: Amino Acids, Aminotransferases, Glutamate dehydrogenase, Protease

**Introduction**

Proteins constitute an essential component of all living cells. If carbohydrate metabolism represents the driving force of life, the protein metabolism represents life itself. Essentially, proteins are responsible for the maintenance of structural and functional organization of the cells. They play a vital role in respiration, enzyme catalysis, transport of materials, and regulation of metabolism, movement and body defense. The total protein content of the cell includes both structural and soluble portions, of which the former plays main role in cellular architecture and the latter in cellular metabolism.

Proteins have always been an interesting biochemical tool for insect biochemists because of their prominent role in development, morphogenesis and the intermediary metabolic pathways in insects. Mihaeslu *et al.* [1] observed biochemical difference in the eggs of *Bombyx mori* and *Philosamia ricini* with regard to their total proteins and amino acids. The first ever observation on insect haemolymph proteins was made by Lauffer [2] in the silkworm *B. mori*. Subsequently a series of detailed physico-chemical studies on haemolymph proteins of *B. mori* appeared [3].

Tojo *et al* [4] made a detailed account of storage proteins while Sasaki *et al.* [5] investigated the intracellular transport and secretion of fibroin in the posterior fat body of *B. mori*. A novel approach in silkworm research is the manipulation of biochemical machinery through exogenous modulators that could boost the silk production. The development of the worm is depending on metamorphosis process which is a dynamic biochemical process [28]. The growth of silkworm during metamorphosis is accompanied by the increase in the bodyweight and accumulation of various biochemical constituents like proteins, aminoacids and enzymes like proteases, glutamate dehydrogenase and aminotransferases [29]. Since, the silkworm is an economically important insect, several insect physiologists attempted to elucidate the role of biochemical constituents in silk protein synthesis and egg formation [30]. More importantly, the parameters of protein metabolism have been extensively examined because of their role in development, morphogenesis and in the intermediary metabolism [31, 32]. A novel approach in silkworm research is the manipulation of biochemical machinery through exogenous modulators that could boost the silk production. This obviously, included the administration of certain neuro-humoral factors, vertebrate hormones and various other chemicals like cyclic AMP and prostaglandins, which could have a profound influence on the growth rate,

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larval life cycle and fecundity [6, 7]. Significantly, the positive impact of vertebrate thyroxine on silkworm biology, especially in improving the pre- and post cocoon parameters is well documented [8]. Another vertebrate hormone, namely prolactin induced improvement in the growth and reproductive potential of silkworms [7]. The dietary administration of vertebrate sex hormones like ethynyl estradiol and norethindrone to the silkworm increased the larval weight, cocoon and shell weights, female pupal and adult weights, but the larval, pupal periods and the egg-hatchability were significantly reduced [9].

These investigations opened up alternative strategies for improving the economic parameters of the sericulture industry by regulating the biochemical machinery. One such option is the ultrasound, whose impact on larval life in *Drosophila* has been reported [10]. Further, it was reported that ultrasound irradiation does not cause any detectable deterioration in behavioral responses such as mating, oviposition, larval development and pupation in insects [11]. In view of its harmless nature, ultrasound has been used as an exogenous modulator in the present investigation for the manipulation of protein metabolism in the fat body silkworm and to examine the possibility of its utility in sericulture.

### Material and Methods

Multivoltine x bivoltine hybrid variety of the silkworm, (Pure Mysore x NB<sub>4</sub>D<sub>2</sub>) *Bombyx mori* L used in the present investigation were obtained from the Central Seed Farm at Tondavada, a suburb of Tirupati, A.P., India.

### Ultrasound treatment

Silkworm eggs were irradiated with ultrasound waves, 10-12 h after hatching (blue-egg stage) by water bag method used as experimental samples while those not exposed to Ultrasound waves were treated as Controls. Prior to exposure, the eggs were kept in a sealed, water-filled polythene cover, smeared with gel so as to prevent the diversion of ultrasonic waves by water bag method. The larvae that emerged from the exposed (experimental) and unexposed (control) eggs were used for the experiment. Biochemical assays were carried out on the fifth instar larvae because of their large size and easy isolation of tissues in adequate quantities since it is the stage that culminates in cocoon-spinning. The duration of exposure was standardized by exposing the eggs to varying intensities of ultrasound waves, at different time intervals, viz. 2, 5, 10, 15, 20, 25, and 30 minutes. Promising results were obtained at 1 MHz, continuous wave of ultrasound at an intensity of 9W/Cm<sup>2</sup> for 2 minutes. The larvae that

emerged from the exposed (experimental) and unexposed (control) eggs were used in the investigation.

### Tissue separation

Fat body was isolated by dissecting the larvae in ice-cold silkworm Ringer [12] were used for the biochemical assay.

### Analysis of protein metabolism and enzyme assay

Day-to-day changes in biochemical parameters of protein metabolism such as total, soluble proteins and free amino acid, the activity levels of protease, aminotransferases, and glutamate dehydrogenase were examined in the fifth instar larvae. The protein content was estimated by the method of Lowry *et al.* [1], the free amino acid content by the method of Moore and Stein [14] as described by Colowick and Kaplan [15] and the protease activity by the method of Davis and Smith [16]. The activities levels of aminotransferases, viz, aspartate aminotransferase (AAT) and alanine aminotransferase (AIAT) were estimated by the method of Reitman and Frankel [17] as described by Bergmeyer and Bruns [18] and the activity of Glutamate dehydrogenase (GDH) was assayed by the method of Lee and Lardy [19].

### Statistical Analysis

Standard deviation was calculated using the following formula:

$$\frac{\sum x^2 - \frac{[\sum x]^2}{n}}{n - 1}$$

Where,

$\sum x$  = individual observation

n = total number of observations

Student's 'T' test was calculated by using the following formula:

$$X_1 - X_2$$

$$t = \frac{\quad}{\sqrt{s^2 [1/n_1 + 1/n_2]}}$$

$$S_2 = \frac{1}{n_1 + n_2 - 2} [n_1 s_1^2 + n_2 s_2^2]$$

Where,

X<sub>1</sub> = mean of the first set of observations

X<sub>2</sub> = mean of the second set of observations

S<sub>1</sub> = standard deviation of the first set of observations

S<sub>2</sub> = standard deviation of the second set of observations

n<sub>1</sub> = number of observations of the first set

n<sub>2</sub> = number of observations of the second set

### Results and Discussion

The levels of total and soluble proteins recorded an increasing trend in the fat body from day 1 to day 6 during the development of fifth instar larvae. The levels of total proteins increased from 43.48 mg to 81.07 mg in fat body (26% rise). Ultrasound has an elevatory effect on total protein content (Table 1). The levels of soluble proteins increased from about 27.77 mg to 60.04 mg. Ultrasound in general caused an elevation in the levels of soluble proteins also, with varying intensities (Table 1). The levels of free amino acids showed a similar trend during fifth instar. The impact of ultrasound on free amino acid levels is positive (Fig. 1).

The activity levels of protease recorded an upward trend through out the fifth instar. The elevation is more pronounced in the fat body (0.053 to 0.08  $\mu$ moles). Ultrasound caused an elevation in the enzyme activity in the selected tissue (Table 2). The activity levels of aspartate aminotransferase enzyme activity decreased in fat body (0.58 to 0.46  $\mu$ moles of pyruvate formed/mg protein/h). The impact of ultrasound is elevatory (Table 2). Alanine aminotransferase activity showed a similar trend during fifth instar development. The enzyme activity decreased from 2.33 to 1.96  $\mu$ moles of pyruvate formed/mg protein/h in the fat body. Ultrasound caused an elevation in the activity notwithstanding its minor fluctuations in controls. The glutamate dehydrogenase (GDH) activity was decreased in the fat body (0.44 to 0.22  $\mu$ moles of formazon formed/mg protein/h) during the fifth instar development. Ultrasound caused an elevation in GDH activity levels (Fig. 2).

The impact of ultrasound on protein metabolism is profound as evidenced by upsurge in the levels of all the biochemical parameters examined. Though, increased levels of proteins were observed in silkworm tissue, these parameters were not analyzed with reference to ultrasound. However, some earlier investigations [20] attempted to elucidate the effect of ultrasound on protein synthesis. Obviously, the intensification of these two behaviors is of paramount importance for the fifth instar larvae. In fat body, the proteins are used for the synthesis of silk proteins, viz, fibroin and sericin [21]. The Increased levels of total, soluble and structural proteins in silkworm tissue indicates the growth-promoting nature of ultrasound when applied in lower dosages and indicate a promising future for the sericultural industry. Apparently, ultrasound seems to enhance the protein synthesis in general, with a bias towards the silk proteins in fat body. Amino acids are the building

blocks of the proteins. Ultrasound irradiation caused an elevation in the levels of free amino acids. The silkworm and other lepidopteran insects are known to contain unusually large amounts of free amino acids [22]. Insect metamorphosis is a dynamic process involving both histogenesis and histolysis [23]. Obviously, 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. Continuous increase in the levels of free amino acids following ultrasound-treatment is attributable to the synthesis of amino acids from non-protein sources like glucose and fatty acids [24].

Protease activity levels recorded a continuous increase throughout the fifth instar development as reported earlier in silkworm and other insects [25]. The positive impact of ultrasound on enzyme activity indicates its ability to degrade proteins by activating proteolytic enzymes. Histolysis seems to be more pronounced in fat body as evidenced by increased turn over of amino acids. This could probably bring about the degeneration of fat body during pupal stage, leaving the fatbody that forms the bulk of pupal weight. The presence of aspartate (AAT) and alanine (AIAT) aminotransferase activity was detected in fat body of silkworm as reported in earlier investigations [26]. Ultrasound caused an elevation in the activity levels of both AAT and AIAT in silkworm tissues (Table 2), indicating the increased turnover of amino acids and glutamate formation during metamorphosis in silkworm. The higher levels of free amino acids observed in the present investigation (Table 1) support this assumption. Ultrasound has an elevatory effect on GDH activity in all the tissue of silkworm (Table 2). Some reports are available on GDH activity in silkworm, *Bombyx mori* [26]. The enhanced activity of GDH in fat body is indicative of increased oxidation of glutamate in this tissue. The  $\alpha$ -ketoglutarate generated by this enzyme is probably used-up in ensuring sperm mobility in silkworm [27]. The actual mechanism of ultrasound irradiation on protein metabolism is not clear.

The role of ultrasound in protein metabolism needs special mention in economically viable insects like the silkworm, in view of its profound and positive impact on biochemical parameters. Under its influence entire biochemical machinery in silkworm is geared up to synthesise silk proteins in fat body during fifth instar development. While the former are used up as the raw materials for the cocoon, the latter are used for generating a muscular mechanism necessary for spinning the cocoon at the end of fifth instar. The

increase in the concentration of amino acids with concomitant increase in the levels of total and soluble proteins in fat body under the impact of ultrasound reflects this fact. Metabolically fat body seems to occupy a pre-eminent position during metamorphosis in silkworm. It may be concluded that under the impact of ultrasound protein metabolism is stimulated to achieve greater turnover of silk proteins, greater spinning activity, and consequently greater sericultural output.

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**Table 1: Day-to-day changes in fatbody protein metabolism during the 5<sup>th</sup> instar of *Bombyx mori* under the impact of ultrasound (9W/Cm<sup>2</sup> for 2 minutes). Each value is the mean ± Standard Deviation (SD) of six separate observations. For each observation, tissue from at least 10 larvae was pooled. The percent changes for all days were calculated taking control as the reference**

		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Total Protein (mg proteins/g wet weight)	Control	35.19 ± 9.49	43.48 ± 6.21	49.69 ± 6.21	55.9 ± 6.21	57.97 ± 9.48	64.54 ± 10.0
	Treated	43.48 ± 6.21	51.76 ± 9.48	62.11 ± 6.21	68.32 ± 6.21	74.53 ± 6.21	81.07 ± 5.73
	%	23.6	19.0	24.9	22.2	28.6	26.6
	Change t-Test	1.2849 NS	1.2649 NS	2.4494 NS	2.4494 NS	2.5297 NS	2.4812 NS
Soluble Proteins (mg proteins/g wet weight)	Control	18.63 ± 6.21	24.84 ± 6.21	28.98 ± 9.49	31.06 ± 6.21	43.48 ± 5.07	55.9 ± 6.21
	Treated	27.77 ± 9.49	30.98 ± 6.11	35.19 ± 15.63	43.47 ± 6.21	57.97 ± 9.48	60.04 ± 15.62
	%	49.1	24.7	21.4	39.9	33.3	7.4
	Change						

Free amino acids (mg of tyrosine equi/g wet weight)	t-Test	0.6327 NS	1.2208 <sup>NS</sup>	0.5681 <sup>NS</sup>	2.4491 <sup>NS</sup>	2.2141 <sup>NS</sup>	0.4264 <sup>NS</sup>
	Control	22.51 ± 0.94	22.50 ± 0.94	23.91 ± 0.46	24.85 ± 0.38	25.32 ± 0.38	26.26 ± 0.47
	Treated	24.22 ± 0.71	25.32 ± 1.23	25.47 ± 0.97	26.26 ± 0.38	26.38 ± 0.96	27.67 ± 0.47
	% Change	7.6	12.5	6.5	5.7	4.2	5.4
	t-Test	2.5208 NS	3.1387*	2.4943 <sup>NS</sup>	3.6742*	1.7173 <sup>NS</sup>	3.6742*

**Table 2: Day-to-day changes in fatbody enzyme contents during the 5<sup>th</sup> instar of *Bombyx mori* under the impact of ultrasound (9W/Cm<sup>2</sup> for 2 minutes). Each value is the mean ± Standard Deviation (SD) of six separate observations. For each observation, tissue from at least 10 larvae was pooled. The percent changes for all days were calculated taking control as the reference**

Enzyme activity		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Protease (µmoles of tyrosine equivalents/mg protein/h)	Control	0.05 ± 0.001	0.055 ± 0.002	0.059 ± 0.003	0.06 ± 0.002	0.06 ± 0.002	0.07 ± 0.002
	Treated	0.053 ± 0.002	0.05 ± 0.002	0.06 ± 0.005	0.07 ± 0.003	0.078 ± 0.002	0.08 ± 0.004
	% Change	6.0	-9.1	1.7	16.7	30.0	14.3
	t-Test	2.6726 NS	1.2629 <sup>NS</sup>	1.9706 <sup>NS</sup>	4.1109*	7.1118**	5.0000**
	Aspartate aminotransferase (µmoles of pyruvate formed/mg protein/h)	Control	0.52 ± 0.02	0.56 ± 0.02	0.54 ± 0.02	0.48 ± 0.02	0.43 ± 0.03
Treated	0.58 ± 0.04	0.64 ± 0.02	0.62 ± 0.02	0.56 ± 0.02	0.52 ± 0.02	0.46 ± 0.02	
% Change	11.5	14.3	14.8	16.7	20.9	21.1	
t-Test	2.3237 NS	4.8989**	4.8989**	4.8989**	4.1109*	4.8989**	
Alanine aminotransferase (µmoles of pyruvate formed/mg protein/h)	Control	2.28 ± 0.02	2.16 ± 0.05	2.10 ± 0.09	1.92 ± 0.06	1.82 ± 0.02	1.74 ± 0.04
	Treated	2.33 ± 0.03	2.22 ± 0.02	2.17 ± 0.01	2.12 ± 0.02	2.02 ± 0.06	1.96 ± 0.02
	% Change	2.2	2.8	3.3	10.4	10.9	12.6
	t-Test	2.3237 NS	4.8989**	4.8989**	4.8989**	4.1109*	4.8989**

<b>Glutamate dehydrogenase (<math>\mu</math>moles of formazon formed/mg protein/h)</b>	t-Test	2.5298 NS	1.8371 <sup>NS</sup>	1.3804 <sup>NS</sup>	4.9734 <sup>**</sup>	5.3164 <sup>**</sup>	8.5205 <sup>**</sup>
	Control	0.41 $\pm$ 0.02	0.35 $\pm$ 0.02	0.32 $\pm$ 0.02	0.27 $\pm$ 0.02	0.22 $\pm$ 0.02	0.18 $\pm$ 0.02
	Treated	0.44 $\pm$ 0.02	0.38 $\pm$ 0.02	0.34 $\pm$ 0.02	0.3 $\pm$ 0.02	0.26 $\pm$ 0.02	0.22 $\pm$ 0.02
	% Change	7.3	8.6	6.3	11.1	18.2	22.2
	t-Test	1.7960 NS	1.8371 <sup>NS</sup>	1.2247 <sup>NS</sup>	1.8371 <sup>NS</sup>	1.9756 <sup>NS</sup>	2.4494 <sup>NS</sup>

NS - Not significant at 0.05 level; \* Significant at 0.05 level; \*\* Significant at 0.01 level

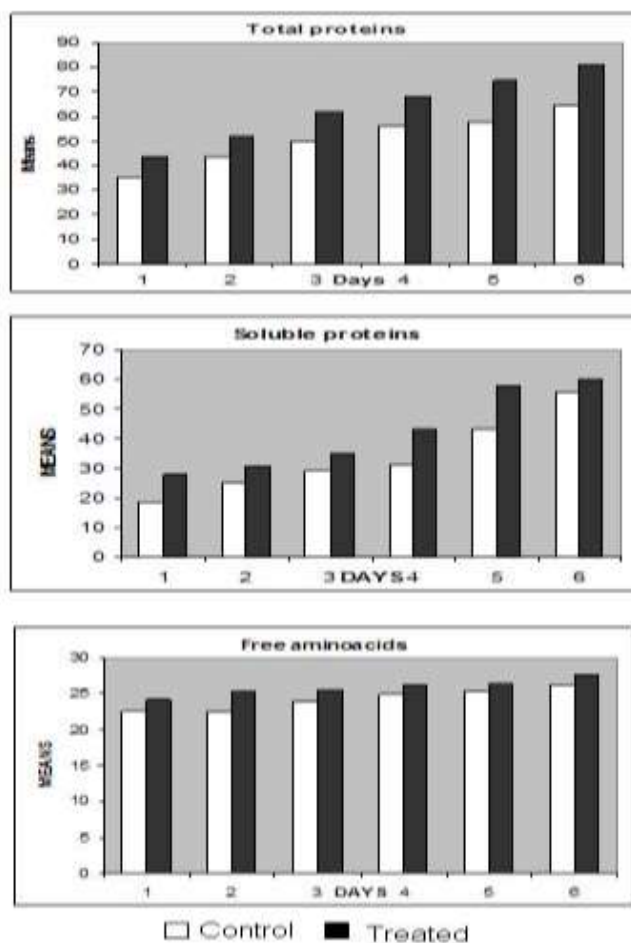


Fig. 1: Impact of ultrasound (9W/Cm<sup>2</sup> for 2 minutes) on fatbody protein metabolism in the silkworm *Bombyx mori* during fifth instar development

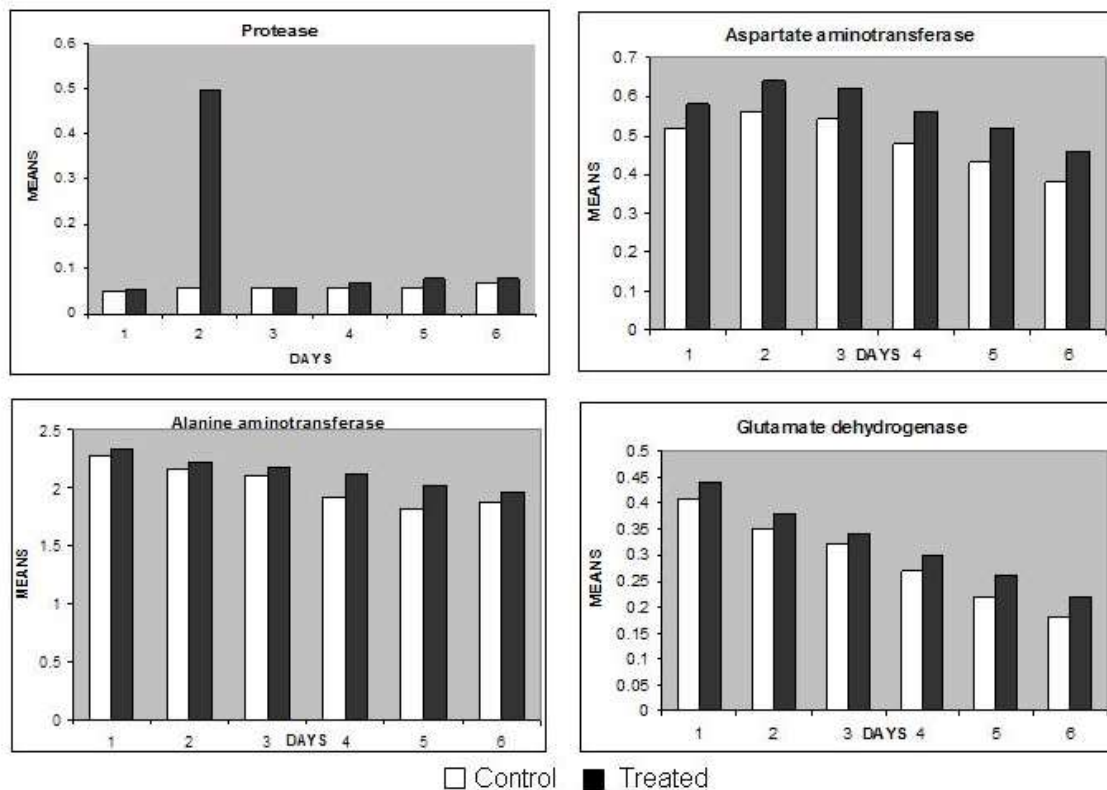


Fig. 2: Impact of ultrasound ( $9W/Cm^2$  for 2 minutes) on fatbody enzyme activities in the silkworm *Bombyx mori* during fifth instar development

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