Effect of ultrasound on protein metabolism in the silkworm, *Bombyx mori* (L.)

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ABSTRACT

The parameters of protein metabolism, such as the levels of soluble, structural and total proteins, free amino acids, and the activity levels of protease, aspartate and alanine aminotransferases and glutamate dehydrogenase were assayed in the hemolymph, silk-gland, muscle and fat-body on different days of the 5th instar larva of the silkworm, *Bombyx mori*, following exposure of the silkworm eggs to 1 MHz continuous wave of ultrasound at an intensity of $9W/cm^2$ for 2 minutes. Ultrasound was found to promote the accumulation of proteins, which include silk proteins as well, while retarding proteolysis and turnover of proteins towards the release of amino acids, keto-acids etc. Changes in the levels of these biochemical constituents are correlated with the events of histogenesis and histolysis associated with metamorphosis. It may be inferred that protein metabolism is stimulated by ultrasound, resulting in greater turnover of silk proteins, spinning activity and silk output.

INTRODUCTION

Insect metamorphosis is a dynamic biochemical activity. Chen (1971) highlighted the role of biochemical constituents in insect metamorphosis. 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 (Horie *et al.,* 1971; Mathavan *et al.,* 1984). The growth of silkworm during metamorphosis is accompanied by the increase in the body weight and accumulation of various biochemical constituents like proteins, amino acids and enzymes like proteases, aminotransferases and glutamate dehydrogenase (Pant and Jaiswal, 1981; Bannikov *et al.,* 1982; Wangerli and Xuting-Sen, 1982; Bose *et al.,* 1989; Siva Prasad and Murali Mohan, 1990). More importantly, the parameters of protein metabolism have been extensively examined because of their role in development, morphogenesis and intermediary metabolism (Tojo *et al.,* 1980; Ogawa and Tojo, 1981; Sasaki *et al.,* 1981; Robert and Rutt, 1982; Sarangi, 1985; Ravikumar and Sarangi, 2004).

A novel approach in silkworm research is the manipulation of biochemical machinery through exogenous modulators that could boost the silk production. This included the administration of certain neurohumoral factors, vertebrate hormones and various other chemicals like cyclic AMP and prostaglandins, which could have a profound influence on the growth rate, larval life cycle and fecundity (Singh and Dutta, 1980; Bharathi and Govindappa, 1987; Thyagaraja *et al.,* 1991; Bharathi, 1993). Significant positive impact of vertebrate thyroxine on silkworm biology, especially in improving the pre- and post-cocoon parameters is well documented (Bharathi *et al.,* 1986;

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Krishnamurthy *et al.*, 1987; Chaudhuri and Medda, 1992). Another vertebrate hormone, namely prolactin, induced improvement in the growth and reproductive potential of silkworms (Bhaskar *et al.,* 1983; Bharathi *et al.,* 1984, 1986). The dietary administration of vertebrate sex hormones like ethynyl estradiol (EE) and norethindrone (NE) 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 (Saha and Khan,1977).

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 (Child *et al.*, 1981). 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 (Koehler *et al.,* 1986). Ultrasound has been used as an exogenous modulator of several parameters in both vertebrate and invertebrate models. However, the mechanism of action of ultrasound in these models has not yet been resolved. A few studies are also available on ultrasound effects on silkworm. Samal *et al.* (2013) examined ultrasound sonication effects on the properties of silkworm fibroin protein. Murali Mohan and Siva Prasad (2013) studied the effect of ultrasound on economic characters of the silkworm and found improvement in several characters including silk output upon exposure to ultrasound. Since the silk fiber is composed of the protein fibroin, it is important to examine the effect of ultrasound on the silkworm protein metabolic profile. Taking this cue, ultrasound was used in the present investigation for the manipulation of protein metabolism in the silkworm and to examine the possibility of its utility in sericulture.

MATERIALS AND METHODS

The present investigation was carried out on the hybrid of Pure Mysore variety (Multivoltine) and NB_4D_2 variety (Bivoltine) of the silkworm *Bombyx mori.* The silkworm eggs in blue-egg stage (10-12 h old) were exposed to ultrasound at a frequency of 1 MHz and an intensity of 9 W /cm² for 2 min, using an ultrasound equipment, model WIPRO–GE Alpha–Logic–100. Prior to exposure, the egg-card was kept in a sealed, water-filled polythene bag, smeared with gel so as to prevent the diversion of ultrasonic waves. The bag was suspended in the ultrasonic bath tank. Water can be continuously circulated inside the tank, and its level can be maintained more or less at a fixed height. A transducer was kept at the base of the tank, and it transmits ultrasonic energy to the eggs in a focused way. 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/cm² for 2 minutes. The larvae that emerged from the exposed (experimental) and unexposed (control) eggs were used in the investigation in the 5th instar, which accounts for all the silk biosynthesis and secretion into the lumen of the silk-gland. The treatment of eggs with ultrasound was replicated six times separately. Tissues from at least ten silkworms that emerged from each treated lot of eggs were pooled for biochemical estimations. Daily changes in biochemical parameters of protein metabolism such as proteins, free amino acids, and the activity levels of the enzymes protease, aspartate and alanine aminotransferases, and glutamate dehydrogenase were observed in the 5th instar larvae. Tissues such as hemolymph, silk-gland, muscle and fat-body, isolated by dissecting the larvae in ice-cold silkworm Ringer (Yamaoka *et al*., 1971) were used for the biochemical assays. The total protein content as well as the soluble protein content was estimated by the method of Lowry *et al.* (1951). The trend of changes in the structural protein levels

was obtained by subtracting the soluble protein levels from the total protein levels in both control and experimental worms and then calculating the percent changes for each day of the 5th instar against the respective controls. The free amino acid content was estimated by the method of Moore and Stein (1954) as described by Colowick and Kaplan (1957) and the protease activity by the method of Davis and Smith (1955). The activity levels of aspartate aminotransferase (AAT) and alanine aminotransferase (AlAT) were estimated by the method of Reitman and Frankel (1957) as described by Bergmeyer and Bruns (1965) and the activity of glutamate dehydrogenase (GDH) was assayed by the method of Lee and Lardy (1965).

Statistical treatment of data

The difference in physiological activity between the control and ultrasound-treated (experimental) groups 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. Percentage changes were calculated from control to treated and presented along with the statistical test.

RESULTS

Tables 1 to 8 and Figures 1 to 8 highlight the changes in the biochemical parameters, viz., proteins, free amino acids, protease, aminotransferases and glutamate dehydrogenase and the effect of ultrasound on these parameters in the 5th instar larvae of *Bombyx mori*.

The levels of total and soluble proteins recorded an increasing trend in all the tissues from the 1st day to the 6th day of the instar. The extent of increase varied from tissue to tissue. The levels of total proteins registered a maximum increase of 15.4% on 1st and 4th days in hemolymph, 92.4% in silk-gland, 71.4% in muscle and 28.6% in fat-body on the 6th day of the 5th instar. Thus, ultrasound treatment elevated the total protein level in all the tissues. However, the increase was not progressive and consistent in hemolymph on different days of the instar (Table 1).

The levels of soluble proteins showed a steady increase from the 1st day to the 6th day of the instar. However, in the worms treated with ultrasound, lower increases in the levels of soluble proteins were evident in all the tissues. This trend was inconsistent in the hemolymph, with greater increases on certain days and lower increases on the other days. The changes in hemolymph were statistically not significant (Table 2).

The structural protein levels showed slight increases in hemolymph on different days of the instar. In the remaining tissues their levels increased progressively throughout, reaching the maximum by the 6th day. High increases were recorded in the silk-gland (169.7%) and muscle (332.7%) on the 6th day (Table 3).

Under the impact of ultrasound, the levels of total free amino acids showed a progressive increase in different tissues on all days of the instar. When the percent increases were calculated against the controls for each day, the increases were found to be relatively small and there was no discernible trend. Except in hemolymph (from the 3rd day), the increases in all other tissues were not statistically significant (Table 4).

Table 1. Day-to-day changes in the levels of total proteins (expressed as mg protein/g wet weight of the tissue or 1ml of hemolymph) during the 5th instar development of *Bombyx mori* under the impact of ultrasound.

Tissue	Indices	1st Day		2nd Day		3rd Day		4th Day		5th Day		6th Day	
		Control	Exptl.†	Control	Exptl.†	Control	Exptl.†	Control	Exptl.†	Control	Exptl.†	Control	Exptl.†
Hemolymph	Mean	77.85	89.84	82.39	94.40	84.47	96.32	86.12	99.37	89.02	101.86	91.09	104.34
	SD	±3.13	±3.18	±1.89	±2.48	±2.48	±3.12	±3.79	±2.48	±1.89	±2.48	±1.89	±2.48
	% change		15.4		14.6		14.0		15.4		14.4		14.6
	t-test		$P < 0.05$		$P < 0.05$		$P < 0.05$		$P < 0.05$		$P < 0.05$		$P < 0.05$
Silk gland	Mean	80.89	124.20	82.38	136.45	84.88	146.84	99.37	175.86	113.87	211.21	124.22	238.96
	SD	±5.97	±10.4	±7.0	±9.49	±9.48	±9.48	±6.21	±8.97	12.62	±8.97	±11.4	±9.48
	% change		53.5		65.6		73.0		76.9		85.5		92.4
	t-test		$P < 0.001$		$P < 0.001$		$P < 0.001$		$P < 0.001$		$P < 0.001$		$P < 0.001$
Muscle	Mean	37.27	53.76	43.48	62.11	47.62	72.46	55.90	86.95	62.11	99.32	64.50	110.53
	SD	±6.27	±9.48	±6.21	±10.42	±9.48	±9.49	±6.21	±11.42	±10.42	±6.21	±10.0	±12.00
	% change		38.9		42.9		52.2		55.6		59.9		71.4
	t-test		$P < 0.001$		$P < 0.001$		$P < 0.001$		$P < 0.001$		$P < 0.001$		$P < 0.001$
Fat body	Mean	43.48	50.57	55.90	66.32	35.19	43.48	49.69	62.11	64.54	81.07	57.97	74.53
	SD	±6.21	±9.48	±6.21	±6.21	±9.49	±6.21	±6.21	±25.0	±10.0	±5.73	±9.48	±6.21
	% change		16.0		18.6		23.6		25.0		25.6		28.6
	t-test		$P < 0.05$		$P < 0.01$		$P < 0.01$		$P < 0.01$		$P < 0.01$		$P < 0.01$

Note: Exptl.† stands for experimental (treated with ultrasound). 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 by taking the respective controls as the reference.

Changes are significant at least at 5% level.

Table 2. Day-to-day changes in the levels of soluble proteins (expressed as mg proteins/g wet weight of the tissue or 1ml of hemolymph) during the 5th instar development of *Bombyx mori* under the impact of ultrasound.

Tissue	Indices	1st Day Control	1st Day Exptl.†	2nd Day Control	2nd Day Exptl.†	3rd Day Control	3rd Day Exptl.†	4th Day Control	4th Day Exptl.†	5th Day Control	5th Day Exptl.†	6th Day Control	6th Day Exptl.†
Hemolymph	Mean	21.11	23.18	26.08	28.15	28.57	31.05	31.47	33.54	35.61	37.68	39.75	44.72
	SD	±2.48	±1.89	±2.48	±3.12	±2.48	±2.48	±1.55	±2.03	±1.55	±1.89	±2.48	±2.48
	% change		9.8		7.9		8.7		6.2		5.8		12.5
	t-test		NS		NS		NS		NS		NS		NS
Silk gland	Mean	31.05	55.90	35.19	64.18	43.48	68.32	49.69	72.46	60.04	86.96	72.46	99.38
	SD	±5.12	±6.21	±4.63	±9.48	±6.21	±6.12	±5.07	±7.75	±10.55	±6.21	±11.63	±6.2
	% change		80.0		82.4		57.1		45.8		44.8		37.2
	t-test		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001
Muscle	Mean	30.24	45.05	31.05	45.55	37.27	47.62	43.48	54.49	51.76	64.18	56.18	74.53
	SD	±7.13	±4.21	±4.12	±8.48	±4.21	±5.48	±3.10	±5.07	±5.75	±5.48	±6.18	±8.42
	% change		48.9		46.7		27.8		25.3		24.0		32.7
	t-test		P < 0.001		P < 0.001		P < 0.05		P < 0.05		P < 0.05		P < 0.001
Fat body	Mean	18.63	27.77	24.84	35.98	28.98	35.19	31.06	37.47	43.48	50.97	55.9	71.04
	SD	±3.21	±4.49	±4.21	±5.11	±4.49	±5.63	±3.21	±4.21	±5.07	±5.48	±5.21	±6.62
	% change		49.1		44.84		21.4		20.6		17.2		27.1
	t-test		P < 0.001		P < 0.001		P < 0.05		P < 0.05		P < 0.05		P < 0.01

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

Table 3. Day-to-day changes in the levels of structural proteins (expressed as mg protein / g wet weight of the tissue or 1ml of hemolymph) during the 5th instar of *Bombyx mori* under the impact of ultrasound.

Tissue	1st Day Control	1st Day Exptl.†	2nd Day Control	2nd Day Exptl.†	3rd Day Control	3rd Day Exptl.†	4th Day Control	4th Day Exptl.†	5th Day Control	5th Day Exptl.†	6th Day Control	6th Day Exptl.†
Hemolymph	56.74	66.66 (17.5)	56.31	66.32 (17.8)	55.90	65.27 (18.5)	54.65	65.83 (20.4)	53.41	64.18 (20.2)	51.34	59.62 (16.1)
Silk gland	49.84	68.30 (37.0)	47.19	72.27 (53.1)	41.40	78.52 (89.7)	49.68	103.4 (108.1)	53.83	124.25 (130.8)	51.76	139.58 (169.7)
Muscle	7.03	8.71 (23.9)	12.43	16.56 (33.2)	10.35	24.84 (40)	12.42	32.46 (161.3)	10.35	35.14 (239.5)	8.32	36.00 (332.7)
Fat body	24.85	22.80 (-8.2)	31.06	30.34 (-2.3)	6.21	8.29 (33.5)	18.63	24.64 (32.2)	21.06	30.1 (42.9)	2.07	3.49 (68.6)

Note**:** Exptl.† stands for experimental (treated with ultrasound). Each value was obtained by subtracting the values of soluble proteins from the values of total proteins. The data was not given statistical treatment as it represents derived data. However, percent changes in their levels in the ultrasound-treated larvae were calculated and presented in parentheses.

Table 4. Day-to-day changes in the levels of total free amino acids (expressed as mg of tyrosine equivalents/g wet weight of the tissue or 1ml of hemolymph) during the 5th instar of *Bombyx mori* under the impact of ultrasound.

Tissue	Indices	1st Day		2nd Day		3rd Day		4th Day		5th Day		6th Day	
		Control	Exptl.†	Control	Exptl.†	Control	Exptl.†	Control	Exptl.†	Control	Exptl.†	Control	Exptl.†
Hemolymph	Mean	4.16	4.58	4.99	5.81	5.26	7.00	5.99	8.00	6.10	8.16	6.25	8.25
	SD	±0.36	±0.37	±0.75	±0.24	±0.59	±0.14	±0.10	±0.09	±0.08	±0.05	±0.07	±0.04
	% change		10.1		16.4		33.1		33.6		33.8		32.0
	t-test		NS		NS		P < 0.001		P < 0.001		P < 0.001		P < 0.001
Silk gland	Mean	30.48	34.07	33.76	36.11	35.63	37.70	37.05	38.45	37.98	39.39	38.92	40.80
	SD	±2.34	±2.17	±2.93	±3.94	±1.94	±2.70	±1.38	±1.77	±1.38	±1.94	±1.47	±2.74
	% change		11.8		6.9		5.8		3.8		3.7		4.8
	t-test		NS		NS		NS		NS		NS		NS
Muscle	Mean	24.38	26.26	15.81	17.2	26.73	28.60	27.70	29.54	28.14	30.48	29.22	31.89
	SD	±1.98	±1.94	±0.97	±0.99	±1.47	±2.46	±2.43	±2.38	±1.83	±2.47	±2.71	±2.94
	% change		7.7		8.8		7.0		6.6		8.3		9.1
	t-test		NS		NS		NS		NS		NS		NS
Fat body	Mean	22.51	24.22	22.50	25.32	23.91	25.47	24.85	26.26	25.32	26.38	26.26	27.67
	SD	±0.94	±1.71	±1.54	±2.23	±1.46	±2.97	±1.38	±2.38	±0.98	±0.96	±0.97	±1.07
	% change		7.6		12.5		6.5		5.7		4.2		5.4
	t-test		NS		NS		NS		NS		NS		NS

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

The activity levels of protease recorded slight elevations in all the tissues throughout the instar, both in the control and experimental silkworms. In general, the elevations were found to be progressively lowered as the instar progressed from the 1st day to the 6th day in the case of hemolymph, silk-gland and muscle, while the percent increase was progressive with the days of the instar. The basic levels of protease activity were more or less similar in all the four tissues. Except in the muscle, the increases in activity in all the other tissues were not statistically significant (Table 5).

The activity of aspartate aminotransferase (AAT) maintained relatively constant levels in the hemolymph both in the control and experimental silkworms on all days of the instar without any perceptible variation. In silk-gland and muscle the enzyme activity recorded marginal percent increases on all the days of the instar both in the control and experimental silkworms, but without any particular trend. These small increases were not statistically significant. However, in the case of fat-body, the AAT activity showed progressive percent increases in the silkworms under the impact of ultrasound as the instar progressed, and the changes were statistically significant (Table 6).

The alanine aminotransferase (AlAT) activity by and large showed a similar trend as of AAT, with marginal percent increases under the impact of ultrasound on all days of the instar. However, in hemolymph, silk-gland and muscle, these slight increases got progressively lowered as the instar progressed. These percent increases were statistically not significant. However, in the case of fat-body, the AlAT activity showed a trend that was similar to the one exhibited by AAT activity, with progressive percent increases in the silkworms under the impact of ultrasound as the instar progressed. These changes were statistically significant from the 4th day onwards (Table 7). Although not statis-

tically significant, the percentage changes in glutamate dehydrogenase (GDH) activity progressively rose in all the tissues of the silkworms under the influence of ultrasound. These increases were marginal and statistically not significant in the case of silk-gland and fat-body, and significant only on the 5th and 6th days of the instar in the muscle. In the hemolymph the percent increases were statistically significant from the 3rd day onwards (Table 8).

DISCUSSION

In the present study, ultrasound was in general found to have a stimulatory effect on the protein metabolism. Since proteins are the chief organic constituents regulating the biochemical events in the cell, it appears logical that ultrasound has a stimulatory effect on the protein metabolism. Although increased levels of proteins were observed in silkworm tissues (Zaidi and Khan, 1979: Tojo *et al.*, 1980; Siva Prasad and Murali Mohan, 1990), these parameters have not been hitherto analyzed with reference to ultrasound in the silkworm. However, reports are available on the effect of ultrasound on protein synthesis with reference to differential tissue response in other animals (Lele *et al.,* 1973).

Proteins perform multiple functions. The hemolymph proteins are implicated in ecdysis, growth of reproductive organs and salivary glands, formation of hemocytes and chitin (Zaidi and Khan, 1979; Gakhar and Maleyvar, 1985). In muscle, most of these proteins are contractile that facilitate feeding and spinning behaviors of silkworm (Siva Prasad and Murali Mohan, 1990). Obviously, the intensification of these two behaviors is of paramount importance in 5th instar larvae. The feeding behavior is more pronounced in early stages and is responsible for the uptake of the nutrients, while the spinning behavior manifests at the end of the 5th instar and is responsible for spinning of the cocoon. As such, the prevalence of higher levels of structural

Table 5. Day-to-day changes in the levels of protease activity (expressed as µmoles of tyrosine equivalents/mg protein/h) during the 5th instar of *Bombyx mori* under the impact of ultrasound.

Tissue	Indices	1st Day		2nd Day		3rd Day		4th Day		5th Day		6th Day	
		Control	Exptl.†	Control	Exptl.†	Control	Exptl.†	Control	Exptl.†	Control	Exptl.†	Control	Exptl.†
Hemolymph	Mean	0.031	0.036	0.033	0.037	0.036	0.04	0.04	0.044	0.04	0.048	0.048	0.054
	SD	±0.01	±0.02	±0.01	±0.01	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02
	% change		16.1		12.1		11.1		10.0		20.0		12.5
	t-test		NS		NS		NS		NS		$P < 0.05$		NS
Silk gland	Mean	0.64	0.70	0.67	0.73	0.67	0.73	0.7	0.76	0.74	0.8	0.8	0.84
	SD	±0.01	±0.01		±0.02	±0.03	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02
	% change		9.8	±0.015	8.9		8.7		8.6		8.1		5.0
	t-test		NS		NS		NS		NS		NS		NS
Muscle	Mean	0.06	0.08	0.06	0.075	0.06	0.07	0.06	0.07	0.07	0.08	0.07	0.08
	SD	±0.002	±0.003	±0.001	±0.002	±0.001	±0.002	±0.002	±0.002	±0.005	±0.006	±0.004	±0.007
	% change		33.3		25.0		16.7		16.7		14.3		14.3
	t-test		$P < 0.01$		$P < 0.05$		$P < 0.05$		$P < 0.05$		NS		NS
Fat body	Mean	0.05	0.053	0.055	0.058	0.059	0.06	0.065	0.07	0.07	0.08	0.07	0.08
	SD	±0.001	±0.002	±0.002	±0.002	±0.003	±0.005	±0.002	±0.003	±0.006	±0.005	±0.005	±0.005
	% change		6.0		5.4		1.7		8.33		14.3		14.3
	t-test		NS		NS		NS		NS		NS		NS

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

Table 6. Day-to-day changes in the levels of aspartate aminotransferase (AAT) activity (expressed as µmoles of pyruvate formed/mg protein/h) during the 5th instar of *Bombyx mori* under the impact of ultrasound.

Tissue	Indices	1st Day Control	1st Day Exptl.†	2nd Day Control	2nd Day Exptl.†	3rd Day Control	3rd Day Exptl.†	4th Day Control	4th Day Exptl.†	5th Day Control	5th Day Exptl.†	6th Day Control	6th Day Exptl.†
Hemo-lymph	Mean	5.06	5.11	5.1	5.17	5.06	5.11	5.04	5.1	5.02	5.08	5.00	5.05
	SD	±0.02	±0.02	±0.02	±0.03	±0.04	±0.04	±0.02	±0.02	±0.02	±0.02	±0.02	±0.03
	% change		1.0		1.4		1.0		1.2		1.2		1.0
	t-test		NS		NS		NS		NS		NS		NS
Silk gland	Mean	0.7	0.74	0.74	0.77	0.77	0.79	0.82	0.84	0.84	0.87	0.88	0.92
	SD	±0.02	±0.03	±0.03	±0.02	±0.02	±0.02	±0.02	±0.02	±0.03	±0.03	±0.02	±0.02
	% change		5.7		4.1		2.6		2.4		3.6		4.6
	t-test		NS		NS		NS		NS		NS		NS
Muscle	Mean	1.47	1.52	1.40	1.48	1.38	1.46	1.34	1.4	1.32	1.38	1.30	1.34
	SD	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02	±0.03	±0.02	±0.02	±0.02	±0.02	±0.02
	% change		3.4		5.7		5.8		4.5		4.5		3.1
	t-test		NS		NS		NS		NS		NS		NS
Fat body	Mean	0.52	0.58	0.56	0.64	0.54	0.62	0.48	0.56	0.43	0.52	0.38	0.46
	SD	±0.02	±0.04	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02	±0.03	±0.02	±0.02	±0.02
	% change		11.5		14.3		14.8		16.7		20.9		21.1
	t-test		NS		P < 0.05		P < 0.05		P < 0.05		P < 0.01		P < 0.01

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

Table 7. Day-to-day changes in the levels of alanine aminotransferase (AlAT) activity (expressed as μmoles of pyruvate formed/mg protein/h) during the 5th instar of *Bombyx mori* under the impact of ultrasound.

Tissue	Indices	1st Day Control	1st Day Exptl.†	2nd Day Control	2nd Day Exptl.†	3rd Day Control	3rd Day Exptl.†	4th Day Control	4th Day Exptl.†	5th Day Control	5th Day Exptl.†	6th Day Control	6th Day Exptl.†
Hemolymph	Mean	1.43	1.49	1.32	1.37	1.26	1.29	1.21	1.23	1.15	1.17	1.11	1.13
	SD	±0.1	±0.13	±0.09	±0.08	±0.03	±0.02	±0.04	±0.04	±0.05	±0.05	±0.03	±0.03
	% change		4.2		3.8		2.4		1.7		1.7		1.8
	t-test		NS		NS		NS		NS		NS		NS
Silk gland	Mean	2.33	2.38	2.42	2.47	2.54	2.57	2.62	2.67	2.76	2.78	2.82	2.95
	SD	±0.02	±0.02	±0.02	±0.02	±0.04	±0.03	±0.02	±0.03	±0.02	±0.02	±0.03	±0.04
	% change		2.2		2.1		1.2		1.9		0.7		4.6
	t-test		NS		NS		NS		NS		NS		NS
Muscle	Mean	1.96	1.98	1.9	1.93	1.72	1.86	1.64	1.75	1.55	1.67	1.5	1.58
	SD	±0.02	±0.02	0.02	±0.03	±0.02	±0.06	±0.03	±0.05	±0.02	±0.04	±0.02	±0.02
	% change		1.0		1.6		8.1		6.7		7.7		5.3
	t-test		NS		NS		NS		NS		NS		NS
Fat body	Mean	2.28	2.33	2.16	2.22	2.10	2.17	1.92	2.12	1.82	2.02	1.74	1.96
	SD	±0.02	±0.03	±0.05	±0.02	±0.09	±0.01	±0.06	±0.02	±0.02	±0.06	±0.04	±0.02
	% change		2.2		2.8		3.3		10.4		10.9		12.6
	t-test		NS		NS		NS		P < 0.02		P < 0.02		P < 0.02

Footnote is the same as in Table 1, except that changes are significant at 2% level. NS – Not significant

Table 8. Day-to-day changes in the levels of glutamate dehydrogenase (GDH) activity (expressed as µmoles of formazon formed/mg protein/h) during the 5th in-star of *Bombyx mori* under the impact of ultrasound.

Tissue	Indices	1st Day Control	1st Day Exptl.†	2nd Day Control	2nd Day Exptl.†	3rd Day Control	3rd Day Exptl.†	4th Day Control	4th Day Exptl.†	5th Day Control	5th Day Exptl.†	6th Day Control	6th Day Exptl.†
Hemolymph	Mean	0.48	0.51	0.43	0.48	0.36	0.44	0.32	0.40	0.25	0.38	0.17	0.32
	SD	±0.02	±0.02	±0.02	±0.03	±0.02	±0.02	±0.02	±0.03	±0.02	±0.02	±0.02	±0.02
	% change		6.2		11.6		22.2		25.0		52.0		88.2
	t-test		NS		NS		P < 0.01		P < 0.01		P < 0.001		P < 0.001
Silk gland	Mean	1.51	1.56	1.61	1.67	1.68	1.74	1.74	1.8	1.82	1.89	1.93	2.01
	SD	±0.02	±0.02	±0.04	±0.02	±0.01	±0.02	±0.02	±0.02	±0.02	±0.04	±0.02	±0.56
	% change		3.3		3.7		3.6		3.5		3.8		4.1
	t-test		NS		NS		NS		NS		NS		NS
Muscle	Mean	0.51	0.57	0.46	0.52	0.43	0.48	0.38	0.42	0.34	0.40	0.28	0.38
	SD	±0.02	±0.03	±0.03	±0.03	±0.03	±0.02	±0.02	±0.03	±0.02	±0.02	±0.02	±0.02
	% change		11.8		13.0		11.6		10.5		17.7		35.7
	t-test		NS		NS		NS		NS		P < 0.05		P < 0.001
Fat body	Mean	0.41	0.44	0.35	0.38	0.32	0.34	0.27	0.30	0.22	0.26	0.18	0.22
	SD	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02
	% change		7.3		8.6		6.3		11.1		18.2		22.2
	t-test		NS		NS		NS		NS		NS		NS

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

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proteins during the 5th instar (Table 3) is indicative of strengthening of the muscle tissue for increased efficiency of feeding behavior during the larval development. Further, the increases in the levels of structural proteins coupled with the increase in the levels of total and soluble proteins is indicative of the ongoing histolysis associated with the degenerative metamorphic changes and delegation of proteins towards the spinning activity as well as the formation of the silk proteins in the silk-gland. In silk-gland, the proteins are used for the synthesis of silk proteins, viz., fibroin and sericin (Horie *et al.,* 1971). The phenomenal increase in the levels of structural proteins (Table 3) in silk-gland and muscle is indicative of the continuous synthesis and accumulation of the silk proteins. The fat-body seems to act as the storage organ similar to that of liver in vertebrates (Price, 1973). Further, hemolymph presumably acts as a transitory repository of biochemical constituents, into which the tissues leak out their biochemical constituents and retrieve them when needed. Thus, a dynamic biochemical exchange mechanism seems to operate in silkworm and other insects, facilitating the exchange of substances between the fat-body and hemolymph (Martin *et. al.,* 1971; Noguchi *et al.,* 1974; Sarangi, 1985; Nagata and Kobayashi, 1990; Ravikumar and Sarangi, 2004). Ultrasound presumably enhances the absorption of soluble proteins from the hemolymph and fat-body. The mechanism by which ultrasound causes changes in silkworm is not yet known. The Increased levels of total, soluble and structural proteins in silkworm tissues indicate 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 the silk-gland and contractile proteins in the muscle. Interestingly, the greater accumulation of structural proteins in the muscle (332.7%) following the ultrasound treatment deserves some attention, and it is indicative of the promising role of ultrasound in consolidating protein levels in tissues. Such consolidation could provide the required tensile strength to the muscles, facilitating active spinning of the cocoon at the end of 5th instar.

Amino acids are the building blocks of proteins. Ultrasound irradiation caused an elevation in the levels of free amino acids in all the tissues, although to a relatively smaller degree. The silkworm and other lepidopteran insects are known to contain unusually large amounts of free amino acids (Siva Prasad and Murali Mohan, 1990; Sinha *et al.,* 1991). Insect metamorphosis is a dynamic process involving both histogenesis and histolysis (Horie and Watanabe 1983; Anderson 1984). The levels of structural, soluble and total proteins in hemolymph, silk-gland, muscle and fat-body indicate the presence of histolytic activity in different tissues in the 5th instar larvae and the consequent build-up of proteins in the tissues. The constructive activity in one tissue appears to match the lytic activity in some other tissue. While histogenesis occurs in silk-gland, muscle and fat-body and proteins accumulate in the hemolymph, histolysis seems to proceed in other tissues. Obviously, the amino acid pool in the silkworm is derived both from proteins through histolysis and from non-protein sources like carbohydrates and lipids through *de novo* synthesis. 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 (Bose *et al.,* 1989), although to a smaller degree. Given the importance of silkworm, it is presumed that amino acids are crucial for the synthesis of fat-body, particularly during larval-pupal transition. 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.* (1974). Through its elevatory effect, ultrasound seems to trigger certain metabolic

events (Pant and Jaiswal, 1981), such as transamination, lipogenesis, maintenance of homeostasis, energy metabolism, formation of hemocytes etc, by actively mobilizing the amino acid pool from hemolymph and fat-body during metamorphosis.

Proteases are a group of proteolytic enzymes that hydrolyze proteins into amino acids (Chen, 1971). Protease activity levels recorded an overall increase during the 5th instar development. Greater enzyme activity was observed in silk-gland followed by muscle, fat-body and hemolymph. Protease activity has been reported in silkworm and other insects (Eguchi and Iwamoto, 1975; Bharathi and Miao Yungen, 2003). Further, ultrasound caused an elevation in its activity to varying degrees in different tissues. The presence of non-intestinal protease activity in silkworm tissues is attributed to its role in proteolysis, characterizing insect metamorphosis (Chen, 1971). 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 silk-gland, muscle and fat-body as evidenced by increased turnover of amino acids in these tissues. This could probably bring about the degeneration of silk-gland during pupal stage, leaving the fat-body that forms the bulk of pupal weight. The significance of protease activity *vis-à-vis* muscle degeneration needs to be elucidated with appropriate histochemical and biochemical investigations.

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 (Lehninger, 1993). The presence of aspartate (AAT) and alanine (AlAT) aminotransferase activity was detected in hemolymph, silk-gland, muscle and fat-body of silkworm as reported in earlier investigations (Pant and Jaiswal, 1981; Bannikov *et al.,* 1982; Urbesekf 1989; Siva Prasad and Murali Mohan, 1990; Vankata Rami Reddy *et al.,* 1992). Ultrasound caused an elevation in the activity levels of both AAT and AlAT in silkworm tissues (Tables 6, 7), indicating increased turnover of amino acids and glutamate-formation during metamorphosis in the silkworm. The elevation in the levels of free amino acids, although to a moderate degree, observed in the present investigation (Table 4) support this assumption. This indicates the crucial role for aminotransferases in protein synthesis in silkworm tissues (Fukuda, 1960). The increase in the levels of both total and soluble proteins (Tables 1, 2), *vis-à-vis* aminotransferase activity (Tables 6, 7) highlights the role of both AAT and AlAT in protein metabolism of silkworm. The impact of ultrasound on aminotransferase activity and protein synthesis is positive, but differs in sensitivity from tissue to tissue. Further, ultrasound seems to enhance protein synthetic activity more through AlAT than through AAT. Presumably AlAT is more sensitive to ultrasound compared to AAT. The role of ultrasound in the manipulation of various metabolic events like glucogenesis, gluconeogenesis, biological oxidations, histolysis and histogenesis (Pant and Jaiswal, 1981; Venkata Rami Reddy *et al.,* 1992) by elevating the levels of aminotransferases needs to be ascertained. Such an approach could be used to shorten the life cycle of silkworm.

Glutamate dehydrogenase (GDH) is an allosteric enzyme localized mainly in the mitochondrial part of the cell and facilitates the transfer of amino groups of amino acids to α-ketoglutarate by transamination, forming L-glutamate with the release of ammonia (Lehninger, 1993). It ensures the availability of α-ketoglutarate to citric acid cycle, and thus connects protein metabolism with carbohydrate metabolism. Ultrasound showed an elevatory effect on GDH activity in all the tissues of silkworm (Table 8). Some reports are available

on GDH activity in *Bombyx mori* (Venkata Rami Reddy *et al*., 1992). The enhanced activity of GDH in different tissues in the present study is indicative of increased oxidative deamination of glutamate in these tissues. The α-ketoglutarate generated by this enzyme is probably used-up in ensuring sperm mobility in silkworm (Osanai *et al*., 1987). Ultrasound could activate this process and thus may increase the sexual potential of silkworm.

As stated earlier, the mechanism of ultrasound irradiation on silkworm is not yet known. However, ultrasound is known to cause an elevation in the temperature, which in turn alters the rate of protein synthesis by optimizing the activity of enzymes (Woeber, 1965). The increased activity levels of aminotransferases, protease and GDH under the impact of ultrasound indicate this possibility. This aspect warrants elucidation in silkworm.

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 the entire biochemical machinery in silkworm is apparently geared up to synthesise silk proteins in silk-gland and contractile proteins in muscle during 5th 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 5th instar. The increase in the concentration of amino acids with concomitant increase in the levels of total, soluble and structural proteins in silk-gland and muscle under the impact of ultrasound reflects this fact. Metabolically, silk-gland seems to occupy a preeminent position during the metamorphosis in silkworm, closely followed by the muscle. Through active deamination of amino acids, facilitated by AAT, AlAT and GDH, silk-gland presumably meets some of its energy requirements by way of enhanced intermediary metabolism. The

growth and metamorphosis of silkworm are characterized by both constructive (histogenesis) and destructive (histolysis) events, probably triggered by ultrasound, as evidenced by the increased protease activity coupled with increased titers of free amino acids in silkworm tissues. 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 silk output.

CONCLUSIONS

Exposure of silkworm eggs to appropriate doses of ultrasound in terms of intensity and duration of exposure would lead to acceleration of protein metabolism with reference to protein and free amino acid levels and the activity levels of protease, aspartate and alanine aminotransferases and glutamate dehydrogenase. The enhancement in protein metabolism would in turn result in the improvement of economic characters of the silkworm, having a positive impact on the profitability of Sericulture.

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