Global Journal of Biotechnology & Biochemistry 5 (3): 182-188, 2010
ISSN 2078-466X

Regulation of Protein Metabolism and Enzyme Activities under the Influence of Ultrasound Treatment in the Muscle Cells of Silkworm, *Bombyx mori*.

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Abstract: The present study was carried out to explain the effect of enhancement of protein metabolism and enzyme activities in muscle cells of the silkworm, *Bombyx mori* L during the fifth instar larva stage. The parameters of protein metabolism, such as the levels of soluble, total proteins and free amino acids and the activity of protease, glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase were assayed to know 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 and enzyme activities are stimulated to achieve greater turnover of silk proteins, greater spinning activity and consequently greater sericultural output.

Key words: Amino Acids · Aminoransferases · *Bombyx mori* · Glutamate dehydrogenase · Muscle cells · Protease · Proteins · Ultrasound

INTRODUCTION

The mulberry silkworm, *Bombyx mori* L. is an economically important insect in the silk industry. The development of the worm is depending on metamorphosis process which is a dynamic biochemical process [1]. 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, glutamate dehydrogenase and aminotransferases [2, 3]. 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 [4]. More importantly, the parameters of protein metabolism have been extensively examined because of their role in development, morphogenesis and in the intermediary metabolism [5, 6].

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, larval life cycle and fecundity [7, 8]. Significantly, the positive impact of vertebrate thyroxin on silkworm biology, especially in improving the pre-and post cocoon parameters is well documented [9]. Another vertebrate hormone, namely prolactin induced improvement in the growth and reproductive potential of silkworms [10].

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 [11]. 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 [12]. In view of its harmless nature, ultrasound has been used as an exogenous modulator in the present investigation for the manipulation of protein metabolism

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and the measurement of enzyme activities in the specific tissue i.e., muscle cell which is an indicator of changes in the silkworm.

MATERIALS AND METHODS

Multivoltine x bivoltine hybrid variety of the silkworm, (Pure Mysore x NB_4D_2) *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. 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. The duration of exposure was standardized by exposing the eggs to varying intensities of ultrasound weaves, 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 $9W/Cm^2$ for 2 minutes. The larvae that emerged from the exposed (experimental) and unexposed (control) eggs were used in the investigation.

Tissue Separation: Muscle tissue was isolated by dissecting the larvae in ice-cold silkworm Ringer [13] 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.* [14], the free amino acid content by the method of Moore and Stein [15] as described by Colowick and Kaplan [16] and the protease activity by the method of Davis and Smith [17]. The activities levels of aminotransferases, viz, aspartate aminotransferase (AAT) and alanine aminotransferase (AlAT) were estimated by the method of Reitman and Frankel [18] as described by Bergmeyer and Bruns [19] and the activity of Glutamate dehydrogenase (GDH) was assayed by the method of Lee and Lardy [20].

RESULTS

The levels of total and soluble proteins recorded an increasing trend in the muscle tissue from day 1 to day 6 during the development of fifth instar larvae. The levels of total proteins increased from about 37 mg to 65 mg (76% increase) in muscle. Ultrasound has an elevatory effect on total protein content. The impact ranged from tissue to tissue. The elevation is about 39% to 71% in muscle (Table 1 and Fig. 1). The levels of soluble proteins increased from about 30 mg to 64 mg in muscle.

Table 1: Day-to-day changes in muscle protein metabolism during the 5th instar of *Bombyx mori* under the impact of ultrasound ($9W/Cm^2$ 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	37.27 ± 6.27	43.48 ± 6.21	47.62 ± 9.48	55.9 ± 6.21	62.11 ± 12.42	64.50 ± 10.0
	Treated	51.76 ± 9.48	62.11 ± 12.42	72.46 ± 9.49	86.95 ± 12.42	99.32 ± 6.21	110.53±12.00
	% Change	38.9	42.9	52.2	55.6	59.9	71.4
	t-Test	2.2135 NS	2.3237 NS	3.2068*	3.8721*	4.6484*	5.1006**
Soluble Proteins							
(mg proteins/g wet weight)	Control	30.24 ± 10.13	31.05 ± 6.12	37.27 ± 6.21	43.48 ± 5.07	51.76 ± 7.75	64.18 ± 9.48
	Treated	31.05 ± 6.21	45.55 ± 9.48	47.62 ± 9.48	49.49 ± 5.07	64.18 ± 9.48	74.53 ± 12.42
	% Change	2.7	46.7	27.8	13.8	24.0	16.1
	t-Test	0.1180 NS	2.2135 NS	1.5811 NS	1.2247 NS	1.6036 NS	1.1471 NS
Free amino acids (mg of tyrosine							
equi/g wet weight)	Control	24.38 ± 0.98	15.81 ± 0.47	26.73 ± 0.47	27.70 ± 0.43	28.14 ± 0.38	29.22 ± 0.71
	Treated	26.26 ± 0.94	27.2 ± 0.94	28.60 ± 0.46	29.54 ± 0.38	30.48 ± 0.47	31.89 ± 0.94
	% Change	7.7	72.0	7.0	6.6	8.3	9.1
	t-Test	2.4516 NS	2.2895 NS	4.9076**	1.7775 NS	6.1388**	3.9000*

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

Table 2: Day-to-day changes in muscle enzyme contents during the 5th instar of *Bombyx mori* under the impact of ultrasound (9W/Cm^2 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	0.055 ± 0.002	0.0586 ± 0.001	0.058 ± 0.001	0.062 ± 0.002	0.066 ± 0.002	0.07 ± 0.002
	Treated	0.066 ± 0.003	0.07 ± 0.002	0.07 ± 0.002	0.074 ± 0.002	0.078 ± 0.002	0.084±0.002
	% Change	18.2	19.5	20.7	19.4	18.2	20.0
	t-Test	1.1030 NS	7.6485**	9.3541**	7.3484*	7.3484**	7.3484**
Aspartate aminotransferase (µmoles of pyruvate formed/mg protein/h)	Control	1.47 ± 0.02	1.40 ± 0.02	1.38 ± 0.02	1.34 ± 0.03	1.32 ± 0.02	1.30 ± 0.02
	Treated	1.52 ± 0.02	1.48 ± 0.02	1.46 ± 0.02	1.4 ± 0.02	1.38 ± 0.02	1.34 ± 0.02
	% Change	3.4	5.7	5.8	4.5	4.5	3.1
	t-Test	2.3348 NS	4.8989**	4.8989**	2.5298 NS	3.0534*	2.4494 NS
Alanine aminotransferase (µmoles of pyruvate formed/mg protein/h)	Control	1.96 ± 0.02	1.9 ± 0.02	1.72 ± 0.02	1.64 ± 0.03	1.55 ± 0.02	1.5 ± 0.02
	Treated	1.98 ± 0.02	1.93 ± 0.03	1.86 ± 0.06	1.75 ± 0.05	1.67 ± 0.04	1.58 ± 0.02
	% Change	1.0	1.6	8.1	6.7	7.7	5.3
	t-Test	1.2247 NS	1.5811 NS	3.8340*	3.1378*	1.2247 NS	3.8987*
Glutamate dehydrogenase (µmoles of formazon formed/mg protein/h)	Control	0.51 ± 0.02	0.46 ± 0.03	0.43 ± 0.03	0.38 ± 0.02	0.34 ± 0.02	0.28 ± 0.02
	Treated	0.57 ± 0.03	0.52 ± 0.03	0.48 ± 0.02	0.42 ± 0.03	0.40 ± 0.02	0.38 ± 0.02
	% Change	11.8	13.0	11.6	10.5	17.7	35.7
	t-Test	3.6742*	2.4494 NS	2.2135 NS	2.2135 NS	3.0822*	6.1237**

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

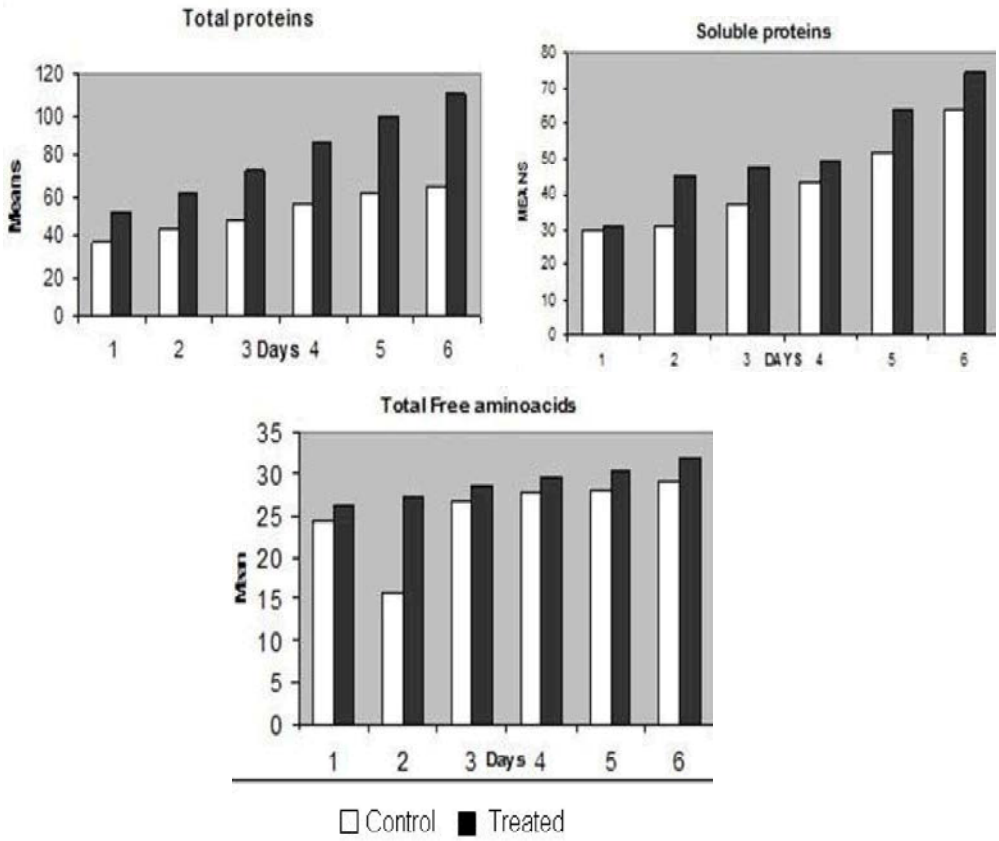

Fig. 1: Impact of ultrasound (9W/Cm^2 for 2 minutes) on muscle protein metabolism in the silkworm *Bombyx mori* during fifth instar development

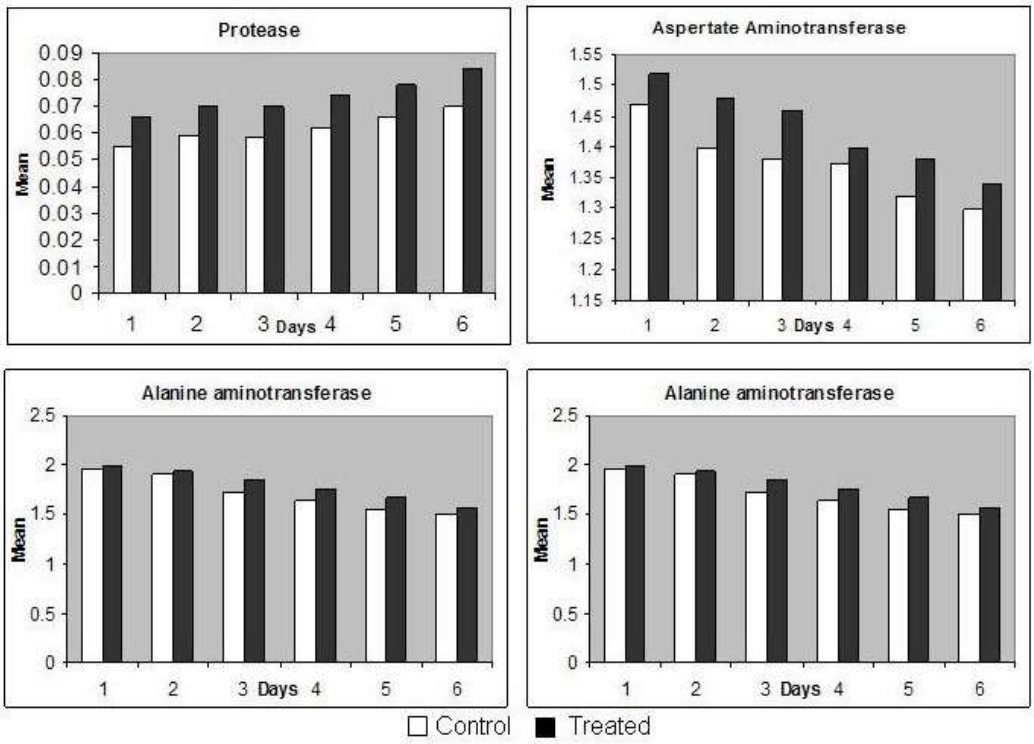

Fig. 2: Impact of ultrasound (9W/Cm^2 for 2 minutes) on muscle enzyme activities in the silkworm *Bombyx mori* during fifth instar development

Ultrasound in general caused an elevation in the levels of soluble proteins also, with varying intensities. The range of increase is about 3 to 47% in muscle (Table 1 and Fig. 1). The levels of free amino acids showed a similar trend during fifth instar. The level of increase is about 24 to 29 mg in muscle (20% rises). The impact of ultrasound on free amino acid levels is positive. The range for increase is about7to 72% in muscle (Table 1 and Fig. 1).

The activity levels of protease recorded an upward trend through out the fifth instar. The elevation is more pronounced in muscle (0.05 to 0.07 µmoles). Ultrasound caused an elevation (2 to 30% increase) in the enzyme activity in muscle tissues examined (Table 2 and Fig. 2). The activity levels of aspertate aminotransferase (AAT) decreased in muscle (1.47 to 1.34 µmoles/mg protein/h) during fifth instar development. The impact of ultrasound is elevatory, with an overall increase of 3% to 5% in muscle (Table 2 and Fig. 2). Alanine aminotransferase (AlAT) activity, showed a similar trend during fifth instar development. In muscle, the enzyme activity declined from 1.96 to 1.5 µmoles/mg protein/h. Ultrasound caused an elevation in the activity notwithstanding its minor fluctuations in controls. The over all elevation is about 1 to 8% in muscle (Table 2 and Fig. 2). The glutamate dehydrogenase (GDH) activity declined in muscle (0.51 to 0.28µmoles/mg protein/h) during the fifth instar development. Ultrasound caused an elevation in GDH activity levels. The elevation is pronounced in muscle (10% to 35%) (Table 2 and Fig. 2).

DISCUSSION

The impact of ultrasound on protein metabolism is profound as evidenced by upsurge in the levels of all the biochemical parameters examined. Since, proteins are the chief organic constituents regulating the biochemical events in the cell, the immediate target of ultrasound seems to be the protein metabolism. Though, increased levels of proteins were observed in silkworm tissues, these parameters were not analyzed with reference to ultrasound. However, some earlier investigations [21] attempted to elucidate the effect of ultrasound on protein synthesis particulary in the selective tissue (Muscle). The proteins perform multiple functions. In muscle, most of these proteins are contractile that facilitate feeding and spinning behaviours of silkworm [3]. The feeding behaviour is more pronounced in early stages and is responsible for the uptake of the nutrients, while the

spinning behaviour manifests at the end of the fifth instar and is responsible for spinning the cocoon. Ultrasound seems to enhance the protein synthesis in general, with a bias towards the silk proteins in silkgland (data not shown) and contractile proteins in muscle.

Amino acids are the building blocks of the proteins. Ultrasound irradiation caused an elevation in the levels of free amino acids in muscle tissue. 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 [2]. Given the importance of silkworm, it is presumed that amino acids are crucial for the synthesis of fatbody particularly, during larval-pupal transition. It is likely that amino acids are mobilized from other tissues into the silkgland and fatbody via the haemolymph, as suggested by Noguchi *et al.* [24].

Proteases are a group of proteolytic enzymes that hydrolyse proteins into amino acids [1]. Protease activity levels recorded a continuous increase throughout the fifth instar development. Greater enzyme activity was observed in muscle. Protease activity has been reported in silkworm and other insects [25]. The presence of non-intestinal protease activity in silkworm tissues is attributed to its role in proteolysis, characterizing insect metamorphosis [1]. The positive impact of ultrasound on enzyme activity indicates its ability to degrade proteins by activating proteolytic enzymes. 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 [26]. The presence of AAT and AlAT activity was detected in muscle of silkworm as reported in earlier investigations [3, 27]. Ultrasound caused an elevation in the activity levels of both AAT and AlAT in silkworm muscle tissues (Table 2 and Fig. 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 and Fig. 1) support this assumption. This indicates the crucial role for aminotransferases in protein synthesis in silkworm tissues [28]. The increase in the levels of both total and soluble proteins (Tables 1 and Fig.1), vis-à-vis aminotransferase activity (Tables 2 and Fig. 2) highlights the role of both AAT and AlAT in protein metabolism of silkworm. The role of aminotransferases in manipulation of various metabolic events like glucogenesis, gluconeogenesis, biological oxidations, histolysis and histogenesis [27] by elevating the levels of aminotransferases needs to be ascertained. Such an approach could bring down the durtion of life cycle of silkworm by triggering appropriate metamorphic changes.

Glutamate dehydrogenase 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 [26]. Ultrasound has an elevatory effect on GDH activity in muscle tissue of silkworm (Table 2 and Fig.2). The enhanced activity of GDH in silkgland is indicative of increased oxidation of glutamate in this tissue while its decrease in muscle indicates a lower rate of oxidative deamination of amino acids in these tissues [27]. The α-ketoglutarate generated by this enzyme is probably used-up in ensuring sperm mobility in silkworm [29]. Ultrasound could activate this process and thus may increases the sexual potential of silkworm. The actual mechanism of ultrasound irradiation on protein metabolism is not clear. However, it is known to cause an elevation in the temperature, which in turn alters the rate of protein synthesis by optimizing the activity of enzymes [30]. The increased activity levels of aminotransferases, protease and GDH under the impact of ultrasound bear strong testimony to this assumption. This parameter warrants further elucidation 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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