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Antioxidant effect of Tryptophan on biochemical parameters in the haemolymph and fat body of final instar larvae of silk insect, *Bombyx mori*

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ABSTRACT

Ageing is a natural life process whose manifestations are familiar and unambiguous. Oxidative modification of cellular molecules by reactive oxygen species and impaired antioxidant mechanism play unique role in a variety of age-associated degenerations. As a defence, cells have developed antioxidant defence system of a group of enzymes including catalases and peroxidases destroying toxic molecules. The natural antioxidant mechanism of an organism may be insufficient and external dietary administration of anti oxidant compounds play vital role in defence against ageing. In the present study, the antioxidant effects of reducing amino acid tryptophan in the final instar larvae of silkworm, *Bombyx mori*. The turnover of total protein, amino acid and glucose was evaluated. The total haemolymph protein of treated larvae showed 34-94% increase when compared to normal and the pattern of the changes in the levels of fat body protein was same but with a change of 12 fold. The total content of free amino acids in the haemolymph of normal and treated larvae increased gradually from the period of 0 h to 96 h with a peak value at 96 h and then decreased. The total content of free amino acids in the fat body is much less than that found in the haemolymph. The peak glucose levels in the total larval haemolymph were almost 28 times to that found at the early stage in normal larvae and approximately 12 times in tryptophan treated larvae. The fat body glucose level showed a consistent reduction in the treated larvae.

1. INTRODUCTION

Ageing is a universal process and has been defined as the progressive loss of function accompanied by decreasing fertility and increasing mortality and disability. Altered response to therapeutic interventions might be considered in any future definitions of ageing [1]. Oxidative modification of DNA, proteins, lipids and small cellular molecules by reactive oxygen species (ROS) along with impaired antioxidant mechanism play some role in a wide range of common diseases and age-related degenerative conditions. Oxidant damage by ROS is also associated to photo ageing radiation toxicity, cataract formation and muscular degeneration [2]. Once free radicals are initiated, they can propagate by involving in chain reactions with other less reactive types, the resulting chain reaction compounds generally survive longer in the body and thus increase the potential for cellular damage. To protect molecules against toxic free radicals and other ROS, cells have developed antioxidant defense system that include the enzymes super oxide dismutase (SOD), which dismutates superoxide; catalase (CAT); glutathione reductase and glutathione peroxidase, which destroy toxic peroxides and small molecules including glutathione [3]. The rise in catalase activity during larval development was directly related to the formation of pro-oxidant in the larva and checks the deleterious effect of aging through its antioxidant effect [4]. External sources of antioxidant vitamins like vitamin C, vitamin E and phytochemicals from plant rich diets provide important protection against oxidant damage [5]. Dietary antioxidant have been demonstrated to be protective through the activation of hermetic pathways, including vitagenes and proteosomal activity degrading oxidatively modified proteins [6].

In addition to this there is an inverse relationship between dietary intake of antioxidant rich foods and incidence of a number of diseases. Therefore search into the determination of antioxidant capacity of different compounds become important. Theoretically, the ageing process pertains to single individual; therefore studies concerned with the effect of ageing on physiological function should ideally be conduct on the same individual during its entire lifespan. Accepting the universality of ageing process in multi cellular animals, it is easier to use insects as experimental animals. Since larvae are the representatives of juvenile form they exhibit maximum protection against ageing activities [7].

The reducing amino acids like tryptophan and tyrosine are potent inhibitors of lipid peroxidation and oxidative stress.

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In addition to this tryptophan is an important precursor of tryptophan hydroxylase enzyme and directly involved in the biogenesis of the neurotransmitter serotonin, the decreased level of which increases neurodegenerative changes in central nervous system [8]. Results of various studies suggest that the total content of haemolymph proteins increases during larval development and the increase is most rapid during the time approaching pupation. Different developmental pattern of proteins was also identified in different animals including mammals and some of these are homologous to insect proteins also. In a study about the relationship between the proteins of the haemolymph and fat body during development of *Pieris brassicae* revealed that the fat body was in fact selectively storing two major haemolymph proteins [9]. Insects are known to contain an unusually large amount of free amino acids whose total concentration in some species has been estimated to be more than thirty times higher than that in other groups of animals. It is found that the ten amino acids *viz.* arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine, valine have been proved to be essential for insect growth and the free amino acid concentrations will change with regard to the physiological state of insect [10]. In insects as in other animals, glucose has a central place in carbohydrate metabolism. Insect haemolymph often contains so much trehalose that the sugar can easily be obtained in crystalline form. In *B. mori* during the fifth larval instars the glycogen content of the fat body rises from 5 to 20% of dry weight [11]. Chitin synthesis in insects involves utilization of glucose derived from trehalose. Variations in the glucose levels during larval development of *Spodoptera mauritia* revealed that the feeding stage of larva was characterized by low levels of glucose in the tissue, whereas the same increased sharply in the non feeding stage [12]. In the present study, a reducing amino acid tryptophan was administered through the feed material to the final instar larvae of silkworm, *Bombyx mori*, in order to evaluate its antioxidant effects on larvae, specifically on the physiological and biochemical alterations as well as the turnover of total protein, amino acid and glucose.

2. MATERIALS AND METHODS

The bivoltine hybrid of silkworm, *Bombyx mori* L Elite-CSR 2x4 was used for the present study. The silkworm rearing was undertaken by procuring newly hatched larvae in the rearing bed fed with tender mulberry leaves at 80-90% humidity and 27°C room temperature for five molts. The duration of fifth instar was normally about 6-7 days and the larvae started to spin cocoon by the end of this stage. The present work was done on the fifth instar larvae, beginning from the newly molted stage and continued till the last day of the instar, just before spinning began. The larval period was divided into seven chronologically identified stages: i.e., 0h, 24h, 48h, 72h, 96h, 120h and 144h. After the fourth molt, the larvae were segregated into two sets. One set was fed with mulberry leaves dipped in 5mM tryptophan solution and drained in air for half an hour. The other set of larvae were fed with leaves dipped in distilled water and drained in air for half an hour.

For various biochemical estimations pooled haemolymph samples were extracted from appropriate number of both normal and treated larvae separately. For the estimations of fat body samples the tissue was homogenized and diluted to appropriate volume with water for all assays except enzymes. The analysis was carried out at 24 hour intervals in on the basis of unit volume in the case of haemolymph and on the basis of unit weight of fresh tissue in the case of fat body. The pooled haemolymph and fat body samples were isolated from the larvae of each set. The tissues were stored at $^{-}20°$ C until the estimations were carried out.

The estimation of protein was done using crystalline bovine serum albumin (Fraction V, Sigma) as standard [13]. From the homogenized tissue, the protein was precipitated with trichloroacetic acid. The precipitate was then successively extracted with ethanol- chloroform, ethanol - ether and finally ether at room temperature. The final residue left was dissolved in 1 N sodium hydroxide. The blue colour developed was measured against a reagent blank at 540 nm in a Shimadzu UV 250 spectrophotometer. The total free amino acid content in the tissues was estimated by the method of Lee and Takahashi [14]. The homogenized tissue was precipitated with 10% sodium tungstate and 2/3 N sulphuric acid and centrifuged at 2000 rpm for 20 min. The resultant supernatant was used for the amino acid estimation. The colour developed was measured at 540 nm against the reagent blank. Glucose was estimated according to Morgan [15]. Homogenates of the tissues were deproteinised by 0.3 N barium hydroxide and 5% zinc sulphate and filtered. The filtrate was then used for the estimation and the blue colour developed was measured at 540 nm against a reagent blank. The data were subjected to statistical analysis to evaluate whether the variations are significant between normal and treated insects using student's t test method.

3. RESULTS AND DISCUSSION

The fifth instar larval period of silkworm was found to last for six days and the body of larvae attained a length of 6-7cm and this larval period is divided into feeding (0 - 120h) and non feeding stage (120 - 144h). The larva began feeding after 12 hours of its penultimate molt. From 48 h to 96 h the larva was in the active feeding and growing stage. After 120h the larval food intake showed a decline and by 144h the larvae completely stopped feeding and were ready to start spinning the cocoon. The larvae administered with tryptophan showed a similar pattern in growth rate except a considerable delay in commencement of spinning and comparatively bigger and stouter in size. The haemolymph was slightly green in color in normal larvae but showed a brownish tinch in the treated, and became more viscous in the case of both normal and treated with development. The fat body cells were creamy white in color, moderately aggregated and freely suspended in the haemolymph.

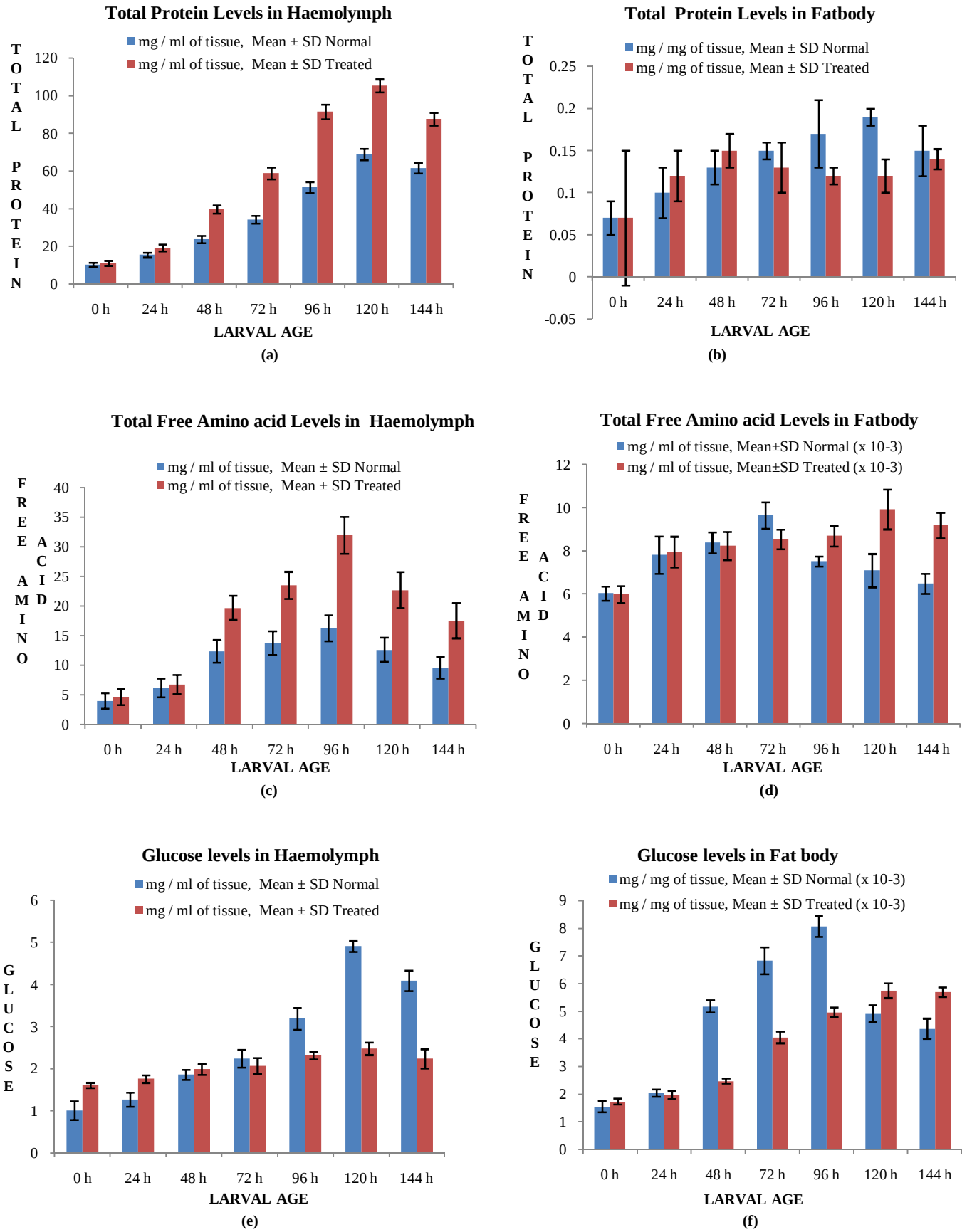

Fig. 1: The levels of total protein, free amino acids and glucose in the haemolymph and fat body of final instar *Bombyx mori* larvae of during its developmental stages

3.1 Total Protein

The levels of total protein in the haemolymph and fat body of the normal and treated larvae during its developmental stages are given Figures 1a and 1b. Total protein in the haemolymph of the normal and treated larvae showed a sharp increase up to 120h followed by a gradual decline. The total haemolymph protein of treated larvae showed 34-94% increase when compared to normal and maintained a relatively steady level between 96h and 144h in contrast to a 10% reduction in protein content during final stages of the normal. The normal larvae showed 11 fold increases in total fat body protein recording the peak value at 120h. In the treated larvae, the pattern of the changes in the levels of fat body protein was same but in a reduced level.

It has been established that the proteins are synthesized in the fat body and released into the haemolymph, which are subsequently sequestered into the fat body and stored there depending upon the physiological condition of the insects [16]. A group of abundant proteins is synthesized in silkworm larval peripheral fat body tissues and transported into the haemolymph to be eventually stored as granules in the pupal perivisceral fat body tissues for adult development during the non-feeding stage. From the morphogenetic point of view, investigations of the haemolymph proteins are of particular interest because they provide us with an adequate background to judge the synthetic activity associated with the differentiation process in the developing organism. The protein concentration of insect haemolymph is generally higher than that of the internal fluids of other invertebrates and is almost similar to that of the blood of man [17]. The results of the present study demonstrate that the total protein levels in both normal and treated larvae increase during the development of the final instar with a slight decline before pupation. The increase in concentration of protein in the early stages of larval period is in accordance with the active feeding and increased growth rate of the larva. In the case of spinning insects it is necessary to maintain a high level of tissue protein in the final instar as it's the main source for cocoon production [18]. The fat body of insects plays a central role in the synthesis, storage and translocation of proteins. It has already been shown that in larvae the fat body functions both as a storage centre for fat, carbohydrate and protein and is the principal site for intermediary metabolism. Insect haemolymph is an extracellular fluid bathing the fat body and therefore allow an easy exchange of metabolites with the latter. The difference in protein content between haemolymph and fat body can be explained in the context of its synthesis in the fat body and released into the haemolymph and are subsequently sequestered into the fat body and stored there depending upon the physiological condition of the animal [9, 19].

3.2 Total Free Amino Acids

The titre of total free amino acids per unit volume/weight of the haemolymph and fat body in the developmental stages of the normal and treated larvae are given in Figures 1c and 1d. The total content of free amino acids in the haemolymph of the larvae gradually increased, recording the peak value at 96h followed by a sharp dip. The tryptophan treated larvae showed a similar pattern of changes but with a higher magnitude. The increase was more significant during the feeding stage. The total content of free amino acids in the fat body is much less than that found in the haemolymph. There was a slight difference in the pattern and magnitude of the changes between normal and treated larvae when compared to haemolymph. Free amino acid titre of treated larvae was high during the beginning and final stages of larval development.

Insects are known to contain an unusually large amount free amino acids whose total concentration in some species has been found to be more than thirty times higher than that in other groups of animals [17]. Estimates of the protein content of other animals in the plasma showed that it is almost equal to that found in the haemolymph of insects, but in the case of free amino acid concentration of the blood of insects it is about 20 to 50 times higher than that in mammals [12]. In the present study, the total content of free amino acids in the haemolymph of normal and treated larvae increased gradually from the period of 0h to 96h with a peak value at 96h and then decreased. This is in conformation of the general observation that the concentration of free amino acid increases during the growth of the larva followed by a decline towards pupation. The change in the amino acid pool will directly influence the protein turnover and thus obviously reflects the physiological state of the organism. The decline in the level of free amino acid towards pupation observed in the present study apparently indicates a positive balance in protein storage during larval development. The treated larvae showed a high level of amino acid titre in haemolymph than that of normal. The difference was more prominent during the feeding stages of larvae which clearly indicate a positive correlation with the high protein titre in the same period. Similar observation was also reported in thyroxine treated fifth instar larvae of tsar silkworm, *Antheraea mylitta* [7]. It was noticed that the total content of free amino acids in the fat body of silkworm is much less than that found in the haemolymph. Any depletion or repletion of amino acids during the development of the larvae indicates a shift in the equilibrium between synthesis and degradation of body proteins.

3.3 Glucose

The changes in the concentration of glucose in the haemolymph and fat body of both normal and treated larvae during the developmental stages of the fifth instar larvae are presented in and Figures 1e and 1f. The levels of haemolymph glucose of both normal and treated larvae recorded a marked increase during the developmental period recording a peak value at 120h followed by a slight reduction towards pupation. The peak glucose levels in the total larval haemolymph were almost 28 times to that found at the early stage in normal larvae and approximately 12 times in tryptophan treated larvae. The fat body glucose levels also exhibited similar variation during the development of the larvae as seen for haemolymph. It was observed that treated larvae maintained a higher level of glucose than that of normal during the feeding stage followed by a dip on non feeding stage. Glucose

forms a predominant carbon source of chitin, a participant in energy metabolism and the substrate for protein and lipid synthesis in insects. The results of the present study revealed that the feeding stage of larva was characterized by low level of glucose in the tissue, which was increased sharply in the non-feeding stage. In the feeding stage, the larva undergoes rapid growth and glucose is utilized for anabolic purposes, whereas in the non-feeding stage, glucose is stored as an energy resource for the synthesis and degradation of tissues. Similar results have been reported during the larval development of *S. mauritia* [12]. The fat body levels of glucose also exhibited similar variation to that of the haemolymph during the larval development. Insect chitin, which accounts for a major part of cuticle, is a polymer of N- acetylglucosamine units. The final instar larvae of silkworm undergo larval – pupal transformation at the end of the instar. During this stage rapid reorganization of integumentary structures takes place for which a ready availability of its precursor molecules such as glucose is required. The high titre of glucose in tissues of the larvae at the end of the instar may be explained in the above context.

4. CONCLUSION

The present study has evaluated the physiological and biochemical changes in the haemolymph and fat body of larvae of silkworm, *Bombyx mori* during its final instar development on the administration of antioxidant amino acid, tryptophan. The result revealed that, tryptophan exerts significant increase in the biochemical parameters in the haemolymph compared to fat body. It is also evident that, tryptophan fed larvae exhibit a short delay in the commencement of cocoon spinning indicating the tendency to retain the juvenile form proving its antioxidant property.

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