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Profiling of Catalase and Hydrogen Peroxide Activity in Tryptophan Administered final instar Larvae of *Bombyx mori* L.

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ABSTRACT

Ageing is a universal, biological and natural phenomenon in all living organism. Oxidant damage by reactive oxygen species along with impaired antioxidant mechanism play a vital role in common and age related degenerative diseases in animals. To protect molecules against toxic free radicals and other ROS, cells have developed antioxidant defence system that include the enzymes super oxide dismutase (SOD), which dismutates toxic superoxide and glutathione peroxidase. Despite of the absence of glutathione peroxidase (GPx) in insects, the catalase mediates the main mechanism for hydrogen peroxide (H_2O_2) breakdown. The natural antioxidant mechanism can be inefficient and hence dietary intake of anti oxidant compounds become important. In the present study, a reducing amino acid tryptophan was administered through the feed material of final instar larvae of silkworm, Bombyx mori, in order to study its antioxidant effects on the growth and longevity of the larvae. The profiles of pro-oxidant H_2O_2 and antioxidant enzyme catalase during the development of the larvae were evaluated. H_2O_2 levels in haemolymph and fat body of the normal larvae increased gradually and recorded a peak value at 72h and decreased thereafter. Catalase activity haemolymph of normal larvae significantly increased from the beginning of fifth instar larval period and recorded a peak value at 72h followed by a sharp dip.

***Key words**: Bombyx mori, tryptophan, ageing, H_2O_2, catalase, ROS*

INTRODUCTION

Ageing 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. To protect molecules against toxic free radicals and other ROS, cells have developed antioxidant defence system that include the enzymes super oxide dismutase (SOD), which dismutates toxic superoxide and glutathione peroxidase. External sources of antioxidant vitamins like vitamin C, vitamin E and phytochemicals from plant rich diets provide important protection against oxidant damage[2]. However, the natural antioxidant mechanism can be inefficient and hence dietary intake of anti oxidant compounds become important. 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.

Ageing is the process pertains to individuals and hence elucidation of age related physiological activities should ideally be carried on the same individual during its entire lifespan and further the cellular and molecular level patterns can rarely be obtained without killing the organism.

As ageing process in multi-cellular animals is universal, such difficulties can be tide over by using insects as experimental animals. Despite of the absence of glutathione peroxidase (GPx) in insects, the catalase mediates the main mechanism for H_2O_2 breakdown. So insects are most useful in analyzing the effect of H_2O_2. It was found that H_2O_2 alone and in combination with other substances cause deleterious damages in the living tissue and the direct interaction of transition metal (copper) to DNA in the presence of H_2O_2 caused destabilization and fragmentation of naked DNA[3]. In most eukaryotic cells super oxide dismutase exists in two different forms, cytosolic type containing copper and zinc and a mitochondrial type containing manganese[4] . The natural combination of dismutase and catalase contributes to remove H_2O_2 and thus has a true cellular antioxidant activity. Catalase is highly effective in the breakdown of H_2O_2 at high concentrations and also has the unique characteristic that it can degrade H_2O_2 by catalytic or by peroxidatic mechanism[5]. Antioxidants may offer resistance against oxidative stress by scavenging free radials, inhibiting lipid peroxidation and or some other mechanism[6]. All organisms possess antioxidant defence system, which protect against oxidative damage and numerous damage removal and repair enzymes, which remove and repair damaged molecules. However, the natural antioxidant mechanism can be inefficient, dietary intake of antioxidant compounds become important[7]. Superoxide dismutases (SOD) are metallic enzymes that are essential for dismutation of O_2 to H_2O_2 and O_2. SODs are important initial components in the cellular defence against oxygen toxicity. In most eukaryotic cells SOD exists in two forms, a cytosolic type containing copper and zinc and a mitochondrial type containing manganese[4, 8]. Glutathion peroxidase is believed to play an important role in the breakdown of H_2O_2, because it is much more efficient than catalase at low concentrations of the substrate[9].

Catalase is primarily localized with in peroxisomes and to a lesser extent in mitochondria which is the main site of H_2O_2 production from O_2 dismutation. Catalase is highly effective in the breakdown of H_2O_2 at high concentrations and also has a unique characteristic that it can degrade H_2O_2 by catalytic mechanism[5]. In a recent study on protective effects of catalase over expression on UVB - induced apoptosis in normal human keratinocytes, pointed out that catalase over expression has a protective role against UVB irradiation by preventing DNA damage mediated by the late ROS increase[10]. Catalase activity revealed differences in time related changes across a 24 h period that are more obvious in peak levels between the brain, kidney and liver tissues of mouse[11]. Their results also showed significant organ differences with the highest catalase activity in liver compared with kidney and brain. This might be related to several factors such as their respective physiological function, the risk of exposure to oxidative damage and the balance between synthesis and degradation of proteins during normal metabolism.

In the present study, a reducing amino acid tryptophan was administered through the feed material of final instar larvae of silkworm, *Bombyx mori*, in order to study its antioxidant effects on the growth and longevity of the larvae. The specific objectives of the study are to evaluate the physiological and biochemical changes during the development of the final instar larvae on the administration of antioxidant amino acid, tryptophan. The profiles of pro-oxidant H_2O_2 and antioxidant enzyme catalase during the development of the larvae were evaluated.

MATERIALS AND METHODS

The experimental animal

The silkworm *Bombyx mori* L. belongs to the Phylum Arthropoda, Class Insecta and Order Lepidoptera. The bivoltine silkworm hybrid, Elite- CSR 2x4 was used for the study. The silkworm rearing was undertaken by procuring newly hatched larvae immediately after their brushing from Serifed, Palakkad, Kerala. The rearing house and all the appliances were disinfected in advance with chlorine dioxide/bleaching powder to free the rearing environment and the surroundings from pathogens. The rearing bed was made up by chopped leaves having the size of half to one cm. squares and covered with blue polythene sheet. Clean wet sand in trays was placed around the rearing bed to ensure 80-90%

humidity and the rearing room temperature was maintained at 27°C. Tender mulberry leaves with more moisture content, protein and carbohydrates were used for feeding the worms in the initial stages of rearing and were fed three times a day.

The duration of 5th instar was normally about 6-7 days and the larvae started to spin cocoon by the end of this stage. During 5th instar the larvae were fed with fully matured mulberry leaves and for the last two days it was coarse leaves. The present work was done on the fifth instar larvae, beginning from the newly moulted 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, 24 h, 48h, 72h, 96h, 120h and 144h. After the fourth moult 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.

Biochemical analysis

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 and total volume in the case of haemolymph and on the basis of unit weight of fresh tissue and total 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 ˉ20° C until the estimations were carried out.

Estimation of Hydrogen peroxide level and Catalase activity

Both estimations were done based on the formation of soluble coloured peroxotitanium complex in a reaction of $H_2O_2$with potassium titanium oxalate and the measurement of the orange/ yellow colour developed, at 410 nm[12]. For estimation of H_2O_2, the homogenized tissue was deproteinised with 20% trichloroacetic acid (TCA) and centrifuged at 3000 rpm for 10 min. 0.5 ml of the protein- free solution was mixed with 1.5 ml of acidic potassium titanium oxalate reagent and the bright yellow colour formed was measured at 410 nm in a spectrophotometer. The determination of catalase activity was based on estimating the amount of residual H_2O_2 in the assay mixture after incubation of a known amount of H_2O_2 with the enzyme extract for a fixed time interval. 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.

RESULTS

The H_2O_2 levels estimated per unit fresh tissue in the haemolymph and fat body of both normal and treated larvae are given in Figure 1 and Figure 2. H_2O_2 levels per unit volume haemolymph of the normal larvae increased gradually and recorded a peak value at 72h and decreased thereafter. H_2O_2 level at the final stage of the larvae was almost one half of that recorded in the previous stage. A different pattern of change was found in treated larvae where the values reached a maximum at 48h and then dropped down sharply. The pattern of change in H_2O_2 levels of the normal and treated larval fat body was more or less similar to that observed per unit volume of haemolymph.

The activities of the antioxidant haemoprotein catalase per unit fresh tissue was estimated in the haemolymph and fat body of both normal and treated larvae and are presented in Figure 3 and 4 respectively. Catalase activity of per unit volume haemolymph of normal larvae significantly increased from the beginning of fifth instar larval period and recorded a peak value at 72h followed by a sharp dip. There was a significant reduction in catalase activity in the treated larvae when compared with normal. The activity of catalase in fat body of normal larvae remained more or less the same throughout its development except that it was low in its initial stage. The activity of catalase in the treated larvae was high in the active feeding stages of the larvae but declined sharply towards the end of larval development.

Fig. 1: Changes in the hydrogen peroxide levels in haemolymph during final larval instar of *B. mori* on administration of tryptophan

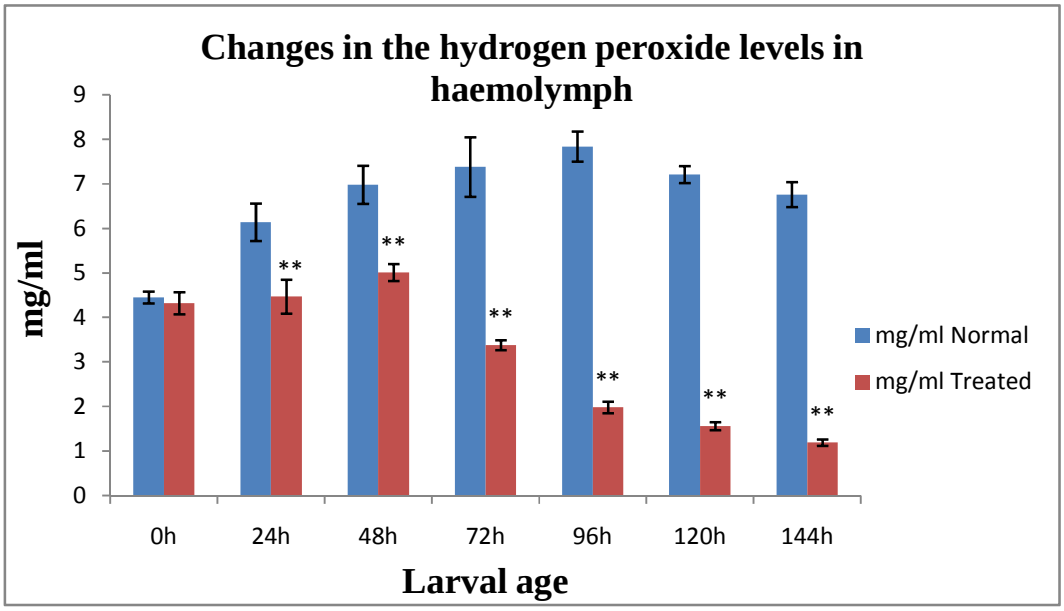


Fig. 2: Changes in the hydrogen peroxide levels in fat body during final larval instar of *B. mori* on administration of tryptophan

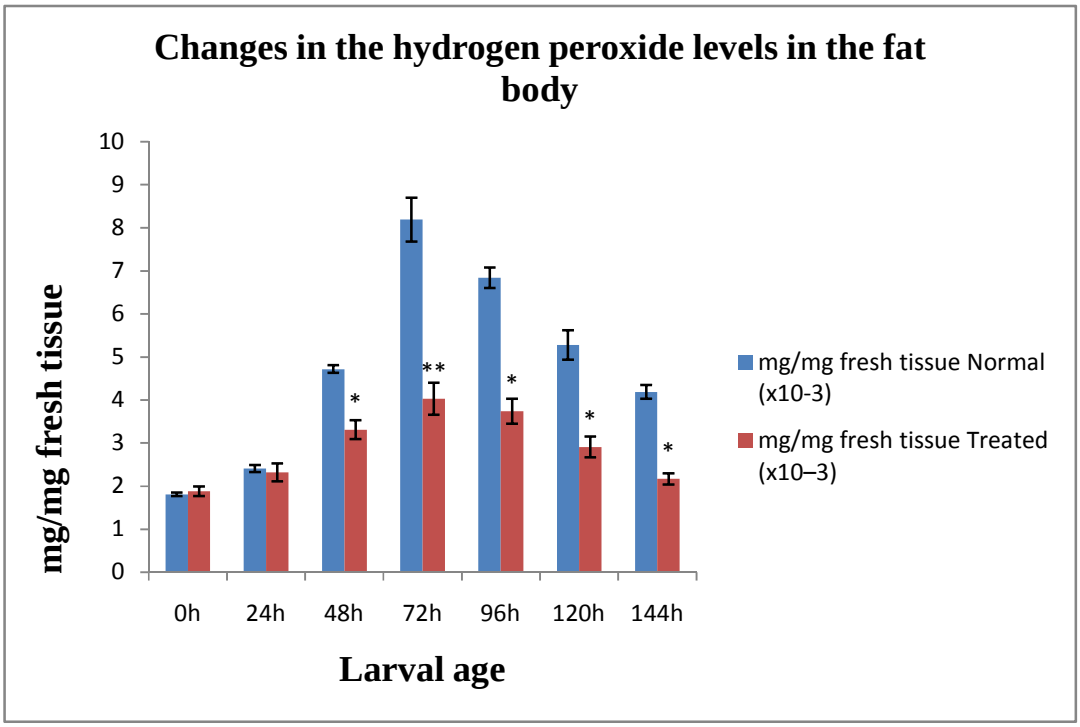


Fig. 3: Changes in the catalase activity levels in haemolymph during final larval instar of *B. mori* on administration of tryptophan

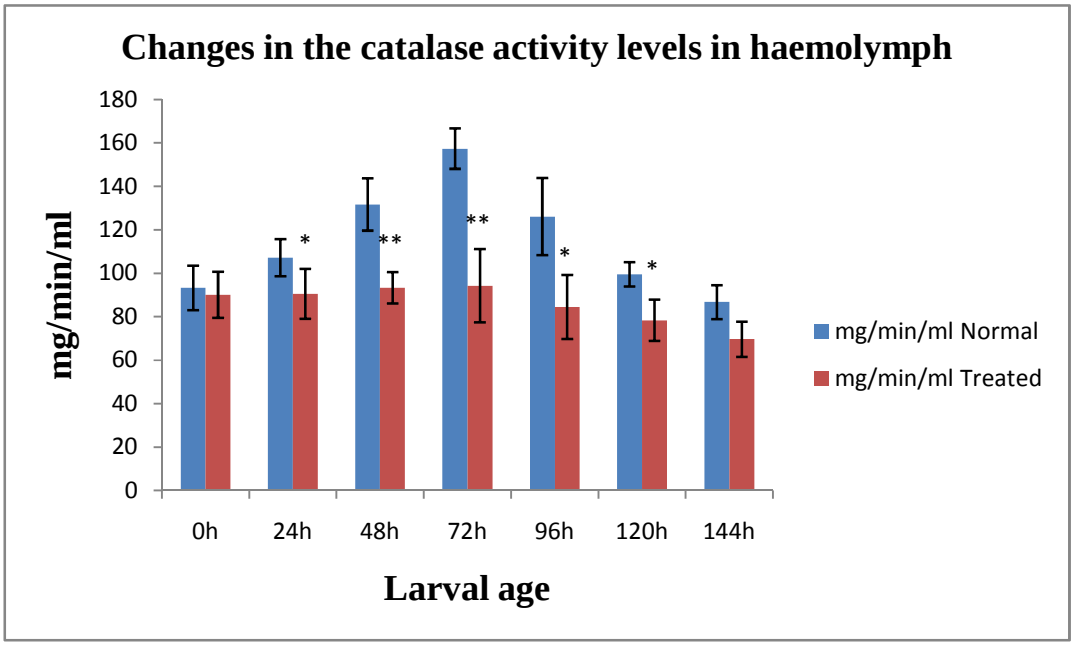
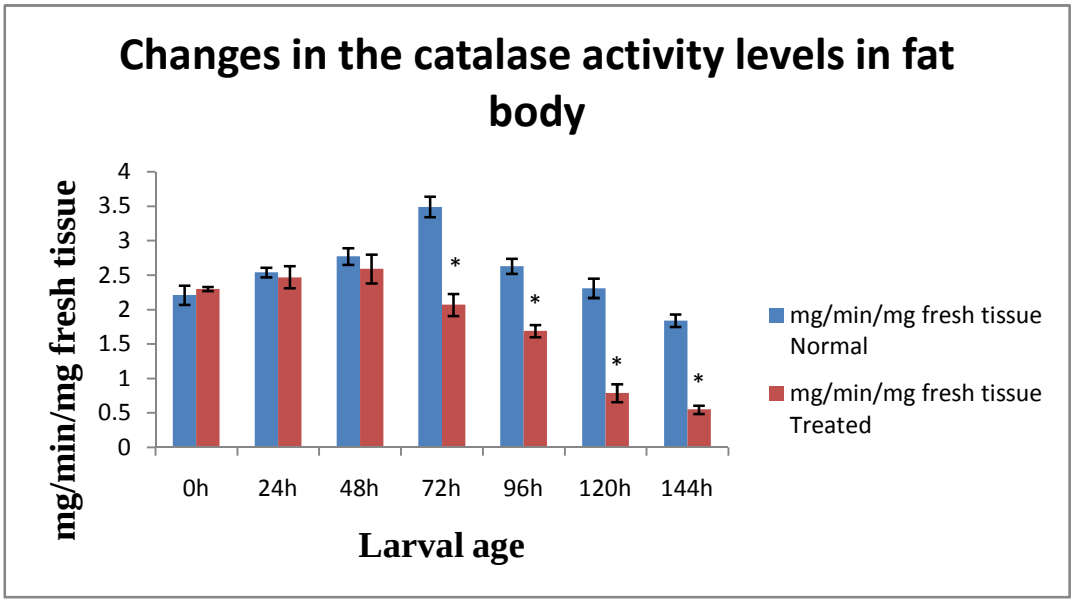


Fig. 4: Changes in the catalase activity levels in fat body during final larval instar of *B. mori* on administration of tryptophan



DISCUSSION

Accumulation of hydrogen peroxide and a decline in catalase activity is associated with ageing process of organisms. Accumulation of tryptophan residues in the transmembrane domains of integral membrane proteins performs vital antioxidant functions[13]. The accumulations of histological biomarkers of ageing were also delayed after tryptophan restriction in some organs. The tryptophan hydroxylase activity is affected by ageing process, which can drastically affect the levels of serotonin in brain[14]. The dietary effects of non-enzymatic antioxidant such as carotenoides, selenium and beta carotenoids have been demonstrated in organisms[15,16].

Cytosolic enzyme catalase is a component of antioxidant defense system that reduces hydrogen peroxide to water and protect the cell from oxidative damage[17]. Generation of H_2O_2 associated with life expectancy is proved by different workers[9,18]. H_2O_2 is normally eliminated from the cells by the activity of catalase and peroxidases. Catalase is primarily localized within peroxisomes, and to a lesser extent in mitochondria, which is the main site of H_2O_2 production[5]. *In vitro* studies on the role of reactive oxygen species on fibroblasts from patients with systemic sclerosis revealed that H_2O_2 act as a mediator of a positive autocrine feed back mechanism of ROS generation. The effects of H_2O_2 and H_2O_2 with the antioxidant agent N-acetyl-1-cystein or catalase on mean arterial pressure and heart rate of rats and found that H_2O_2 produced as a result of oxidative reaction causes misfunction of organs[19]. They also concluded that even 1.0 to 1.5 μmol doses of H_2O_2 can produce long lasting bradycardia.

The development of the final instar larvae of *B. mori* is marked by distinctive feeding and non- feeding stages. At the end of the non- feeding stage, the larva is transformed into a pupa with entirely different morphological features. The larvae of *B. mori* maintain a high level of H_2O_2 in its feeding stages and showed a sharp dip in the non- feeding stage. H_2O_2 levels in the tryptophan treated larvae showed a conspicuous reduction apparently indicating its scavenging action on the free radicals. Similar reduction in peroxide level on the administration of mixed antioxidant supplemented diet in adults of *Zaprionus paravittiger*, after the administration of mixed antioxidant supplemented diet[20]. Studies have been conducted on *Bombyx mori* larvae and proved that the administration of ascorbic acid and tyrosine caused significant changes in the organism's body by scavenging the free radicals[21].

Although steady state level of oxidative stress depends on both pro-oxidant generation and antioxidant defenses, most of the studies pertaining to ageing have focused on antioxidant defences. Catalase activity has been studied in a variety of insects[22,23]. Catalase activity increased with age and decreased during the latter part of life. Studies on catalase activity and lifespan of flies have shown that complete lack or reduced level of catalase activity does not affect their lifespan. An over expression of catalase (about 50%) had no effect on the lifespan of flies, nor did it improve their viability to an experimentally enhanced level of oxidative stress induced by parquet intake or hyperoxia[24]. The increased levels of catalase activity in the haemolymph of normal and treated larvae in the active feeding stages and its decline in the non-feeding stages observed in the present study can be explained in the above context. The treated larvae exhibited a significant reduction in haemolymph catalase activity which is in tune with the reduced H_2O_2 levels in the tissue. The results of the present study also demonstrate that there is a corresponding increase in the antioxidant activity with an increase in pro-oxidant generation. Studies on the variation of catalase activity in brain, kidney and liver of adult male mice showed that the activity was highest in the liver compared with kidney and brain[11]. The difference in the levels of enzymes in different tissues have been attributed several factors such as their respective physiological functions, the risk of exposure to oxidative damage and the balance between synthesis and degradation of proteins during normal metabolism. The reduced levels of catalase activity in the treated larvae compared to the normal also indicate a reduced pro-oxidant generation in the latter.

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