ANTIOXIDANT ENZYMES AS DEFENSE MECHANISM AGAINST OXIDATIVE STRESS IN MIDGUT TISSUE AND HEMOCYTES OF *Bombyx mori* LARVAE SUBJECTED TO VARIOUS STRESSORS

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In this study, larvae of silkworm Bombyx mori were subjected to low temperature, hypoxia, and viral infection to evaluate stressor-mediated oxidative stress (OS) and the induction of antioxidant enzymes (AOEs). Exposure to cold, hypoxia, and nuclear polyhedral virus for 24 h resulted in a significant increase in hydrogen peroxide generation with concomitant increase in lipid peroxidation (LPO) and protein carbonyl levels in midgut and hemocytes. AOEs such as superoxide dismutase and catalase also increased significantly in both the tissues and the increased AOEs reverted to control values during recovery. Ontogenic stages of the larvae showed a diminishing ability of the tissues to overcome OS induced by the stressors. A significant increase in AOE activity during short stress period indicated a possible transitory defense mechanism to avoid OS-induced cell damage.

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Keywords: oxidative stress; free radicals; antioxidant enzymes; lipid peroxidation; protein carbonyl

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INTRODUCTION

Reactive oxygen species (ROS), a by-product of oxidative metabolism in aerobic cells, are produced following the exposure of cells and tissues to various stressors (Droge, 2002). An imbalance between the production of ROS and reactive intermediate species in biological systems is termed oxidative stress (OS). Radical species such as superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), peroxy ($RO_2^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$), and nonradical species such as hydrogen peroxide (H_2O_2), singlet oxygen (1O_2), and peroxynitrite ($ONOO^-$) in excess cause protein oxidation and lipid peroxidation (LPO; Nordberg and Arner, 2001; Dalle-Donne et al., 2002). Protein oxidation is a covalent modification of proteins, which is induced mainly by ROS or by the secondary by-products of OS. Oxidative damage to proteins is considered a key indicator of OS, and protein carbonyl content is a general measure of OS (Davies, 1999). Membrane phospholipids are also subjected to oxidants and LPO involving chain reactions that are initiated by the abstraction of hydrogen atoms of unsaturated fatty acyl chain (Cadenas, 1989; Davies, 1995).

Living organisms, thus, require regulatory systems for protection from ROS. One such system is antioxidant enzymes (AOEs), wherein the primary defense against superoxide anions and hydrogen peroxide is mediated by the action of superoxide dismutase (SOD) and catalase (CAT) (Joanisse and Storey, 1996; Imlay, 2008; Li et al., 2011; Sim and Denlinger, 2011). SOD removes $O_2^{\cdot-}$ through the process of dismutation to O_2 and hydrogen peroxide, whereas CAT breaks down hydrogen peroxide into H_2O and O_2 (Kashiwagi et al., 1997). Hydrogen peroxide accumulation and decreased antioxidant state have been reported in cells under cold stress (O’Kane et al., 1996; An and Choi, 2010). On the contrary, activation of AOEs to overcome the cold-induced OS has been reported by Lalouette et al. (2010). In general, ROS are implicated in the heat stress signal transduction pathway and defense mechanism (Pnueli et al., 2003), and the ontogenic stage has a relevance in increased AOEs in the Oriental fruit fly *B. dorsalis* subjected to thermal stress (Jia et al., 2011). Increased production of ROS in hypoxic environments, however, remains controversial (Zuo and Clantron, 2005), since the general understanding is that hypoxia leads to increased oxidant production. During hypoxic conditions, oxygen demand exceeds its supply and can promote the disruption of cellular homeostasis and can trigger many cellular responses (Synder and Chandel, 2009). Anoxia/hypoxia is an overwhelming cellular response in most overwintering insects and usually coincides with an increased antioxidant capacity (Sim and Denlinger, 2011). Herbivorous insects often face the challenge from ROS of plant origin (Krishnan and Kodrik, 2006) and from pathogens (Doke et al., 1996), since plant ROS function as a deterrent against insects and pathogens. OS-induced free radicals are considered as pathogenic molecules in viral diseases (Maeda and Akaike, 1991) and lepidopteran larvae infected with virus have shown considerable upregulation of antioxidant mechanism against OS (Lee et al., 2005). Increased level of OS and decreased AOE activities are reported in two of the lepidopteran cell lines infected with virus *Autographa californica* multiple nuclear polyhedrovirus (Wang et al., 2001), whereas mosquito cells rescue themselves from Dengue virus infection through antioxidant defense (Chen et al., 2011).

The midgut and hemocytes of insects are considered to be highly metabolic, and the midgut is usually susceptible to oxidative injury during food digestion with strong redox potential; the oxidising condition often causes the production of ROS (Krishnan and Kodrik, 2006). High ROS concentration impairs the absorption of ingested nutrients and can cause oxidative damage to the midgut cells (Bi and Felton, 1995). On the other hand, insect hemocytes play an important role in immunity and the respiratory burst

of hemocytes is often associated with SOD during immune reactions (Minakami and Sumimotoa, 2006; Kavanagh and Reeves, 2007).

The main aim of the work was to evaluate the role of AOE in maintaining the homeostasis of ROS in silkworm, *Bombyx mori*, larvae under various stressors. In the present study, we analyzed the altered protein oxidation and LPO levels, activities of AOE SOD and CAT in the midgut and hemocytes of two instars. We report increased AOE in both instars to overcome OS when larvae were stressed for a short time, with low temperature, hypoxia, and virus. Our results clearly indicated a possible transitory defense mechanism afforded by AOE to lessen the OS-induced cellular damage.

MATERIALS AND METHODS

Chemicals

Thiobarbituric acid (TBA), horseradish peroxidase, and dinitrophenylhydrazine (DNPH) were purchased from Sigma–Aldrich, (St. Louis, MO). Hydrogen peroxide, Triton X-100, epinephrine, 3,5,3',5'-tetramethylbenzidine (TMB), sodium dodecyl sulfate (SDS), acetic acid, butanol, pyridine, and tetra methoxy propane (TMP) were purchased from Sisco Research Laboratory (Mumbai, India).

Insects and Experimental Design

The present study was approved by the Institutional Animal Ethics Committee, Bangalore University, Bangalore, India. The second instar larvae were procured from Kunigal seed area, Karnataka, India, and were maintained in laboratory throughout the larval stages and were fed ad libitum on M5 variety mulberry leaves (Vyjayanthi and Subramanyam, 2002a, 2002b). The uniformly grown healthy larvae of IV and V instars were used in all experiments and were maintained at 24–25°C with relative humidity of 70–75%. They were made into six groups and each group consisted of hundred. Experimental animals of group I were not subjected to any stress and were considered as control. Group II larvae were subjected to cold treatment at 5°C for 24 h, whereas group III was also subjected to cold treatment and maintained at room temperature for an additional period of 12 h as recovery period. Group IV was subjected to hypoxia for 24 h and group V larvae were subjected to hypoxia for the same period and were allowed to recover for an additional period of 12 h. Hypoxia was induced by closure of four pairs of posterior spiracles with dental wax and during recovery period all the spiracles were in open state. Group VI larvae were inoculated with 10 µl of 1×10^6 *B. mori* nuclear polyhedra virus (BmNPV) suspension per gram body weight. Larvae that were injected with 10 µl of insect ringer served as sham.

Midgut epithelial cells were isolated by micro dissection and collagenase treatment. Hemolymph was collected in a precooled 2 ml vial containing 5 mg thiourea by gentle incision on caudal horn of the larvae and hemocytes were separated by centrifuging the diluted hemolymph at 3,000 rpm for 10 min in cold. Cold phosphate buffer of pH 7.4 was used for the tissue homogenate preparation and for the separation of hemocytes or for the isolation of midgut epithelial cells.

Hydrogen Peroxide Generation

Hydrogen peroxide, a by-product of reactions catalyzed by oxidase, was determined according to Josephy et al. (1982). Two hundred microliters of tissue extract was added to

incubation mixture that contained 100 nmol of 3, 5, 3', 5'-TMB and horseradish peroxidase in acetate buffer (0.2 M, pH 5.0). The absorbance was measured at 700 nm and hydrogen peroxide concentration was expressed as micromole per milligram protein per minute.

LPO Level

Malanodialdehyde (MDA), a product of LPO was determined as described by Ohkawa et al. (1979). In brief, 200 μ l of tissue extract was added to 8.1% SDS, vortexed and incubated for 10 min. Three hundred seventy-five microliters of 20% acetic acid and 0.6% TBA were added to the reaction mixture and placed in a boiling water bath for 60 min. The samples were allowed to cool and 1.25 ml butanol:pyridine mixture (15:1, v/v) was added and centrifuged at 640 g for 5 min. Absorbance was measured at 532 nm using 1,3,3-TMP as standard. MDA concentration was expressed as nanomole per milligram protein.

Protein Carbonyl Level

Protein carbonyl (PrC) was measured by the method of Uchida and Stadtman (1993). 0.1% DNPH in 2 N HCl was added to 800 μ l of tissue extract. Samples were held in the dark for 1 h. The protein was precipitated with 20% trichloroacetic acid and centrifuged. The pellet was washed thrice with ethanol and ethyl acetate (1:1, v/v) and was dissolved in 2 ml of 8 M guanidine hydrochloride, and centrifuged. The supernatant was used to measure the absorbance at 365 nm and the PrC level was calculated using a molar absorption coefficient of 22,000 M⁻¹ cm⁻¹. The results were expressed as micromolar per milligram protein.

SOD (E.C. 1.15.1.1) Activity

SOD activity was measured according to Misra and Fridovich (1972) with slight modification. Briefly, 100 μ l of 5% tissue extract was added to 880 μ l of carbonate buffer (0.5 M, pH 10.2). Twenty microliters of epinephrine (30 mM in 0.05% acetic acid) was added to the mixture and measured spectrophotometrically (Genway, UK) at 480 nm for 4 min. SOD activity was measured as the amount of enzyme that inhibits oxidation of epinephrine by 50%, which is equal to 1 unit.

CAT (E.C. 1.11.1.6) Activity

CAT was determined by method of Aebi (1984). Briefly, 100 μ l enzyme sample with 10 μ l of absolute alcohol was incubated for 30 min at 0°C followed by addition of 10 μ l Triton X-100. An aliquot of 50 μ l was taken in 1.25 ml of 0.066 M H₂O₂ in phosphate buffer and decrease in absorbance was measured at 240 nm for 60 sec in a spectrophotometer. An extinction coefficient of 43.6 M cm⁻¹ was used to determine enzyme activity and was expressed as 1 μ mol of H₂O₂ degraded per minute per milligram protein.

Statistical Analysis

Data are shown as mean $\pm$ SD of six observations. Changes between the groups were analyzed by MANOVA and further tested by Bonferroni post hoc test using Statistical

Package for Social Science (SPSS) software (Huberty and Olejnik, 2006) and $P < 0.05$ was considered significant. Statistically significant data are presented in the text.

RESULTS

H_2O_2 Generation

A decrease of temperature from 27 to 5°C for 24 h duration resulted in a significant increase in hydrogen peroxide generation in the midgut tissue and hemocytes in IV and V instar larvae. In the present experiment, 24 h was considered the stress period since H_2O_2 production was found to be optimal at 18 h onward. Hypoxia induced by spiracular closure also resulted in a significant increase in H_2O_2 in the midgut and hemocytes of both instars. Increased H_2O_2 generation induced by low temperature and hypoxia reverted to base value within 12 h of the recovery period, irrespective of the instars in both of the tissues studied. Increased peroxy radical generation in midgut cells and hemocytes was observed on viral infection to larvae by hypodermal injection (Fig. 1A and B). V instar

Figure 1. Hydrogen peroxide concentration in silkworm *B. mori* midgut tissue (A) and hemocytes (B) subjected to cold, cold recovery, hypoxia, hypoxia recovery, and viral infection. Data are means $\pm$ SE ($n = 6$). $P < 0.05$ was considered significant. Values between the stressors are represented in lower cases (a, b, c, d, bc). Those not sharing the same letters are significant.

Figure 2. MDA level in silkworm *B. mori* midgut tissue (A) and hemocytes (B) subjected to cold, cold recovery, hypoxia, hypoxia recovery, and viral infection. Data are means $\pm$ SE ($n = 6$). $P < 0.05$ was considered significant. Values between the stressors are represented in lower cases (a, b, c, d, ac, bc). Those not sharing the same letters are significant.

larvae showed a significantly higher generation of H_2O_2 over IV instar irrespective of the treatment in the tissues studied. Sham-operated larvae were not significantly different from the control group in terms of the H_2O_2 generation in both the tissues.

LPO Level

Free radical induced LPO was observed in midgut tissue and hemocytes of the silkworm larvae. Significant increase in MDA content was observed on cold exposure, hypoxia, viral infection, and also during the recovery period in midgut tissue and hemocytes of both instars (Fig. 2A and B). However, MDA did not show any correlation with the larval stage in the tissues studied.

PrC Level

PrC level as a marker of protein oxidation increased in the midgut tissue as well as in the hemocytes on exposure to cold, hypoxia, and viruses. The increased PrC content due

Figure 3. PrC level in silkworm *B. mori* midgut tissue (A) and hemocytes (B) subjected to cold, cold recovery, hypoxia, hypoxia recovery, and viral infection. Data are means $\pm$ SE ($n = 6$). $P < 0.05$ was considered significant. Values between the stressors are represented in lower cases (a, b, c). Those not sharing the same letters are significant.

to stress remained the same during the recovery period as well in both the instar larvae (Fig. 3A and B).

SOD Activity

SOD activity in midgut tissue of silkworm larvae under cold stress significantly increased in the IV instar larvae and the increased activity returned to its control value in the recovery period of 12 h. The exposure to hypoxia and viral infection also increased SOD activity significantly in IV instar. Increased SOD activity in the midgut tissue of V instar larvae was also observed upon exposure to all stressors. The extent of increase was relatively higher in the cold when compared to hypoxia and viral infection (Fig. 4A). SOD activity under cold, hypoxia, and viral treatment was also significantly increased in the hemocytes of IV and V instar larvae (Fig. 4B). However, the enzyme activity in both midgut and hemocytes of V instar larvae was less than IV instar larvae under various experimental conditions (Fig. 4A and B).

Figure 4. SOD activity in midgut tissue (A) and hemocytes (B) of IV and V instar silkworm *B. mori* to cold, cold recovery, hypoxia, hypoxia recovery, and viral infection. Data are means $\pm$ SE ($n = 6$). $P < 0.05$ was considered significant. Values between the stressors are represented in lower cases (a, b, c, ac). Those not sharing the same letters are significant.

CAT Activity

CAT activity was significantly enhanced in midgut tissues of *B. mori* larvae subjected to all stressors and its activity was found to be higher in IV instar compared to those of V instar per assay (Fig. 5A). It was also significantly increased in the hemocytes of IV and V instar larvae subjected to various stressors (Fig. 5B). However, activity in the IV instar was significantly higher than that in V instar larvae (Fig. 5A and B).

DISCUSSION

A cell's early response to different stressful stimuli is to defend against and recover from the insult. Depending on the level and mode of stress, different survival mechanisms are mounted and the reactions are highly conserved in evolution; antioxidant defense is one among the survival mechanisms against oxidative injury (Fulda et al., 2010). In the present study, the lepidopteran larvae were subjected to stressors such as low temperature, hypoxia, and a known pathogen. In the study, midgut epithelium and hemocytes experienced OS, as evidenced by a significant increase in the H_2O_2 level during

Figure 5. CAT activity in midgut tissue (A) and hemocytes (B) of IV and V instar silkworm *B. mori* to cold, cold recovery, hypoxia, hypoxia recovery, and viral infection. Data are means $\pm$ SE ($n = 6$). $P < 0.05$ was considered significant. Values between the stressors are represented in lower cases (a, b, c). Those not sharing the same letters are significant.

exposure to stressors. H_2O_2 is one of the entities of ROS, with the other being superoxide radical. Hydrogen peroxide is typically produced in peroxisomes as a by-product of reactions catalyzed by oxidases (Kinnula et al., 1992) and, in addition, H_2O_2 is released into the cytoplasm via leakage from a range of organelles. Increased H_2O_2 generation upon exposure to low temperature, as evidenced in the present study, is similar to the studies on *Ostrinia nubilalis* subjected to low temperature. Increased levels of H_2O_2 and radical properties of melanin accounted for the cold hardiness of this particular insect on exposure to low temperatures (Kojic et al., 2009). Hypoxia can induce elevation in ROS, especially H_2O_2 (Giaccia et al., 2004), and increased production of H_2O_2 in cardiomyocytes helps in hypoxia-induced ischemia during preconditioning (Zhang et al., 2002). Although elevation in ROS by hypoxia is a known phenomenon, the real mechanism involved has not been explained (Zuo and Clantrou, 2005). In our experiments, hypoxia was induced by the closure of the last four pairs of spiracles. We consider the above condition as hypoxia rather than anoxia because the insect's oxygen demand is not met. In our preliminary experiments, we observed an 18% decrease in oxygen consumption when

the last four pairs of spiracles were closed. A significant increase in H_2O_2 generation in both midgut and hemocytes during hypoxic stress may be reasoned as “reductive stress,” involving NADH oxidase or ubiquinone as discussed by Clanton (2005). Cells experience OS following infection with virus (Schweizer and Peterhans, 1999), and such OS mainly occurs by viral challenge at the cell surface rather than viral replication (Kaul et al., 2000). Although ROS is produced in the cytoplasm by oxidases, mitochondria were presumed to be a major source of ROS in virally infected cells (Wang et al., 2001) and oxygen free radicals contribute to pathogenesis in several viral infections (Simula and De Re, 2010; Chen et al., 2011). In the present study, a significant increase in H_2O_2 generation in the midgut and hemocytes of *B. mori* larvae infected with NPV was observed. Increase in H_2O_2 generation was also reported on viral infection in whole silkworm infected with BmNPV (Li et al., 2011). Hydrogen peroxide can peroxidase the unsaturated lipid of the cell membrane (Fridovich, 1978) and cause the oxidative modification of proteins involving the formation of carbonyl groups in the side chains of certain amino acid residues (Stadtman, 1992; Dalle-Donne et al., 2002). A substantial increase in ROS generation was found in the midgut and hemocytes of both instar larvae and it was concomitant with a significant increase in LPO and protein oxidation, thereby implying that stressors such as hypoxia, cold, and viral infection caused OS leading to damage of the plasma membrane structure and cellular proteins. The correlative relationship among ROS production and oxidative damage in the present study is consistent with the predictions of the OS hypothesis.

Stress induces a rapid production of ROS and their elimination by the antioxidant system is essential for the survival of the animal (McArdle and Jackson, 2000). In dipteran insects, low temperature significantly increased the AOE (Jia et al., 2011); hypoxia induced by diapause also showed a relationship with free-radical formation and AOE (Jovanovica-Galovic, 2007) and an increase in AOE was also found in silkworm on pathogenic infection (Wang et al., 2001; Krishnan et al., 2002). Viral infection activates the translation of host genes causing responses involving unfolded proteins (Patramool et al., 2011), whereas alteration in SOD 1 following Rift Valley fever virus (RVFV) infection and simultaneous activation of p38 mitogen activated protein kinase (p38 MAPK) were implicated for improving survival of infected cells (Narayanan et al., 2011). Mosquito cells use antioxidant mechanism to survive Dengue virus infection (Chen et al., 2011) and upregulated glutathion S-transferase (GST) found to be responsible for the survival of mosquito cells infection with Dengue virus (DENV) (Lin et al., 2007). Exposure to low temperature, hypoxia, or inoculation with BmNPV resulted in a significant increase in AOE in the fourth and fifth instar larvae. Increased SOD and CAT activities in the fifth instar were relatively less when compared to the fourth instar. A concomitant increase in H_2O_2 in both tissues was also evident, especially in the fifth instar, due to increased OS associated with the ontogenic stage of silkworms. Variations in antioxidant system in ontogenesis have been reported in the beetle *Tenebriomolitor* (Gulevsky et al., 2006a, 2006b). In contrast to the present findings, studies on lepidopteran larvae infected with NPV have indicated a reduction of AOE and reasoned it for increased LPO (Li et al., 2011). The current study was restricted to the immediate response of larval oxidative and antioxidant system subjected to the stress exposure and on recovery. An increase in the ROS and oxidative products were observed along with a simultaneous increase in AOE during the stress period and reversal during recovery period. The present study clearly indicates enhanced AOE, which may function as an immediate defense mechanism to overcome the oxidative insult induced by single exposure to cold, hypoxia, and virus.

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