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[Note]

Oxidative Stress and Antioxidant Defences in the Tasar Silkworm *Antheraea mylitta* D: Challenged with *Nosema* Species

Karmabeer Jena*, Jay Prakash Pandey, and Ajit Kumar Sinha

Silkworm Physiology Laboratory, Central Tasar Research and Training Institute (Central Silk Board), P.O. Piska-Nagri, Ranchi 835303, India

Abstract

This study was designed to find out the effect of *Nosema* spore on oxidative damages and antioxidant defence in the midgut of tasar silkworm *Antheraea mylitta*. Higher level of lipid peroxidation (LPX) and total hydroperoxides indicate the resultant oxidative stress in the *Nosema* exposed specimen. Increased superoxide dismutase (SOD) suggests activation of physiological mechanism to scavenge the superoxide radical produced during *Nosema* infection. Higher activities of catalase and glutathione-S-tranferase on 18th d indicate adaptive behaviour of the tissue against oxyradicals. The results suggest that *Nosema* infection is involved in altering the active oxygen metabolism by modulating LPX and reactive oxygen species (ROS), which is indicative of pebrine disease disorder.

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Introduction

Tropical tasar silkworm, *Antheraea mylitta* D. (Lepidoptera: Saturniidae) is a commercially important wild polyphagus sericigenous insect. It is an important component of Asian non-mulberry sericulture industry. Since tasar silkworms are reared outdoors, they are more vulnerable to various diseases such as pebrine, virosis, bacteriosis and mycosis. Among these, pebrine disease which is caused by *Nosema* sp. infects almost all stages and ecoraces of the tasar silkworm by both primary (transovarial) and secondary (peroral) infections. In insect, *Nosema* infection takes place after ingestion of mature spores that germinate in the midgut by polar tube extrusion and injection of the sporoplasm inside the epithelial cell cytoplasm (Higes *et al*., 2007). As ingestion is the main entry route of many pathogens, the intestinal epithelium is the first line of defense against invasion and dissemination of pathogenic microorganisms. In this view insect innate immune system plays a vital role in the defense against microorganisms (Medzhitov and Janeway, 1997). One of the most immediate epithelial responses in mammals to combat the pathogen is the generation of antimicrobial reactive oxygen species (Cohn *et al*., 1994; Geiszt *et al*., 2003). It is to be noted that ROS can affect both entomopathogens and the host tissues, and may lead to damages of cellular biomolecules such as lipids, proteins and nucleic acids.

To protect against these toxic cellular environment, insect possess a suit of antioxidant defence mechanisms, comprised of both enzymatic as well as non-enzymatic components.

***Corresponding author.**
Karmabeer Jena
Central Tasar Research & Training Institute, Nagri, Ranchi-835303, Jharkhand, India
Tel: +91-9471517167 / FAX: +91-651-2557629
E-mail: jenakb2010@gmail.com

The major enzymes involved in the process are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST). Superoxide radicals ($O_2^{\bullet -}$) are dismutated by SOD to hydrogen peroxide (H_2O_2) which is reduced to water and molecular oxygen by CAT. Further, GPX catalyzes the reduction of H_2O_2 to water and organic peroxide to alcohols using reduced glutathione GSH as a source of reducing equivalent. GR regenerates reduced glutathione (GSH) from oxidized glutathione (GSSG), which is a scavenger of ROS as well as a substrate for the other enzymes. GST conjugates xenobiotics with GSH for excretion. The non-enzymatic component consists of small organic molecules such as GSH, and vitamin C (Felton and Summers,1995; Krishnan and Kodrik,2006; Krishnan *et al*., 2009; Buyukguzel *et al*., 2010; Zhao and Shi 2009, 2010).

Sufficiently large details are available on larval growth rate (Rath *et al*., 2003), cocoon characters (Velide *et al*., 2013), total haemocyte count (Madhusudhan *et al*., 2011) and excretory products (Renuka and Samitha, 2012) during infection. However very scant information exists regarding the damages caused by *Nosema* spores to the host tasar silkworm and the mechanism adopted by this insect to protect itself. The present study has been therefore designed to quantify the oxidative damages (LPX and total hydroperoxide) and antioxidant defence (SOD, CAT and GST) in midgut of tasar silkworm *A.mylitta* infected by *Nosema* spores.

Materials and Methods

Isolation of spores and Insect infection

Nosema spores were isolated from diseased larvae of Daba ecoraces homogenized in 0.6% K_2CO_3, filtered and the filtrate was centrifuged at 3000 rpm for 15 min. Spores were purified on discontinuous sucrose gradient (25, 50 and 75%) by centrifugation at 4000 rpm for 10 min.. The spores were collected from the sediment and washed in distilled water thrice and stored as stock at 4°C in 0.85% NaCl until use. They were then suspended in distilled water and counted using haemocytometer. The stock solution was diluted to obtain an inoculum dosage of 10^6 spore's mL^{-1}. Daba TV healthy fifth instar (after fourth moult) larvae were starved for 3-4 h to induce hunger and than fed on the *Terminalia arjuna* leaves smeared with inoculum dosage.

Sample preparation

The midguts were dissected out, thoroughly washed in ice-chilled phosphate buffer (50 mM, pH 7.0) to remove haemolymph, muscle tissues and fat body contamination. Tissue samples (midguts) were homogenized in ice-cold buffer (50 mM phosphate buffer, pH 7.0). Homogenization was carried out in an ice-chilled motor driven Teflon Potter-Elvejhen homogenizer and centrifuged at 8000 x g for 15 min at 4°C. The supernatant was used for biochemical analysis.

Estimation of lipid peroxidation

LPX level was assayed by measurement of malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids hydro peroxides were determined by the TBA reaction as described by Bar-Or *et al*. (2001). Briefly, the reaction mixture containing 0.1 mL of sample, 0.9 mL of 0.8 % aqueous solution of TBA (in 20% TCA). Then the mixture was heated at 95°C for 60 min and cooled under room temperature. The supernatant was read at 532 nm after removal of any interfering substances by centrifuging at 4000 x g for 10 min. The amount of MDA formed was calculated by using an extinction coefficient of $1.56 \times 10^5 M^{-1} cm^{-1}$ (Wills 1969), and expressed as nmol MDA/mg protein.

Estimation of total hydroperoxides

Total hydroperoxides were determined spectrophotometrically according to the method of ferrous oxidation with xylenol orange (FOX1) (Wolff, 1994). Hydroperoxides oxidize ferrous to ferric ions selectively in dilute acid and the resultant ferric ions can be determined by using ferric sensitive dyes as an indirect measure of hydroperoxide concentration. Xylenol orange binds ferric ions with high selectivity to produce a coloured (blue-purple) complex. The absorbance was read at 560 nm after removal of any flocculated material by centrifugation at 4000 x g for 10 min. The signal was read against an H_2O_2 standard curve.

Superoxide dismutase

Super oxide dismutase activity was estimated by Kono, (1978). The reaction mixture consisted of 50 mM sodium carbonate, 25 µM NBT, 0.6% Triton X 100 and 0.1 mM EDTA. The reaction was initiated by addition of 1 mM hydroxylamine-hydrochloride. The rate of NBT reduction was recorded at 560 nm. The control was simultaneously run without tissue homogenate. One unit of SOD is defined as the amount required inhibiting the photoreduction of NBT by 50%. The specific activity of SOD was expressed as unit/mg protein.

Catalase

Catalase activity was determined according to Aebi, (1974). The method is based on the decomposition rate of H_2O_2 by the enzyme. The assay mixture contained 2.9 mL of 12 mM H_2O_2 and 0.1 mL of sample (100 µg protein). Absorbance was measured at 240 nm and CAT activity is expressed as nkat/ mg protein (1 katal = 1 mol sec^{-1}).

Glutathione-S-transferase

Glutathione-S-transferase activity was measured according to Habig *et al*. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. Assay mixture contained 2.7 ml of 100 mM phosphate buffer (pH 7), 0.1 mL of 30 mM GSH, 0.1 mL of 15 mM CDNB and 0.1 mL of sample (100 µg protein). The change in absorbance was recorded at 340 nm and enzyme activity was expressed as nmol CDNB conjugate formed/ min/ mg protein using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Protein assay

The protein content was estimated by the Bradford (1976) method using bovine serum albumin as standard.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Difference between control and treatment was analyzed by Student's t-test. Differences were considered statistically significant when $p<0.05$. Further inter relationship was analysis by correlation.

Results

Lipid peroxidation

A significant increase in LPX level was observed in the midgut of *A.mylitta* larvae on 6th and 18th day after the insects were inoculated with *Nosema* spore, compared to controls (Fig-1A, $p < 0.05$). Also an insignificant increase was also seen on 12th day of the treatment in comparison to respective controls ($p > 0.05$).

Total hydroperoxides

Total hydroperoxide level significantly increased on 6^{th} day of treatment (Fig-1B, $p < 0.05$) while an insignificant increase was recorded on 12^{th} and 18^{th} day in relation to controls (Fig-1B, $p > 0.05$).

Superoxide dismutase

A significant increase in SOD activity to was seen up to (1.55 fold) on the 6^{th} day, 1.98 fold on 12^{th} day and 1.89 fold on the 18^{th} day in the larvae exposed to *Nosema* spores (Fig-1C, $p < 0.05$) in relation to control (Fig-1C).

Catalase

CAT activity showed a significant increase on 18^{th} day of exposure compared to control larvae (Fig-1D, $p < 0.05$). However, no significant change in CAT activity was recorded on 6^{th} and 12^{th} day of post inoculation.

Glutathione-S-transferase

GST activity in the midgut of infected larvae significantly increased on 18^{th} day after inoculation in comparison to the control (Fig-1E, $p < 0.05$). However, on 6^{th} and 12^{th} day the GST activity no significant changes were seen in the infected larvae (Fig-1E).

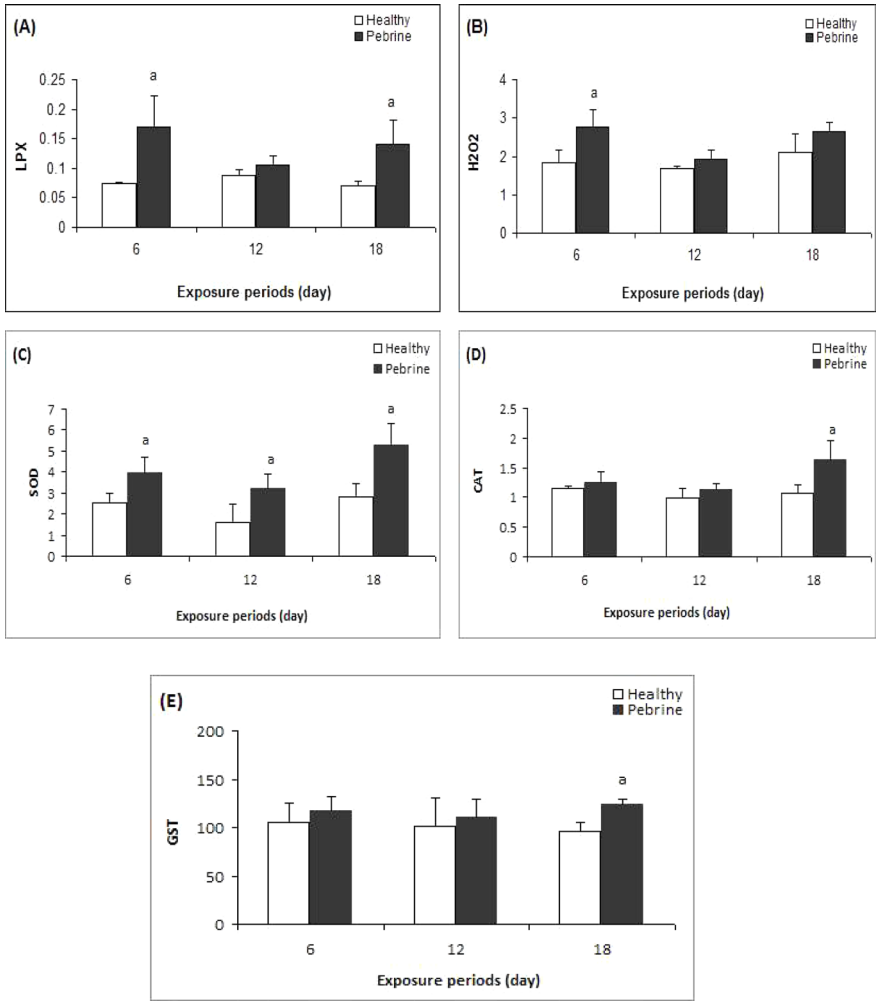

Fig. 1. (A) Lipid peroxidation (nmol TBARS/ mg protein), (B) total hydroperoxide (µmol/ mg protein), (C) catalase (µkat/ mg protein), (D) superoxide dismutase (Unit/mg protein) and (E) glutathione-S-transferase (nmol CDNB conjugate formed/min/mg protein) in midgut of tasar silkworm *A. mylitta*. Data expressed as mean ± SD (n = 3). Symbols [a] indicate significant difference between control and *Nosema* infected at $p < 0.05$.

Discussions

LPX serves as an indicator of oxidative damage in cells and tissues (Pampanin *et al*., 2005). An enhanced level of MDA (lipid peroxidation product) in the midgut of larvae was observed in response to *Nosema* infection (Fig-1A). Similar to this higher level of LPX was also detected in insects during viral (Wang *et al*., 2001) and bacterial infection in insects (Dubovskiy *et al*., 2008). It is understood that one of the most immediate immune response of the gut involves the production of ROS to fight microbial infection both in mammals (Kinnula *et al*., 1992; Geiszt *et al*., 2003) and insects (Ha *et al*., 2005a,b; Ryu *et al*., 2010). The increased immune response during infection could be the possible reason for the increase in the rate of ROS formation, resulting in oxidative stress. The observed higher level of total hydroperoxide (H_2O_2 and other water soluble hydroperoxides, Wolff, 1994) in infected larvae (Fig-1B) as compared to healthy may indicate increased formation of $^{\bullet}OH$ by Fenton reaction, thereby, enhancing the LPX level in midgut of larvae. This is also evident from a significant positive correlation between total hydroperoxide and LPX in midgut tissues samples (Fig-2A, $p < 0.05$). In *A. mylitta* reduction of larval weight (Madhusudan *et al*., 2012), cocoon weight (Velide *et al*., 2013) was also observed during *Nosema* infection. This may be due to an increase in the production of ROS and oxidative damage in response to diseases.

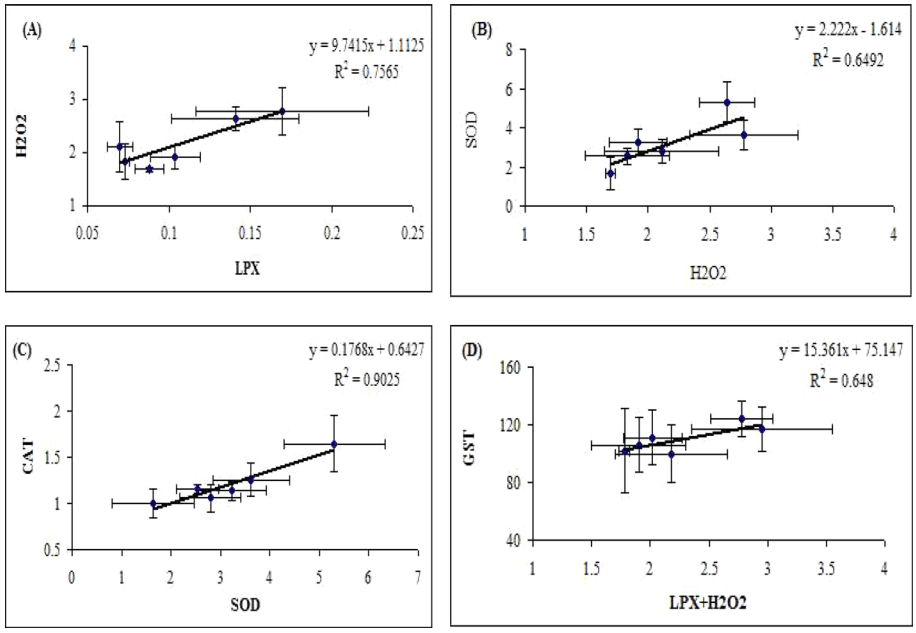

Fig. 2. Correlation between (A) LPX vs H_2O_2, (B) H_2O_2 vs SOD, (C) SOD vs CAT, (D) LPX + H_2O_2 vs GST

Determination of antioxidant status of larva exposed to *Nosema* is important to understand the toxic mechanism and predict the damage potential in the organism. An increased oxidative stress suggests elevated activity of antioxidant enzymes, thereby protecting animals from oxidative stress (Halliwell and Gutteridge, 2001). SOD is a crucial antioxidant enzyme, which dismutates $O_2^{\bullet -}$ to H_2O_2. In the present study significant increase in SOD activity was observed in the midguts of infected larvae (Fig-1C), which suggests active production of $O_2^{\bullet -}$ radicals during pathogenic infection. Similarly, higher SOD activity was also observed in midgut of insect (*Galleria mellonella*) infection with bacterial pathogen (Dubovskiy *et al*., 2008). The induction of SOD during *Nosema* infection may indicate that it helps in inhibiting the oxygen radical accumulation.

The principal H_2O_2 scavenging enzyme CAT showed increased level in midguts of *A.mylitta* in response to *Nosema* infection (Fig-1D). This suggests that during infection period cell might elevate the rate of H_2O_2 production in tissues, and as a consequence, an elevation of CAT may be seen. Similarly, upregulation of CAT activity was observed in honey bee *Apis mellifera* infected with *Nosema ceranae* (Dussaubat *et al*., 2012). However, in early stages (newly hatched larvae) a biphasic response of CAT activity was observed in whole body mass of pebrinised larvae (Madhusudhan *et al*., 2012). Further, CAT activity has been shown to be a key enzyme of the *Drosophila* defense system during pathogenic infection in the gut epithelia as reported by Ha et al. (2005b). The enhanced activity of CAT prevents the accumulation of ROS as evidenced by lesser total hydroperoxides in midgut tissues of *Nosema* infected larvae in comparison with 6^{th} day.

GSTs are involved in the detoxification of both reactive intermediates and oxygen radicals (Van der Oost *et al*. 2003). In the present study, increased GST activity (Fig-1E), suggests formation of oxidative damage products which might lead to increased GST expression in order to protect the tissues against oxidative stress generated by *Nosema* spores. In support of this observation a significant positive correlation observed between oxidative stress indices (such as LPX and total hydroperoxides) and GST activity (Fig-2D, $p < 0.05$). Induction of GST in insect after exposure of *Nosema* is well documented in honey bee (Dussaubat *et al*., 2012).

The present study demonstrates that *Nosema* spore can significantly modulate LPX and ROS production in the midgut of tasar silkworm, which is a disorder of pebrine diseases. To protect against oxidative stress, SOD, CAT and GST activities get activated in midgut of *Nosema* infected larvae. This is indicated by a significant positive correlation observed between oxidative stress indices and antioxidant enzymes (Fig-2A-D). Despite this protective response, *Nosema* infected larvae have

reduction of growth and development compared to healthy ones (Rath *et al*., 2003), suggesting that they are unable to cope with the physiological stress of *Nosema* spore over the long period.

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