

Generation of cytotoxic molecules and oxidative stress in haemolymph of pebrinised tasar silkworm *Antheraea mylitta* Drury

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Abstract

The present study was carried out to investigate the effects of microsporidial infection on redox regulation mechanism and oxidative stress in tasar silkworm *Antheraea mylitta*. High level of superoxide radical ($p < 0.05$), nitric oxide ($p < 0.001$) and lipid peroxidation ($p < 0.001$) was observed in haemolymph of pebrinised larvae, which indicated the resultant generation of cytotoxic molecules and oxidative damage. Increased phenol oxidase (PO) activity in haemolymph of pebrinised larvae indicated the activation of immune defences during pathological conditions. In addition, higher level of glutathione-S-transferase (GST) activity and reduced glutathione (GSH) level observed in pebrinised larvae indicated adaptive behaviour of tissue against toxic oxyradicals ($p < 0.05$). Conversely, low level of ascorbic acid (ASA) content suggested that the larvae might have used these compounds to counteract stress in tissues or low uptake under microsporidial infection ($p < 0.05$). Present findings provide new insights into the cellular and biochemical bases of host-pathogen interactions in tasar silkworm *A. mylitta*.

Key words

Antioxidants, Cytotoxic molecules, Oxidative stress, Tasar silkworm

Introduction

Antheraea mylitta is an economically important silk worm species due to its silk quality, and great demand in the local as well as international markets. Since tasar silkworms are reared at outdoor condition, are more susceptible to various pathogens such as virus, bacteria, fungus and microsporidia. Among these, *A. mylitta* is very often infected with microsporidia (genus *Nosema*), which infects almost all stages and ecoraces of the tasar silkworm. *A. mylitta* infected with *Nosema* sp. show extended developmental period, reduced size and larval weight (Rath *et al.*, 2003), silk gland (Renuka and Shamitha, 2012), reduction of protein level (Madhusudhan *et al.*, 2011), oxidative damages (Jena *et al.*, 2014) and causes crop loss up to 20-25% (Sahay *et al.*, 2000).

Insect haemocytes play a vital role in defence against pathogens through phagocytic mechanism, nodule formation

and encapsulation (Lavine and Strand, 2002; Nappi and Christensen, 2005; Kwon *et al.*, 2014). In phagocytosis, pathogens are internalised and degraded through hydrolytic enzymes and reactive oxygen species (ROS) (Lavine and Strand, 2002; Nappi and Christensen, 2005; Cerenius and Soderhall, 2004). In aerobic organisms ROS are generated in response to both external and internal stimuli (Halliwell and Gutteridge, 2001) and the reactive oxygen intermediates produced during the process are highly toxic to microbes. Also ROS are recognized to have an important role in immune defense and could play multiple functions in many biological processes (Lambeth, 2004). Like many other animals, tasar silkworm contains a complex of antioxidant and detoxifying enzymes whose action is directed to scavenge ROS. *A. mylitta* possesses an antioxidant defense system that consists of both enzymatic and non-enzymatic components. The enzymatic components are superoxide

dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST). Superoxide radical ($O_2^{\cdot-}$) radical is dismutated by SOD to hydrogen peroxide, which is reduced to water and molecular oxygen by catalase. Further, glutathione-S-transferase conjugates xenobiotics with reduced glutathione for excretion. The non-enzymatic components consist of small organic molecules such as reduced glutathione and vitamin C (Jena *et al.*, 2013a and b, Sahu *et al.*, 2014, Jena *et al.*, 2014).

A lot of information is available on the incidence, epidemiology and its impact on productivity of tasar silkworm (Sahay *et al.*, 2000; Rath *et al.*, 2003; Madhusudhan *et al.*, 2011; Renuka and Samitha, 2012). However, little is known about *Nosema* spore causing host damage and the mechanisms by which tasar silkworm protect themselves. After the infection, insects rapidly mount an immune response involving different molecular pathways, among these the production of ROS is also a key feature of this protective response (Ha *et al.*, 2005a). A concurrent elimination of residual ROS is also observed to protect the host (Ha *et al.*, 2005b), since the homeostasis of redox (reduction-oxidation) balance mediated by antioxidant enzymes is essential to the host survival. Therefore, the present study has been designed to quantify the cytotoxic molecules (NO, $O_2^{\cdot-}$), oxidative damages (LPX), immune defence enzyme (PO) and antioxidant defences (GST, ASA and GSH) in haemolymph of tasar silkworm *A. mylitta* infected by *Nosema* spores.

Materials and Methods

Animal maintenance and sample preparation : During first crop rearing (July-August, 2014) the bivoltine Daba ecorace of *A. mylitta* were utilised for present study. The pebrinised and non-pebrinised larvae were segregated based on black pepper spot on the integument of pebrinised larvae. Microscopic examination of haemolymph samples was done to confirm pebrine disease by detecting the pebrine spore. To obtain samples of haemocytes and plasma, hemolymph was collected in pre chilled (4°C) eppendorf tubes coated with 0.02% phenylthiourea (PTU) to inhibit denaturing or blackening of haemolymph. Then haemolymph was centrifuged at 4°C for 5 min at 3000g for separation of haemocyte and plasma. Pellet containing haemocyte was used for $O_2^{\cdot-}$ and nitric oxide (NO) analysis and supernatant containing plasma was used for other biochemical analyses. As PTU inhibits the phenoloxidase (PO) activity, therefore sample was collected separately in pre chilled (4°C) eppendorf tubes containing without PTU to study PO and assay was conducted immediately.

Biochemical estimations : Lipid peroxide (LPX) level was assayed by measuring malondialdehyde (MDA), a

decomposition product of polyunsaturated fatty acids. LPX was determined by the TBA reaction as described by Baror *et al.* (2001). Generation of intra-haemocyte superoxide anion was estimated spectrophotometrically by nitro blue tetrazolium (NBT) reduction reaction modified after (Song and Hsieh, 1994). Intra-haemocyte NO level was estimated spectrophotometrically by Griess reaction (Green *et al.*, 1982). The PO activity was measured according to Asokan *et al.* (1997) by using L-DOPA as a substrate. Ascorbic acid content was determined according to the method of Mitusi and Ohata (1961). Estimation of reduced glutathione was done by Ellman, (1959). Glutathione-S-transferase activity was measured according to Habig *et al.* (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. The protein content was estimated by the Bradford (1976) method using bovine serum albumin as standard.

Statistical analysis : Results were expressed as mean \pm standard deviation (SD). Difference between pebrinised and non-pebrinised was analyzed by Student's t-test. Differences were considered statistically significant when $p < 0.05$. Further inter relationship between biochemical indices were analysed by correlation.

Results and Discussion

Both nitric oxide and superoxide anion are principal cytotoxic agents engaged in destruction of intracellular parasites or microbes (Nappi and Christensen, 2005). These cytotoxic agents are generated as immune response in insects and considered as important defence molecules. In invertebrates, haemocytes mediated cytotoxicity through the production of free radicals was reported by several authors (Cerenius and Soderhall, 2004; Nappi and Christensen, 2005). In the present study, significantly higher level of superoxide anion ($p < 0.05$) and nitric oxide ($p < 0.001$) were observed in pebrinised larvae (0.578 ± 0.169 OD/ml and 1.29 ± 0.099 μ M nitrite/ml) in comparison to non pebrinised (0.359 ± 0.04 OD/ml and 0.623 ± 0.14 μ M nitrite/ml) larvae (Fig-1A & B). This finding is correlated with the findings of Faraldo *et al.* (2005) who reported the enhanced production of NO in blowfly haemolymph after yeast inoculation.

Like other organisms, insects maintain a balance between generation and neutralization of ROS under normal physiological conditions. However, during infection excess production of ROS and reactive nitrogen species (RNS) exceeds organisms scavenging capacity and attack cell biomolecules (Halliwell and Gutteridge, 2001). MDA, a major oxidation product of peroxidized polyunsaturated fatty acids, has been used to determine the degree of lipid peroxidation and as a biological marker of oxidative stress (Rael *et al.*, 2004). Besides, under environmental stress, ultraviolet irradiation, bacterial infections, antibiotics and

pesticides exposure may also increase the ROS level remarkably and result in oxidative stress in insects (Lopez-martinez *et al.*, 2008; Buyukguzel and Kalender, 2009). An enhanced level of MDA (lipid peroxidation product) was observed in the haemolymph of pebrinised larvae (0.527 ± 0.095 nmol TBARS mg^{-1} protein) as compared to non-pebrinised larvae (0.254 ± 0.052 nmol TBARS mg^{-1} protein, Fig-1C, $p < 0.001$). Similarly, higher level of LPX was detected in insect during viral (Wang *et al.*, 2001), bacterial (Dubovskiy *et al.*, 2008) and microsporidial infection (Jena *et al.*, 2014). It is well established that, in insects the most immediate immune response in tissues involves the production of free radicals to fight against microbial infection (Ha *et al.*, 2005a; Ryu *et al.*, 2010). The increased immune response during infection could be the possible reason for the increase in the rate of ROS/RNS formation, resulting in oxidative stress. The observed higher level of total $\text{O}_2^{\cdot-}$ and NO in infected larvae (Fig. 1A & B) as compared to non-pebrinised may indicate the increased formation LPX level in tissues of larvae. This is also evident from a significant positive correlation between free radicals ($\text{O}_2^{\cdot-}$ and NO) and LPX in haemolymph samples (Table 1, $p < 0.05$). In *A. mylitta* reduction of larval growth, silk gland weight, cocoon weight (Rath *et al.*, 2003) was also observed during *Nosema* infection. This may be due to an increase in the production of ROS/RNS and oxidative damage in response to diseases.

PO is an important humoral defense component in insects. Its activation results in induction of a number of potent bioactive molecules which assist various defensive mechanisms such as phagocytosis, cell adhesion and formation of melanin deposits (Soderhall and Cerenius, 1998). Generally, PO is present in silkworm plasma in an inactive prophenoloxidase (proPO) state. In the present study, significant induction of plasma PO was detected in

pebrinised silkworm larvae (3.48 ± 0.417 μmol Dopachrome formed mg^{-1} protein) when compared to non-pebrinised (1.314 ± 0.3 μmol Dopachrome formed mg^{-1} protein) larvae (Fig. 1D, $p < 0.001$), indicating responses of proPO to active stage. It has been reported that, melanization process is accompanied by the release of ROS (Dubovskii, *et al.*, 2010). This is also evident from the observed significant positive correlation between free radicals ($\text{O}_2^{\cdot-}$ and NO) and PO in haemolymph samples (Table 1, $p < 0.05$). Earlier studies showed that in silkworm the induction of proPO activation and melanisation reactions take place in response to fungal pathogen (Rajitha and Savithri, 2013). Further, enhanced level of GST activity was also observed in haemolymph (Fig. 1E, $p < 0.05$) of pebrinised larvae (48.78 ± 9.83 nmol CDNB conjugate formed min^{-1} mg^{-1} protein) when compared to non-pebrinised (29.67 ± 8.2 nmol CDNB conjugate formed min^{-1} mg^{-1} protein) indicates the formation of oxidative damage products which might modulates GST expression in order to protect the tissues against oxidative stress. To support this hypothesis a significant positive correlation was observed between stress indices (such as LPX, NO and PO) and GST activity (Table 1, $p < 0.05$). Earlier studies also reported the increased GST activity in honey bee after exposure of *Nosema* (Dussaubat *et al.*, 2012). The increase in the levels of these defense enzymes may have positively influence on the survivability of animals upon challenge with infectious pathogens.

GSH is the main non-protein thiol that responsible for maintaining cellular homeostasis and the cellular redox balance (Halliwell and Gutteridge, 2001). Significant increase in GSH content in pebrinised silkworm (8.48 ± 2.46 nmol mg^{-1} protein) as compared to non-pebrinised larvae (5.91 ± 2.02 nmol mg^{-1} protein Fig. 1F, $p < 0.01$), suggests that GSH accumulation may help counteract oxidative damage caused by *Nosema* in *A. mylitta*. Earlier studies

Table 1 : Correlation matrix of cytotoxic molecules, oxidative damages and antioxidant defence indices in hemolymph of tasar silkworm larvae. Gray colours are significant at $P < 0.05$. NS-Not significant

Biochemical indices	$\text{O}_2^{\cdot-}$	NO	LPX	PO	GST	GSH	ASA
$\text{O}_2^{\cdot-}$	1						
NO	NS	1					
LPX			1				
PO				1			
GST	NS				1		
GSH						1	
ASA		NS	NS		NS		1

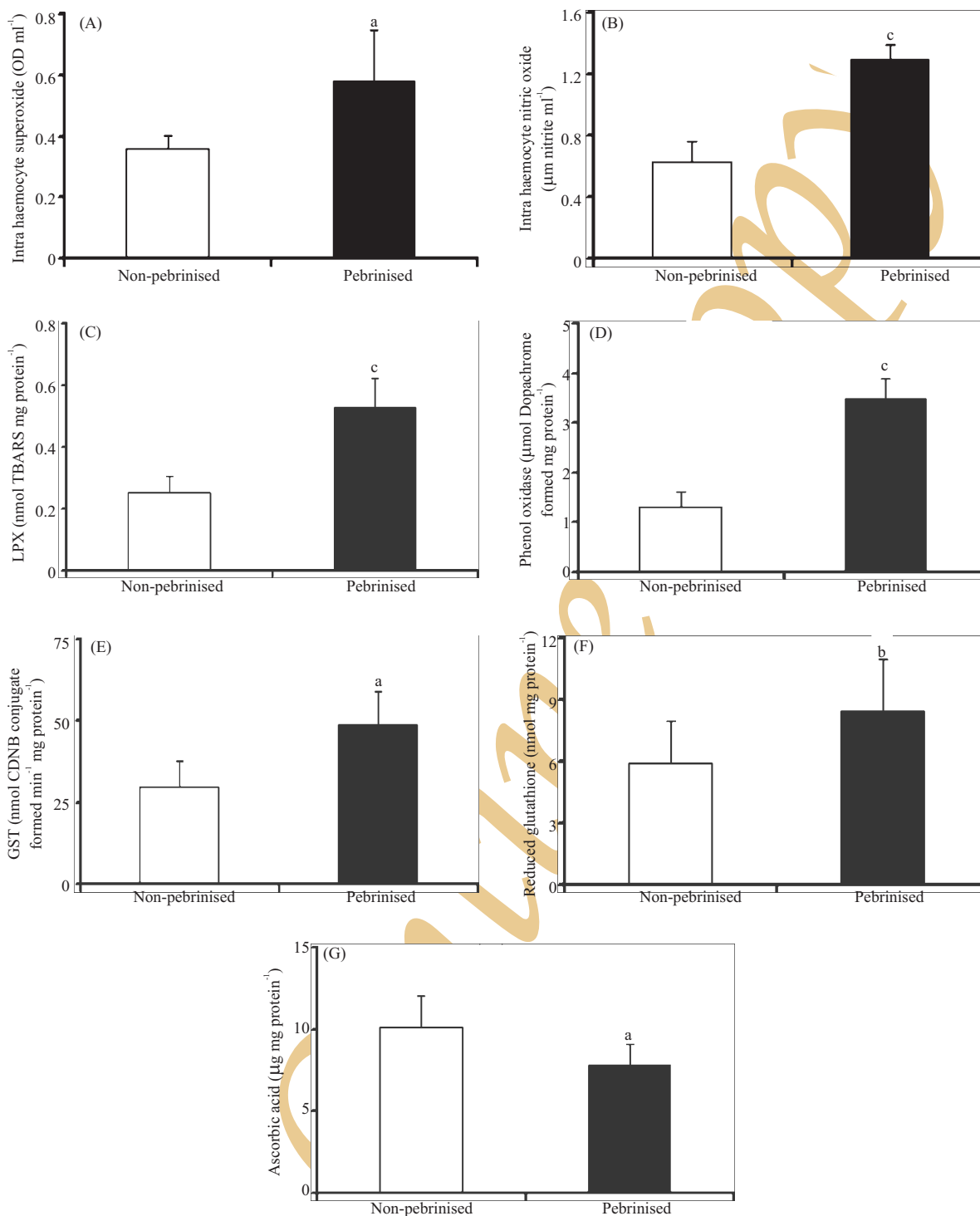


Fig. 1 : (A) Superoxide radical, (B) Nitric oxide, (C) Lipid peroxidation, (D) Phenol oxidase, (E) Glutathione-S-transferase, (F) Reduced glutathione and (G) Ascorbic acid level in non-pebrinised and pebrinised haemolymph of tasar silkworm *A. mylitta*. Data expressed as mean \pm SD (n = 5). Symbols indicate the significant difference between non-pebrinised and pebrinised at ^aP < 0.05, ^bP < 0.01 and ^cP < 0.001

suggested that GSH can act as a physiological defense against pathogen-induced cytotoxicity (Morries *et al.*, 2013). Higher level of GSH along with GST, suggests that the regulation of ROS level, generated due to protozoan toxicity in *A. mylitta*, is efficiently achieved using the glutathione pathway. ASA is known to directly scavenge ROS (Halliwell and Gutteridge, 2001). It also acts as a crucial micronutrient and performs various vital physiological functions (Wilson and Poe, 1973). Low ASA concentrations were observed in pebrinised larvae ($7.84 \pm 1.24 \mu\text{g mg}^{-1}$ protein) when compared to non-pebrinised ($10.11 \pm 1.92 \mu\text{g mg}^{-1}$ protein, Fig-1G, $p < 0.05$). It may indicate either due to dietary deficiency or utilization of ASA in response to elevated ROS generation during infection. Further, during *Nosema* infection low feeding and low growth rate of the silkworm was also reported by Rath *et al.*, (2003).

In conclusion it may be inferred that the significant induction of $\text{O}_2^{\bullet-}$, NO and oxidative damage in response to microsporidia spores suggests the toxic nature of pathogen to *A. mylitta*. A positive response accompanied by elevated activity of PO, GST & GSH in the diseased specimen indicates the potential of animal to protect against the oxidative stress generated by the spores. Furthermore, low ASA indicates their utilization in scavenging action for free radicals or low uptake during infection. However, as a whole the tasar silkworm comes under stress condition during microsporidia infection.

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