Research Article

Effect of Thermal Stress on Antioxidant Responses in *Bombyx mori*

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Abstract

Thermal stress alters the redox equilibrium in insects leading to oxidative damage and induction of antioxidant enzymes. In the present study, antioxidant responses of silkworm breeds (polyvoltine: Nistari & Sarupat; bivoltine: SK6 & SK7) exposed to thermal stress (35 & 40^0 C; 75 ± 5% relative humidity) were analyzed. The reactive oxygen species (ROS) were estimated by assessing hydrogen peroxide (H_2O_2) and lipid peroxidation in haemolymph; and induced antioxidant enzymes *viz.*, catalases (CAT), superoxide dismutases (SOD) and ascorbate peroxidases (APOX) were determined. Significant enhancement of H_2O_2 levels were observed in bivoltine and polyvoltine silkworms exposed to 35 & 40^0 C. Lipid peroxidation assayed by malondialdehyde (MDA) formation indicated augmented plasma MDA levels only in bivoltine silkworms. Further, significant increase in antioxidant enzyme (CAT, SOD & APOX) levels were observed in the silkworm breeds at high temperatures. Breed-specific response was observed in *Bombyx mori* to counter the thermal stress induced oxidative damage by antioxidant enzymes.

Keywords: *Bombyx mori*, Thermal stress, ROS, Oxidative damage, Antioxidant enzymes

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Introduction

Mulberry silkworm, *Bombyx mori* L. is an economically important poikilothermic insect producing silk. Environmental factors, especially temperature plays a very prominent role in the normal growth and development of silkworm and could be reared optimally at 25-28^{o}C. Any deviation from the ambient temperature for conducting normal physiological and metabolic processes is often considered as thermal stress, which is often associated with an array of physiological stress responses leading to enhanced reactive oxygen species (ROS) generation causing oxidative damage [1, 2]. Although, ROS such as hydroxyl radical (OH·), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), differential lipid radicals and peroxides are usually generated during the process of respiration, augmented levels were observed in response to abiotic or biotic stress [3]. Excessive ROS elicits severe damage to cellular constituents (lipids, proteins and nucleic acids). However, membrane lipids owing to the presence of polyunsaturated fatty acids (PUFA) are highly susceptible to ROS induced peroxidation (lipid peroxidation) leading to disruption of the cell membrane integrity. Elevated levels of lipid peroxidation (LPO) are indicative of oxidative stress and hence considered as a biochemical marker [4, 5].

Like any other insects, silkworms also exhibit enzymatic and non-enzymatic antioxidative defense mechanisms to counter the oxidative damage. The most imperative antioxidant enzymes include superoxide dismutases (SOD), catalases (CAT), peroxidases (POX) and glutathione S-transferases (GST) in silkworm; while the non-enzymatic components comprise of glutathione, tocopherols and ascorbic acid [1, 6, 7]. SOD plays a prominent role in combating ROS by catalyzing the conversion of superoxide anion to hydrogen peroxide and molecular oxygen [8]; CAT and POX transforms hydrogen peroxide to water and oxygen molecules, whereas GSTs are involved in conjugation of reduced glutathione with xenobiotics and further elimination.

The physiological and biochemical responses of insects to temperature have gained significant attention over the last couple of decades owing to the global warming and climate change [9, 10]. Considerable information on induction of diverse heat shock proteins (HSPs) in response to thermal stress is reported in polyvoltine and bivoltine silkworms [11]. Thermal stress induced catalase activities in different tissues of silkworm were also elucidated in bivoltine silkworms [6, 7]. However, reports on oxidative enzymatic defense mechanisms in poly- and bivoltine silkworms at high temperatures are scant.

Materials and Methods

Experimental insects

Silkworm breeds (Nistari, Sarupat, SK6 and SK7) experimented in the present study were obtained from Silkworm Breeding and Genetics Laboratory, Central Sericultural Research & Training Institute, Berhampore, West Bengal. The silkworm breeds were reared on fresh mulberry leaves (S-1635 variety) during July-August following standard rearing conditions till fourth instar (Temperature: 25-28^0 C; RH: 85-90% for young age, 75% during IV instar).

Temperature treatment and Sample collection

Fifth instar larvae, immediately after IV moult were subjected to thermal stress treatments (35 and 40 ± 1^0 C; 75 ± 5% RH) in an environmental chamber for 6 hours each day; subsequently the larvae were reared under optimum temperature (25-26^0 C) upto fifth day, whereas the control batches were maintained continuously at optimum temperature. Three replications were maintained for each treatment and each replicate comprised hundred larvae. Haemolymph was collected from control and treated silkworm larvae by pricking first proleg on the fifth day of V instar. Haemolymph was collected into pre-chilled sterile microfuge tubes containing a pinch of phenylthiourea and samples were centrifuged at 5000*g* for 10 min at 4^0 C. The supernatant was transferred to fresh microfuge tubes and haemolymph protein was quantified by Lowry's [12] method employing bovine serum albumin as standard. The same were stored at -80^0 C till further experimentation.

Hydrogen peroxide estimation

Hydrogen peroxide generated due to thermal stress was measured according to Loreto and Velikova [13]. The assay mixture contained haemolymph plasma (125 µl) with equal quantity of 10 mM potassium phosphate buffer (pH 7.0) and 500 µl of 1 M potassium iodide. The absorbance was measured spectrophotometrically at 390 nm and read against H_2O_2 standard calibration curve.

Lipid peroxidation

Lipid peroxidation assay was conducted by assessing lipid peroxide production in terms of malondialdehyde (MDA) equivalents [14]. Haemolymph plasma (2.5 mg protein) was added to 1.5 ml of TBA-TCA reagent [0.5% Thiobarbituric acid was prepared in 20% (w/v) Trichloroacetic acid] and incubated at 95^0 C in waterbath for 25 min. Reaction was terminated by cooling on ice for 3-5 min and the reaction contents were centrifuged at 15000*g* for 5 min at 4^0 C. Absorbance of the supernatant was read at 532 nm and MDA concentration was calculated using molar extinction coefficient (155 $mM^{-1}cm^{-1}$).

Catalase assay

Catalase activity in haemolymph plasma was estimated as described by Sinha [15]. The assay components included 100 µl of phosphate buffer (0.1 M, pH 7.4) and 500 µl of sample to which 500 µl of 0.2 M H_2O_2 was added. The reaction was terminated by adding 500 µl of $K_2Cr_2O_7$/glacial acetic acid reagent (1:3) and the absorbance was read at 540 nm immediately.

Superoxide dismutase assay

Superoxide dismutase (SOD) was performed as described by Kono [16]. The assay components comprised 1.3 ml of 50 mM sodium carbonate buffer (pH 10), 500 µl of 96 µM nitroblue tetrazolium (NBT) and 100 µl of 0.6% Triton X-100. The reaction was initiated by adding 100 µl of 20 mM hydroxylamine hydrochloride (pH 6) and incubated for 2 min at room temperature before adding 70 µl of plasma sample. The percentage inhibition in the rate of NBT reduction was recorded as an increase in absorbance at 540 nm.

$$\% \text{ inhibition of NBT reduction, } y = 100^* [\Delta A/\text{min (Blank)} - \Delta A/\text{min (Test)}]/\Delta A/\text{min (Blank)}$$

One unit of the enzyme activity is defined as the concentration of enzyme necessary to inhibit the absorbance at 540 nm of chromogen formation by 50% in one minute under the assay conditions. SOD activity was expressed as SA = mol unit activity/mg protein.

Ascorbate peroxidase assay

Ascorbate peroxidase (APOX) activity was determined according to the method outlined by Nakano and Asada [17] with slight modifications. The reaction mixture included 750 µl of phosphate buffer (100 mM, pH 7.0), 150 µl of 5.0 mM ascorbate, 300 µl of 0.5 mM H_2O_2 and haemolymph plasma (500 µg protein). APOX activity was assessed by evaluating the reduction in optical density due to ascorbic acid at 290 nm. One mole of dehydroascorbate is produced by oxidation of one mole of ascorbate by one mole of H_2O_2. One unit of enzyme activity was calculated as the amount of enzyme necessary to oxidize 1.0 µM of ascorbate/min/mg of protein.

Statistical analysis

All the assays were conducted atleast twice utilizing independent biological samples with a minimum of three replications. Data presented are the mean values of replicates corresponding to each treatment and controls. Data was analyzed using Student's *t*-test.

Results and Discussion

The generation of ROS was assessed by estimating H_2O_2 levels in haemolymph plasma of polyvoltine and bivoltine controls and thermal stressed silkworm larvae. H_2O_2 levels varied insignificantly among the tested breeds reared at optimum temperature, while the imposed high temperature (35^0 C) resulted in significantly (P<0.01) elevated levels of H_2O_2 in all the breeds. Similar trend was observed in Sarupat, SK6 and SK7 silkworm larvae exposed to 40°C; however, the H_2O_2 levels were reduced in Nistari (**Figure 1**). The hydrogen peroxide contents were reported to vary under optimum conditions as well as in response to abiotic stresses in different insects. Increment in H_2O_2 levels at extreme temperatures (9°C and 24°C) was reported in *Galerucella placida* [18]. Significant variations in peroxide contents across different races and seasons were elucidated in *Apis mellifera* [19]. Tissue and voltinism specific hydrogen peroxide content variations were reported in *Antheraea mylitta* [20].

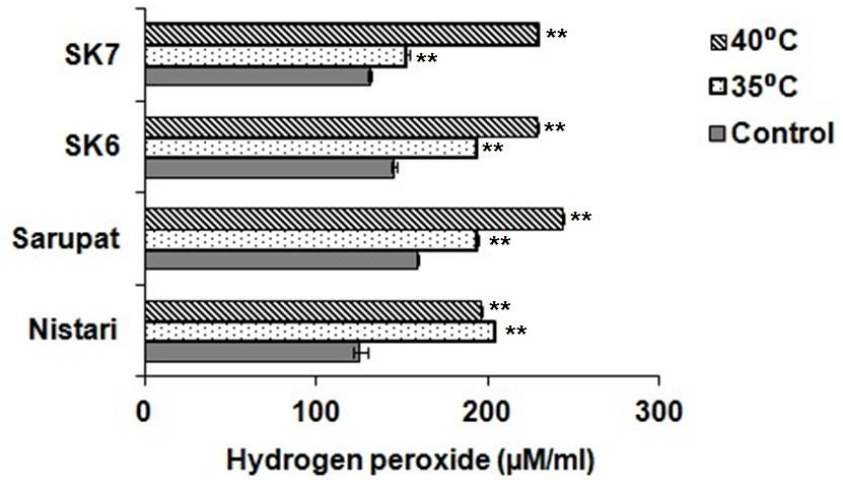

Figure 1 Effect of temperature on H_2O_2 content in haemolymph of different silkworm breeds. (Data are represented as mean ± SD; * and ** represents data is statistically significant at P<0.05 and P<0.01, respectively)

ROS induced oxidative damage of lipids results in production of thiobarbituric acid reactive substances (TBARS) which can be estimated by using thiobarbituric acid that reacts with malondialdehyde, a resultant end product generated during peroxidation of PUFA [5]. Significant variation in LPO was recorded among all the breeds reared at optimum temperature. LPO increased significantly in Sarupat (P<0.05) and SK7 (P<0.01) exposed to 35°C; while, only SK7 exhibited significant (P<0.006) enhanced levels of MDA over control at 40°C (**Figure 2**). Lipid peroxidation is also reported to augment in different insects *viz*., *Scapharca broughtonii*, *Panonychus citri*, *Bactrocera dorsalis*, *Antheraea mylitta* at higher temperatures [4, 21-23].

Superoxide dismutases (SOD) catalyze the dismutation of superoxide radicals by adding or removing an electron, thereby converting them to a less damaging species i.e., molecular oxygen or hydrogen peroxide. SOD activity varied significantly in all the silkworm breeds reared at optimum temperature and was least in Sarupat. SOD activities enhanced significantly (P<0.05 or P<0.01) in all the breeds exposed to thermal stress (35°C and 40°C) and the maximum activity was recorded in SK6 (**Figure 3**). These findings corroborate with the SOD activities reported in *B.*

dorsalis and *A. mylitta* [4, 23]. However, a 1.18 fold reduction was observed in Sarupat exposed to 40°C over 35°C which could be attributed to the silkworm breed response to the increased thermal stress.

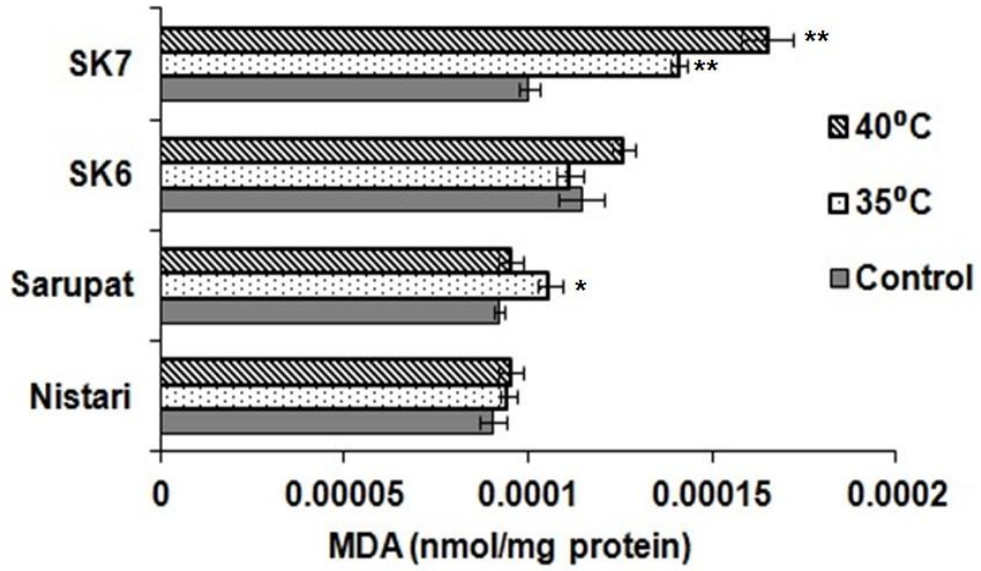

Figure 2 Effect of temperature on MDA levels in haemolymph of different silkworm breeds. (Data are represented as mean ± SD; * and ** represents data is statistically significant at $P<0.05$ and $P<0.01$, respectively)

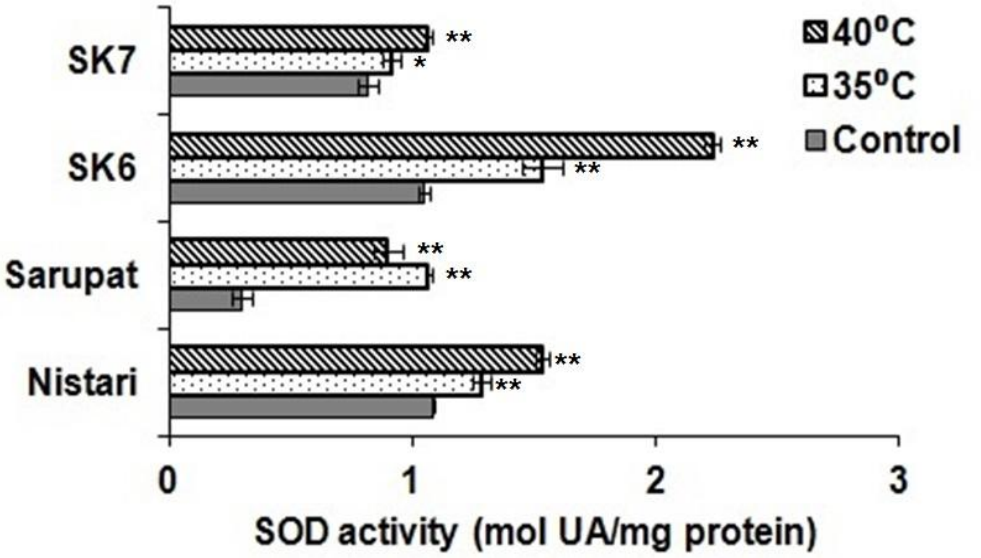

Figure 3: Effect of temperature on SOD activity in haemolymph of different silkworm breeds. (Data are represented as mean ± SD; * and ** represents data is statistically significant at $P<0.05$ and $P<0.01$, respectively)

Catalases primarily assist in removal of hydrogen peroxide, thereby stabilizing the internal milieu. Insects being devoid of selenium-reliant glutathione peroxidases are exclusively dependent on catalases for scavenging hydrogen peroxide [24, 25]. In the present study, enhanced catalase activities were observed in bivoltine silkworms in comparison to the polyvoltines at optimum temperature. Catalase activity augmented significantly ($P<0.01$) in bivoltines as well as polyvoltine breeds over their respective controls (**Figure 4**) at high temperatures (35 and 40°C). Catalase activity reduced at 40°C in comparison to 35°C in SK6. The highest and lowest catalase activities at 40°C were observed in SK7 and SK6, respectively. Similar trends of catalase activity were reported in the fat body of bivoltine breeds (CSR4, CSR2, JROP, KA and NB_4D_2) at 35 and 40°C. Further, it was shown that haemolymph catalases significantly increased across JROP, KA and NB_4D_2, whereas the midgut catalases elevated only in JROP and NB_4D_2 [7]. Elevated catalase activities were also reported in testes homogenates of *A. mylitta* pupae exposed to thermal stress [23].

The levels of ascorbate peroxidases (APOX) across bivoltine and polyvoltine silkworms reared at optimum temperature ranged between 0.014-0.024 mmol UA/mg protein and no significant variation was observed among the

breeds. Sarupat, SK6, SK7 exhibited significantly (P<0.01) higher levels of APOX at 35°C in comparison to controls, whereas the levels remained same in Nistari (0.0175 ± 0.0003 mmol UA/mg protein). The highest APOX activity was recorded in SK7 (0.051 ± 0.001 mmol UA/mg protein) exposed to 35°C and the same remained unaltered even at 40°C. A significant (P<0.01) increment in APOX activity was noticed across the breeds exposed to 40°C in comparison to their respective controls and the maximum levels of APOX were observed in Sarupat and SK7 (**Figure 5**). Similarly, higher levels of APOX were previously reported in *Chilo suppressalis* (Lepidoptera: Crambidae) exposed to 34°C [26]. In contrast, APOX activity in coleopteran insect *Galerucella placida* was highest at 9°C which further reduced with increase in temperature upto 21°C [18]. In another study, it was reported that APOX is more efficient than CAT in countering peroxides and significantly varied across different seasons in *Apis mellifera* [19].

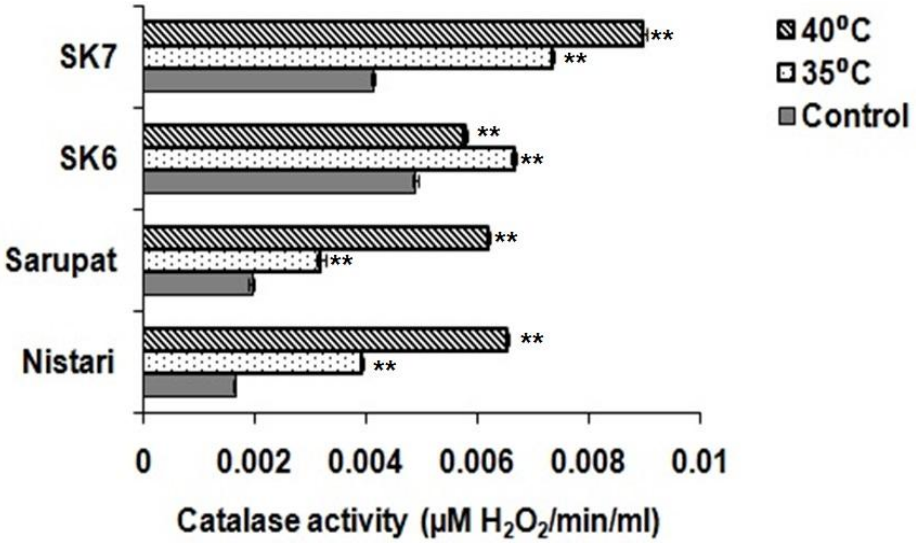

Figure 4 Effect of temperature on catalase activity in haemolymph of different silkworm breeds. (Data are represented as mean ± SD; * and ** represents data is statistically significant at P<0.05 and P<0.01, respectively)

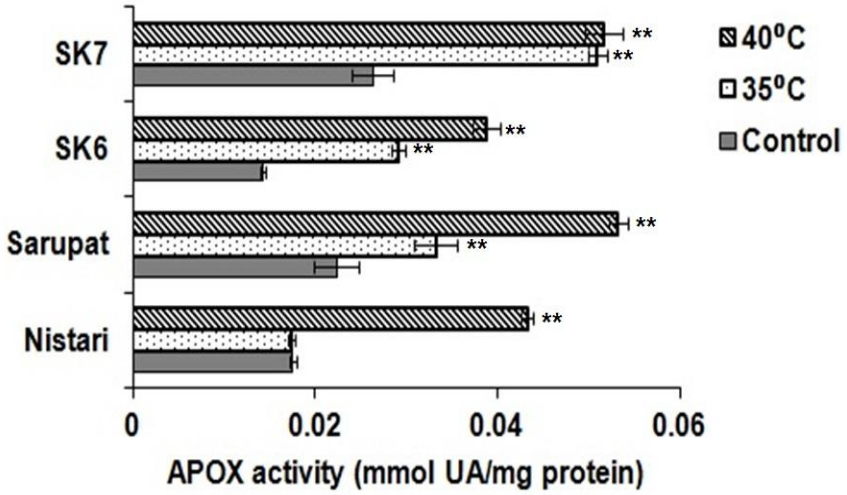

Figure 5 Effect of temperature on APOX activity in haemolymph of different silkworm breeds. (Data are represented as mean ± SD; * and ** represents data is statistically significant at P<0.05 and P<0.01, respectively)

Conclusion

Bivoltine and polyvoltine breeds of *B. mori* exhibited considerable differences in antioxidant defense mechanisms in response to thermal stress. Significant elevated levels of H_2O_2 with concomitant increased LPO in SK7 indicated higher oxidative damage than any other tested breeds. In response, catalase and APOX levels were elevated in SK7 as enzymatic antioxidant defense mechanism to lower thermal stress induced oxidative damage. The other bivoltine breed, SK6 exhibited maximum SOD levels in response to elevated H_2O_2 and LPO among the tested breeds. ROS generation also enhanced in the experimented polyvoltine breeds at imposed temperatures. However, oxidative

damage in terms of lipid peroxidation was observed only in Sarupat indicating Nistari is more resistant to thermal stress. In conclusion, a breed-specific antioxidative defense response was observed against ROS and oxidative damage elicited due to thermal stress.

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