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Expression of BmApaf-1 and BmDredd Genes and Antioxidant Responses of Silkworm (*Bombyx mori* L.) Exposed to Thermal Stress

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A B S T R A C T

High temperature is one of the major environmental stresses, which is an important reason for altering the physiological responses such as surplus production of reactive oxygen species and consequent oxidative damage among animals especially in insects. This ROS could stimulate intrinsic as well as extrinsic pathways of apoptosis. The present study envisages appraising the impact of thermal stress on the expression of apoptotic related genes BmApaf-1 and BmDredd and the response of antioxidant enzymes (SOD, CAT, POD, GPX, GR, GST) in the silk gland of silkworm. For this study the fifth instar larvae grouped in to control (28±1ºC) and heat exposed group (40±2ºC) and analyzed the expression of BmApaf-1 and BmDredd mRNA by real time PCR using Actin as the internal control. Based on the results we observed a significant 1.7 fold upregulation of BmApaf-1 and 1.5 fold upregulation of BmDredd genes under thermal stress and the antioxidant enzymes showed increased activity against thermal stress to alleviate the consequent oxidative stress.

***Key words*:** Silkworm, Thermal stress, Antioxidant enzymes, BmApaf-1, BmDredd

Silkworms are economically important insects that are thermally sensitive, rearing at agreeable temperatures between 25-28 °C. Temperature, humidity and photoperiod fluctuations are all important factors in the growth and development and production of high-quality cocoons [1]. Elevated temperature has a direct impact on cell organizations, metabolic processes [2] and physiological activities of silkworm [3]. Normally the endogenous oxidative metabolism as well as from different exogenous sources results the formation of ROS, which consists of partly reduced metabolites of oxygen such as superoxide anion (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radicals [4-5].

Although ROS does have a range of functions, for example an ideal level of intracellular ROS needed for the mechanism of cellular homeostasis [6], where as a slight amount of ROS render as a signaling molecule in the survival pathways [7]. Excessive production of reactive oxygen species (ROS) can cause oxidative stress [8]. The status of oxidative stress is defined as the disparity between the formation of ROS and the regulation of antioxidant defense system [9].

Antioxidant enzymes and non-enzymatic antioxidants are used to counteract the generation of high amounts of ROS [10]. Superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST), are included in the category of enzymatic antioxidants [11]. Whereas ascorbic acid, vitamin E and reduced glutathione (GSH) are non-enzymatic antioxidants [12]. Each enzyme plays their own role to combat oxidative stress. Superoxide anion can be converted to molecular oxygen and hydrogen peroxide by SOD [11], although the catalytic enzyme CAT [13] and POX [14] convert hydrogen peroxide to molecular oxygen and water. GPX engaged in the detoxification of H_2O_2 as well as reduction of organic hydroperoxides and lipid peroxides, these actions are facilitated by reduced glutathione (GSH,) which functions as a hydrogen donor. The enzyme glutathione reductase liable for the reversion of reduced glutathione (GSH) from oxidized glutathione (GSSG) [11]. The hydroperoxides formed by the byproduct of lipid peroxidation, can be removed by the enzyme GST. The activity of antioxidants may not be enough to control the excessive ROS produced and it will lead to oxidative stress [15].

The stress induced cells become apoptotic or necrotic, due to the structural changes occurred especially in the lipids, proteins and nucleic acids**.** These cellular injuries and the intensity of lipid peroxidation determine the depth of oxidative stress [16]. Unwanted and potentially perilous cells are being eliminated by the physiological process called apoptosis [17]. Apoptosis plays different roles such as tissue homeostasis [18], regulation of development and organ differentiation process, and confrontation against environmental stress [19]. It has two important pathways, intrinsic and extrinsic [20]. ROS shows

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important roles in extrinsic [21] as well as intrinsic pathways [22]. Intrinsic pathway also known as mitochondrial apoptotic pathway [23], triggered by intracellular signals and extrinsic pathway activated by the transmembrane receptor mediated interactions [24]. In intrinsic pathway, mitochondria release cytochrome C makes an apoptosome complex with Apaf-1 and ATP, which activates caspase-9. The activated caspase-9, activate caspase-3 and commence apoptotic process [23]. In the extrinsic pathway, plasma membrane carrying cell death receptors and that have been activated by the binding of extracellular ligands [25] and finally activates the effector caspases [26].

BmApaf- 1(*Bombyx mori* apoptotic peptidase activating factor 1) is similar to mammalian Apaf-1 [27-28] and BmDredd (*Bombyx mori* death related ced-3/Nedd2-like protein) is an initiator caspase homologue [29] shows high similarity with mammalian initiator caspases [30]. Normally insect larval specific tissues are detached during programmed cell death mechanism [31]. In silkworm silk gland is the silk producing larval specific organ [32], which can be decadent quickly following the spinning stage [31]. The present study was conducted to find out the effect of thermal stress in the activity of antioxidant enzymes and the expression of BmApaf-1 and BmDredd genes in the silk gland of silkworm.

MATERIALS AND METHODS

Experimental animals and protocol

Bombyx mori larvae were procured from National Silkworm Seed Organization, Central Silk Board, Pallatheri, Palakkad, Kerala. The rearing was carried out according to the method of [33]. The larvae were reared on an appropriate temperature 25±1 °C and 70-85% relative humidity. They were fed with fresh mulberry leaves *ad libitum.*

In the fifth instar stage the larvae were grouped into control group and test group. The test group larvae were treated with 40±2 °C temperature in 1 hour per/day during the fifth instar stage. The silk gland of fifth instar larvae were selected as the experimental tissue and it was dissected for examining the activity of antioxidant enzymes and gene expression pattern.

Determination of oxidative stress markers

Tissue homogenization was done with an ice-cold PBS buffer of 7. 4 pH, centrifugation at 10000 RPM for 30 min at –4 °C. After centrifugation the supernatant was kept in pre-chilled eppendorf tubes at -20 °C, used for further studies. The quantification of protein was determined by using Lowry's method [34] in which bovine serum albumin (fraction v, sigma) was taken as standard. The quantity of lipid peroxidation was determined by the method of [35]. 1.0 ml of the tissue homogenate of silk gland was mixed with a mixture of TBA-TCA and HCl reagent. Then the samples were kept in a boiling water bath for 15 minutes and after cooling the samples, then they were centrifuged for up to 10 minutes. The supernatant was used for the estimation of various stress markers. Concentration of lipid peroxidation expressed in terms of µM MDA produced /mg protein.

Catalase enzyme

The Luck [36] was used to determine catalase enzyme activity by drop in absorbance induced by H_2O_2 breakdown at 240 nm, which results in the formation of water and oxygen. Enzyme activity expressed in terms of 1 µM of H_2O_2 consumed /min/mg protein.

SOD enzyme activity

The approach of Marklund and Marklund [37] could be used to determine SOD activity at 420 nm. This approach is based on the amount of enzyme needed to block the autoxidation of pyrogallol by 50%. The activity expressed in units/ml.

Peroxidase enzyme activity

The activity of peroxidase enzyme at 420 nm was determined by the method of Reddy *et al.* [38], in which oxidation of substrate catechol was done with the presence of H_2O_2. The change in absorbance measured in µmoles/min/mg protein.

Glutathione peroxidase

The glutathione peroxidase enzyme is used to decompose H_2O_2 or other peroxides as well as oxidize GSH in to GSSG. The activity of this enzyme was measured at 412 nm using the method of Rotruck *et al.* [39].

Glutathione reductase (GR)

Glutathione activity was measured at 340 nm using the method of David and Richard [40], in which glutathione was reduced to generate reduced glutathione (GSH) in the presence of NADPH, and NADPH was oxidized to form NADP+. One unit of glutathione reductase activity was defined as the reduction of 1 micromole of glutathione per minute per mg of protein (µM NADPH oxidized /minute/mg protein).

Glutathione S-transferase

According to the method of Habig *et al.* [41], 2, 4-dinitro-chlorobenzene (CDNB) react with reduced glutathione (GSH), to turn out a yellow product with 340-360 nm absorbance. Activity of enzyme related with the formation of product and it can be calculated by the increase in absorbance at 340 nm. The unit expressed in µmoles of 2, 4-dinitrophenyl glutathione formed /min/mg protein.

mRNA expression studies

Silk gland tissues were dissected out using an ice-cold PBS buffer. TRIZOL (Invtrogen) reagent method was used for the isolation of total RNA from desired tissues and quantity measured by using Qubit™ 4 fluorometer. The 1-5 µl of total RNA was used as template for first strand cDNA synthesis using iScript cDNA synthesis kit (Biorad) according to the manufacturers' instructions.

Table 1 Primers for real time PCR analysis

Genes	Primer (5'-3')	Reference
BmApaf-1	F: GGTTTGCTCGTAATGGAC	[43]
	R: CAGGACCAGTGGAGGCT	
BmDredd	F: AGTGACAGAAATGCTTGGAAC	[43]
	R: AAATGGGAACCTGAGGATG	
BmActin	F: CGGGAAATCGTTCGTGAT	[31]
	R: ACGAGGGTTGGAAGAGGG	

Real time PCR

RT-PCR was used to examine gene expression levels in this investigation. (Table 1) lists all primer sequences utilized in this study. The Biorad CFX Connect RT PCR apparatus and i Taq Universal SYBR Green Supermix were used to prepare RT-PCR experiments according to the manufacturer's instructions. 2µl of cDNA, 10µl of SYBR Green Supermix, 1µl of each gene specific primer and 4µl of ddH2O were used in each 20 µl reaction volume. The thermal cycler programme featured 5 minutes at 95 °C, followed by 10 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C and 10 s at 55 °C for 35 cycles. Every

measurement was repeated three times. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ technique, which was described by Livak *et al.* [42]. *BmActin* was used as internal control.

Statistical analysis

The differences among control group and treated group were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test using Origin statistical software and Microsoft Excel, the result obtained indicated as mean ± SEM (Standard error of mean). P values less than 0.05 considered as statistically significant.

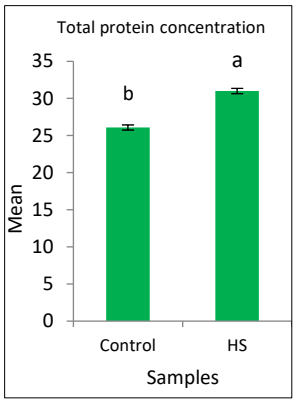

Fig 1 Effect of temperature on the protein concentration (mg/ml) of SG tissue. Those bars having different letters indicates significant difference (P < 0.05). The results represented as the mean ± SEM

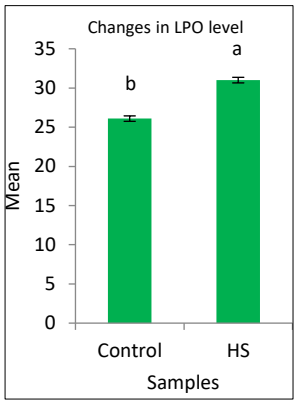

Fig 2 Effect of temperature on the level of MDA (µmoles/ min/ mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different. Data expressed as mean ± SEM

RESULTS AND DISCUSSION

1. Total protein concentration of silk gland

Analysis of total soluble protein concentration in the silk gland showed significant increase in treated (2.16) group compared to the control (1.89).

2. Changes in LPO levels

MDA concentration indicates the level of lipid peroxidation in response to thermal stress, and it also describes the level of oxidative stress in the silk gland tissues of *B. mori* as shown in (Fig 2). The present study showed that there is a significant increase in the level of LPO in the treatment group compared to control.

3. Activities of antioxidant enzymes

The activity of SOD showed a significant increase in the test group compared to the control (Fig 3). The activity of catalase in the silk gland tissues of the control and test group was analyzed and found significant increase in the heat-exposed group compared to the control (Fig 4). The activity of POD enzyme in control and treated larvae of silk gland shows a very low level of activity in the control group but a significantly increased activity was found in the treated sample (Fig 5). GPX activity was significantly enhanced in the silk gland of *B. mori* larvae by exposure to temperature. Test group shows increased activity of GPX than the normal group (Fig 6). Analysis of GST activity showed discrepancy among the both examined groups, significantly elevated activity was found in the treated group than the normal group (Fig 7). Glutathione reductase shows lowest activity in the normal group but significantly increased activity was found in the treated sample (Fig 8).

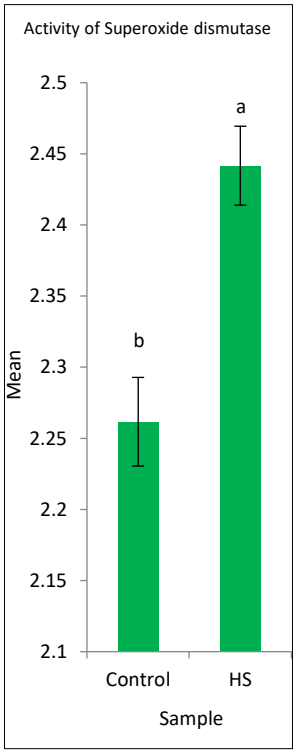

Fig 3 Effect of temperature on the activity of SOD (µmoles oxidized/min/mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different. Data expressed as mean ± SEM

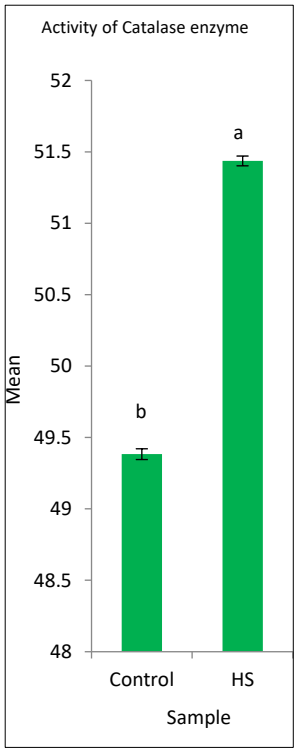

Fig 4 Effect of temperature on the activity of CAT (µmoles of hydrogen peroxide consumed/min/mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different. Data expressed as mean ± SEM

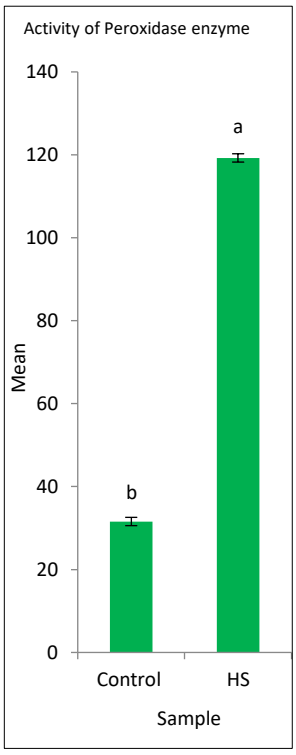

Fig 5 Effect of temperature on the activity of POD (µmoles /min/mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different. Data expressed as mean ± SEM

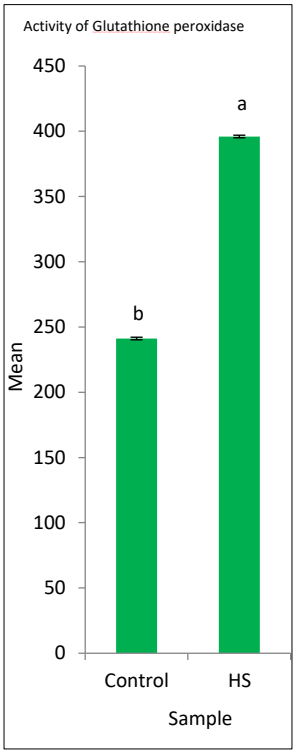

Fig 6 Effect of temperature on the activity of GPX (µmoles NADPH oxidized /min/mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different. Data expressed as mean ± SEM

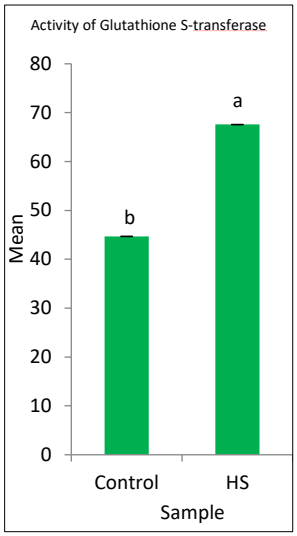

Fig 7 Effect of temperature on the activity of GST (µmoles /min/mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different.
Data expressed as mean ± SEM

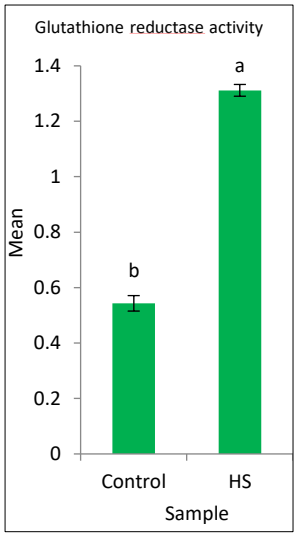

Fig 8 Effect of temperature on the activity of GR (µmoles of NADPH oxidized /min/mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different.
Data expressed as mean ± SEM

Effect of thermal stress on the expression of BmApaf-1 and BmDredd genes

To evaluate the expression of mRNA levels of BmApaf-1 and BmDredd genes in silk gland tissues of the fifth instar larval stage by RT-PCR. The silk gland tissue showed change in the expression of BmApaf-1 and BmDredd genes in both control and test groups. The larvae exposed to thermal stress showed 1.7 fold significant upregulation of BmApaf-1 and 1.5 fold significant upregulation of BmDredd gene compared with control (Fig 9).

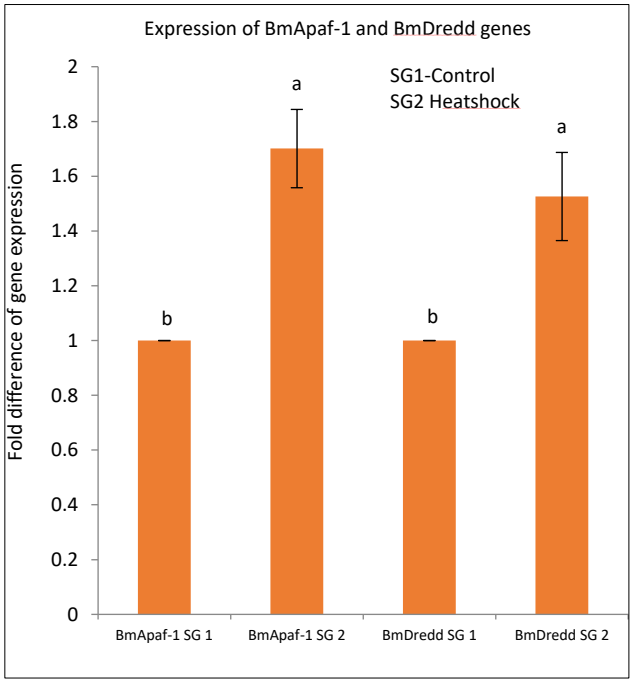

Fig 9 Effect of temperature on the expression of apoptotic related genes in SG tissue of *B. mori.* BmActin were used as the internal control to normalize the data that were represented as mean ±SEM by the three independent measurements. The 2^-ΔΔCT method was used to calculate the fold difference of gene expression. The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different

Temperature is one of the major abiotic factors that influences the growth and development of silkworm and production of excellent quality cocoons [44]. Rates of biological as well as physiological processes have been adversely affected by high temperature [45], which will be reflected in the quality of cocoons [46]. The present study was intended to investigate the quantity of protein, level of oxidative stress, response of antioxidant enzymes and expression of apoptotic related genes against induction of thermal stress in the silk gland of silkworm. The quantity of protein in the larvae showed a significant increase in the silk gland in response to high temperature compared to the control. The results indicated that the larvae treated with high temperature exhibited elevated metabolic rate of proteins in the silk gland and may be due to the synthesis of new stress proteins. A similar inclination was demonstrated in tasar silkworm *Antheraea mylitta* D [47].

An exact steadiness exists between ROS production and activity of antioxidant enzymes, nevertheless this will be disturbed by environmental stress like high temperature disclosure, insect's exhibit consequent physiological responses lead to the generation of surplus ROS, which is able to smash up the structure of proteins, lipids, DNA and RNA [7] and also disrupt the cell membrane fluidity and in turn apoptosis [48].

Cellular and tissue level oxidative stress has been established by measuring the products formed by lipid peroxidation in vertebrates as well as in invertebrates. The products of lipid peroxidation is a sign of oxidative damage to lipids and in addition, it also triggers the regulation of antioxidant resistance mechanisms' [49]. An oxidative attack on the peroxidized polyunsaturated fatty acids gives the product MDA, an indicator of the extent of oxidative damage of lipids [50]. An elevated level of MDA concentration in the silk gland of fifth instar larvae was observed in response to thermal stress (Fig 2). Our results obviously demonstrated that in *B. mori*, thermal stress was accompanied by lipid peroxidation and other responses to oxidative stress. A Similar trend of increase in the levels of MDA was reported in the posterior silk gland, midgut and fat body of silkworm *B. mori* on exposure to azaserine [51]. More over the same trend was also reported in some other insects such as citrus red mite *Panonychus citri* [52], oriental fruit fly *Bactrocera dorsalis* [3], and predatory mite Neoseiulus cucumeris [53].

Antioxidant enzymes work synergistically to alleviate the oxidative stress engendered by surplus ROS inside the cell [53]. SOD, CAT, GST, GSH-Px, are enzymatic antioxidants and GSH is the non-enzymatic antioxidant. Among these antioxidants SOD is the most prominent ubiquitous metalloenzyme facilitating the diminution of elevated levels of superoxide radical induced by low or high temperatures [54]. The current study has revealed an increase in SOD levels in silk glands of fifth instar larvae exposed to temperature compared with control. This implies that the level of SOD was altered with changing environmental temperature. A parallel trend of result was demonstrated by Jena *et al.* [49], who observed that, a significantly elevated level of SOD in pupal testes of tropical tasar silkworm *Antheraea mylitta* under thermal stress. Ali *et al.* [55] reported that another insect *Mythimna separata* induced with high temperature showed significantly increased levels of SOD.

CAT is responsible for the degradation of H_2O_2 [49]. In the present study CAT activity was increased up on thermal stress in the fifth instar larvae (Fig 4). The result indicated that the relation between H_2O_2 production and CAT activity was influenced with thermal stress. Such a case was reported by [56], the CAT level in *Chilo suppressalis* larvae exposed to thermal stress significantly elevated over control. The result is

also consistent with the findings of Jena *et al*. [49], they revealed that the activity of CAT is enhanced in the testes of pupa on exposure to thermal stress.

Glutathione s-transferases are known to be an important multifunctional enzyme play major role in the removal of toxic LPO products [53], detoxification of xenobiotics, protection from attack of oxidative stress and also help in isomerization and intracellular transportation [57]. In the present experiment, enhancement of GST activity was determined under thermal stress compared to the control. This suggests that GST actively eliminated the lethal LPO products formed on thermal stress [58].

POX enzyme is able to convert H_2O_2 into water and molecular oxygen [14], although it works together with GSTs and metabolizes the lipid peroxides [3]. In the present study, peroxidase enzyme shows increased activity under thermal stress. Similar observations were done by [53], [58]. These observations indicate the necessity of POX activity for scavenging the ROS produced under thermal stress.

Glutathione peroxidase is able to reduce free radical damage by metabolizing lipid peroxidation products and H_2O_2 using reduced glutathione as substrate [59]. The present data obtained in the GPx activity in *B. mori* larvae treated with temperature determined a significant increase compared with control. This data suggests that GPx takes major action against ROS produced during thermal stress [60].

The homodimeric flavoprotein GR is capable of synthesizing reduced glutathione (GSH) from oxidized glutathione (GSSG) using NADPH as a reducing cofactor. This bears different functions including coping against oxidative stress and also the biosynthesis of protein and DNA by maintaining a balanced ratio of GSH and GSSG [61]. In our study GR showed an increased activity in thermal stress induced groups than control [62].

Under stress conditions cells are capable of producing ROS and they could be a reason for inducing apoptosis [56]. ROS is capable of stimulating intrinsic as well as extrinsic pathway of apoptosis [21]. In the present study there was an enhanced production of MDA in the treated group indicates the lipid membrane damage due to ROS produced during the exposure of thermal stress. The present study implies an elevated expression of BmApaf-1 and BmDredd in the thermal stress exposed group compared to control. BmApaf-1 is involved in the intrinsic pathway of apoptosis. Chen *et al*. [63] reported that H_2O_2 induced oxidative stress in BmN-SWU1 cells showed an increased expression of BmApaf-1. Previous studies proved that BmDredd is actively participated in the apoptosis process; such a case was reported by Wang *et al*. [64]. An elevated expression of BmDredd was shown in the emodin induced treatment of BmN –SWU1 cells. UV treated BmN cells also showed increased expression of BmDredd in silkworm *Bombyx mori* [65].

CONCLUSION

In conclusion, thermal stress is able to produce oxidative stress in the silk gland of *Bombyx mori* larvae and it may cause lipid membrane damage. The antioxidant enzymes make an effort to reduce the oxidative stress by increasing their activity. The significant 1.7 fold increase of BmApaf-1 and 1.5 fold increase of BmDredd genes in heat induced silk glands may be the indication of activation of apoptotic pathway. Further investigations are required to learn more about impact of thermal stress on the apoptotic pathways in the silk gland.

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