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Comparative Study of Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) Activity Level in Pupal Developmental Stage of Selected Two Voltine Groups of Silkworm *Bombyx mori* L.

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A B S T R A C T

In this present research study, the superoxide dismutase and glutathione peroxidase contents were determined in pupal developmental stage of the Silkworm, *Bombyx mori* L. The two bivoltine (CSR_2 & NB_4D_2) and two multivoltine races (Pure Mysore & C.nichi) were selected for the study and rearing was conducted as per the standard rearing method and after the rearing good cocoons were selected for further assessment. The male and female sex were separated during 5th instar just after resuming from 4th moult before feeding to identify the male and female pupae on 3rd day after spinning. The fat body samples were extracted from male and female from 3rd day to till last of pupal duration. The extracted samples were kept at -20^{o}C for preservation and utilization for the analysis of enzyme profiles viz. Superoxide dismutase (SOD), Glutathione peroxidase (GPx) as per the standard estimation procedures. The results showed stage-specific significantly higher trend levels of glutathione peroxide contents in the female and male pupae as compared to the superoxide dismutase level on (p<0.05). However, a significantly higher level of glutathione peroxidase activity was observed in female pupa on 3rd day of CSR_2 breed (p<0.05). In the case of male pupae of four selected races, significantly lower level of superoxide dismutase activity was observed (p<0.05). Correlation analysis clearly revealed differences in the way the enzyme activity level is equilibrated for a particular stage and developmental pattern. Hence, in this research study, it is very clearly that, the results obtained could be the effect of intensive metabolic transformation that takes place in tissues of the non-diapause generation and causes increased production of reactive oxygen species, such as hydroperoxides. The results of this study suggesting that antioxidants plays a key and fundamental role in the total defense mechanisms and also protecting cells against reactive oxygen species (ROS).

Key words: *Bombyx mori* L, Superoxide dismutase, Glutathione peroxidase, Activity level, Fat body, Pupal stage

Silkworm *Bombyx mori* L. is a very important economic insect and also a model organism for laboratorial tool to study its genetic, breeding, proteomic, genomic, biochemistry, physiology, etc. As per physiological activity of the insect is concerned, there are several numbers of enzymes play various activities as documented in many literatures. Reactive oxygen species, such as superoxide dismutase and glutathione peroxide are cause oxidative stress leading to the damage of biomolecules such as proteins, lipids, and nucleic acids, resulting in disturbance of homeostasis and cellular death if not eliminated (Hermes-Lima and Zenteno-Savin 2002). Cells have interdependent antioxidant defense mechanisms that protect against damage from oxidative stress. Insects possess an antioxidant defense system such as superoxide dismutase, catalase, glutathione peroxidase, catalase, glutathione reductase, and glutathione-S-transferase, reduced glutathione and vitamin C.

Silkworms are an incredibly significant model organism among the economically beneficial insects for researchers next to Drosophila, belonging to the order Lepidoptera of

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phylum Arthropoda. It is calling as holometabolic insects, during metamorphosis silkworms pass from egg, larva, pupa, and adult stages to complete their life cycle. It has been documented that silkworm pupae have excellent antioxidant potential to scavenge free radicals and good antityrosinase activity and also high levels of palmitic acid, oleic acid, stearic acid, linoleic acid, and palmitoleic acid in profiles of fatty acids (Meetali *et al.* 2014).

Among the silkworm species, antioxidant defense systems have been studied in the mulberry silkworm, *Bombyx mori* L. Zhao and Shi (2009) observed differences in antioxidant enzymes (superoxide dismutase, catalase, and xanthin oxidase) and hydrogen peroxide levels in univoltine and polyvoltine strains of *B. mori*. They also studied the effect of chilling on the status of hydrogen peroxide and the activities of superoxide dismutase, catalase, and xanthin oxidase of diapause and non-diapause eggs (Zhao and Shi 2010). It is noteworthy that, the enzymatic basis of development in pupal stage is the most consequences for the expression of commercial characters. There are two different enzymes are considered as major indices such as, superoxide dismutase, and glutathione peroxidase to emphasize the cellular, sub cellular level of activity in fat body tissue is a major organ system from which the energy storage distributed to the different cells of the organisms for the developmental, behavioural, morphological and physiological adaptation under different situation. Variation in the antioxidant defense in the mitochondria of the European corn borer, *Ostrinia nubilalis*, has been studied in diapause and post-diapause stages (Jovanovic-Galovic *et al.* 2007). Krishnan *et al.* (2002) observed high superoxide dismutase activity in hemolymph of *Bombyx mori* L due to bacterial infection. Therefore, the present study was undertaken in order to investigate the activity levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in male and female pupae of four races (Two bivoltines and two polyvoltines) of silkworm *Bombyx mori* L.

MATERIALS AND METHODS

Two bivoltine races viz. CSR_2 and NB_4D_2 and two multivoltine races viz. Pure Mysore and C. nichi were selected for the study and the layings were prepared and the eggs were incubated at 25±1°C temperature and 80 – 85% relative humidity for about 10 days till their hatching and reared by adopting methods described by Tazima (1978), Krishnaswami (1978) and the good cocoons were selected for further assessment. The male and female sex were separated during 5th instar just after resuming from 4th moult before feeding to identify the male and female pupae on 3rd day after spinning. The fat body samples were extracted from male and female from 3rd day to till last of pupal duration. The extracted samples were kept at -20ºC for preservation and utilization for the analysis of enzyme profiles viz. Superoxide dismutase (SOD), Glutathione peroxidase (GPx) as per the standard estimation procedures are detailed as follows.

Superoxide dismutase (SOD) activity (EC. 1.15.1.1)

Superoxide dismutase (SOD) was assayed according to the method of Kakkar *et al.* (1984), Preparation of enzyme extract: the sample (0.5g) was grinded with 3.0 ml of potassium phosphate buffer, centrifuged at 2000 rpm for about 10 minutes and the supernatant were used for the assay. The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of nitroblue tetrazolium (NBT), 0.2 ml of enzyme preparation and water in a total volume of 2.8 ml. the reaction was initiated by the addition of 0.2 ml of nicotinamide adenine dinucleotide (NADH), the mixture was incubated at 36ºC for 90 seconds and arrested by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was then shaken with 4.0 ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560nm in a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme in which 50% inhibition of NBT reaction in one minute.

Glutathione peroxidase (GPx) activity (EC. 1.11.1.9)

Glutathione peroxidase (GPx) activity was measured by the procedure of Flohe and Gunzler (1984), the reaction mixture consisted of 0.3 ml of phosphate buffer (0.1 M 7.4) 0.2 ml of glutathione (GSH) (2 mM). 0.1 ml of sodium azide (10mM), 0.1 ml of H_2O_2 (mM) and 0.3 ml of tissue homogenate was incubated for 15 minutes at 37°C. Reaction was stopped by addition of 0.5ml of TCA (5%). The mixture was centrifuged at 1500x g for 5 min and to the supernatant 0.7 ml of 5, dithiobis (2- nitro -benzoate) (DTNB) (0.4 mg/ml) and 0.2 ml of phosphate buffer (0.1 M, pH 7.4) was added. After vortexing absorbance was recorded at 420 nm.

RESULTS AND DISCUSSION

The activity profiles of enzymes in fat body tissue of pupae of CSR_2, NB_4D_2, PM and C. nichi strains in the present investigation to correlate the comparative study under taken to narrate the relationship among the two voltine groups. The active profile of fat body superoxide dismutase level and glutathione peroxidase activity level in male and female pupae of CSR_2, NB_4D_2, PM, C. nichi, reveals the distinct changes among races in all the days of progressive development of the pupae commencing from day 3 till the completion of life duration. However, significant variation of SOD and GPx level was observed among male and female pupae of both the bivoltine and the multivoltine races (Table 1-2). The statistical ANOVA revealed highly significant variation between two races in days (P<0.05% and CD@5%). The SOD enzymatic level of both male and female pupae of selected races expressed in (Table 1), on 3rd day of male pupae of CSR_2, NB_4D_2, PM & C. nichi of 1.56±0.001, 1.40±0.002, 1.11±0.003 and 1.03±0.003 respectively, mean value of female pupae on 3rd day of 1.93±0.06, 1.72±0.06, 1.47±0.02 and 1.42±0.04 of CSR_2, NB_4D_2, PM and C.nichi respectively and the SOD level form 3rd to last day of pupal duration was slightly increasing in order in both case of male and female pupa.

Data in (Table 2) represents, the mean value, F value and CD@5% of four races in relation to male and female pupae, on the 3rd day of female pupae of CSR_2 race show

higher GPx level followed by female pupae of NB_4D_2, PM and C.nichi of 4.93±0.04, 4.21±0.03, 4.20±0.06 and 4.03±0.05 respectively. GPx level of Male pupae of CSR_2, NB_4D_2, PM and C.nichi of 4.45±0.02, 4.21±0.03, 3.57±0.02 and 3.42±0.05 respectively. From 3rd day onwards Glutathione peroxidase activity level is gradually in increasing order till the completion of pupal period (till moth emerges).

Table 1 Changes in fat body superoxide dismutase and glutathione peroxidase activity level of male and female pupae of selected silkworm races (Each in unit/ observation are expressed mg protein)

Races / Days	CSR_2		NB_4D_2		PM		C.nichi	
	Male	Female	Male	Female	Male	Female	Male	Female
3	1.56 ± 0.001	1.93 ± 0.062	1.40 ± 0.002	1.72 ± 0.061	1.11 ± 0.003	1.47 ± 0.023	1.03 ± 0.003	1.42 ± 0.043
4	1.62 ± 0.002	1.98 ± 0.036	1.47 ± 0.002	1.82 ± 0.049	1.16 ± 0.002	1.52 ± 0.034	1.08 ± 0.003	1.47 ± 0.026
5	1.68 ± 0.002	2.02 ± 0.043	1.51 ± 0.003	1.94 ± 0.072	1.23 ± 0.002	1.57 ± 0.051	1.13 ± 0.002	1.54 ± 0.043
6	1.74 ± 0.002	2.08 ± 0.016	1.56 ± 0.003	1.98 ± 0.012	1.26 ± 0.003	1.63 ± 0.016	1.19 ± 0.003	1.59 ± 0.067
7	1.80 ± 0.002	2.12 ± 0.042	1.63 ± 0.003	2.06 ± 0.059	1.32 ± 0.003	1.68 ± 0.071	1.25 ± 0.003	1.66 ± 0.059
8	1.84 ± 0.001	2.17 ± 0.061	1.68 ± 0.003	2.09 ± 0.072	1.37 ± 0.002	1.74 ± 0.016	1.30 ± 0.003	1.71 ± 0.043
9	1.88 ± 0.002	2.24 ± 0.052	1.76 ± 0.002	2.16 ± 0.028	1.43 ± 0.003	1.79 ± 0.043	1.38 ± 0.002	1.77 ± 0.019
10	1.91 ± 0.002	2.28 ± 0.061	1.82 ± 0.003	2.21 ± 0.043	1.47 ± 0.003	1.84 ± 0.049	0.000	0.000
11	1.95 ± 0.001	2.32 ± 0.049	1.86 ± 0.003	2.26 ± 0.073	1.51 ± 0.002	1.88 ± 0.041	0.000	0.000
12	1.99 ± 0.002	2.35 ± 0.043	1.91 ± 0.002	2.29 ± 0.019	1.57 ± 0.003	1.97 ± 0.023	0.000	0.000
F-test	*	*	*	*	*	*	*	*
C.D@ 5%	0.005	0.007	0.007	0.014	0.008	0.008	0.007	0.007

Table 2 Changes in fat body superoxide dismutase and glutathione peroxidase activity level of male and female pupae of selected silkworm races (Each in unit/ observation are expressed mg protein)

Races / Days	CSR_2		NB_4D_2		PM		C.nichi	
	Male	Female	Male	Female	Male	Female	Male	Female
3	4.45 ± 0.024	4.93 ± 0.044	4.21 ± 0.035	4.73 ± 0.064	3.57 ± 0.024	4.20 ± 0.065	3.42 ± 0.053	4.03 ± 0.053
4	4.48 ± 0.033	4.96 ± 0.053	4.26 ± 0.073	4.76 ± 0.043	3.60 ± 0.054	4.22 ± 0.044	3.45 ± 0.033	4.05 ± 0.065
5	4.52 ± 0.014	5.00 ± 0.035	4.30 ± 0.064	4.80 ± 0.034	3.64 ± 0.043	4.26 ± 0.025	3.49 ± 0.023	4.10 ± 0.024
6	4.56 ± 0.034	5.08 ± 0.074	4.34 ± 0.083	4.84 ± 0.074	3.69 ± 0.033	4.29 ± 0.084	3.52 ± 0.084	4.14 ± 0.094
7	4.60 ± 0.023	5.13 ± 0.064	4.48 ± 0.054	4.88 ± 0.056	3.72 ± 0.084	4.33 ± 0.093	3.57 ± 0.074	4.18 ± 0.043
8	4.64 ± 0.044	5.18 ± 0.024	4.52 ± 0.064	4.92 ± 0.044	3.75 ± 0.023	4.38 ± 0.014	3.60 ± 0.023	4.21 ± 0.024
9	4.67 ± 0.076	5.22 ± 0.015	4.57 ± 0.023	4.97 ± 0.085	3.78 ± 0.043	4.41 ± 0.044	3.64 ± 0.014	4.25 ± 0.075
10	4.73 ± 0.053	5.25 ± 0.075	4.61 ± 0.014	5.01 ± 0.064	3.82 ± 0.073	4.47 ± 0.074	0.000	0.000
11	4.77 ± 0.035	5.29 ± 0.044	4.59 ± 0.070	5.07 ± 0.042	3.85 ± 0.063	4.50 ± 0.014	0.000	0.000
12	4.81 ± 0.091	5.34 ± 0.064	4.69 ± 0.064	5.11 ± 0.034	3.90 ± 0.033	4.54 ± 0.074	0.000	0.000
F-test	*	*	*	*	*	*	*	*
C.D@ 5%	0.015	0.013	0.066	0.012	0.011	0.012	0.009	0.010

Each values are the mean ± SD of 3 replications, P<0.05%: Significant (*), P>0.05%: Non significant (NS)

Pupa looks on chrysalis period Pupa looks after development

Plate 1 Transformation of pupa from chrysalis stage to last day

The SOD and the GPx enzymatic activity level in fat body tissue of male and female pupae from on chrysalis stage (Plate 1) to completion of pupal period, the glutathione peroxidase (GPx) level is showed higher activity level compare to superoxide dismutase (SOD) activity level of selected races. Further, the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the fat body tissue of male and female pupae of all the four selected breeds/races were showed bivoltine pupae compare to pupae of multivoltine (Fig 1-4). However, the CSR_2 showed relatively more active profile of an enzyme compare to NB_4D_2, PM and C.nichi races.

GPx an important enzyme plays a logical role in the reactive oxygen species to tide over the stress situation of the cells, organ and organismic behaviour, it has been attributed for the manifestation of longevity parameters and interaction of physiological activities of living cells. The pupal stage from day 3, the enzyme modulation is in the direction of order of increase till the day of completion of pupation period. The day to day pattern of changes revealed an interesting observation and maintained consistency in all

the days of pupal development irrespective of the sex. The significance differences association among the breeds, the multivoltine and bivoltine substantiates $P<0.05\%$ significance was noticed. The differences among the male and female pupae in relation to the glutathione peroxidase activity in fat body tissue, the female pupae of the bivoltine breeds observed comparatively more in the activity profiles compare to male pupae of multivoltine strains.

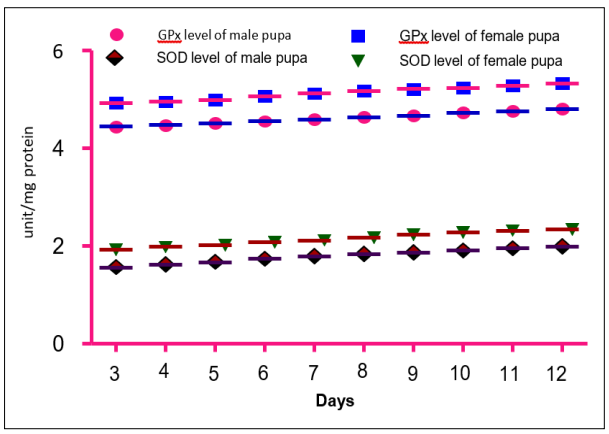

Fig 1 Comparative level of GPx and SOD of female and male pupae of CSR2 silkworm breed

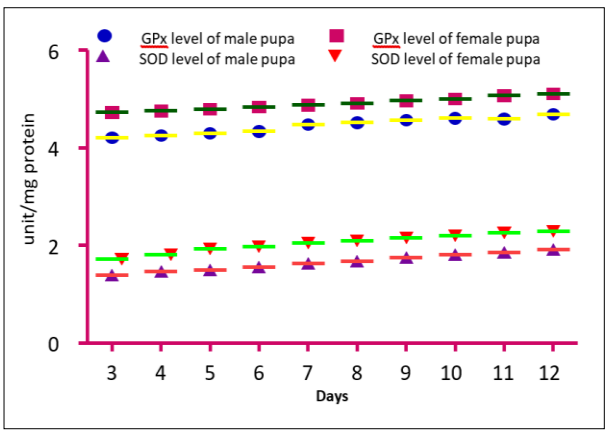

Fig 2 Comparative level of GPx and SOD of female and male pupae of NB4D2 silkworm breed

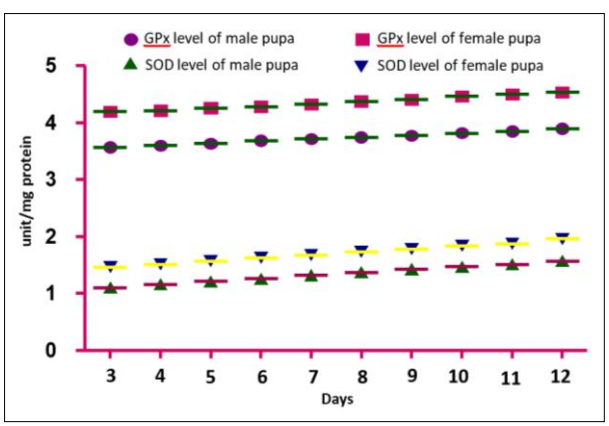

Fig 3 Comparative level of GPx and SOD of female and male pupae of PM silkworm race

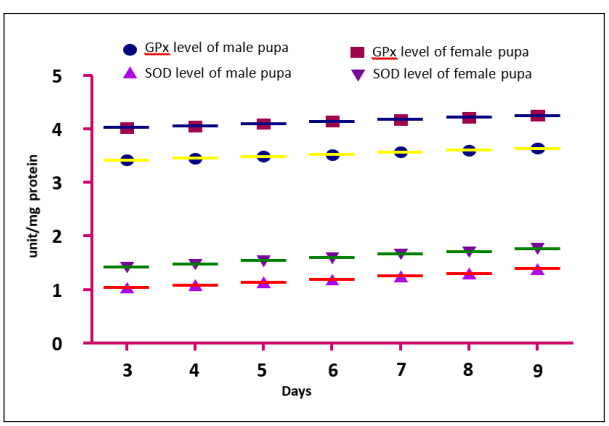

Fig 4 Comparative level of GPx and SOD of female and male pupae of C. nichi silkworm race

Fig 1-4 Changes in fat body SOD and GPx level in male and female pupae of bivoltine and multivoltine silkworm races/breeds

Glutathione peroxidase (GPx) is a well-known key selenoenzyme that functions as an antioxidant (Flohe *et al.* 1972). This selenoprotein catalyzes the reduction of harmful peroxides by glutathione and protects the lipid membranes and other cellular components against oxidative damage and at its catalytic site catalyses the reduction of hydrogen peroxides and hydroperoxides to non-toxic products. The reducing equivalent of glutathione is used as a substrate to form oxidized glutathione (Bruce *et al.* 1982). The enzyme with catalytic site includes a selenocysteine residue in which the selenium undergoes a redox cycle involving the selenol as an active form that reduces hydrogen peroxides and organic peroxides. The selenol is oxidized to form selenenic acid which reacts with reduced glutathione (GSH) to form selenenyl sulfide adduct. Further, Zhao and Shi (2009) observed the variation of antioxidant enzymes (superoxide dismutase, catalase, and xanthin oxidase) and hydrogen peroxide levels in univoltine and polyvoltine strains. They noticed that the metabolism of hydrogen peroxide exhibited significant differences between univoltine and polyvoltine strains and between embryonic and pupal stages. Moreover, few research's results suggested that, cellular antioxidants are involved in both the protection of cells and the regulation of redox levels during the pre-adult stages of *Ostrinia nubilalis* (Wiley-Liss 2004). A comparison study of peroxides and antioxidant defense in diapause and non-diapause *A. mylitta* showed that the elevation of all the oxidative and antioxidative components (with the exception of glutathione-S-transferase activity in 5th instar larvae) occurred in the non-diapausing generation (Jena *et al.* 2013).

Yuta *et al.* (2019) reported seven types of superoxide dismutase (BmSOD1 to BmSOD7) in *Bombyx mori*. Among them, BmSOD4 and BmSOD5 was decreased from the first instar larva to pupa. The decreased activity might be due to the sensitivity of SOD isoforms to various reagents including the product of dismutation reaction, hydrogen peroxide and metal ions (Hodgson and Fridovich 1975). Glutathione peroxidase (GPx) is involved in protecting the

organism from oxidative damage. The increased activity of enzymes may protect exposed species against ROS. It also involved in the reduction of lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water (Kannan *et al*. 2011).

However, superoxide dismutases are metalloproteins and play a vital role in protecting cells against oxidative damage and it reacts with superoxide radicals and converts them to H_2O_2 catalyzed by catalase (Kakkar *et al*. 1984). In the results obtained, suggests the fat body glutathione peroxidase of male and female pupae of all the selected four silkworm breeds/races maintained almost a similar and consistent level of phase response in all the days of pupation period. Further, glutathione peroxidase and superoxide dismutase activity level of fat body tissue of said races were increased with age was observed. SOD and GPx activity level of the fat body in pupal stage difference was found between male and female. Because, in the pupal stage fat body accumulation is very high and metabolism of insect also high in this stage. However, knowledge of SOD with relevant to the silkworm, *Bombyx mori* is so far very limited till date as there were very few attempts made by researchers. Hence, it's very difficult to draw a solid conclusion with relevant to the enzymatic activity level is concerned with relevant to the silkworm *Bombyx mori* L.

Based on the results obtained from the present research study can draw a conclusion that, there is an evident of clear difference in the expression of enzyme activity level in the male and female pupae of selected silkworm *Bombyx mori*. Further, there is also observed differences among the male and female pupae in relation to the glutathione peroxidase activity in fat body tissue, the female pupae of the bivoltine breeds observed comparatively more in the activity profiles compare to male pupae of multivoltine strains. Moreover, glutathione peroxidase and superoxide dismutase activity level of fat body tissue of said races were increased with age was observed. Further, results obtained under the present study can conveniently be utilized for further research and developmental studies in the Sericulture Industry and research Institutes.

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