Antioxidative Response in a Silkworm, *Bombyx mori* larvae to Dichlorvos insecticide

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ABSTRACT

Introduction: Antioxidant defense components protect insects by scavenging reactive oxygen species, leading to oxidative stress. We therefore investigated the effect of an organophosphate insecticide, dichlorvos, on superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione S-transferase (GST) and glutathione reductase (GR) as antioxidative biomarkers in silk worm, *Bombyx mori* (L.), larvae. **Methods:** The newly hatched larvae were reared on mulberry leaves containing 0.01. 0.1, 1 and 5ppm dichlorvos. **Results:** The leaf diet with lowest concentration of dichlorvos did not significantly influence the activity of antioxidative biomarkers. Dichlorvos at 0.1 and 1ppm significantly resulted in increased SOD, GPx and GST activity, CAT and GR activity were decreased. Highest concentration of dichlorvos 5ppm significantly decreased SOD and CAT activity compared to control group, whereas GPx and GR activity were significantly increased than the control groups. **Conclusion:** Over all the date suggest that increased activity of SOD, GPx and GST could be involved in free radicals scavenging in *B.mori* larvae leading to oxidative stress by dichlorvos insecticide.

Key words: Antioxidative biomarkers, GPx, GST, Insecticide toxicity, Organophosphate, Reactive oxygen species.

INTRODUCTION

The silkworm, *Bombyx mori* (L.) is an economically important insect. India is the second largest producers of all commercially exploited insets silk next to the China.[1] Mulberry is a major commercial crop and widely grown as food plant for silkworm *B. mori*.[2] Mulberry, *Morus* spp is infested by a number of insect pests and thrips among these *Pseudodendrothrips mori* is one of the major pest that cause huge damage to mulberry plants.[3] For the control of thrips organophosphate pesticide like dimethoate and dichlorvos are commonly used.[4-5]

As a consequence of pesticide usage in the past couple of years, sericulture faces many common problems such as sensitivity of the silkworm, *Bombyx mori* (L.), to many pesticides, and the death of silkworm.[6-7] This is largely due to the application of pesticides in the control of pests in crop fields that are close to mulberry plantations and also to the direct control of mulberry pests.[8] Apart from the indirect effect of pesticides on silkworm, the presence of pesticide residue and other xenobiotics in the mulberry orchards could also affect the biology of the silkworm. The effect of such residues and contamination may cause the formation of free radical leads to cellular damage. It has been reported that exposure to various exogenous and endogenous sources reactive oxygen species (ROS) such as superoxide radicals hydroxyl radical, hydrogen peroxide, and hydroperoxides are generated.[9-10]

Exogenous sources including pesticides and prooxidant allelochemicals, pose a serious challenge to herbivorous insect species during host interactions. However, insects have evolved a complex antioxidant mechanism to overcome the toxic effects of ROS. The antioxidant defense is primarily constituted by the enzymatic actions of superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), reduced glutathione (GSH) and glutathione reductase (GR).[11-12]

Very few studies have been carried out on the oxidative response to herbicides and other chemical induced oxidative stress in wax moth *G. mellonella,* silkworm *Antheraea*

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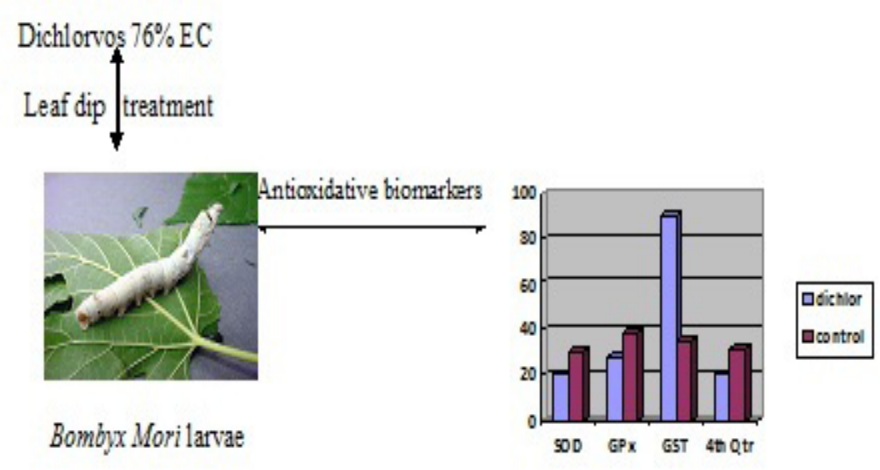

Graphical Abstract

mylitta,[13-14] However, little information is available on nontarget effects of pesticide on the silkworm. Hence the aim of the present study was to understand the antioxidant response of silkworm *B. mori* to dichlorvos pesticide, which is used for the control of thrips in the mulberry plant.

MATERIALS AND METHODS

Silkworm sources and rearing

The eggs of the silkworm, *Bombyx mori* was gifted from the Regional Sericulture Research Station, Salem, Tamilnadu, India. Silkworms were reared under laboratory conditions at 25 ± 2°C, 70-85% RH, and a photoperiod of 12:12 (L: D) h. All silkworms were fed twice per day with fresh *M. alba* leaves harvested from an irrigated mulberry orchard at Regional Sericulture Research Station, which had not been treated with any pesticides.

Insecticide and Chemicals

Commercial grade of dichlorvos, 76% EC was obtained from Enjay Marketing Services Pvt. Ltd., Maharashtra, India. Thiobarbituric acid, bovine serum albumin (BSA), reduced glutathione (GSH), 5, 5-dithio-bis (2-nitrobenzoicacid) (DTNB) and tricholoroacetic acid (TCA) were purchased from Hi-Media Chemicals Ltd., (Hi-Media Laboratories, LBS Marg, Mumbai, India). Phenylmethanesulphonyl fluoride (PMSF), dithiothreitol (DTT), horse radish peroxidase, H_2O_2, ascorbic acid and phenol red were purchased (SRL, SISCO Research Laboratories PVT.LTD. Mumbai, India). All other chemicals were of analytical grade.

Experimental design

Based on the method of Zhang *et al.*[15] we used leaf dipping bioassay. Briefly, Commercial-grade insecticide which is in a liquid form was first diluted in 1 ml of ethanol and accomplished with distilled water to prepare solutions of the required concentrations at 0.01, 0.1, 1, and 5 ppm. These concentrations were tolerated by *B. mori* larvae, which are confirmed by our previous bioassay study (data not shown). For leaf dip assay fresh mulberry leaves were washed by tap water and then air-dried. After veins were cut, leaves were dipped in to four concentrations of insecticide dilutions for 5 s. Then, the air dried treated mulberry leaves were chopped to strips ≈2 mm in length and placed in the petri dish (9 cm in diameter). Fifteen third instars larvae were placed in each of petri dish. Silkworm larvae were allowed to feed on the treated mulberry leaves for 24 h. Control larvae were reared on the mulberry leaves treated with an equal volume of distilled water.

Enzyme preparation

Ten third instars of *B. mori* larvae were randomly selected form the bioassay. The larvae were pooled and homogenized in 0.1M ice cold phosphate buffer (pH 7.0). Homogenates were centrifuged at 4°C for 12,000 rpm for 10 min. The enzyme homogenate was immediately used to assay the antioxidant biomarkers.

Biochemical analysis

Total protein

Protein content of the tissue homogenate was estimated according to the method of Lowry *et al.*[16] using BSA as a standard. Protein content was expressed as µg/g tissue wet weight.

Superoxide dismutase (SOD) activity assay

Total SOD (EC 1.15.1.1) activity was determined according to Marklund and Marklund[17] method assaying

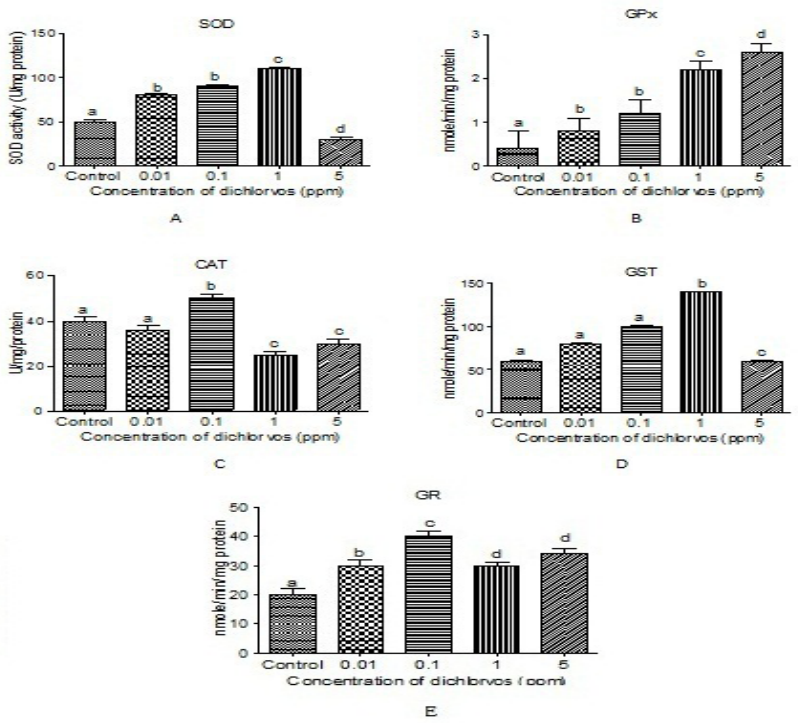

Figure 1: Effect of dichlorvos on antioxidant enzyme activity of *B. mori* larvae. A. SOD; B. GPx; C. CAT; D. GST and E. GR activity

Bars represents the Mean±SE. Means followed by the same letters are not significantly different (P>0.05; Kruskal-Wallis test).

the autooxidation and illumination of pyrogallol at 440 nm for 3 min. One unit total SOD activity was calculated as the amount of protein causing 50% inhibition of pyrogallol autooxidation. The total SOD activity was expressed as units per miligram of protein ($U.mg^{-1}$).

Glutathione peroxidase (GPx) activity assay

GPx (EC1.11.1.9) activity was measured with H_2O_2 as substrate according to method of Paglia and Valentine.[18] This reaction was monitored indirectly as the oxidation rate of NADPH at 340 nm for 3 min. Enzyme activity was expressed as nanomoles of NADPH consumed per minute per milligram of protein, using an extinction coefficient of 6220 $M^{-1}cm^{-1}$.

Catalase (CAT) activity assay

CAT (EC1.11.1.6) activity was spectrophotometrically measured according to the Luck method.[19] CAT activity was by the rate of decomposition of H_2O_2 by CAT. CAT activity was expressed as µmol of H_2O_2 decomposed /min/mg protein.

Glutathione S-transferase (GST) activity assay

GST (EC 2.5.1.18) activity was assayed by measuring the formation of the GSH and 1-chloro-2, 4-dinitrobenzene (CDNB) conjugate Habig *et al.*[20] The increase in absorbance was recorded at 340 nm for 3 min. The specific activity of GST was expressed as nanomoles GSH-CDNB conjugate formed per minute per milligram protein using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

Glutathione reductase (GR) activity assay

Glutathione reductase (EC 1.8.1.7) activity in erythrocytes was determined by following the oxidation of NADPH to NADP during the reduction of oxidized glutathione Goldberg and Spooner.[21] GR activity was expressed as nmol of NADPH oxidized/min/mg protein. All assays were corrected for nonenzymatic reactions using corresponding substrate in phosphate buffer (0.1M, pH 7.0).

Statistical analysis

The data are summarized as means ± their respective standard error (SE). Differences among experimental groups were calculated by using a one-way analysis of variance (ANOVA), followed by Kruskal-Wallis test. In all experiments, the value $P<0.05$ was used as the standard of statistical significance for hypothesis testing. The data were analyzed using the Prism Graph-Pad software (Version-6.0).

RESULTS

Biochemical assay

SOD activity was significantly increased by increasing the concentrations of dichlorvos up to 1 ppm. Dichlorvos at 1 ppm increased SOD activity from 50. ± 1.81 in control to 110.21 ± 2.30 U/mg protein. For 5 ppm of dichlorvos, a significant reduction was recorded in comparison with control and other concentrations of the insecticide (Figure 1A). The low concentrations of dichlorvos significantly decrease the GPx activity. However, a high concentration of this insecticide (1 and 5 ppm) dramatically increases the GPx activity in the larvae from 0.45 ± 0.21-2.21 ± 0.04 and 2.60 ± 0.22 nmol/mg protein/min respectively (Figure 1B).

Catalase activity was found to decline in low concentration to high concentration compare to control. However, it was surprising to observe that there was increased in CAT activity at 0.1 ppm from 40 ± 1.20 in control to 50.16 ± 0.87 U/mg protein (Figure 1C). GST activity had the same trend as SOD-its activity was increased by increasing the concentrations of dichlorvos up to 1 ppm. The low concentrations of dichlorvos significantly increased the GST activity of the larvae. Dichlorvos at 1 ppm increased GST activity from 60. ± 0.32 in control to 140.21 ± 0.47 nmol/min/mg protein. For 5 ppm of dichlorvos, a significant reduction was recorded in comparison with control and other concentrations of the insecticide (Figure 1D).

The activity of GR was significantly increased at 0.01 and 0.1ppm as compared to control. However the GR activity was decreased from 40 ± 0.64-28.70 ± 01.3 nmol/min/mg protein at high concentration of 1 and 5ppm (Figure 1E).

DISCUSSION

Dichlorvos is the common insecticide used for sucking pests control in mulberry. Apart from its direct effect on target insects, other non-target species especially *Bombyx mori* may also exposed to it. In this study, we showed that dichlorvos induced oxidative response in *B.mori* larvae. The results indicate that despite the increased dichlorovs doses induced oxidative stress at cellular level, activity of antioxidant enzyme showed dose dependent increase up to lethal dose.

Of the five tested enzymes, SOD, GST, GPx and GR showed the highest increase of activity. SOD is an antioxidant enzyme which catalyze the dismutation of the toxic superoxide (O_2^-) adical into either molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). The activity of SOD in *B. mori* at 1ppm was 1.5 times higher than in control, respectively. This suggests that SOD enzyme, play a crucial role in defense against ROS during toxic action of dichlorovs. An increased activity of SOD in acerola juice revealed that SOD plays an important role in dismutation of superoxide radicas.[22] Mahmud *et al*[23] also found a potential antioxidant activity of SOD against carbon tetrachloride induced liver damage in rats. Previous study reported that induction of SOD activity is the main response to organophosphate toxicity and other dietary prooxidant exposure in lepidopteran insectS[9,24]

CAT catalyzes the conversion of hydrogen peroxide to water and molecular oxygen.[25] Hence, the increase in CAT activity after treatment of dichlorvos in *B. mori* could be expected in order to scavenge hydrogen peroxide. Whereas in our study CAT activity was decreased at 1ppm and 5ppm as compare to control. The decrease of CAT activity at increasing doses, may be due to the fact that CAT is known to be inhibited by the accumulation of superoxide anion during destruction processes, which may be caused by dichlorvos insecticide.[26] Suggest that increased production of free radicals may lead to depletion or inactivation of CAT enzyme. CAT in the midgut of *Lymantria dispar* larvae fed on an unfavorable plant has shown similar results.[27] Decreased activity of CAT also was detected due to high level of superoxide radical generation during oxidative stress in the acute stage of bacteriosis in *G. mellonella*.[28]

GSTs are a family of enzymes that catalyze the addition of the tripeptide glutathione to endogenous and xenobiotic substances which have electrophilic functional groups. They play an important role in the detoxication and metabolism of many xenobiotic and endobiotic compounds.[29] The present data obtained in the GST activity in *B. mori* larval after treatment with dichlorvos showed a significant increase in the dose dependent manner and in the 5ppm, GST activity decreased to be with slight insignificant changes with control. This finding may prove that GST is involved in the inactivation of toxic lipid peroxidation products accumulated during destructive processes caused by dichlorvos insecticide. Our results are in accordance with those obtained in *G. mellonella* midgut in the early stage of bacteriosis[28]. GPx is another important antioxidant enzyme; which reduces free radical damage by metabolizing H_2O_2-H_2O. In the present study GPx activity was 5 times increased in the treatment than control activity. Suggest that GPx is involved in free redial scavenging in the *B.mori* in response to pesticide exposure. Similar study were reported by Anderson and Paris-Palacios *et al*.[30,31] who found that GPx play a crucial role in intracellular protection against the reactive oxygen

species in *Culex pipiens* and *Anopheles multicolor*.

Glutathione reductase catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell.[32] GR activity was 2 times increased in the present study at 0.1ppm compared to control activity. Similar result was reported by Karthi and Shivakumar,[33] suggest that increased activity of GR in response to pyrethroid treatment may involve in oxidative response in *S.litrua* larvae. Pena-Llopis *et al*[34] have reported elevated GR activity under a stress condition.

CONCLUSION

In conclusion, the result of the present study demonstrated that antioxidant enzyme marker such as, SOD, GST, GPx and some lesser degree of GR were involved in the free radical scavenging in response to dichlorvos exposure in silk worm larvae *B. mori*.

CONFLICT OF INTEREST

The authors declare that we have no conflicts of interest.

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ABBREVIATION

SOD: Superoxide dismutase

GPx: Glutathione peroxidase

CAT: Catalase

GST: Glutathione S-transferase

GR: Glutathione reductase

PPM: Parts per million

ROS: Reactive oxygen species

EC: Emulsifiable concentrate.

Highlights of the Paper

- Dichlorvos at 0.1 and 1 ppm significantly increased the SOD, GPx and GST activity.
- GPx and GR activity were increased at 5 ppm concentration than the control groups.
- SOD and CAT activity was decreased at 5 ppm concentration.

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