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RESEARCH REPORT

Influence of sericin in alleviating the hydrogen peroxide induced oxidative stress in silkworm *Bombyx mori*: role of the amino acids

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Abstract

Sericin is an important peptide derived from silk fibre spun by the silkworm *Bombyx mori* and has various biological activities. The aim of the present study was to characterize the major constituents of sericin that are providing cytoprotective effect against hydrogen peroxide-induced cell damage in midgut epithelial cells and hemocytes of silkworm. Extracted sericin was subjected to LCMS analysis for amino acid composition. Isolated cells of midgut and hemocytes were incubated with sericin or with mixture of serine and aspartic acid prior to suboptimal concentration of hydrogen peroxide treatment. Sericin as well as amino acid mixture reduced the activity of antioxidant enzymes triggered by hydrogen peroxide, inhibited oxidative derivatives such as protein carbonyl and malondialdehyde and increased antioxidant capacity in both the cells studied. Furthermore, sericin and amino acid mixture significantly decreased intracellular reactive oxygen species as assessed by fluorescent detection. These results suggest that major constituent amino acids of sericin defend midgut epithelial cells and hemocytes against oxidative damage by scavenging reactive oxygen species rather than activating antioxidant enzyme system thereby inhibiting cell damage.

Key Words: amino acids; antioxidant enzymes; *Bombyx mori*; oxidative stress; reactive oxygen species; sericin

Introduction

Reactive oxygen species (ROS) are produced during normal cellular metabolism or are derived from exogenous sources, and play an important role in cellular homeostasis. Exogenous sources, including prooxidant allelochemicals, severely affect herbivorous insects during host interactions and stress such as starvation may contribute to endogenous sources of ROS accumulation. Unchecked or increased levels of ROS can cause severe damage to various cellular compartments, including DNA, protein and lipids, thereby causing oxidative injury. Oxidative injury to the cell membrane results in structural changes and increased permeability to ions and fluids (Nagasaka *et al*., 2004). Nonetheless, insects have evolved a complex antioxidant mechanism to overcome the toxic effects of ROS (Krisnhnan and Kodrik, 2006). Antioxidant defence is primarily contributed by antioxidant enzymes (AOEs) such as peroxidase (POX, E.C. 1.11.1.7), superoxide dismutase (SOD, E.C. 1.15.1.1) and catalase (CAT, E.C. 1. 11. 1.6) (Felton and Summer, 1995). Glutathione peroxidase (GPx) reduces H_2O_2 and hydroperoxides, thereby scavenging oxidative radicals in cell membranes, whereas SOD converts O_2^- to molecular O_2 and H_2O_2 (Maiorino *et al*., 2003; Imlay, 2008). H_2O_2 is subsequently scavenged by CAT, resulting in the production of water and molecular oxygen (Kashiwagi *et al*., 1997).

Several naturally occurring macromolecules (Balsano and Alisi, 2009) and amino acids have been found to be effective as antioxidants (Wu *et al*., 2003a; Atmaca, 2004; Liu *et al*., 2004; Movahedian *et al*., 2006; Selvaraju and Subbashinidevi, 2011). Among the macromolecular proteins that combat oxidative stress (Elisa *et al*., 2008; Medina-Navarro *et al*., 2010), the silk protein sericin role as an antioxidant is commendable because of the presence of polyphenols and flavanoids (Devi *et al*., 2011; Prasong, 2011). Sericin is an active biomolecule with several implications as a therapeutic agent (Khudaiderdier, 1997; Aramwit and Sangcakul, 2007; Li *et al*., 2008) and component of cosmetics (Padamwar and Pawar 2004; Patel and Modasiya, 2011). Silk protein filament has two protein fractions, fibroin, a fibrous component and glue-like sericin that holds the fibroin components together and often discarded as waste product in silk industry. Sericin exhibits

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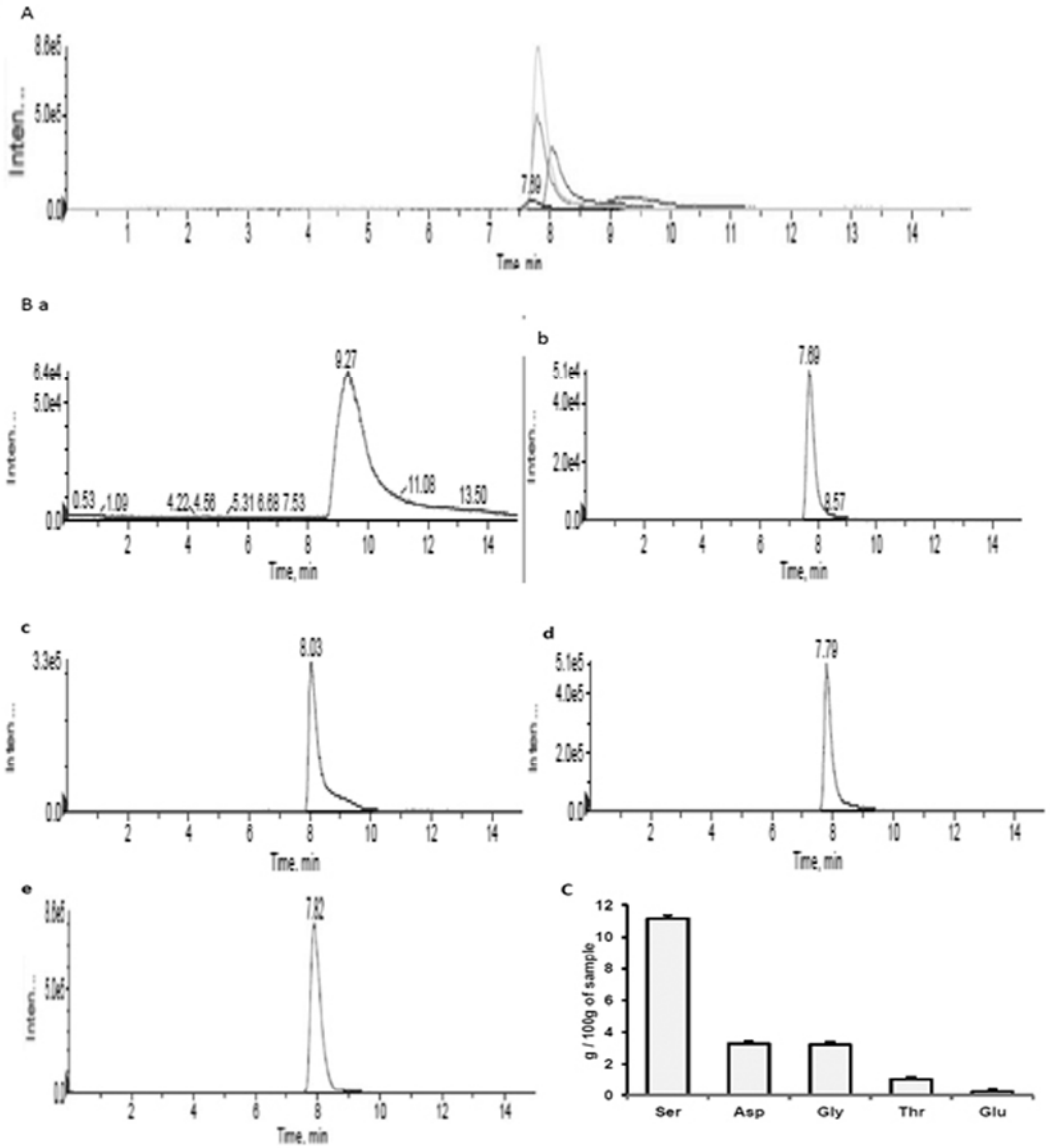

Fig. 1 (A) LCMS spectra of sericin showing prominent amino acids. (B) Spectra of the major amino acids serine (a), aspartic acid (b), glycine (c), threonine (d) and glutamic acid (e). The X-axis is retention time and the Y-axis is intensity in cps. (C) Prominent amino acid composition of sericin protein in *B. mori*.

several pharmacological effects, such as enhancing digestion and cryoprotection. Dietary sericin reduces lipids and ameliorates glucose tolerance in rats fed a high fat diet and also acts as an anticoagulant upon sulphonation (Kato *et al*., 1998; Sasaki *et al*., 2000; Tsujimoto *et al*., 2001; Okazaki *et al.,* 2010). Sericin is specifically synthesized in the middle silk gland of the silkworm *B. mori.* It is a polypeptide with 18 amino acids, most of which have strong polar side groups such as hydroxyl, carbonyl and amino groups (Wu *et al*., 2007) and is especially rich in serine (~32 %) (Kwang *et al*., 2003). In general, amino acids found in proteins have the potential to interact with free radicals if the energy of the radicals insult is high (Elias *et al*., 2008) and antioxidant activity of proteins in radical mediated oxidation reactions may be due to their ability to act as radical trapping devices (Ostdal *et al*., 2002).

The silkworm *Bombyx mori* is a monophagus insect known worldwide because of the lustrous silk it produces in the final instar of the larval stage. One of the reason for the decline in silk production and the survivability of highly domesticated mulberry silkworm is due to food contaminated with pesticides (Vyjayanthi and Subramanyam, 2002a, b). In an earlier study, we demonstrated a transient increase in antioxidant defence mechanisms in silkworms stressed with short-term exposure to low temperature, hypoxia and viral infection (Micheal and Subramanyam, 2013). The purpose of the present study was to assess the effect of sericin on

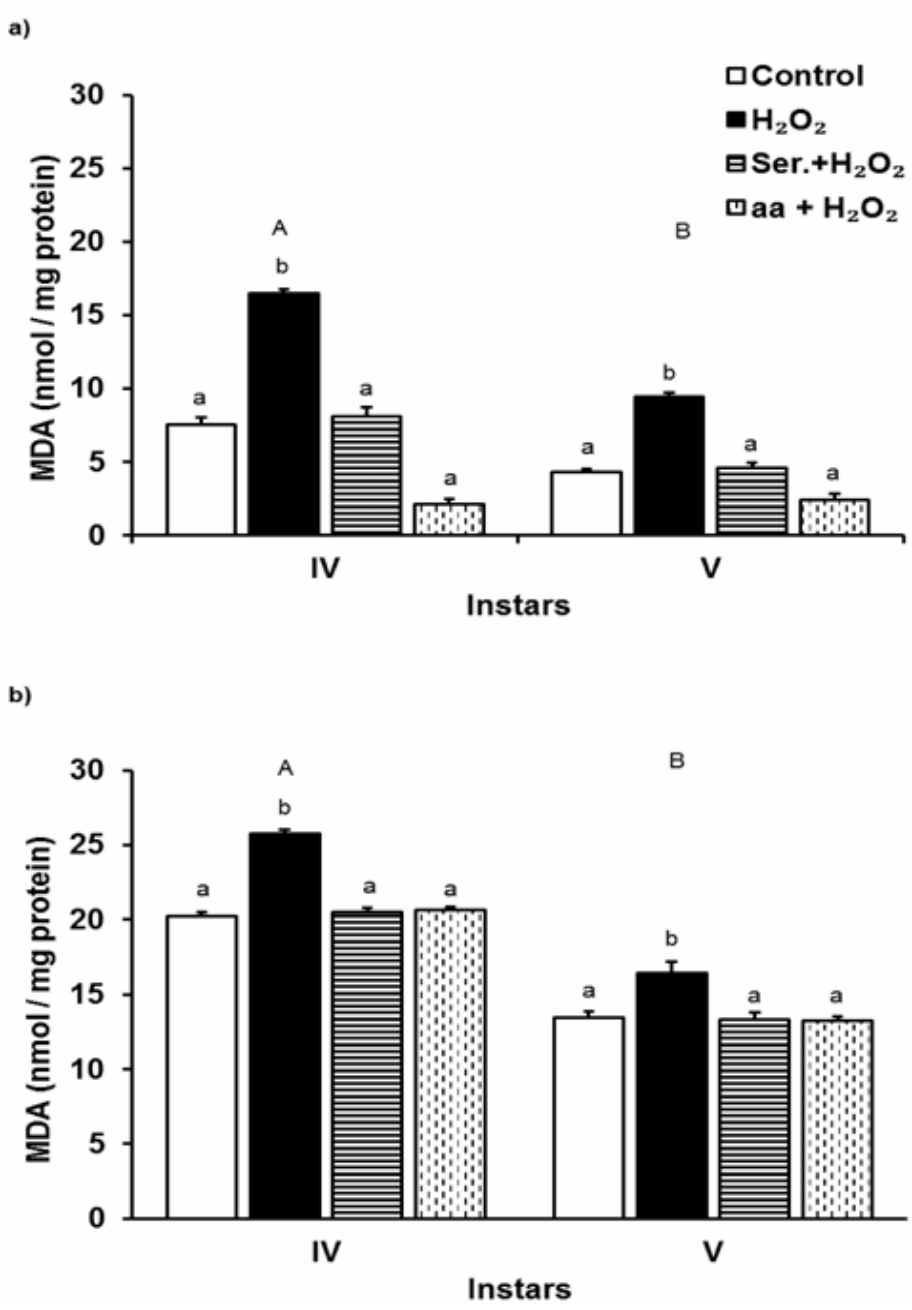

Fig. 2 Malondialdehyde levels in silkworm *B. mori* midgut epithelial cells (a) and hemocytes (b) treated with hydrogen peroxide and pre-incubated with sericin or amino acid mixture prior to hydrogen peroxide treatment. Data are shown as mean ± SE (n = 6); $p < 0.05$ was considered significant. Values between the treatments are represented by lower case letters (a, b) and between the instars are represented by upper case letters (A, B). Those not sharing the same letters are significant.

hydrogen peroxide-induced oxidative stress in the midgut epithelial cells and hemocytes of silkworm and to explore the possible components of antioxidant activity that alleviate oxidative injury caused by transient exposure to hydrogen peroxide. We have demonstrated that the major amino acids of sericin acts as scavenger of radical oxygen species in hydrogen peroxide induced oxidative stress.

Materials and Methods

Chemicals

Thiobarbituric acid (TBA), glutathione reductase, horseradish peroxidase and dinitrophenylhydrazine (DNPH) were purchased from Sigma-Aldrich, (St. Louis, MO, USA). H_2DCFDA was purchased from Molecular Probes (Eugene, USA). Reduced glutathione (GSH), NADPH, t-butylhydroperoxide,

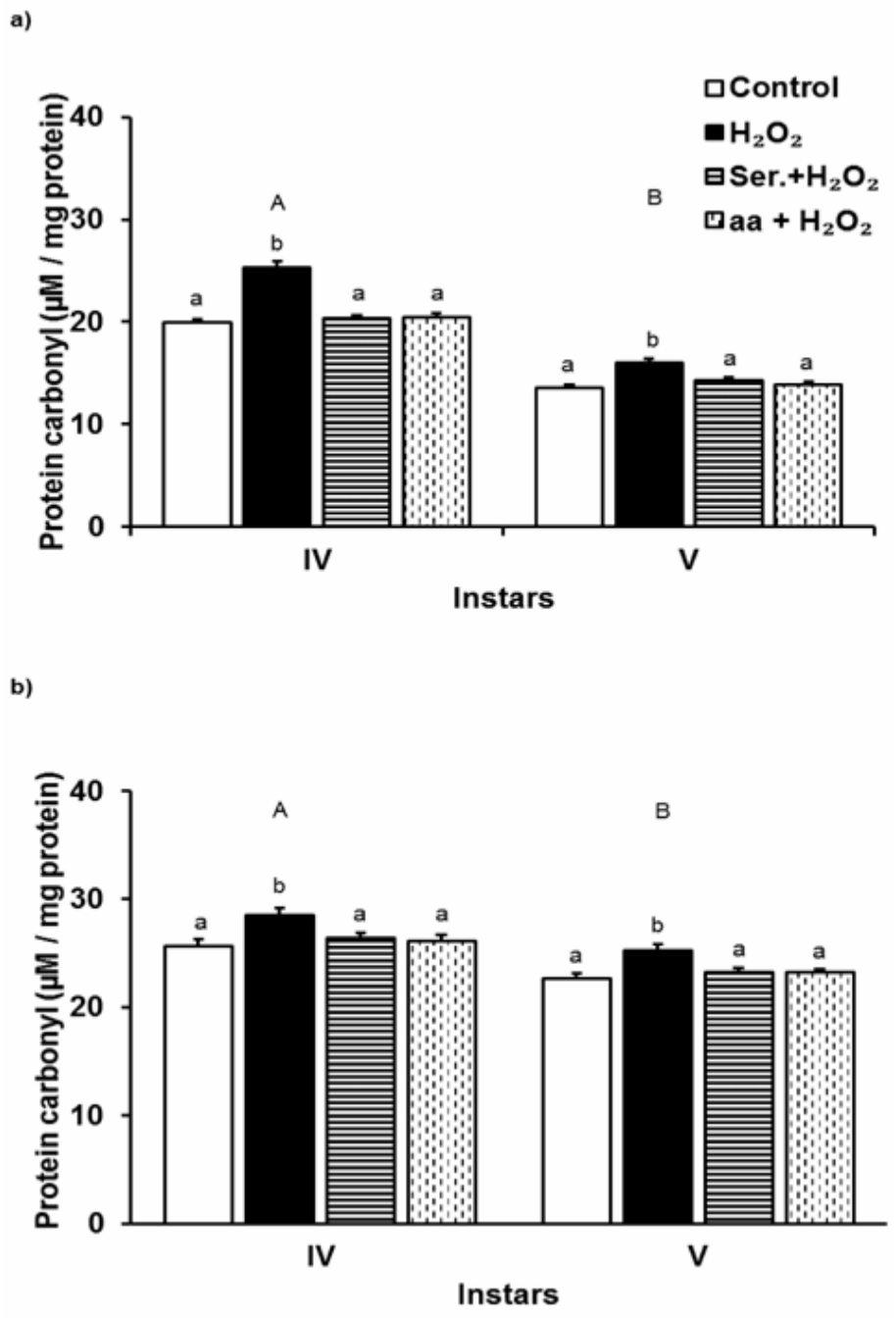

Fig. 3 Protein carbonyl levels in silkworm *B. mori* midgut epithelial cells (a) and hemocytes (b) treated with hydrogen peroxide and pre-incubated with sericin or amino acid mixture prior to hydrogen peroxide treatment. Data are shown as mean ± SE (n = 6); $p < 0.05$ was considered significant. Values between the treatments are represented by lower case letters (a, b) between the instars are represented by upper case letters (A, B). Those not sharing the same letters are significant.

hydrogen peroxide (H_2O_2), Triton X-100, epinephrine, sodium dodecyl sulphate (SDS), acetic acid, butanol, pyridine, tetra methoxy propane (TMP) and 2,4,6-tripyridyl-S-triazine (TPTZ) were purchased from Sisco Research Laboratory (Mumbai, India). L-Aspartic acid and L- Serine from Spectochem (Mumbai, India).

Insects and experimental design

The present study was approved by the Institutional Animal Ethics Committee (IAEC) of Bangalore University, Bangalore, India. Second instar larvae were procured from the Kunigal seed area, Karnataka, India and were maintained in the laboratory throughout the larval stages and were fed

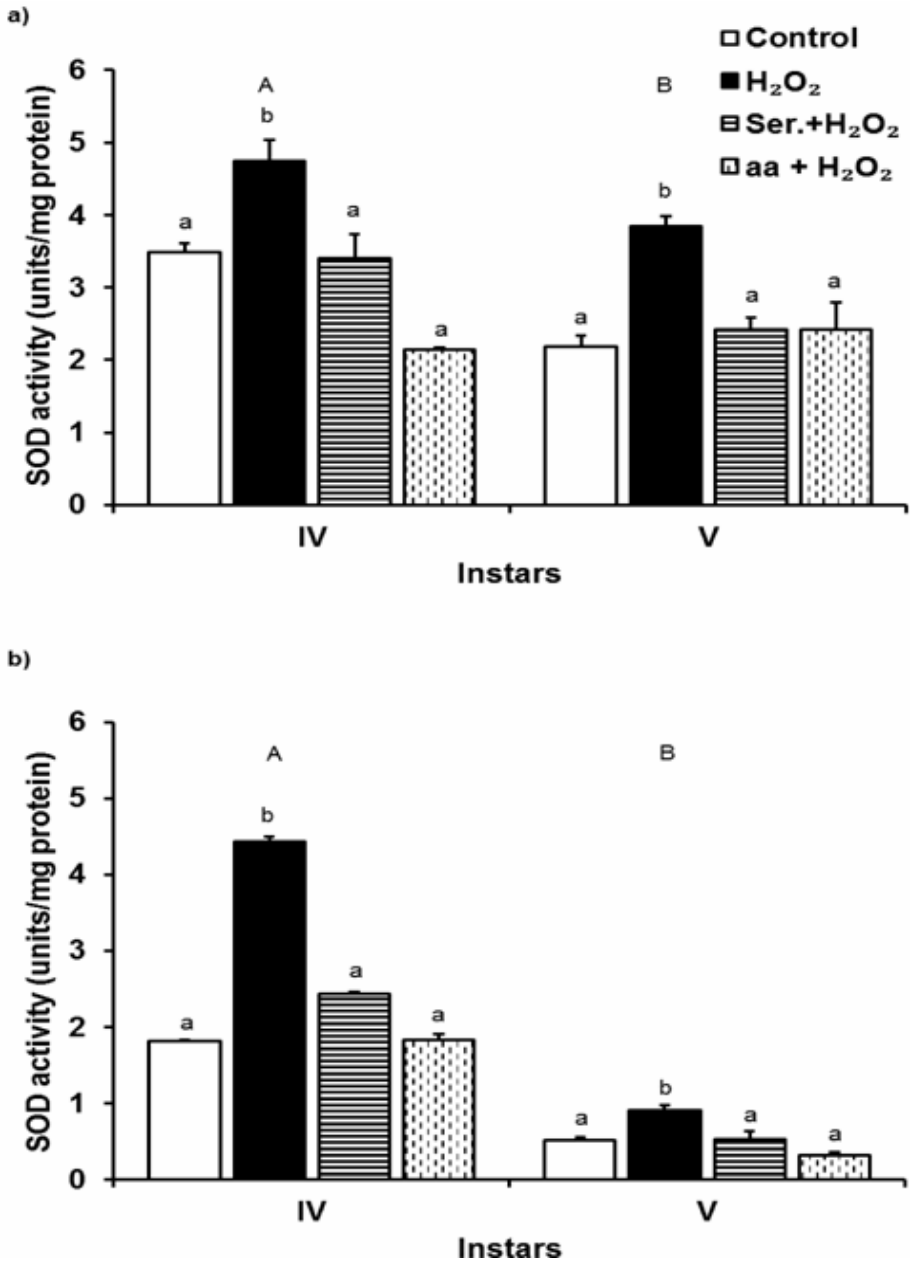

Fig. 4 SOD activity in midgut epithelial cells (a) and hemocytes (b) of IV and V instar of the silkworm *B. mori* treated with hydrogen peroxide and pre-incubated with sericin or amino acid mixture prior to hydrogen peroxide treatment. Data are shown as mean ± SE (n = 6); $p < 0.05$ was considered significant. Values between the treatments are represented by lower case letters (a, b) between the instars are represented by upper case letters (A, B). Those not sharing the same letters are significant.

ad libitum with M5 variety mulberry leaves (Vyjayanthi and Subramanyam, 2002a, b). The uniformly grown healthy larvae of IV and V instars were used in all experiments and were maintained at 24 - 25 ^{o}C with a relative humidity of 70 - 75 %. Midgut epithelial cells were isolated by micro dissection and 1 % collagenase treatment. Dissociated cells were washed repeatedly with cold phosphate buffer and were allowed to stand in cold phosphate buffer of pH 7.4 for further usage. Hemolymph was collected in pre-cooled 2 ml vials containing 5 mg thiourea by gentle incision on the caudal horn of the larvae. Hemocytes were separated by centrifuging the diluted hemolymph at 3,000 rpm for 10 min in cold. Cold phosphate buffer of pH 7.4 was used for the tissue homogenate preparation and for the separation of hemocytes. Isolated cells were exposed to H_2O_2 (20 µM) for 10 min to induce oxidative stress. To evaluate the antioxidant nature of sericin, isolated cells were incubated with 28 mg/ml sericin for 10 min at RT prior to treatment with H_2O_2. The isolated cells were

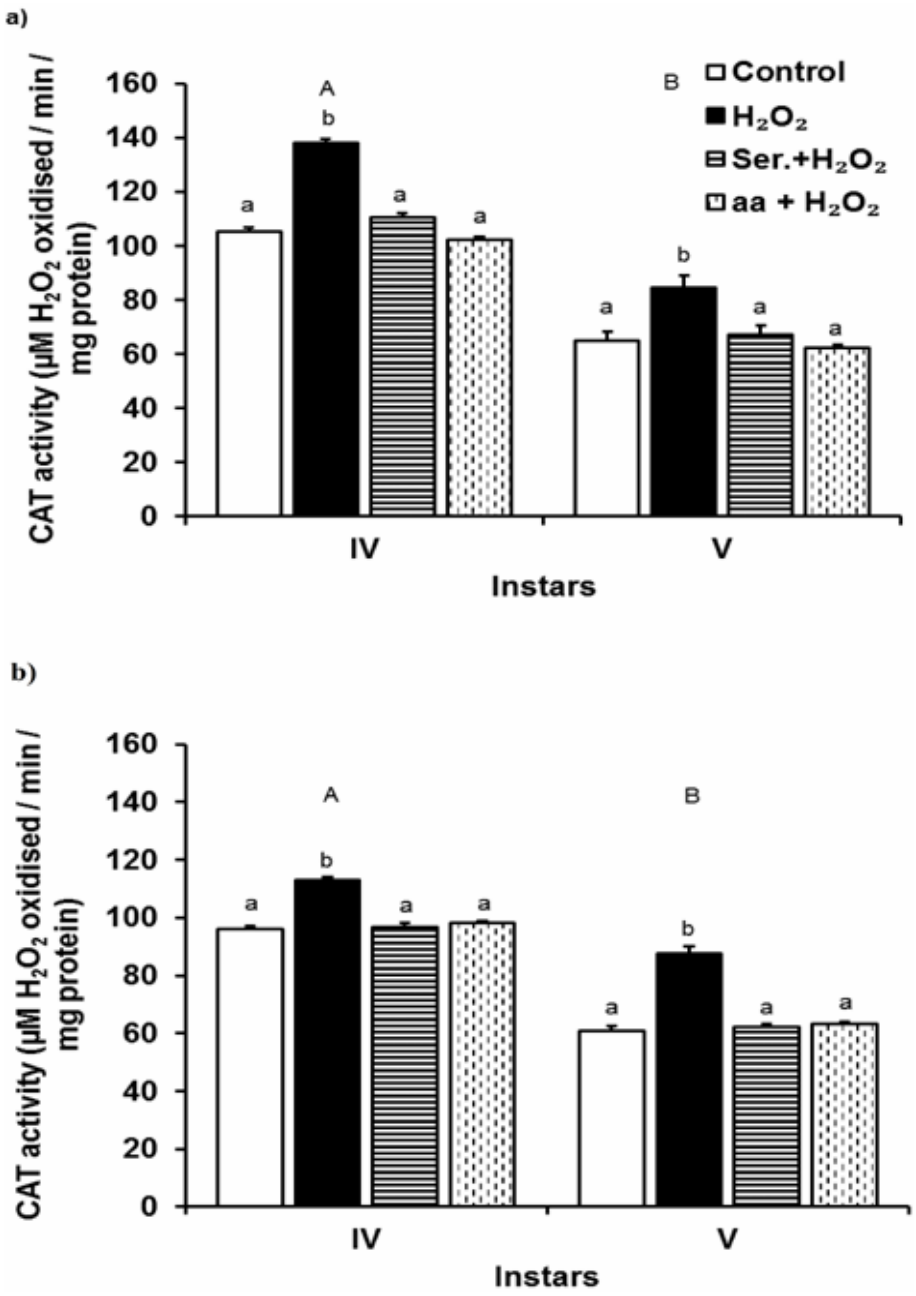

Fig. 5 CAT activity in midgut epithelial cells (a) and hemocytes (b) of IV and V instar of the silkworm *B. mori* treated with hydrogen peroxide and pre-incubated with sericin or amino acid mixture prior to hydrogen peroxide treatment. Data are shown as mean ± SE (n = 6); $p < 0.05$ was considered significant. Values between the treatments are represented by lower case letters (a, b) between the instars are represented by upper case letters (A, B). Those not sharing the same letters are significant.

also incubated with 0.5 mM each of L-serine and L-aspartic acid for 10 min at room temperature prior to treatment with H_2O_2 to access the role of amino acids as antioxidants.

Extraction of sericin

Sericin was extracted from silk cocoon according to (Wu *et al*., 2007) with slight modification. Briefly, multi-voltine cocoons were boiled for several hours in distilled water to extract sericin. The water extract was condensed further by evaporating the water at 50 ºC, and the concentrate was spray dried and collected as a powder. The powder was dissolved in distilled water in a 10:1 ratio (w/v), and was chilled overnight at 4 ºC. Pure chilled ethanol was added to the sericin solution with constant stirring to obtain a final ethanol concentration of 75 % (w/v). The obtained mixture was then kept at -20 ºC overnight,

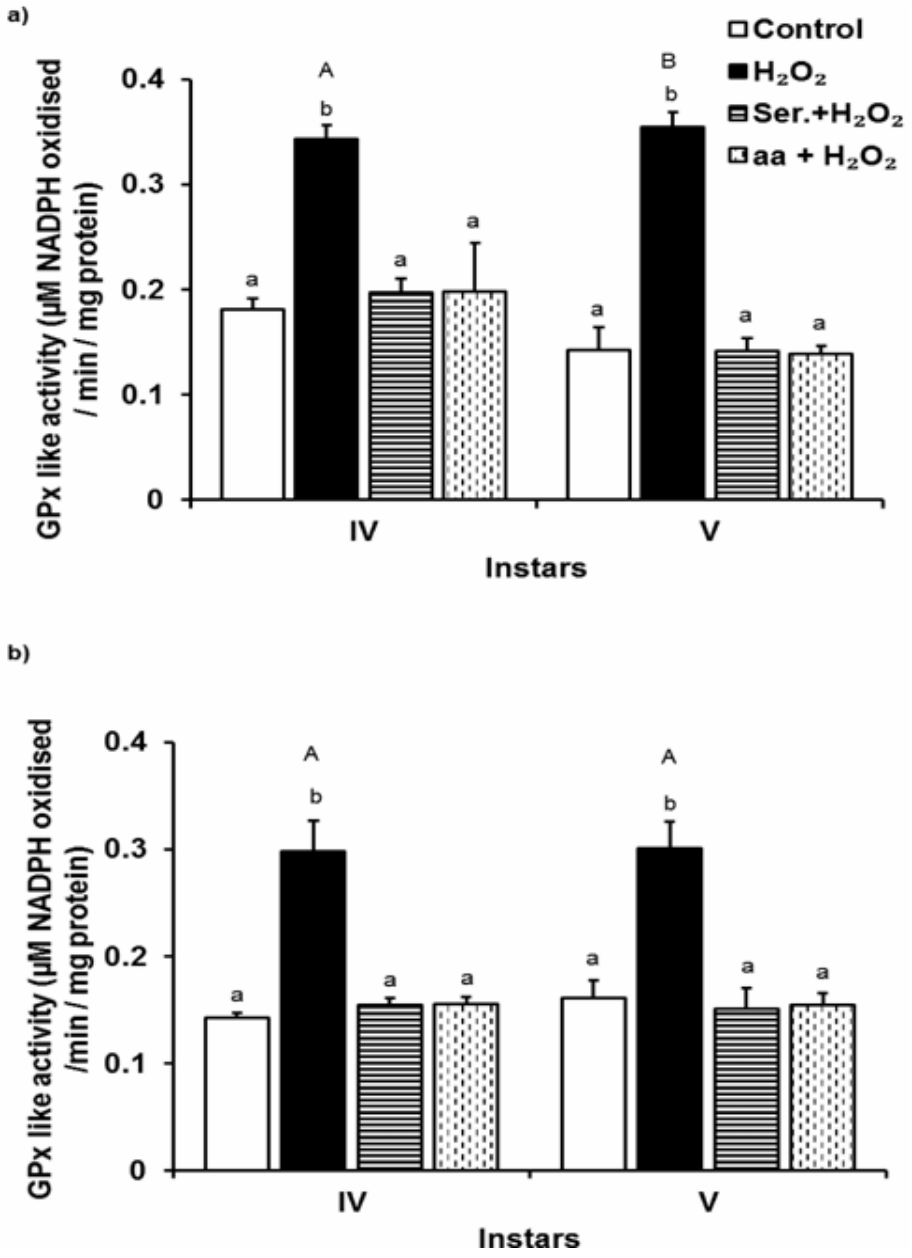

Fig. 6 GPx-like activity in midgut epithelial cells (a) and hemocytes (b) of IV and V instar of the silkworm *B. mori* treated with hydrogen peroxide and pre-incubated with sericin or amino acid mixture prior to hydrogen peroxide treatment. Data are shown as mean ± SE (n = 6); $p < 0.05$ was considered significant. Values between the treatments are represented by lower case letters (a, b) between the instars are represented by upper case letters (A, B). Those not sharing the same letters are significant.

followed by centrifugation at 3500 rpm for 20 min. Alcohol evaporation was performed at 40 ºC and the samples were lyophilized and stored until use.

LC-MS analysis of sericin protein

LC-MS analysis was performed using an API 3000 LC-MS system fitted with a turbo ion spray source and a quadrupole mass spectrometer (Perkin Elmer Sciex, Thornton, Canada). The instrument was operated in positive ion mode with a spray voltage of 5500 V and a source temperature of 475 ºC using a Phenomenex column (2540x6.6 mm) with methanol:water (3:1) as the mobile phase. Data were analysed with Analyst software version 1.4.2.

Lipid peroxidation level

Malondialdehyde (MDA), a product of LPO, was determined as described by Ohkawa *et al*. (1979). In brief, 200 µl of cell homogenates were added to 8.1 % SDS, vortexed and incubated for 10 min. 375 µl of 20 % acetic acid and 0.6 % thiobarbituric acid

were added to the reaction mixture and placed in a boiling water bath for 60 min. The samples were allowed to cool and 1.25 ml of a butanol:pyridine mixture (15:1, v/v) was added and centrifuged at 640 *g* for 5 min. Absorbance was measured at 532 nm using 1,3,3-tetramethoxy propane (TMP) as the standard. The MDA concentration was expressed as nmol/mg protein.

Protein carbonyl level

Protein carbonyl (PrC) was measured according to the method of Uchida and Stadtman (1993). 0.1 % DNPH in 2 N HCl was added to 800 µl of cell homogenate. Samples were kept in the dark for 1 h. The protein was precipitated with 20 % trichloroacetic acid and centrifuged. The pellets were washed thrice with ethanol and ethyl acetate (1:1, v/v) and were dissolved in 2 ml of 8 M guanidine hydrochloride, and centrifuged. The supernatant was used to measure the absorbance at 365 nm and the PrC level was calculated using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}. The results were expressed as µM/mg protein.

S*uperoxide dismutase (SOD, E.C. 1.15.1.1) activity*

SOD activity was measured according to Misra and Fridovich (1972) with slight modifications. Briefly, 100 µl of a 5 % cell homogenate was added to 880 µl of carbonate buffer (0.5 M, pH 10.2). 20 µl of epinephrine (30 mM in 0.05 % acetic acid) were added to the mixture and measured spectrophotometrically (model: Genova MK3, Jenway, UK) at 480 nm for 4 min. SOD activity was measured as the amount of enzyme that inhibits oxidation of epinephrine by 50 %, which is equal to 1 unit.

Catalase (CAT, E.C. 1.11.1.6) activity

Catalase was determined by the method of Aebi (1984). Briefly, 100 µl enzyme samples with 10 µl of absolute alcohol were incubated for 30 min at 0 ^oC followed by the addition of 10 µl Triton X-100. An aliquot of 50 µl was placed in 1.25 ml of 0.066 M H_2O_2 in phosphate buffer and the decrease in absorbance was measured at 240 nm for 60 s in a spectrophotometer. An extinction coefficient of 43.6 M cm^{-1} was used to determine enzyme activity and was expressed as one µmole of H_2O_2 degraded/min/mg protein.

Glutathione peroxidase-like enzyme (GPx, E. C. 1.11.1.9) activity

GPx-like enzyme activity was analysed by the method of Flohe and Gunzler (1984). 50 µl of 0.1 M phosphate buffer (pH 7.0), 100 µl of the enzyme sample, 100µl glutathione reductase (0.24 units) and 100 µl of 10 mM GSH were mixed. The mixture was pre incubated for 10 min at 37 ^oC followed by the addition of 100 µl of 1.5 mM NADPH in 0.1 % $NaHCO_3$. 50 µl of 12 mM t-butylhydroperoxide was added to monitor the hydrogen peroxide independent concentration of NADPH for 3 min. The overall reaction was started by adding 100 µl of pre-warmed H_2O_2, and the decrease in absorption at 340 nm was monitored for 5 min. GPx-like activity was expressed as µm NADPH oxidized/min/mg protein.

Antioxidant capacity

Antioxidant capacity was analysed by the modified method of Benzie and Strain (1996) using ascorbic acid as the standard. FRAP reagent was prepared fresh from acetate buffer (pH 3.6). 10 mM TPTZ diluted with 40 mM HCl and 20 mM ferric chloride solution at a ratio of 10:1:1 (v/v), respectively, were warmed to 37 ^oC prior to use. 100 µl of cell homogenate and 3 ml of the FRAP reagent were vortexed and absorbance was measured at 593 nm at 0 min. The samples were incubated at 37 ^oC and absorbance was recorded after 30 min. The antioxidant capacity of the cell homogenate was expressed in µM Fe^{2+} /100 mg cell mass.

Fluorescent microscopy studies

To assess the intracellular ROS levels in isolated midgut epithelial cells and hemocytes, the cells were loaded with an ROS-sensitive indicator, CM-H_2DCFDA of 20 µM for 10 min at RT. The excess stain was washed off with insect Ringer's solution. In cells, esterase cleaves CM-H_2DCFDA to release CM-H_2DCFH, which is converted to the fluorescent product CM-H_2DCF when exposed to ROS (Xie *et al*., 1999). CM-H_2DCFDA was excited at 520 nm and the emitted light was collected at 570 nm using a fluorescent microscope (Olympus IX 71, Japan). ROS was quantified using Image Pro Express version 6.3 software from stored images.

Statistical analysis

Data are shown as the mean ± SD of six observations. Changes between the groups were analysed by MANOVA and further tested by the Bonferroni post-hoc test using Statistical Package for Social Sciences (SPSS) software (Huberty and Olejnik, 2006) and $p < 0.05$ was considered significant. Statistically significant data are presented in the text.

Results

Composition of silk protein sericin

A flow rate of 0.3 ml/min through the Phenomenex column was used in the LC-MS studies to provide information on the retention times and the endogenous concentrations of various amino acids in the silk protein sericin. The retention times from the samples were compared with synthetic standards, as shown in Figures 1A and B. Among the amino acids, serine was the most abundant, followed by glycine, aspartic acid, threonine and glutamic acid (Fig. 1C).

Oxidative stress markers

Incubation with 20 µM hydrogen peroxide significantly increased MDA, a product of lipid peroxidation, in both midgut epithelial cells and hemocytes irrespective of the instars studied. However, the MDA level was significantly higher in IV instar larvae when compared to V instar larvae. Incubation with sericin or a combination of serine and aspartic acid for 10 min prior to hydrogen peroxide treatment inhibited hydrogen peroxide-induced lipid peroxidation in both midgut epithelial cells and hemocytes (Figs 2a, b).A significant increase in protein carbonyl content (a product of

Table 1 Antioxidant capacity of sericin and the effect of sericin and amino acid mixture to induce antioxidant capacity in midgut epithelial cells and hemocytes of IV and V instar silkworm *B. mori* treated with hydrogen peroxide

Nature of the treatment	Midgut epithelial cells (μM Fe^{2+}/ 100mg cell mass)		Hemocytes (μM Fe^{2+}/ 100mg cell mass)	
	IV	V	IV	V
Control	13.6[a] ±0.4	11.8[a] ±0.9	15.2[a] ±0.3	14.9[a] ±0.3
Incubation with H_2O_2	8.6[a] ±0.6	7.2[a] ±0.5	14.5[a] ±0.4	12.7[a] ±0.5
Incubation with sericin prior to H_2O_2	23.8[b] ±0.4	17.2[b] ±0.7	25.3[b] ±0.4	28.8[b] ±0.4
Incubation with amino acids prior to H_2O_2	36.5[b] ±0.9	17.4[b] ±0.8	24.8[b] ±0.8	23.3[b] ±0.9
FRAP value of sericin	35.8[c] (μM Fe^{2+}/ 100mg sample) ±0.2			

Data are means ± SE (n = 6); $p < 0.05$ was considered significant. Values between the treatments are represented in lower cases (a, b, c). Those not sharing the same letters are significant.

protein oxidation), as a result oxidative stress, was evident following treatment with hydrogen peroxide in both in midgut epithelial cells and hemocytes (Figs 3a, b). Prior incubation with sericin and amino acid mixture significantly inhibited protein oxidation in both types of cells.

Antioxidant enzymes

Incubation of isolated midgut epithelial cells and hemocytes with 20 μM hydrogen peroxide significantly increased SOD activity in both instars, but more so in IV instar over V instar silkworms irrespective of the treatment. Incubation with sericin and serine, aspartic acid mixture prior to hydrogen peroxide treatment inhibited the hydrogen peroxide-induced increase in enzymatic activity. SOD activity in midgut epithelial cells was significantly higher than in hemocytes per assay (Figs 4a, b).

Catalase hydrolyses hydrogen peroxide into H_2O and O_2. To assess the role of sericin as a probable antioxidant, isolated midgut epithelial cells and hemocytes were incubated with 20 μM hydrogen peroxide with or without prior treatment with sericin. CAT activity was found to be significantly higher in IV instar larvae than in V instar larvae of both tissues studied. Incubation with hydrogen peroxide significantly increased CAT activity in midgut epithelial cells as well as in hemocytes. Cells treated with sericin or mixture of aspartic acid and serine before the induction of oxidative stress did not elicit a rise in CAT activity under stress (Figs 5a, b).

A significant increase in GPx-like activity was noted in the context of oxidative stress in both midgut epithelial cells and hemocytes. Prior treatment with sericin or the mixture of aspartic acid and serine prevented the oxidative stress-induced increase in enzymatic activity in both tissues (Figs 6a, b). The instar-dependent decrease in antioxidant enzymes such as SOD and CAT was not observed for the GPx-like enzyme.

Antioxidant capacity of silk protein and a mixture of serine and aspartic acid

The antioxidant capacity of the silk protein sericin was determined by a FRAP assay using ascorbic acid as the standard. The residual antioxidant capacity was found to be 13.6 and 11.8 μM Fe^{2+}/100 mg cell mass in midgut epithelial cells, while in hemocytes it was found to be 14.5 and 14.9 μM Fe^{2+}/100 mg cell mass in IV and V instar silkworms, respectively. Cells incubated with sericin and also with combination of serine/aspartic acid prior to hydrogen peroxide treatment showed significantly increased antioxidant capacity by 0.72 fold and 0.45 fold in midgut epithelial cells, while in hemocytes the increase was 0.74 fold and 1.6 fold in IV and V instar silkworms, respectively (Table 1).

Hydrogen peroxide-induced increase in reactive oxygen species

H_2DCFDA staining was used to study the H_2O_2-induced change in reactive oxygen species in midgut epithelial cells and hemocytes under a fluorescent microscope. Under phase contrast, blebbing of the plasma membrane and swelling of the cells were observed in cells treated with H_2O_2 at concentrations above 20 μM. In contrast, pre incubation with 28 ng/ml sericin or with mixture of 0,5 mM serine and aspartic acid inhibited H_2O_2-induced morphological changes in both types of cells studied (Figs 7, 8). H_2DCFDA stained cells revealed a higher intensity of fluorescence, indicating an increase in ROS upon exposure to 20 μM H_2O_2 (Supplementary Figs S1, S2).

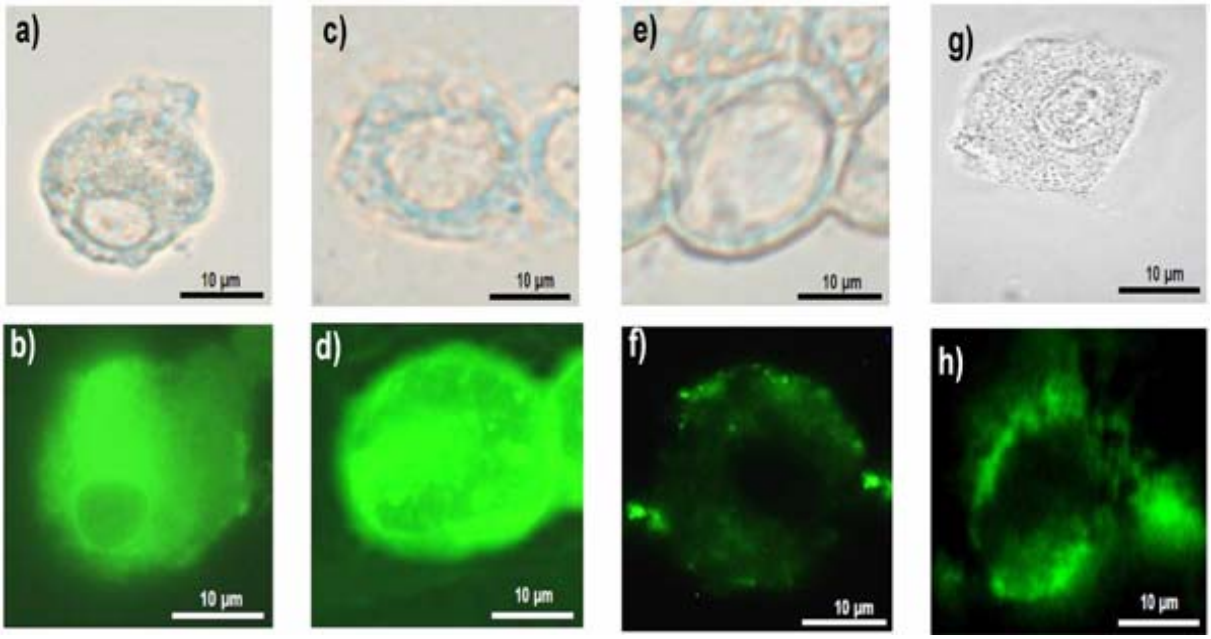

Fig. 7 Effect of sericin and amino acid mixture on the hydrogen peroxide-induced increase in reactive oxygen species in the midgut epithelial cells of the silkworm *B. mori.* (a, b) Normal midgut epithelial cells, (c, d) hydrogen peroxide treated cells, (e, f) cells incubated with sericin prior to hydrogen peroxide treatment and (g, h) cells incubated with amino acid mixture prior to hydrogen peroxide treatment.

Discussion

Hydrogen peroxide is membrane permeable, diffusible, less reactive and longer-lived than $OH^{\cdot}$ or $O_2^{\cdot-}$ (Stone and Yang, 2006). The physiological range of the intracellular hydrogen peroxide concentration appears to be remarkably conserved in different forms of life (Meller, 2000). Notably, among the ROS, hydrogen peroxide is the only species that is generated by several specific enzymes, which suggests that the intracellular concentration of hydrogen peroxide is tightly regulated and may serve several specific cellular functions. It potentiates antioxidant mechanisms; however, if produced in excess, it has deleterious consequences (Veal *et al*., 2007). It has been reported to be less effective as an intracellular signalling molecule when added exogenously than endogenously produced hydrogen peroxide (Choi *et al*., 2005). In the present study, 20 µM hydrogen peroxide, when applied exogenously, caused close to 50 % mortality in midgut epithelial cells and hemocytes with a concomitant increase in ROS, as reflected in the fluorescent microscopy study.

Hydrogen peroxide can cause oxidative modifications in proteins (Stadtman, 1992; Dalle-Donne *et al*., 2002) and peroxidise unsaturated lipids in the cell membrane (Fridovich, 1978). In our experiments, exposure of midgut epithelial cells and hemocytes to 20 µM hydrogen peroxide significantly increased the level of lipid peroxidation products, *i.e.,* MDA; prior treatment with sericin and with a mixture of amino acids was found to inhibit hydrogen peroxide-mediated effects in the cell membrane. Sericin was found to suppress lipid peroxidation in rat brain homogenates (Kato *et al*., 1998) and several amino acids have the property of reducing the lipid peroxidation (Movahedian *et al*., 2006; Selvaraju and Subbashinidevi, 2011). In our present study a mixture of serine and aspartic acid have similar effect of reducing the lipid peroxidation in midgut epithelial cells and hemocytes of silkworm. However, there has been no evidence of a role of sericin as an inhibitor of protein oxidation, as protein thiols were found to inhibit protein hydroperoxide formation (Platt and Gieseg, 2003). The current study clearly indicates that sericin functions as an inhibitor of protein oxidation.

Up-regulation of antioxidant enzymes (AOEs) affords protection against ROS (Fulda *et al*., 2010). In current study, exogenous treatment with H_2O_2 resulted in a significant increase in the antioxidant enzymes SOD, CAT and GPx in the midgut epithelial cells and hemocytes of the silkworm prior to the treatment with sericin or with combination of serine and asparatic acid. Such an increase in SOD (Tsai *et al*., 2011), CAT (Dash *et al*., 2008a) and GPx (Caldinin *et al*., 1998) upon exposure to hydrogen peroxide was observed in fibroblasts. An increase in SOD, CAT and GPx were reported in silkworms infected with Bm NPV and reared on an artificial diet containing $CeCl_3$ (Li *et al*., 2011). The increased AOEs were explained by the fact that $CeCl_3$ acts as scavenger of hydroxyl radicals in pathogenic states. Also, treatment with 4-methylumbelliferone, a model drug, up-regulated the activities of CAT and GPx against oxidative stress in fat body cells of the silkworm (Fang *et al*., 2014). Our

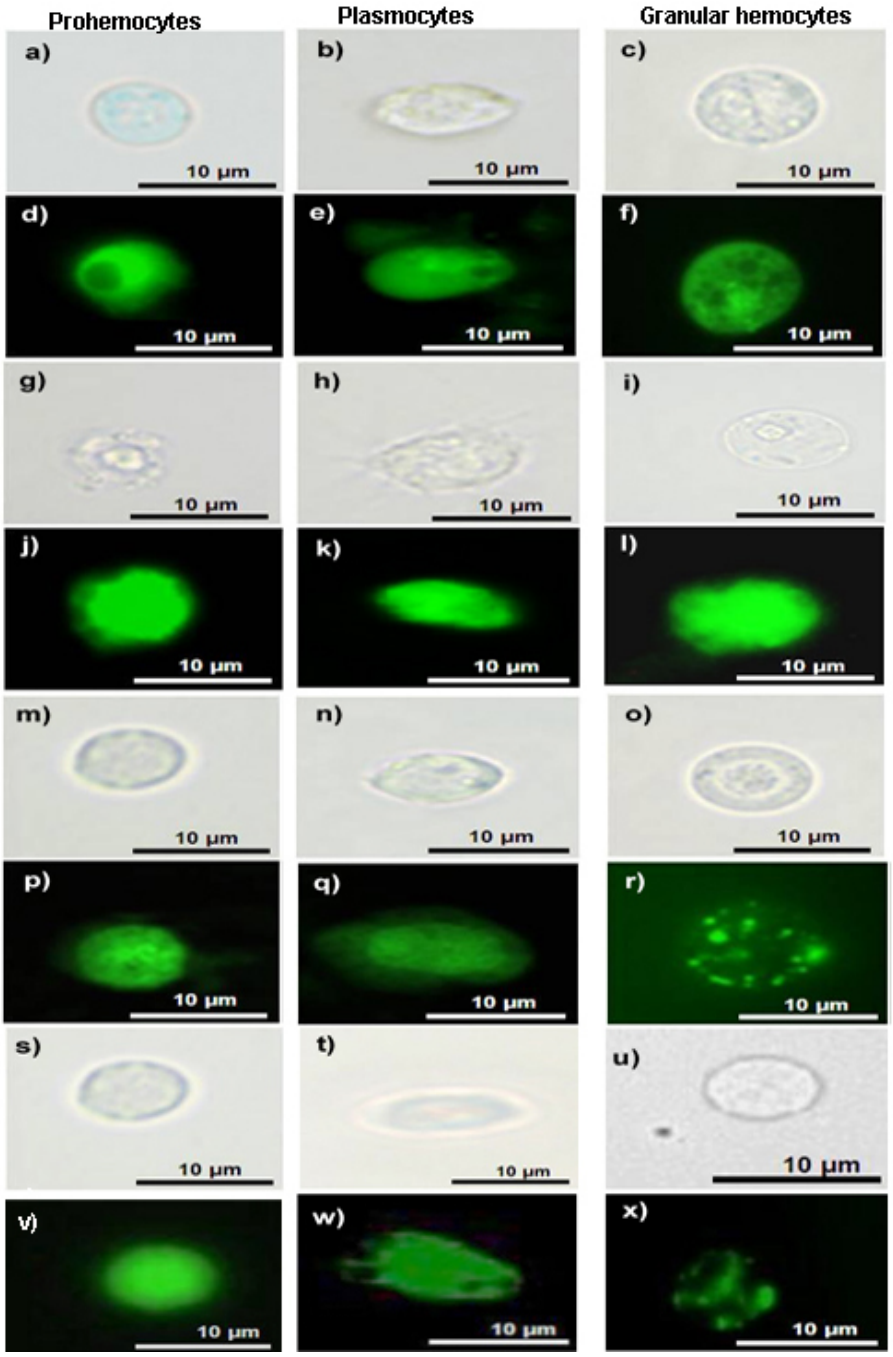

Fig. 8 Effect of sericin and amino acid mixture on the hydrogen peroxide-induced increase in reactive oxygen species in hemocytes of the silkworm *B. mori.* (a - f) Normal hemocytes, (g - l) hydrogen peroxide treated hemocytes, (m - r) hemocytes incubated with sericin prior to hydrogen peroxide treatment and (s - x) hemocytes incubated with amino acid mixture prior to hydrogen peroxide treatment.

results show a significant increase in AOEs upon hydrogen peroxide treatment. A possible explanation for increased AOEs seen in H_2O_2 treated cells, but not in cells pre-treated with sericin and amino acids, is that the increased antioxidant defence reduces more of the generated ROS, such that the ROS signal is not generated or ROS-mediated damage may not occur. In our study, the instar-dependent increase in antioxidant enzymes such as SOD and CAT was not observed for GPx. Similar variations in the antioxidant system as an ontogenic effect has been reported in the beetle *Tenebrio molitor* (Gulevsky *et al*., 2006a, b). In the present study prior treatment with sericin and a mixture of amino acids reduced the AOE activities that were evoked by hydrogen peroxide exposure

alone. Contradictory findings have been reported regarding the effect of sericin and amino acids on AOEs. Sericin elevated SOD, CAT and GPx activity in rat livers treated with alcohol (Li *et al*., 2008, Selvaraju and Subhashinidevi, 2011), while sericin had no effect on SOD in fibroblasts (Dash *et al*., 2008a). It is possible that sericin and amino acids may participate in detoxifying harmful radicals rather than potentiating the activity of AOEs, thereby ameliorating oxidative stress-induced cell damage.

The ability of a compound to reduce Fe^{3+} and Fe^{2+} serves as an important indicator of its potential antioxidant power (Benzine and Strain, 1996; Yen *et al*., 1999). In the present study, treatment with 20 µM H_2O_2 failed to evoke a change in antioxidant capacity in midgut epithelial cells and hemocytes. Perhaps, the elevated antioxidant status upon oxidative stress might have been utilized by the cells to overcome the radicals in the initial period. Nonetheless, prior incubation with sericin and also with combination of serine and asparatic acid significantly increased the FRAP values in both tissues subjected to oxidative stress.

Exposure to hydrogen peroxide in excess of 20 µM induced changes such as swelling and blebbing in both tissues studied. Similar changes in morphology were also observed in fibroblasts (Dash *et al*., 2008a) upon hydrogen peroxide treatment. Furthermore, an increased hydrogen peroxide concentration was reported in keratinocytes irradiated with UVB (Dash *et al*., 2008b). Changes in the intracellular concentration of peroxyl radicals were evident from our fluorescent microscopy studies. The cells labelled with H_2DCFDA showed a higher intensity upon treatment with hydrogen peroxide, and prior incubation with sericin or with amino acids obliterated the accumulation of peroxyl radicals induced by hydrogen peroxide. Our observations are in concurrence with earlier work of Fan *et al*. (2009). It was evident from our study that hydrogen peroxide induces peroxide radical generation and sericin and mixture of serine and aspartic acid may act as a scavenger of free radicals.

The ability of proteins to scavenge free radicals has been reported in several systems (Kong and Xiong, 2006; Sakanaka and Tachibana, 2006; Elias *et al*., 2008). The results of our study substantiate earlier studies and provide evidence for free radical scavenging by sericin and LCMS studies clearly indicate that sericin is rich in serine, followed by aspartic acid, glycine, threonine and glutamine. Evidence for protective role of amino acids against oxidative stress is well documented in several experimental models (Patterson *et al.,* 2003; Movahedian *et al*., 2006; Selvaraju and Subbashinidevi, 2011). In the present study a mixture of serine and aspartic acid 0,5 mM each significantly scavenged ROS thereby prevented protein oxidation and lipid peroxidation without altering the antioxidant enzymes. Serine acts as an antioxidant (Maralani *et al*., 2012), efficient nucleophilic reagent and particularly play a vital role in enzyme catalyst (Anderson *et al*., 1961). Whereas, aspartic acid is known as an antioxidant (Liu *et al*., 2004) and also undergo transamination to function directly or indirectly as an antioxidant. Although L-serine or L aspartic acid alone scavenged ROS to certain extent, a mixture of the amino acids significantly decreased the ROS. Perhaps synergistic effect of serine and aspartic acid may be responsible for the scavenging effects however, such synergistic effects were not found on antioxidation among the compounds used in combination (Wu *et al*., 2003b). Our present result further support antioxidant properties of L-serine (Kitazawa *et al*., 2005) and L- aspartic acid (Chen and Nawar, 1991; Wang and Xiong, 2005). Earlier studies on antioxidant property of sericin were appertained to phenol compound (Prasong, 2011) and flavonoids (Devi *et al*., 2011). However, our results on a mixture of serine, aspartic acid have clearly indicated the scavenging of ROS by these amino acids without altering the antioxidant enzymes. Here we report for the first time that the antioxidant property of sericin as scavenger of free radicals rather than enhancing the activities of AOE's. We suggest that fortification of silkworm diet with a mixture of serine and aspartic acid can alleviate the effects of ROS thereby larvae can with stand environmental born stressors to certain extent. Although, the mechanism of protection offered is through scavenging of free radicals by major constituent hydroxyl amino acids, suboptimal hydrogen peroxide also helped to enhance the endogenous antioxidant machinery as defense against initial ROS signaling or ROS damage.

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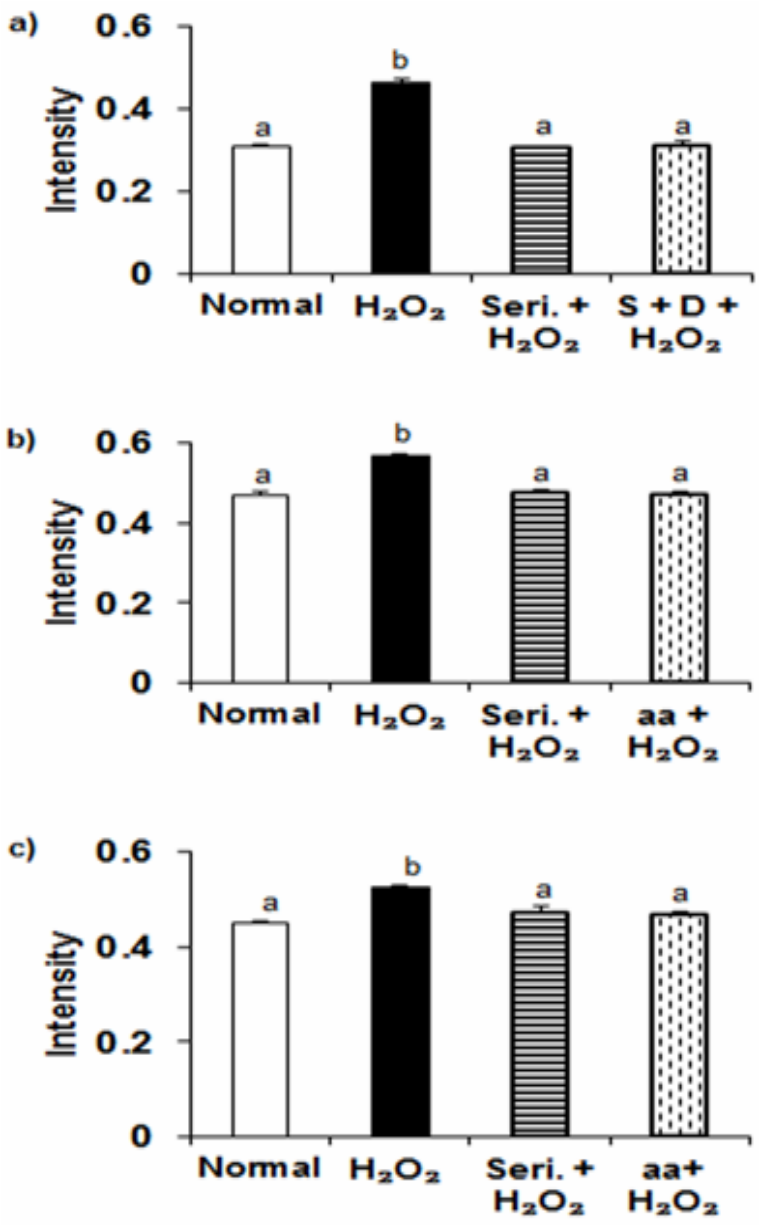

Fig. S1 Effect of sericin and amino acid mixture on the hydrogen peroxide induced increase in reactive oxygen species in hemocytes (a) prohemocytes, (b) plasmocytes and (c) granular hemocytes of the silkworm *B. mori.* Data are shown as mean ± SE (n = 3); $p < 0.05$ was considered significant. Values between the stressors are represented by lower case letters (a, b). Those not sharing the same letters are significant.

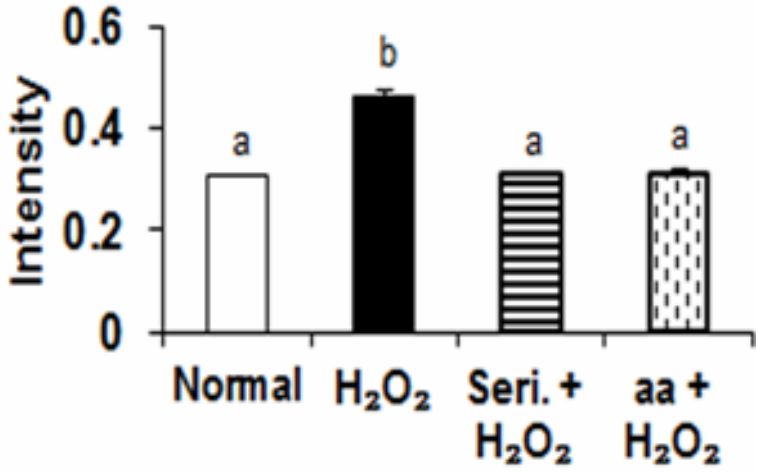

Fig. S2 Effect of sericin and amino acid mixture on the hydrogen peroxide induced increase in reactive oxygen species in midgut epithelial cells of the silkworm *B. mori.* Data are shown as mean ± SE (n = 3); $p < 0.05$ was considered significant. Values between the stressors are represented by lower case letters (a, b). Those not sharing the same letters are significant.