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Antioxidant potential of silk protein sericin against hydrogen peroxide-induced oxidative stress in skin fibroblasts

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The antioxidant potential of silk protein sericin from the non-mulberry tropical tasar silkworm *Antheraea mylitta* cocoon has been assessed and compared with that of the mulberry silkworm, *Bombyx mori*. Skin fibroblast cell line (AH927) challenged with hydrogen peroxide served as the positive control for the experiment. Our results showed that the sericin obtained from tasar cocoons offers protection against oxidative stress and cell viability is restored to that of control on pre-incubation with the sericin. Fibroblasts pre-incubated with non-mulberry sericin had significantly lower levels of catalase; lactate dehydrogenase and malondialdehyde activity when compared to untreated ones. This report indicates that the silk protein sericin from the non-mulberry tropical tasar silkworm, *A. mylitta* can serve as a valuable antioxidant. [BMB reports 2008; 41(3): 236-241]

INTRODUCTION

Free radical theory of aging has given much impetus to the role of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals in the initiation and progression of the aging process (1). Increased levels of ROS can damage various cellular processes. The hydroxyl radical generated from hydrogen peroxide reacting with different transition metals is particularly damaging to DNA, leading to mutagenesis and carcinogenesis (2). Voluminous research has been carried out to discover new antioxidant compounds from plant and animal origin to prevent free radical damage (3-6). Polypeptide with antioxidant property has also been reported in *Chlamys farreri* (7, 8).

Lepidopteran insects of family Bombycidae and Saturniidae produce commercially important silks. The domesticated mulberry silkworm *Bombyx mori* belongs to family Bombycidae whereas the wild non-mulberry silkworms including *Antheraea mylitta* belong to family Saturniidae. Cocoons of silkworms belonging to both families consist of two major proteins, fibroin and sericin. Fibroin and sericin, obtained from mulberry silkworm *Bombyx mori* are now recognized as excellent biomaterials in the field of tissue engineering and therapy. Fibroin, the water-insoluble protein, from mulberry silkworm, has been recognized as a substrate for growth and adherence of cells in culture (9-13). Sericin, the water-soluble component of silk, from the mulberry silkworm, is used as a biomaterial due to its antibacterial and UV resistant properties (14). Sericin is also reported to suppress *in vitro* lipid peroxidation (15) and possesses antitumor properties (16) with no immunogenicity (17).

All the biomaterial related applications of silk proteins involve *in vitro* studies on cells prior to their implantation *in vivo*. The present study reports the antioxidant effects of silk protein sericin obtained from the cocoons of the non-mulberry, tropical tasar silkworm *Antheraea mylitta*, in skin fibroblast cell line AH927 exposed to hydrogen peroxide for 24 hrs.

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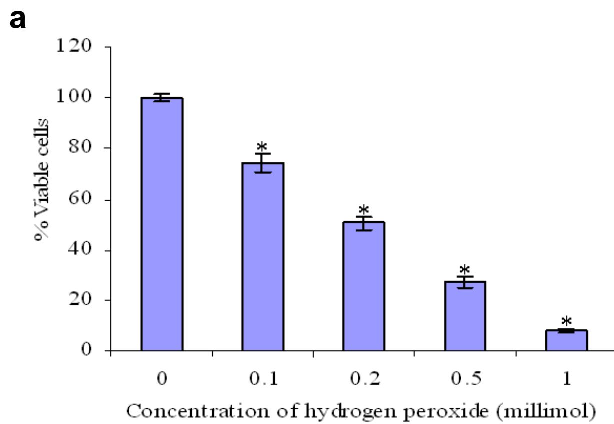
RESULTS

Sensitivity to H_2O_2

Cell viability strikingly decreased in a concentration-dependant manner on treatment with various concentrations of H_2O_2. The LC_{50} for 24 hr exposure was 0.2 mM, indicating that AH927 cells were sensitive to H_2O_2-induced cell damage. Treatment with 0.5 mM H_2O_2 reduced cell viability to 27.3 % and with 1.0 mM decreased the viability to 9.07% (Fig. 1a).

Morphological changes on hydrogen peroxide exposure

Preliminary MTT assay (Fig. 1a) revealed that viability was significantly less at hydrogen peroxide concentrations of 0.5 mM. Propidium iodide staining was used to study the morphological changes in cells at concentration of 0.5 mM H_2O_2 to assess the damage caused by oxidative stress. Under phase contrast microscope, the morphology of the cells exposed to 0.5 mM H_2O_2 were shrunk and rounded as compared to normal cells. Fluorescence staining of cells revealed that cells exposed to 0.5 mM H_2O_2 exhibited nuclear condensation (Fig. 1b).



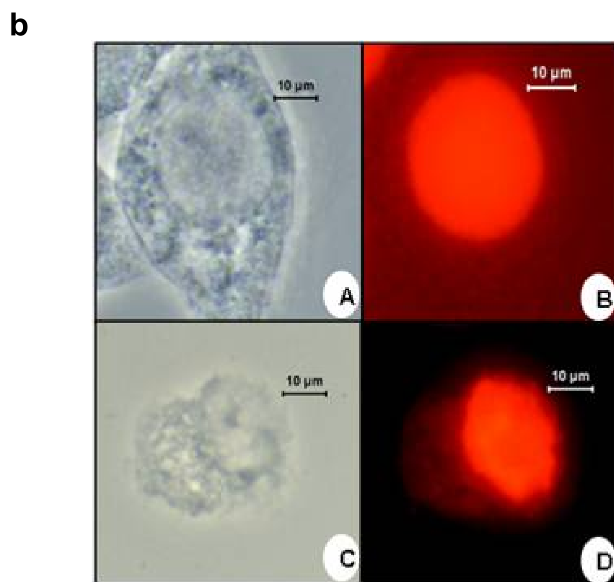


Fig. 1. Hydrogen peroxide stress upon feline fibroblast cells. a) Addition of hydrogen peroxide decreased the cell viability of feline fibroblast cells (AH927) as assessed by MTT assay. Results are expressed as a percentage of the corresponding untreated control as the mean ± SEM. (n = 4), *P < 0.05 (ANOVA followed by Tukeys test). b) Effect of H_2O_2 on morphology of feline fibroblast cells (AH927) (A) Phase contrast micrograph of normal untreated cell, (B) Fluorescence micrograph of normal cell, (C) Phase contrast micrograph of H_2O_2 treated cell, (D) Fluorescence micrograph of H_2O_2 treated cell. Cells were micro photographed at a magnification of 100 ×.

Effect of pre-treatment with sericin on oxidative damage

The effect of pre-treatment of cells with sericin for 24 hrs is given in Fig. 2. One-way analysis of variance revealed that there was an overall significant difference in cell viability between controls, H_2O_2-treated and sericin treated fibroblasts (F = 44.47, $P < 0.001$). Subsequent multiple comparisons by Tukeys test indicated that cell viability was significantly lower

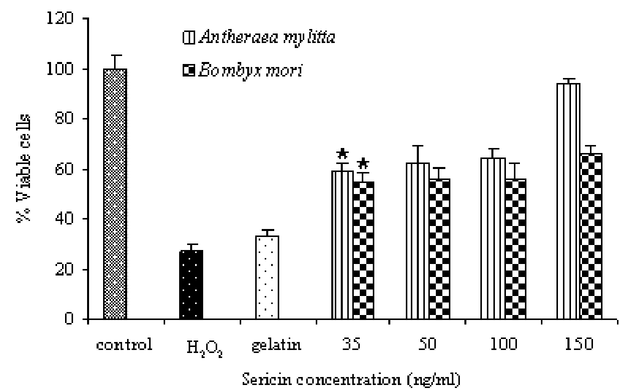


Fig. 2. Effect of silk protein sericin (from mulberry silkworm, *B. mori* and non-mulberry silkworm, *A. mylitta*) treatment on fibroblast (AH927) cell viability. Incubation of cells with sericin was carried out for 24 hrs, prior to the exposure to 0.5 mM hydrogen peroxide for 24 hrs, and cell viability was assayed by MTT. Results are expressed as the mean ± SEM. (n = 4), *P < 0.05 compared to hydrogen peroxide treated cells (one-way ANOVA followed by Tukeys test).

($P < 0.01$) in hydrogen peroxide-treated when compared with control and sericin treated fibroblasts. Pre-incubation with *A. mylitta* sericin from 5 to 30 ng/ml had no protective effect (data not shown). It was observed that concentrations of 35, 50 and 100 ng/ml significantly ($P < 0.05$) increased the cell viability. Cells treated with sericin at 150 ng/ml showed cell viability comparable to that of control group ($P > 0.05$), indicating that pre-incubation with 150 ng/ml restored the cell viability to normal. On the other hand sericin from *B. mori* could not restore cell viability to normal at the same concentration (Fig. 2). Cell viability study also showed that pretreatment of gelatin with the cells for 24 hrs did not show significant protection against H_2O_2 induced oxidative stress.

LDH activity

Fig. 3 depicts the percentage of LDH activity released into the medium in normal cells, cells treated with 0.5 mM hydrogen peroxide for 24 hrs, and cells pre-incubated with 35 ng/ml and 100 ng/ml of sericin from *A. mylitta*. There was a significant increase ($P < 0.01$) in the release of enzyme in hydrogen peroxide treated cells as compared to untreated control and cells pre-incubated with 100 ng/ml of sericin before H_2O_2 treatment.

Catalase activity

The rate of release of catalase activity in various treated cells and control are presented in Fig. 3. Catalase activity was significantly high ($P < 0.01$) in medium of cells treated with hydrogen peroxide (81%) compared to control. The fibroblasts pre-incubated with sericin at both 35 and 100 ng/ml had significantly lower amount ($P < 0.01$) of enzyme activity, indicating the protective effect of silk protein against oxidative stress.

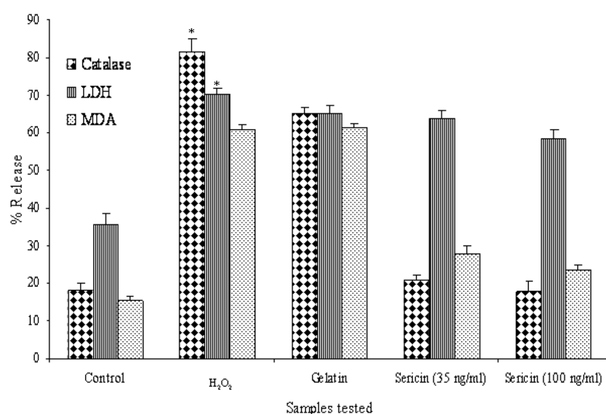


Fig. 3. *A. mylitta* (non-mulberry silkworm) silk protein sericin significantly attenuated catalase, lactate dehydrogenase and malondialdehyde levels in AH927 fibroblast cells exposed to 0.5 mM H_2O_2 on incubation prior to H_2O_2 exposure. Results are reported as the mean ± SEM. (n = 4), $^*P < 0.05$ compared to hydrogen peroxide treated cells. Statistical comparison was made by one-way ANOVA followed by Tukeys test.

Malondialdehyde (MDA) levels in medium

The % of TBARS/ MDA in the media expressed as nmol /ml of the medium is shown in Fig. 3. The products of peroxidation were significantly high ($P < 0.01$) in the media of cells treated with hydrogen peroxide. On the other hand the cells pre-incubated with sericin at 35 and 100 ng/ml had significantly low levels of TBARS ($P < 0.05$).

DISCUSSION

H_2O_2 is particularly attractive as a model oxidant because its cellular actions and its fate has been well studied. In the present study, the protective effects of silk protein sericin, on hydrogen peroxide-induced oxidative damage in feline skin fibroblasts were observed. The mechanism of damage by hydrogen peroxide in fibroblast cultures involves ROS generation (18-20). The level of ROS in cultures pre-incubated with sericin was significantly decreased as indicated by cell viability tests (MTT assay). Among the silk proteins tested, sericin from the non-mulberry silkworm, *A. mylitta*, showed protective effect at a minimum concentration of 35 ng/ml and restored the viability and normal biochemical profile at 150 ng/ml which was not observed in case of sericin of *B. mori*.

To investigate further the effect of *A. mylitta* sericin on H_2O_2-induced oxidative stress, the activities of LDH and catalase were examined. H_2O_2, one of the major reactive oxygen species, is produced at a high rate as a product of normal aerobic metabolism. Hence an acceleration of anaerobic metabolic pathway to cope with oxidative stress is reflected from an increase in LDH activity. The LDH activity of cells subjected to oxidative stress increased significantly when compared to normal ones which indicate loss of membrane integrity (21). Catalase is involved in the decomposition of hydrogen peroxide to water and oxygen and is therefore important in protecting cells against oxidative stress (22). There was a significant increase in catalase activity in hydrogen peroxide stressed cells when compared to normal cells. A similar trend was also detected in case of the intracellular levels of products of lipid peroxidation. Malondialdehyde (MDA) alters the structure and function of the cellular membrane and blocks cellular metabolism leading to cytotoxicity (23). The amounts of LDH, catalase and TBARS were also significantly low in medium of sericin-treated cells when compared to control as well as hydrogen peroxide-treated cells. This study indicates that sericin might be providing protective effect on fibroblast by acting as antioxidant as well as by promoting endogenous antioxidant enzymes *in vitro*.

Indeed, sericin has previously been reported to possess photoprotective effect against UVB-induced acute damage (16) and colon carcinogenesis (24). We report for the first time that cutaneous cell lines like AH927 fibroblasts are effectively protected against oxidative stress by sericin from the cocoons of the tropical tasar silkworm, *A. mylitta*. Sericin from *B. mori* (25) and *A. mylitta* (26, 27) contains high amount of polar amino acids. Because of remarkably high content of hydroxy amino acids (serine and threonine), the hydroxy groups might be responsible for the antioxidant action by chelating trace elements (15). To observe whether the protective effect of sericin is due to its viscous nature, gelatin (a viscous polymer) was taken as control. Interestingly, it is observed that viscous compounds like gelatin did not show any protection against H_2O_2-induced oxidative stress (Fig. 3). The protective effect of sericin may be due to its unique antioxidant potential. Although skin possesses an extensive and most effective network of antioxidant system, many of the free radicals produced by various agents can escape this surveillance, inducing substantial damage to cutaneous constituents, especially when skin defense mechanisms are overwhelmed (28, 29). Consequently, exogenous antioxidants that scavenge ROS and restore normal redox state are supposed to be beneficial (30).

In conclusion, this report suggests that water-soluble silk protein sericin, from a natural source like silkworm cocoon, is an ideal molecule to prevent oxidative stress. The use of antioxidants has been observed to improve cutaneous wound healing significantly (31). Hence sericin may also be used in therapy with other conventional non-enzymatic antioxidants like ascorbic acid, tocopherol, uric acid and beta-carotene.

MATERIALS AND METHODS

Cell culture

The feline fibroblast cell line AH927 (obtained from National Centre for Cell Science, Pune, India) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and streptomycin-penicillin (10 μg/ml). Cells were incubated at 37°C in 5% CO_2 atmosphere. The

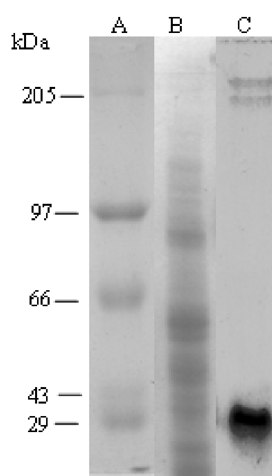


Fig. 4. SDS-PAGE (8 %) analysis of silk protein sericin from cocoon *Antheraea mylitta* and *Bombyx mori*. A: Protein molecular weight standard indicated by the numbers on the left. B: Sericin from *Antheraea mylitta*. C: Sericin from *Bombyx mori*.

cells were sub cultured at intervals of 3 days and those between passages 4-8 were used for the experiments.

Isolation of silk protein sericin from cocoons

Silk protein sericin was isolated from the cocoons of non-mulberry silkworm, *Antheraea mylitta* and mulberry silkworm *Bombyx mori* using a standard protocol (32). In brief, to isolate sericin the cocoons of both species were cut into small pieces and were boiled in the presence of 0.02 M Na_2CO_3 for half an hour. The supernatant was decanted out and was dialyzed to obtain sericin. The protein solution was dialyzed against several changes of Milli Q water. The crude extracts of sericin (including all fractions) were used for further experiments. 8 % SDS-PAGE was done to confirm the presence of proteins (Fig. 4).

Analysis of cell viability

The extent of reduction of the thiazolyl tetrazolium compound (MTT) was utilized to check cell viability (33). Briefly, AH927 cells were seeded in 96-well plates (1×10^4 cells/well) in DMEM medium containing 10% fetal bovine serum and after overnight culture they were treated with different concentrations (0, 0.1, 0.2, 0.5 and 1.0 mM) of hydrogen peroxide (H_2O_2), freshly prepared from 30 % stock solution (Sigma), for 24 hrs with untreated cells serving as control. At the end of incubation the media in wells were removed and replaced with 200 μl of fresh media containing 20 μl MTT solution (5 mg/ml) and incubated at 37°C for 4 hrs. Thereafter, media-containing MTT were removed and 200 μl of DMSO was added to solubilize the formazan crystals formed in the viable cells. Absorbance was recorded in Biorad 550 microplate reader at 595 nm. The extent of H_2O_2 mediated cell death was expressed as the percentage of cell viability in control cells. Each experiment was done in quadruplicates. The mean values and standard error were calculated.

Phase contrast and fluorescence microscopy

For microscopic observations, 10^6 cells were seeded on a square coverslip (22 × 22 mm) placed inside a petri plate and allowed to grow overnight. Subsequently they were treated with 0.5 mM H_2O_2 for 24 hrs. After observing under phase contrast, the cells were washed with ice-cold sterile phosphate buffered saline (PBS). This was followed by fixation in methanol : acetone (1 : 1) for 1 hr. The cells were then rinsed in PBS and incubated with Tween-20 (0.05 %) for 30 min for membrane permeabilisation, followed by RNase (10 mg/ml) treatment for 30 min. After rinsing in PBS, cells were exposed to propidium iodide (1 mg/ml) for 30 mins and the nuclei were visualized under fluorescence microscope Leica DMR-HC (with camera MP-60) at an emission wavelength of 615 nm.

Effect of pre-treatment with silk protein sericin for 24 hrs

As mentioned earlier for assessment of cell viability, the cells were subjected to pretreatment with silk protein sericin individually at different concentrations ranging from 5 to 150 ng/ml or gelatin (150 ng/ml). Preliminary experiments showed that pretreatment with sericin for 24 hours, before subjecting to oxidative stress due to 0.5 mM H_2O_2, gave maximum protection in comparison to 6, 12 and18 hours. The viability was checked by MTT assay after 24 hrs of exposure to hydrogen peroxide.

Preparation of cell homogenate

Cells (1×10^4 cells/ well) were seeded in petri plates (35 mm) and allowed to adhere for 24 hrs before treatment with silk protein sericin. Only sericin from non-mulberry silkworm was used for further biochemical experiments as it offered the best protection (from cell viability assays). Cells were pre-incubated with 35 and 100 ng/ml sericin or 150 ng/ml gelatin (these represent the concentrations where minimum and maximum protective effect was observed for sericin from *A. mylitta* in cell viability assay) for 24 hrs followed by 24 hours oxidative stress induction (hydrogen peroxide treatment). The cells were washed twice with ice-cold PBS and were harvested using 0.025 % Trypsin-0.02 % EDTA solution. The cells were re-suspended in ice-cold PBS and were homogenized. The cell homogenates were then centrifuged for 5 min at 5,000 × *g* and the supernatant of each sample was used for biochemical tests.

Analysis of lactate dehydrogenase (LDH)

Fibroblast damage caused by hydrogen peroxide was quantitatively assessed from the ratio of the enzyme activity released from damaged cells to the activity in undamaged / intact cells, monitored 24 hrs after the induction of stress. The medium collected after stress induction was centrifuged and supernatant used for the assay of enzyme released from damaged cells.

$$\% \text{ release of LDH} = \frac{\text{Activity released into the medium}}{\text{Activity in sonicated cells}} \times 100$$

The reduction of pyruvate by the enzyme on addition of NADH was monitored as the change in absorbance of the reaction mixture containing 20 mM phosphate buffer (pH 7.4), 3.3 mM sodium pyruvate and 2 mM NADH. The enzyme activity was expressed as change in absorbance at 340 nm per minute (34).

Analysis of catalase

Catalase (CAT) was assayed by noting the decrease in absorbance of NADH at 240 nm following the decomposition of hydrogen peroxide (35). The reaction mixture comprised of 10 mM phosphate buffer (pH 7.0), 30 mM hydrogen peroxide and the enzyme. The decrease in absorbance of the mixture was monitored at 240 nm for 5 min, and the activity expressed as change in absorbance per minute. (Extinction coefficient = 0.021). The activity of enzyme released into the medium and those in intact cells were determined as described for LDH analysis and expressed as percentage.

Measurement of lipid peroxidation expressed in terms of thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA)

The concentration of TBARS in the medium after induction of oxidative stress was measured as per the the standard protocol (36). Briefly, 0.5 ml supernatant of each sample was mixed with 0.5 ml thiobarbituric acid reagent (1 : 1, v: v, mixture of 0.67% thiobarbituric acid and acetic acid). The reaction mixture was heated at 95°C for an hour. After cooling, centrifugation was carried out at 1,000 × *g* and the absorbance of the supernatant was measured at 535 nm.

Statistical analysis of data

Each experiment was observed in quadruplicate. The data were presented as mean ± S.E.M. and compared using one-way ANOVA and Tukeys test. $P < 0.05$ or less was considered to be statistically significant.

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