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Effect of Silk Sericin From The Cocoons of Silkworm, Antheraea Mylitta (L) And Bombyx Mori (L) on Hydrogen Peroxide Induced Oxidative Stress in Feline Fibroblasts

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ABSTRACT: *The attempt is concerned with assessment of potential regarding antioxidant activity of Sericin from silk from cocoons of non-mulberry silkworm, Antheraea mylitta And mulberry silkworm, Bombyx mori (L) through the use of hydrogen peroxide induced stress in skin fibroblast cell line culture (AH927). Cells treated with sericin exhibited significant cell viability comparable to that of control group (P >0.05). The fibroblasts pre-incubated with sericin of both the species at 100 ng/ml had significantly decreased (P <0.01) catalase activity.The oxidative stress through hydrogen peroxide was found responsible for significantincrease (P <0.01) in the release of enzyme (LDH) in comparison with untreated control. The sericin treatment definitely serving to restore the original membrane integrity of the cells. The aqueous form of silk sericin, from a natural source like silkworm cocoon, serve as ideal antioxidant source and may be used to treat the cancer cells.*
Keywords: *Antioxidant activity , Fibroblast cell line, Hydrogen peroxide, Oxidative stress, Moracin*

I. INTRODUCTION

The credit of much impetus to the role of reactive oxygen species (ROS) such as superoxide, hydrogenperoxide, and hydroxyl radicals in the initiation and progression of the aging process goes to the free radical theory of aging (1). Increased levels of reactive oxygen species (ROS) is responsible for damage of various cellular processes. The hydrogen peroxide is responsible for release of hydroxyl radical, which in it's turn reacting with different transition metals. The harmful effects of reactive oxygen species (ROS) include damage the DNA, lipid peroxidation, oxidation of proteins and deactivation of enzymes through oxidation of co-factors, leading to mutagenesis and carcinogenesis (2). There is voluminous research with reference to discover new antioxidant compounds from plant and animal origin to prevent free radical damage (3-9). Polypeptide with antioxidant property has also been reported in marine bivalve (Farrer's scallop, also known as the Chinese scallop),*Chlamys farreri* (10, 11). The lepidopteran insects of family Bombycidae and Saturniidae are well known to produce commercially important silks. Domesticated mulberry silkworm, *Bombyx mori* represents family Bombycidae and wild non-mulberry silkworms, *Antheraea mylitta* represents family Saturniidae. The silk fiber of cocoons of silkworms of both the families consist of two major proteins, fibroin and sericin. The fibrion forms central core and sericin forms envelop. Fibroin and sericin, of mulberry silkworm, *Bombyx mori* deserve appreciable physioc-chemical properties and therefore, they are now recognized as excellent biomaterials in the field of tissue engineering, biotechnology and therapy. The fibroin is the water-insoluble protein and sericin is water soluble. The fibroin of silk fibre of mulberry silkworm, has been recognized as a substrate for growth and adherence of cells in culture (12-16) and sericin is used as abiomaterial. This utilization is due to its antibacterial and UV resistant properties of sericin(17). There are some reports on lipid peroxidation through sericin(18), antitumor properties sericin(19) and with no immunogenicity (18).All the biomaterial related applications of silk proteins involve*in vitro* studies on cells prior to their implantation *invivo*. The present attempt was planned to analyse the antioxidant influence of sericin of silk of the cocoons of the mulberry silkworm, *Bombyx mori* and non-mulberry, tropical tasar silkworm *Antheraea mylitta*, in skin fibroblast cell line, exposed to hydrogen peroxide for 24 Hours.

II. MATERIAL AND METHODS

Cell culture of Skin Derived Fibroblast:

The skin fibroblast (feline) cell line (AH927) was procured from National Centre for Cell Science, Pune, India. This skin fibroblast (feline) cell line was used for further processing. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and streptomycin-penicillin (10 µg/ ml) were used as culture medium. The cells were incubated at 37 degree Celsius. The percentage of carbon dioxide in the cabinet of culture was five percent. Cells were sub cultured at intervals of 3 days and those between passages 4-8 were used for furtherexperimentations(19).

Separation and Isolation of Sericin from cocoons:

The sericin was separated and isolated from the cocoons of non-mulberry silkworm, *Antheraea mylitta* and mulberry silkworm *Bombyx mori* through the use of standard protocol described by Sofia, *et al* , 2001 (20 -23). The cocoons of both species (*Antheraea mylitta* and *Bombyx mori)* were cut into smaller pieces. The cocoon pieces were boiled in the solution of 0.02 M Na_2CO_3. This boiling was carried out for half an hour. The supernatant of resulted solution was decanted out and was dialyzed toobtain sericin. The protein solution was dialyzed against several changes of Milli Q water(ultrapure water of Type 1). The crude extracts of sericin (along withall fractions) were used for further experimentations. Further, 8 % SDSPAGE was carried out to confirm the presence of proteins.

Analysis of cell viability:

For the purpose to analyse the cell viability, the extent of reduction of the methyl-thiazolyl tetrazolium compound(MTT) was utilized (36). The cells of culture AH927were seeded in 96-well plates (1×104 cells/well) in DMEM medium containing 10% fetal bovine serum. After the overnight culture, they were treated with different concentrations(0, 0.1, 0.2, 0.5 and 1.0 mM) of hydrogen peroxide (H2O2)for24 hrs. This hydrogen peroxide (H2O2) was freshly prepared from 30 % stock solution (Sigma).The untreated cells were served as control. At the end of incubation,the media in wells were removed and replaced with200 µl of fresh media containing 20 µl MTT solution (5 mg/ml)and incubated at 37 degree Celsius for 4 hrs. Then, the media-containing MTT were removed. Addition of 200 µl of DMSO was made. This was for the purpose to dissolve the formazan crystals formed in the viable cells. The optical density was recorded in Biorad 550 microplate reader at 595nm. The result, extent of H2O2 mediated cell death was expressed as the percentage of cell viability in control cells (24 – 27). Each experiment was repeated for four times for consistency in the results. The collected data was subjected for statistical analysis.

The Phase contrast and fluorescence microscopy:

For the purpose of microscopic observations, 106 cells were seeded on square coverslip (22×22 mm). This coverslip was placed inside a petri plate and allowed to grow. The entire overnight was utilized for the growth of cells. Thereafter, the cells were treated with 0.5 mM H2O2 for 24 hrs. The growth of the cells was observed under phase contrast and then, cells were washed with the ice-cold sterile phosphate buffered saline (PBS). The cells were then fixed in fixative consisting of methanol : acetone (1 : 1). The fixation was carried out for an hour. The cells were rinsed in PBS and incubated with Tween-20 (0.05 %). For the membrane permeabilization, incubation was carried out for 30 minutes. This was followed by RNase (10 mg/ml)treatment for half an hour. After rinsing in PBS, cells were exposed to propidium iodide (1 mg/ml) for half an hour. The cell nuclei were visualized under fluorescence microscope Leica DMR-HC(with camera MP-60). The emission wavelength was consisting of 615 nm (28 and 29).

Influence of pre treatment with silk protein sericin for 24 hrs:

As mentioned earlier for assessment of cell viability, the cellswere subjected to pretreatment with silk protein sericin individually at different concentrations ranging from 5 to 150ng/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 H2O2, gave maximum protection in comparison to 6, 12 and18 hours. The viability was checked by MTT assay after 24 hrs of exposure to hydrogenperoxide(30).

Cell homogenate Preparation:

The cells (1×104 cells/ well) were seeded in petri plates (35 mm)and allowed to adhere for 24 hrs before treatment with silk protein sericin. The strength of sericin of sericinof 100 ng/ml from both non-mulberry(*Antheraea mylitta*)and mulberry silkworm (*Bombyx mori*) was found exhibiting the appreciable protection (from cell viability assays). Therefore, only sericin of 100 ng/ml from both non-mulberry(*Antheraea mylitta*)and mulberry silkworm (*Bombyx mori*) were used for further biochemical experimentations.The 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 for two times with ice-cold PBS and they were harvested through the use of 0.025 %Trypsin-0.02 % EDTA solution. The cells were re-suspended in ice-cold phosphate buffered saline (PBS) and were then homogenized. The cell homogenates were then processed for centrifugation for 5 min at 5,000 × *g* .Supernatant of each sample was used for further biochemical tests (32 – 36).

Analysis of lactate dehydrogenase (LDH)

Quantitative assessment of fibroblast damage caused by hydrogen peroxide was carried out through the ratio of the enzyme activity released from damaged cells to the activity in undamaged / intact cells, monitored for 24 hrs after induction of the stress. The medium
collected after stress induction was centrifuged (for 5 min at 5,000 × *g)* and supernatant used for the assay of enzyme released from damaged cells.

% release of LDH= (released into the medium÷ Activity in sonicated cells)x100

Reduction of pyruvate by the enzyme on addition of NADH was monitored as the change in optical density of the reaction mixture containing 20 mM phosphate buffer (pH 7.4),3.3mM sodium pyruvate and 2 mM NADH. The LDH activity was expressed as change in absorbance at 340 nm per minuteas explained by Bergmeyer and Bernt (37).

Catalase Analysis:

The activity of enzyme catalase (CAT) was carried out through the method described by Maehly and Chance (38). It was assayed by noting the decrease in optical density of NADH at 240 nm following the decomposition of hydrogenperoxide (38). The reaction mixture consisted of 10mM phosphate buffer (pH 7.0), 30 mM hydrogen peroxide and the enzyme source. The decrease in optical density of the reaction mixture was monitored at 240 nm for 5 min. The activity of enzyme LDH was expressed as change in optical density 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 in the unit of percentage.

Lipid Peroxidation Analysis through thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA):

Concentration of thiobarbituric acid reactive substances (TBARS) in the medium after induction of oxidative stress was measured according to the protocol by Niehaus and Samuelson (39). 0.5 ml supernatant of each sample was mixed with 0.5 ml thiobarbituric acid reagent (1 : 1, v: v, mixture of0.67% thiobarbituric acid and acetic acid). The reaction mixture was heated at 95 degree celsius for an hour. After cooling, centrifugation was carried out at 1,000×*g* . The optical density of the supernatant was measured at 535 nm.

Data Analysis:

For the purpose to get consistency in the results, each experiment was repeated for four times. The collected data was subjected for statistical analysis explained by Norman and Baily (40).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.

III. RESULTS AND DISCUSSION

The results pertaining the antioxidant potential of silk protein sericin against hydrogen peroxide-induced oxidative stress in skin fibroblasts are presented in Table – 1 to 3 and explained through the parameters, which include: sensitivity of Fibroblast cells to hydrogen peroxide; morphological changes of the cells on exposure to hydrogen peroxide; effect of Sericin treated before the induction of oxidative stress,, LDH activity, Catalase activity and lipid peroxidation through the levels of thiobarbituric acid reactive substances (TBARS) and Malondialdehyde (MDA).

Sensitivity of Fibroblast Cells to Hydrogen Peroxide:

Viability of fibroblast cells strikingly found decreased in a concentration-dependant manner on treatment with various concentrations of hydrogen peroxide. The LC50 value for 24 hr exposure to hydrogen peroxide was found measured 0.2 mM. This result is indicating that, AH927 cells were sensitive to cell damage induced through hydrogen peroxide treatment. The concentration of 0.5m Mhydrogen peroxide was found reducing the cell viability to the percentage 27.383 (± 4.76) and with that of 1.0 mM hydrogen peroxide decreased the viability to the percentage 8.321 (± 2.49) (Fig. 1). Increase in the concentration of hydrogen peroxide was found significantly affecting on the percent viability of the fibroblast cells. Reduction in percent viability of fibroblast cells treated with hydrogen is responsible for induction of stress.

Morphological changes in Fibroblast cells on hydrogen peroxide exposure:

Preliminary MTT assay in the present attempt revealed in that, viability of fibroblast cells was significantly less at 0.5 mM hydrogen peroxide concentrations. The propidium iodide staining was used for the study on morphological changes in cells at concentration of 0.5 mM Hydrogen peroxide. This method helped further to assess the damage caused by oxidative stress. The observations under phase contrast microscope, the morphology of the fibroblast cells exposed to 0.5 mM Hydrogen peroxide were revealed shrunken and rounded nature of the fibroblast cells. This is in comparison to normal cells. Fluorescence staining of cells revealed that cells exposed to 0.5 mM H2O2 exhibited nuclear condensation.

Effect of Sericin treated before the induction of oxidative stress:

The effect of pre-treatment of cells with sericin for 24 hrs is presented in Table - 2. The pretreatment of gelatin with the cells for 24 hrs did not show significant protection against Hydrogen peroxide induced oxidative stress. The one-way analysis of variance revealed that,there was an overall significant difference in cell viability between controls, H2O2-treated and sericin treated fibroblasts ($F=44.47$, $P < 0.001$). Subsequent multiple comparisons by Tukeys test indicated that, cell viability was significantly lower ($P < 0.01$) in hydrogen peroxide-treated when compared with control and sericin treated fibroblasts. The concentrations of 35, 50and 100 ng/ml sericin were found significantly ($P < 0.05$) increasing the cellviability. Cells treated with sericin of *A. mylitta*, at 150 ng/ml exhibited cellviability comparable to that of control group ($P > 0.05$). This result indicatethat, pre-incubation with 150 ng/ml restore the cell viability to normal. The sericin from *B. mori* was found not responding in the same way to restore cell viability to normal at the same concentration.

LDH activity:

The table – 3 depicts the percentage of LDH activity released into themedium in normal fibroblast cells, fibroblast cells treated with 0.5 mM hydrogen peroxide for 24 hrs, and fibroblast cells pre-incubated with 35 ng/ml and100 ng/ml of sericin from *A. mylitta*. The oxidative stress through hydrogen peroxide was found responsible for significantincrease ($P < 0.01$) in the release of enzyme (LDH) in comparison with untreated control and cells pre-incubated with 100 ng/ml of sericin before hydrogen peroxide treatment.The oxidative stress through hydrogen peroxide is responsible for the loss of membrane integrity of the cells. The sericin treatment definitely serving to restore the original membrane integrity of the cells.

Catalase activity:

Rate of catalase activity in various treated cellsand control are presented in Table - 3. Catalase activity was significantlyhigh ($P < 0.01$) in medium of cells treated with hydrogen peroxide (80.608 %) compared to control(18.434%). The fibroblasts pre-incubated with sericinof both the species of silkworm (*Antheraea mylitta* and *Bombyx mori*) at 100 ng/ml had significantly decreased ($P < 0.01$) catalase activity. Oxidative stress through treating the cells with hydrogen peroxide is responsible for increase in the catalase activity and sericin treatment have had protective influence on oxidative stress.

Lipid peroxidation through the levels of thiobarbituric acid reactive substances (TBARS) and Malondialdehyde (MDA):

The % of TBARS/ MDA in the media expressed as nmol /ml of the medium is shown in Table - 3. The products of peroxidationwere significantly high ($P < 0.01$) in the media of cells treatedwith hydrogen peroxide. On the other hand the cells pre-incubated with sericin of both the species of silkworm (*Antheraea mylitta* and *Bombyx mori*) at 100 ng/mlhad significantly lower levels of TBARS ($P < 0.05$).

The hydrogen peroxide is model oxidant because itscellular actions and its fate has been well studied. In the present attempt, the protective effects of silk sericinof both the species of silkworm (*Antheraea mylitta* and *Bombyx mori*), on hydrogen peroxide-induced oxidative damage in feline skin fibroblasts were observed. The mechanism of damage by hydrogen peroxide in fibroblast cultures involves the production of reactive oxygen species (ROS) (41). Level of the 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 thenon-mulberry silkworm, *A. mylitta*, showed protective effect ata minimum concentration of 35 ng/ml and restored the viability and normal biochemical profile at 150 ng/ml which wasnot observed in case of sericin of *B. mori*. To study further the effect of sericin of *A. mylitta* on hydrogen peroxide induced oxidative stress, the activities of LDH and catalase were examined. 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 in comparison to normalcells. This clearly indicate the loss of membrane integrity through oxidative stress (42). The enzyme catalase is involved in the decomposition of hydrogen peroxide to water and

oxygen. Therefore, the enzyme catalase is important in protecting cells against oxidative stress (43). 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 the lipidperoxidation. Malondialdehyde (MDA) is expert to alter the structure and function of the cellular membrane and blocks cellular metabolism leading to cytotoxicity (44). 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 attempt indicates that sericin of both the species of silkworm (*Antheraea mylitta* and *Bombyx mori*), might be providing protective effect on fibroblast by acting as antioxidant as well as by promoting endogenous antioxidant. Indeed, sericin has previously been reported to possess photo - protective effect against the UVB-induced acute damage and colon carcinogenesis (45). The present attempt report the cutaneous cell lines like AH927 fibroblasts are effectively protected against oxidative stress throughsericin from the cocoons of the tropical tasar silkworm, *A. mylitta*. The sericin of both the species of silkworm (*Antheraea mylitta* and *Bombyx mori*), contains high amount of polar amino acids. Because of remarkably high content of hydroxyl amino acids (like, serine and threonine), the hydroxy groups of amino acids in sericin might be responsible for the antioxidant action by chelating trace elements(15). For the purpose to observe whether the protective effect of sericin is due to its viscous nature, gelatin (a viscous polymer) was taken as control. Surprisingly enough, it is observed that, viscous compoundslike gelatin did not show any protection against oxidative stress through hydrogen peroxide. The protective influence of sericin may be due to its unique antioxidant potential. The skin already possesses extensive and most effective network of the system of antioxidant. Practically, many more 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 (46).The use of antioxidants has been observed to improve cutaneous wound healing significantly (31). Consequently, exogenous antioxidants (like sericin) that scavenge reactive oxygen species (ROS) and restore normal redox state are supposed to be beneficial (30).Conclusively enough, the present attempt suggests that water-soluble silk sericin, from a natural source like silkworm cocoon, serveas ideal molecule to prevent oxidative stress. Use of sericin may also be used in therapy with other conventional non-enzymatic antioxidants. This may open a new avenue for most suitable method for treating the cancer.

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Table – 1 : Cell viability (extent of reduction of the thiazolyltetrazolium compound(MTT) of feline fibroblast cells (AH927) in presence of Hydrogen Peroxide.

Concentration of Hydrogen Peroxide (milli mol)	Percent Viable Cells
0.00	100.000 (±2. 381)
0.10	073.806 (± 7.793)
0.20	051.193 (± 5.95)
0.50	027.383 (± 4.786)
1.00	008. 321 (± 2.492)

- Each value is the mean of three replications.
- Figures in parenthesis with(±) signs are the standard deviations.

Table –2: Influence of sericin on the Cell viability (extent of reduction of the methyl-thiazolyl tetrazolium (MTT) of feline fibroblast cells (AH927) treated with Hydrogen Peroxide.

Group	Percent Viable Cells
Untreated Control	97.048 (± 6.134)
Hydrogen Peroxide Treated	28.311 (± 3.249)
Gelatin Treated	32.502 (± 2.562)
Sericin (*A. mylitta*) (35 ng/ml)	57.179 (± 4.414)
Sericin (*B. mori*) (35 ng/ml)	54.883 (± 4.786)
Sericin (*A. mylitta*) (50 ng/ml)	61.297 (± 7.124)
Sericin (*B. mori*) (50 ng/ml)	55.594 (± 4.451)
Sericin (*A. mylitta*) (100 ng/ml)	63.423 (± 3.531)
Sericin (*B. mori*) (100 ng/ml)	54.652 (± 5.649)
Sericin (*A. mylitta*) (150 ng/ml)	91.287 (± 2.409)
Sericin (*B. mori*) (150 ng/ml)	63.991 (± 6.117)

- Each value is the mean of three replications.
- Figures in parenthesis with(±) signs are the standard deviations.

Table –3 : Influence of sericin on the activity of Catalase, Lactate Dehdrogenase (LDH) and Malondialdehyde (MDA) homogenate of feline fibroblast cells (AH927) treated with Hydrogen Peroxide.

Group	Catalase Activity	LDH Activity	MDA Activity
Untreated Control	18.434 (± 1.426)	35.217 (± 4.092)	15.652 (± 1.873)
Hydrogen Peroxide Treated	80.608 (± 23.912)	69.631 (± 16.655)	60.263 (± 21.374)
Gelatin Treated	64.956 (± 18.268)	64.823 (± 21.319)	61.43 ± 18.969)
Antherea mylitta Sericin (100 ng/ml)	23.748 (± 4.832)	63.391 (± 5.574)	27.387 (± 3.213)
Bombyx mori Sericin (100 ng/ml)	18.083 (± 3.715)	57.913 (± 4.638)	23.478 (± 4.235)

- Each value is the mean of three replications.
- Figures in parenthesis with(±) signs are the standard deviations.