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Evaluation of antioxidant and antimicrobial activity of silk protein sericin extracted from *Bombyx mori* cocoons

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Abstract
Silk proteins are mainly composed of fibroin and sericin and both these have wide range of applications. In the present study the silk protein was extracted from the yellow cocoons of *Bombyx mori* and it was characterized by FT-IR and GC-MS analysis. The IR spectra showed major peaks at 3329.14 cm^{-1} and at 1641.42 cm^{-1} and GC-MS analysis on showed the presence of amino acids such as serine, alanine, glycine and leucine. Further the silk protein was mainly evaluated for its antioxidant and antimicrobial property. The antioxidant activity was studied by the DPPH method and the IC_{50} was found to be 50.4 µg/ml. This showed good scavenging activity against free radicals by silk proteins. An antimicrobial study was studied and highest activity of 250 µg/ml was observed against *Staphylococcus aureus* MTCC 902.

Keywords: silk proteins, antioxidant,; DPPH, antimicrobial activity, sericin

Introduction

For many years silkworm cocoons were used for production of silk fibres especially in India approximately 1,600 tons are processed for the reeling of silk. In the recent decades other application of cocoons mainly on the silk proteins were investigated in detail. Silkworm can be considered as a medicinal insect because of its unique properties. Two major silk proteins are present in coccons -fibroin comprising the major protein and remaining protein is made up of sericin. It was reported that sericin is a water soluble protein with molecular weight ranging from 10 to 310 kDa. It is present in two forms α-sericin and β-sericin and amino composition reveals both polar and non-polar groups the percentage of which varies for each strains [1, 2, 3]. The amino acid composition will influence the antioxidant and other properties and sericin extraction process also plays an indirect contributing factor.

Several methods are followed for recovery of this valuable protein during the silk manufacturing process. These proteins can be recovered both in the degumming process as well as in the reeling process, but the major percentage is extracted at the degumming process. The common conventional methods such as boiling, autoclaving, use of alkalis, soaps, enzymes are been followed for sericin extraction. The selection of the extraction method is to be considered such as to maintain the stability of the protein. One of the limitations in this conventional method is possibility of sericin instability which makes it inapplicable at commercial levels. At present other alternative methods such as use of enzymes, and cold radiation can be utilized for extraction process [4, 5, 6]. This protein has many unique properties like excellent moisturizing property, antioxidant, antibacterial, antitumour and protection against ultraviolet radiation. The silk proteins also have the ability to form crosslinking between them hence can develop into a natural polymer [7, 8, 9]. It can also undergo copolymerization with other polymers and therefore can be used as composites and scaffolds.

During the aging process free radicals are produced by the damaged cells, this high level of reactive oxygen species can be detrimental to health and hence have to be eliminated. The antioxidant helps in maintaining a balance between production and elimination of free radicals. Many synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ) are administered as food supplements to combat this problem. This may cause side effects so extraction of antioxidants from natural resources has to be developed. Antimicrobial property is another important aspect which is studied extensively by many researchers. Even though diverse number of antimicrobial drugs are available still more number of drugs are yet to be explored due to continuous development of microbial resistance [10, 11]. There are different ways in determining the antimicrobial activity. The agar well diffusion method is the most common and simple method. In the present study the silk protein sericin is extracted from cocoons of Bombyx mori and its antioxidant and antibacterial activity was studied.

Materials and Methods

Sample collection

Yellow silkworm cocoons were collected from Sericulture department at Hasthampatti in Salem District, Tamilnadu. The coccons were transferred to the laboratory in a clean air tight container at room temperature.

Degumming of silk proteins

For degumming process the microwave radiation method was followed as described by Mohammad *et al.*, (2010) [12]. The fresh white cocoons was cut into small pieces of approximately 2cm size and added to distilled water in the ratio of 1: 50. The Microwave Equipment model used in this experiment was CE305CF (SUMSUNG) and the following parameter was set (power consumption: 1400 W and operating frequency 2450 MHz). The coccons were exposed to microwave radiation at varying time intervals of 5, 10,

15, and 20 minutes. It was then filtered using a clean muslin cloth.

Characterization of silk proteins

UV-Visible Spectroscopic Analysis

For UV spectrophotometer analysis, the sample was diluted to 1:10 with the ethanol solvent. It was scanned in the wavelength ranging from 260-900nm using Perkin Elmer Spectrophotometer and the characteristic peaks were detected [13].

FT-IR spectroscopic analysis

FTIR analysis was performed using Perkin Elmer Spectrophotometer system, which was used to detect the characteristic peaks ranging from 400 - 4000 cm-1 and their functional groups. The sample was prepared by compressing the sample with potassium bromide [14, 15]. The peak values of FTIR were recorded. Each and every analysis was repeated twice for the spectrum confirmation.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Pre preparation of the sample were performed by exposing the 20g of sample to 40 mL of 2% methanolic NaOH followed by boiling the sample until saponification occurs. At the end of saponification, 50 mL of 14% BF3 was added and the sample was boiled for 5 min. Then, 20 mL of n-heptane was added and boiled for another 1 min [16, 17, 18]. After that, 4 mL of saturated NaCl was added. Finally, it was put in separation funnel and phase separation was observed after 5-10 min (AOAC, 1980). Then, 1 µL of extracted sample was injected into GC (Shimadzu GC-MS QP2010 Plus). This injected sample passed through a capillary column (Teknokroma TR-CN 100).

Determination of antioxidant activity by DPPH

The free radical scavenging activity was determined as per the protocol reported by Brand William *et al*., 1995 [19]. About 39 mg of 2,2 Diphenyl-1-picrylhydrazyl (DPPH) was dissolved in 100 ml of methanol and store at -20 ºC until needed. 0.1 mM of DPPH solution in methanol was prepared and added to the sample at different concentrations (500, 250, 50, 10, and 5 µg/mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was read at 517 nm using a Systronic UV-VIS Spectrophotometer. (Ascorbic acid was used as the reference (1mg/mL). Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability of scavenging the DPPH radical can be calculated by using the following formula. DPPH scavenging effect (% inhibition) = [(absorbance of control-absorbance of reaction mixture)/absorbance of control] X 100.

Determination of antimicrobial activity

Bacterial cultures

The bacterial cultures *Escherichia coli* MTCC 443, and *Staphylococcus aureus* MTCC 902 was purchased from Microbial Type Culture Conditions at Chandigarh in India.

Agar- well diffusion method

Sterile petri plates containing 20 ml nutrient agar medium were seeded with 24hr culture of bacterial strains. Wells were cut and different concentration of sample (500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml) was added [20]. The plates were then incubated at 37 °C for twenty-four hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Gentamicin antibiotic was used as a positive control. The values were calculated using Graph Pad Prism 6.0 software (USA).

Results & Discussion

Degumming Of Silk Proteins

The yellow coccons were cut into small pieces and soaked in distilled water. In the present study, degumming of silk proteins was carried out with microwave assisted radiation. The conventional method was not followed as the longer time duration would be taken for degumming. In microwave radiation the high frequency electric field was transferred to thermal energy and hence it reaches the boiling point at a short duration compared to the conventional boiling method. Another added advantage is homogenous heating of the material and high penetration power. At lower temperature of 4 °C gel formation was observed and the incubation time was directly proportional to gel thickness. In nature the silk proteins are able to form polymers due to conformational changes in silk proteins. As per literature reports by Teramoto *et al*., (2008) & Wang *et al*., (2014) [21, 22] the gel formation was enhanced in lower temperature and this is one of a unique property of the silk proteins.

Characterization of Silk Proteins

The silk protein extracted by the above method was partially purified by dialysis and later both UV spectra and IR spectrum were studied.

UV spectra analysis

UV scans of silk protein solution were done at wave length between 200-800nm. As seen in results for silk proteins obtained with ethanol it gives a peak at 269-358 nm (Fig1). It was observed that recovered sericin gave peak at the same wave length interval as that of the standard sericin. In recovered sericin other impurities may also be present which may probably be the reason for presence of other peaks together with sericin between 269-324 nm. Therefore the peak of recovered sericin may not be as distinct as that of standard sericin. For further studies the sample BMY-6 was used for characterization by FT-IR and GC-MS. [23] Deepti *et al*., 2014 compared the quality and quantity of sericin obtained from four sources, namely mulberry silk cocoons, silk flats, reeling silk waste and woven silk fabric and obtained similar results.

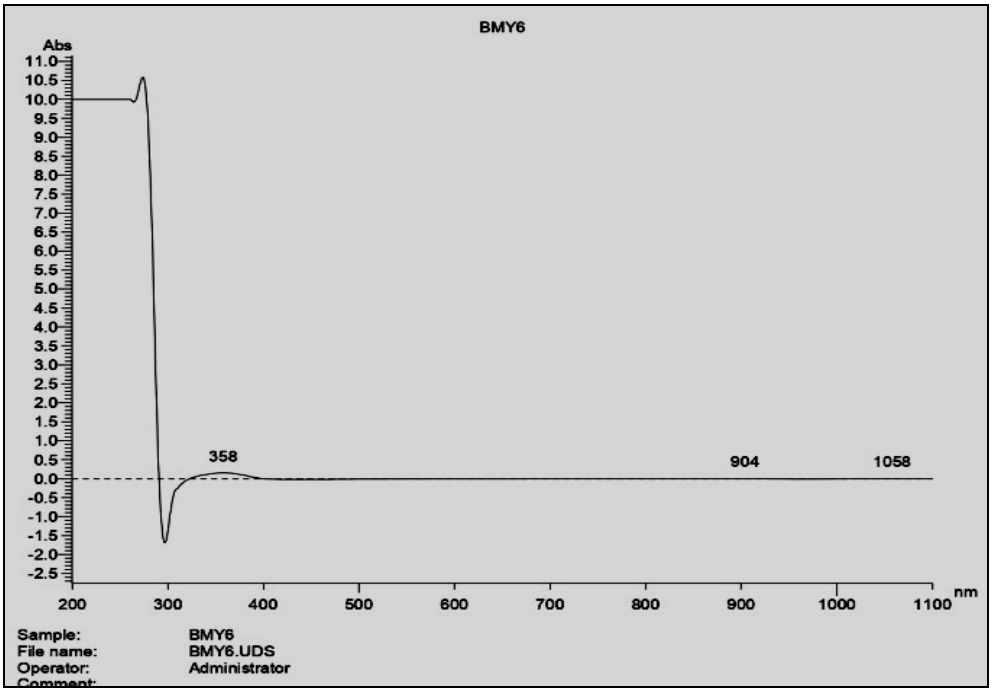

Fig 1: The UV spectra of the silk proteins isolated from yellow coccons (BMY-6)

FT-IR spectroscopic analysis

FT-IR spectrum identified the functional group present in the silk protein based on the peak value in the region of infrared radiation. When the sample BMY-6 was passed into the FT-IR, the functional group of the components was separated based on its ratio. An IR spectrum of the BMY-6 was shown in Figure 2 that showed major peaks at 3329.14 cm^{-1} for O-H Hydrogen bonded-Alcohol usually strong and broad; for triple bond C-H alkynes, possibly N-H Amide at 1641.42 cm^{-1} as strong peak was observed, peak at 651.94 cm^{-1} indicates C-H alkynes. The major peak seen and from 3700 - 2000 cm^{-1} represents N-H stretching vibrations. NH nitrogen hydrogen stretch vibration between 3000-2500 cm^{-1} in combination with highly characteristic, medium at 1641.42cm^{-1} to weak absorption peaks between 2000-2500~I were also present. Similar reports was suggested by Prasong *et al*., (2009) and Kwak *et al*., (2017) [24, 25] who also observed major peaks between 3000-2500 cm^{-1}

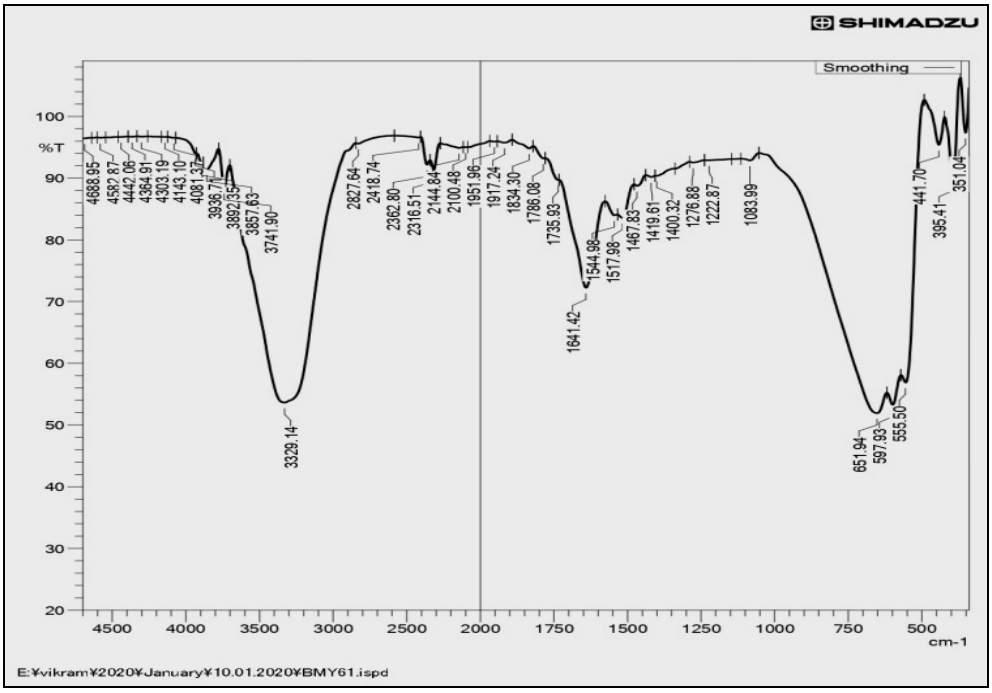

Fig 2: FT-IR spectrum of the BMY-6 isolated from yellow cocoons

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Silk sericin protein containspredominantly amino acid groups like serine, glycine, glutamic acid, aspartic acid, threonine, and tyrosine. From the GC-MS analysis the sample BMY-6 was found to contain amino acids such as serine, alanine, glycine and leucine in higher composition (Figure 3). Twenty peaks were identified by GC-MS analysis based on active principles with their retention time.

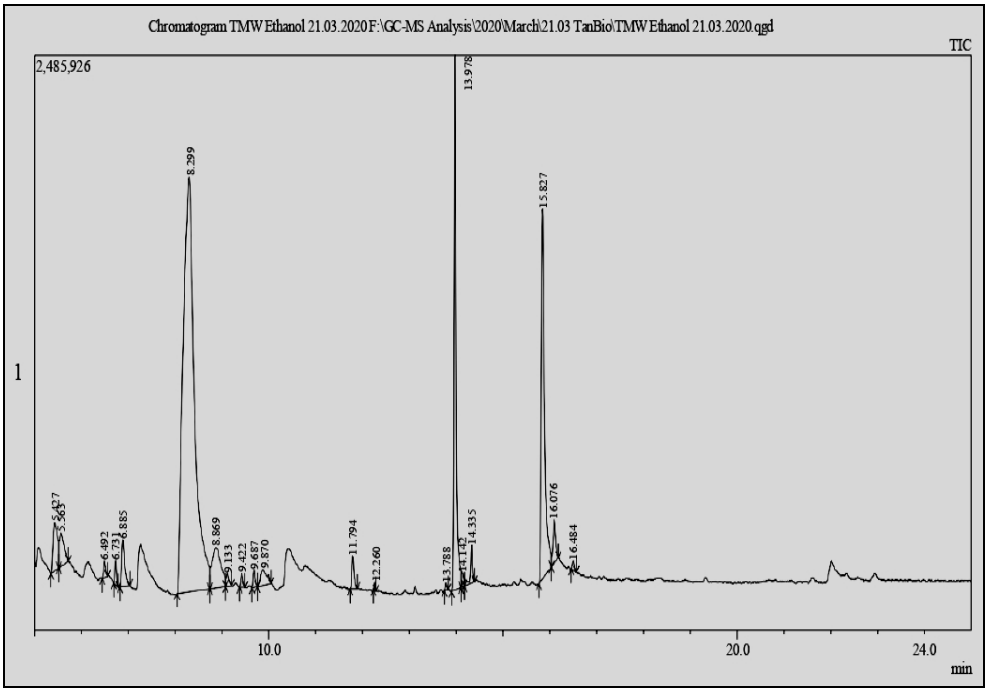

Fig 3: The GC chromatogram of protein present in BMY-6

Determination of Antioxidant Activity by DPPH

Two important characteristic features such as antioxidant and antimicrobial activity are studied as they play a significant role in view of clinical applications. Antioxidant activity is related to removal of free radicals which are toxic in nature and nascent oxygen from the environment. There are number of natural compounds which habour this property and studies by Mina *et al*., (2013); Enqin *et al*., (2019) [26, 27] have showed that silk proteins exhibit free radical scavenging activity. Antibacterial property implies to destroying bacteria which are pathogenic in nature. One hypothesis is that as antioxidant scavenges oxygen it may create an anaerobic environment which make the environment unfavourable for microbial growth. There is no strong scientific proof that both these properties are related but most of the literature cites that many natural compounds exhibit both antioxidant and antimicrobial property.

In the present study, the antioxidant activity of the sample BMY-6 was assayed by DPPH method and the results were tabulated in Table 1 & 2 and figure 4 & 5

Table 1: The estimation of antioxidant activity by DPPH method

Tested sample (µg/ml) concentration	OD Value at517 nm(in triplicates)		
Control	1.485	1.800	1.808
5 µg/ml	1.102	1.126	1.118
10 µg/ml	1.082	1.082	1.081
50 µg/ml	1.080	1.075	1.062
250 µg/ml	1.063	1.051	1.064
500 µg/ml	1.040	1.035	1.033

Control Mean OD value: 1.697

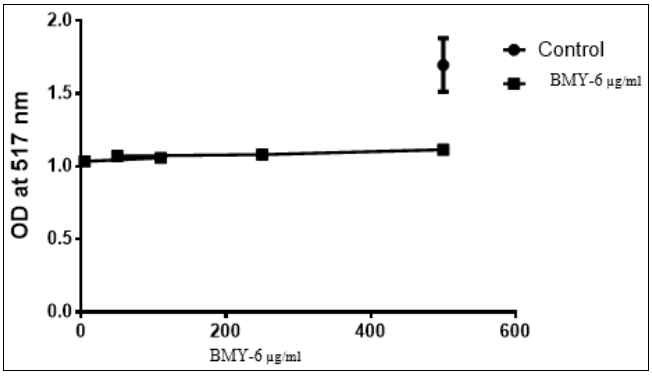

Fig 4: The graph representing the antioxidant activity by DPPH method

Table 2: The table showing the percentage of inhibition of BMY-6 at varying concentration

Tested sample concentration (µg/ml)	Percentage of inhibition	(in triplicates)		Mean value (%)
Control	100	100	100	100
5 µg/ml	35.06	33.64	34.11	34.27
10 µg/ml	36.24	36.24	36.29	36.25
50 µg/ml	36.35	36.65	37.41	36.80
250 µg/ml	37.36	38.06	37.30	37.57
500 µg/ml	38.71	39.01	39.12	38.94

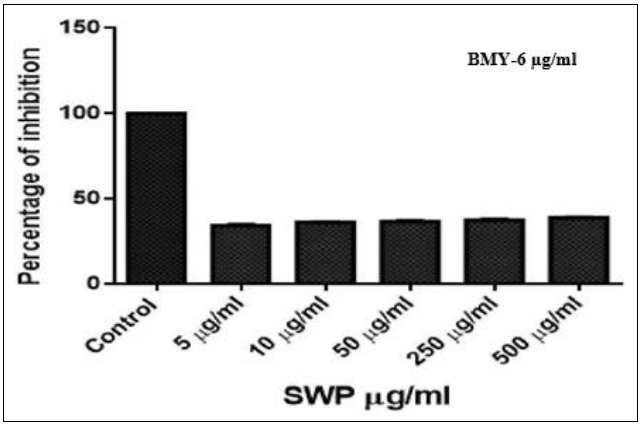

Fig 5: Graphical representation of percentage of inhibition of SWP

The IC_{50} value of an antioxidant is the concentration required to provide 50% inhibition of probe (2, 2-diphenyl - 1-picryl hydrazyl) in DPPH assay. The concentration required to scavenge DPPH radical by 50% was obtained by linear regression analysis of dose-response curve plotting between different concentration and percentage inhibition. IC means inhibition concentration and IC_{50} is the half concentration it means the concentration of antioxidants that inhibit free radical. This is inversely proportional to the free radical scavenging activity. The scavenging activity of free radicals in the sample is due to the presence of molecules which is known as antioxidants. In the present study the IC_{50} was observed at the concentration of 125 µg/ml which indicates antioxidant activity of silk protein as it is a wellknown fact that low IC_{50} value denotes high antioxidant capacity. The scavenging effect of sericin was also discussed by many researchers and the results were similar to Chlapanidas *et al.*, (2013); Miguel M, *et al.*, (2020) [28, 29] who also found sericin to exhibit 80% of free radical scavenging activity.

Determination of antimicrobial activity

The antimicrobial activity against both Gram positive and Gram negative bacteria was investigated for the sample BMY-6 and zone of inhibition was observed.

Table 3: SD± Means of zone of inhibition against the pathogens *Escherichia coli* and *Staphylococcus aureus*

Name of the test organism	Name of the test sample	Zone of inhibition (mm) SD ± Mean				
		500 µg/ml	250 µg/ml	100 µg/ml	50 µg/ml	Gentamicin
E. coli	BMY-6	11±1.0	11±1.0	9.5±0.5	9.5±0.5	13.5±1.5
Staphylococcus aureus		11.5±1.5	13.5±1.5	12.5±1.5	12.5±0.5	14.5±1.5

On comparison with the positive control Gentamicin (10 µg**)** the sample BMY-6 was less sensitive to *E. coli* whereas it was moderately sensitive to *Staphyloccocus aureus*. The reason may be the presence of outer lipid layer in Gram negative bacteria which is absent in Gram positive bacteria. Varying concentration of BMY-6 in µg/ml was used and the higher concentration of 250 µg/mL showed a clear zone of inhibition against *Staphyloccocus aureus*. Senakoon *et al*., (2009) also observed that the degummed eri sericin had antibacterial activity against both *E. coli* and *S. aureus* but of lower concentration of 40 µg when compared to our results. Ahamad, Sayed *et al*., (2018); Esamil M *et al*., (2020) [30, 31, 32] also observed antibacterial activity by the silk proteins extracted from *Bombyx mori*.

Conclusions

For many years silkworm *Bombyx mori* was traditionally used for silk production especially in India because of our customary. In recent years the protein of the silkworm cocoons was studied extensively due to its unique properties. In the present study two important biomedical applications such as antioxidant and antimicrobial activity was investigated. The silk protein was extracted from Bombyx *mori* by microwave assisted radiation. The obtained protein was characterized by FT-IR and GC-MS method. The silk protein sericin has both antioxidant and antimicrobial activity hence can be used clinical after further animal study. This work will create a great opportunity to sericulture industry as in India it is one the prime industry that provide large annual revenue. The silkworm can be called as medicinal insect because of its varied properties.

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