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EXTRACTION AND CHARACTERIZATION OF SERICIN PROTEIN FROM *Bombyx mori*

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Abstract

Silk sericin is a water-soluble macromolecular protein obtained from the raw cocoons of silkworm, *Bombyx mori*. It comprises of about 25% of the total cocoon shell weight and is categorized as a waste by-product in textile and silk industries after the extraction of silk fibers. This study emphasizes on the extraction of sericin from *B. mori* by a conventional salt alkaline method and characterized their physico-chemical properties, thermal stability, anti-bacterial and anti-oxidant activities. The extraction yield of the sericin protein from the cocoon was found to be 24 %. FTIR spectrum shows the presence of functional groups corresponding to the amino acids of sericin depicting its purity. TGA analysis demonstrates the degradation of sericin at 210°C with a weight loss of 57.69%. Sericin exhibited effective antibacterial activity against *E. coli* and *S. aureus*. DPPH assay revealed the antioxidant property of the sericin.

Key words: antibacterial, *B. mori*, extraction, protein, sericin

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1. Introduction

Silk produced by silkworms, consists of two-filaments bound together to form its cocoon (Padamwar and Pawar, 2004). Silk is mainly composed of two proteins, fibroin and sericin apart from salts and fatty acids (Rangi and Jajpura, 2015). Fibroin is a water-insoluble fibrous protein that accounts for about 70-80 wt% of the cocoon. It comprises of both crystalline and amorphous domains which gives them unique physico-chemical properties. Presence of amino acids with larger side chains contributes to its amorphous domains while the short side chains with high percentage of alanine, glycine and serine attributes to its crystalline property (Padamwar and Pawar, 2004). Sericin is the second major protein comprising 20-30 wt% of the cocoon. It is soluble in hot water, acts as natural adhesive for fibroin strands and assist in the formation of fibrous network of cocoon. Sericin consists of 18 different amino acids with a higher content of serine for about 32 mol% (Padamwar and Pawar, 2004).

The presence of polar groups in its amino acid side chains influence their solubility which led to its classification into three fractions A, B and C. The outermost layer (A) consists of fewer fibre filaments which are soluble in hot water; the middle layer (B) has cross fibre filaments and yields amino acids upon acid hydrolysis, and the innermost layer (C) has more longitudinal fibres which requires hot dilute alkali or acid based procedures to separate sericin from silk fibres (Shaw and Smith, 1951; Sprague, 1975; Wang et al., 1985).

Silk proteins adapts various secondary structures such as α-helices, β-sheets and crossed β-sheets (Komatsu, 1996). These proteins possess anti-oxidant, anti-microbial, UV resistant and wound healing capabilities besides their biocompatibility and

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biodegradability (Kato et al., 1998; Siqinzhaorigetu and Sasaki, 2003).

In recent years, the use of sericin has been widely increasing in pharmaceutical, cosmetic, and textile industries (Zhang, 2002). In cosmetic industry, sericin is used for its strong affinity towards keratin. Sericin gel helps to prevent water loss from the upper layer of the skin, and also it is used in hair and skin conditioners. It is also used in skin creams to provide hydration, elasticity, antiaging and antiwrinkle effects (Kumar and Mandal, 2019; Singh, 2014; Singh et al., 2011; Yamada et al., 2001). It also prevents nails from chapping and brittleness (Tsubouchi et al., 2005). Sericin also finds application in food industry for its antioxidant activity and in preventing aging when coated on fruits (Kato et al., 1998).

Sericin has been proved as a good biomaterial when used in the form of polymer foams and membranes due to their easy moisture absorption and gelling properties (Wang et al., 2018). It provides a suppressant effect on colon and skin cancer as demonstrated in a tumor-induced mouse model (Siqinzhaorigetu and Sasaki, 2003). It also serves as a good substratum for the attachment of the primary cultured human skin fibroblast (Voegeli, 1993). A hydrogel made of sericin mixed with fibroin and poly-vinyl alcohol had excellent elasticity, moisture absorption and desorbing properties.

Sericin hydrogel has also been used as a wound dressing material and are shown to promote the collagen production to enhance wound healing process in a rat model (Aramwit and Sangcakul, 2007). The protein has been cross-linked covalently to form 3D pure hydrogel for drug delivery applications (Wang et al., 2014). Sericin nanoparticles prepared by protein de-solvation method was proposed for gene and drug delivery applications without compromising its host compatibility (Das et al., 2014). Sericin is also known for its other excellent properties like photoluminescence, adhesiveness and elasticity (Dong et al., 2019).

Sericin shows anti-apoptotic and anti-oxidant properties as it gradually reduces intracellular reactive oxygen species (ROS) (Micheal and Subramanyam, 2014; Rajput and Kumar, 2015). It shows anti-proliferative effect on peripheral blood mononuclear cells *in vitro*, by reducing the release of interferon gamma (IFN-γ) (Altman et al., 2003). Besides all these excellent properties, there are also reports on adverse immune response and hypersensitivity reactions induced by silk proteins especially by sericin (Chlapanidas et al., 2013).

However, sericin possesses ideal properties to serve as a biomaterial in the field of tissue engineering and regenerative medicine (Dash et al., 2008). In this study, sericin is extracted from *B. mori* cocoons and their physico-chemical, antimicrobial and antioxidant properties were studied to demonstrate their potential as a biomaterial.

2. Material and methods

2.1. Material

The *B. mori* cocoons were bought from the local market of Vellore district, Tamil Nadu. Sodium carbonate, bovine serum albumin, copper sulphate, Folin-Cioalteau phenol agent, microbial culture media and 2,2'-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Himedia, India. Deionized water was used for the extraction process.

2.2. Extraction of sericin

Sericin was extracted from the silk cocoons by a conventional soap-alkaline method (Yun et al., 2013). About 10g (w_i) of cocoons were placed in a solution containing 0.02% (w/v) of sodium carbonate and 0.03% (w/v) of natural organic soap and heated for 15 minutes with periodic stirring. This results in the separation of sericin from the fibers, leaving the sericin in the solution. The solution was then filtered using Whatman filter and dialyzed against distilled water for 3 days in order to remove the traces of impurities. The filtrated was freeze-dried to get sericin powder. The cocoons, obtained after the extraction process were dried at 50°C for 3 days and its weight was measured (w_f) to calculate degumming ratio (%) (Eq. 1).

$$Degumming\ \ ratio\ (\%) = \frac{w_i - w_f}{w_f} \times 100 \qquad (1)$$

2.3. Characterization of the extracted sericin

Total protein content of the extracted powder was determined by Lowry's method, where the protein concentration was estimated against the standard, bovine serum albumin (BSA). The chemical and secondary structural analysis of sericin was determined by Fourier Transform Infrared (FTIR) spectroscopy in attenuated total reflection mode (IR Affinity 1S spectrophotometer, Schimadzu Scientific Instruments, Japan). The thermal property of sericin was examined using a thermal analyzer (SDT Q600, TA Instruments, USA) by heating it from room temperature to 800°C at a scanning rate of 20°C per minute under nitrogen atmosphere.

2.4. Antibacterial and antioxidant property

Effect of sericin extract on the growth of two different bacterial strains, *Escherichia coli* and *Staphylococcus aureus* were measured by agar diffusion assay (ADA) and observed for the formation of inhibitory zones. Briefly, the plates containing nutrient agar were inoculated with 0.1 mL of overnight grown culture (1 x 10^{-5} CFU/mL). The sericin protein at two different concentrations of 1 mg/mL and 2 mg/mL were filled into the wells.

Tetracycline was used as the positive control and it is filled into the same plate and incubated at 37°C for 24 hours. The experiment was performed in duplicate for each bacterial strain and the diameter of zone of inhibition was measured. The antioxidant activity of sericin was measured using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). Approximately, 500 µL of protein solution (20 mg/mL) was mixed with 500 µL of DPPH (20 mg/L) and absolute alcohol was used as the blank. The oxidation reaction was measured using spectrophotometry at 520 nm, after 30 minutes of incubation.

3. Results and discussions

Various methods employing hot-water, urea, urea-mercaptoethanol, sodium chloride and sodium carbonate have been established for the sericin extraction and degumming with the conventional soap-alkaline method was found to be efficient for the extraction of sericin (Yun et al., 2013). Extraction conditions such as temperature, treatment duration and concentration of the chemicals should be taken care to regulate their effect on degumming rate, protein fractionation and amino acid composition (Takasu et al., 2002).

In this study, sericin protein was effectively extracted by soap-alkaline method where the Na_2CO_3 acts as the alkaline source and the soap prevents the re-adsorption of the removed sericin. The degumming ratio was found to be 24% which is quite consistent with the previous study reported by Yun et al. (2013) where 25% was obtained from *B. mori* (Yun et al., 2013). The total protein yield was 6% which is much lower than the degumming ratio. The lyophilized powder contains 1mg/mL of protein as determined by Lowry's method.

FTIR spectrum of sericin is shown in Fig. 1, which displays the characteristic absorption bands of their polypeptide and protein units. The region from 1384 to 1400 cm^{-1} significantly attributes to the amino acid serine, the major amino acid found in sericin (Boulet-Audet et al., 2015). The band at 1068 cm^{-1} corresponds to C-O stretching which distinguishes the presence of sericin from other silk proteins. The strong band at 1384 cm^{-1} was assigned to CH_2 bending. The absorption bands of amides III, II and I at 1234, 1517 and 1626 cm^{-1} respectively further confirmed the secondary structures of sericin (Gupta et al., 2014). The shoulder peak at 1743 cm^{-1} along with the amide I represents the formation of β-sheets and their aggregates and this represents its crystallinity. It has been reported that the secondary structures of the sericin are greatly influenced by the extraction process (Chirila et al., 2016). Therefore, the presence of all important peaks of sericin along with the strong absorption band for amide I reflected the successful isolation of sericin.

The thermal behavior of the extracted sericin was examined using thermogravimetric analysis. Results showed that the sericin degrades with three specific zones (Fig. 2). The initial weight loss of 9.39% below 150°C is due to the presence of adsorbed moisture. The major step of protein degradation occurs at 210°C with a significant weight loss of 57.69%.

This attributes to the breakdown of amino acid side chains and cleavage of peptide bonds leading to the complete degradation of sericin with increasing temperature (Ribeiro et al., 2015). This confirms that sericin is thermally stable and the extraction process did not affect its thermal property.

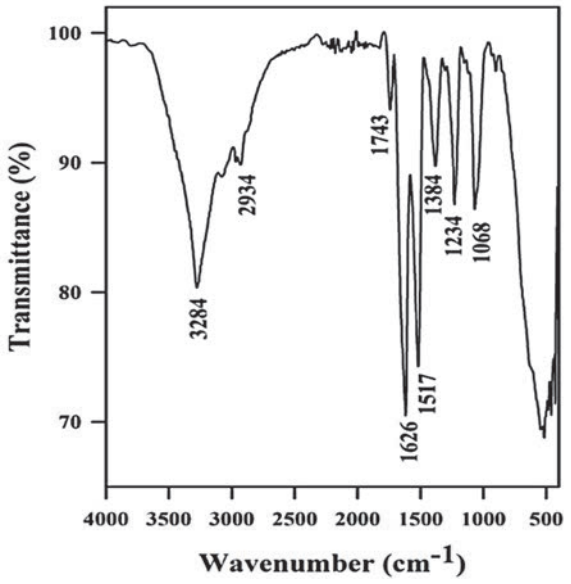

Fig. 1. FTIR spectrum of extracted sericin powder from *B.mori* showing respective peaks of amino acids

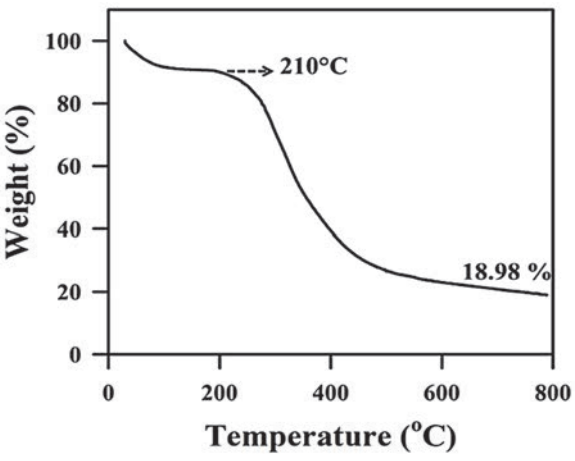

Fig. 2. Thermogravimetric analysis of sericin showing the degradation of amino acids

Assessment of anti-bacterial activity by agar diffusion assay revealed that the sericin inhibits the growth of *E.coli* and *S.aureus* (Fig. 3). The zone of inhibition against gram negative is estimated to be 30 mm for 1 mg/mL and 35 mm for 2 mg/mL concentrations which is greater than control (20 mm). For the gram positive bacteria, the zone of inhibition for 1 mg/mL concentration is 14 mm which is lesser than that of control (21 mm).

However, it increased to 40 mm for the protein at 2 mg/mL concentration indicating that antibacterial activity increases with the increase in protein concentration. This suggests that sericin is more effective towards gram positive bacteria than gram negative bacteria. This difference in their antibacterial activity may be due to their varying compositions and structures of the bacterial cell wall. Sericin exhibits their antibacterial activity by penetrating into the bacterial cells and forms anionic complexes, resulting in the loss of cell integrity (Zhao et al., 2014). Therefore, presence of thick cell wall in gram negative bacteria prevents the penetration of sericin and thus inhibitory effect is low as compared to gram positive bacteria.

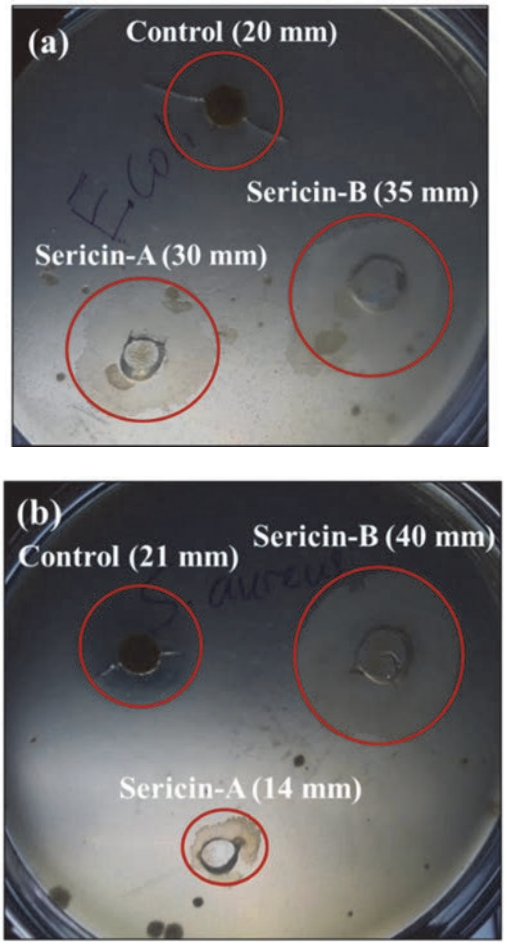

Fig. 3. Anti-bacterial activity of sericin at two concentrations sericin-A (1 mg/mL) and sericin-B (2 mg/mL) against (a) *E. coli* and (b) *S. aureus* after 24 hours of incubation, as measured by agar diffusion assay

Antioxidant activity of proteins is mainly influenced by its structure and amino acid compositions (Sangwong et al., 2016). In order to see the effect of extraction process, anti-oxidant activity of sericin was evaluated by DPPH assay. Previous studies have reported the antioxidant activity of sericin extracted from Thai silk (Prommuak et al., 2008) and few have been reported on mulberry silk. In this study, the anti-oxidant activity of sericin extracted from *B.mori* was investigated.

The change in color of the solution in the presence of sericin confirmed the quenching ability of DPPH radical and it was found to be higher than the control (Fig. 4). This confirms that the extraction process did not affect the amino acid composition of sericin and thus it exhibited free-radical scavenging activity. Sericin with short-chain peptide and low molecular weight possess greater antioxidant activity as compared to the crude protein. Therefore, in this study the lower antioxidant activity with the crude protein may due to its higher molecular weight and thus further fractioning of sericin may result in enhanced antioxidant activity.

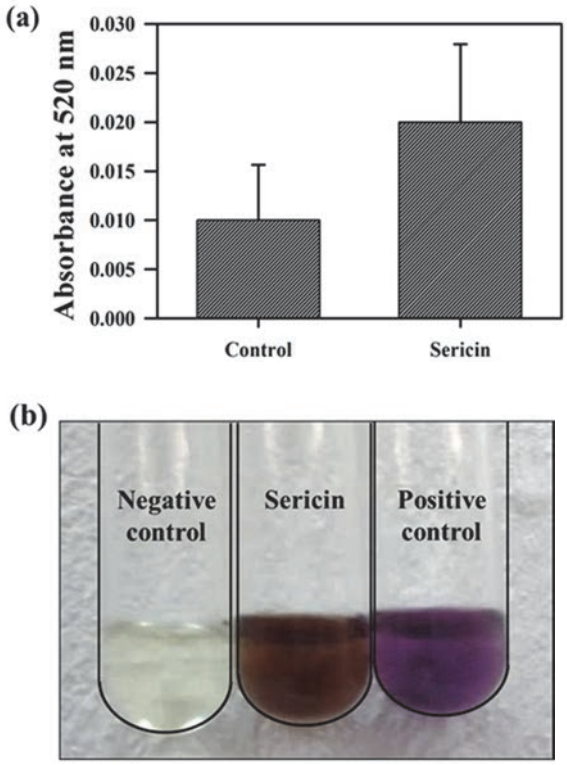

Fig. 4. Anti-oxidant activity of sericin determined by DPPH assay. The absorbance values are represented in (a) and (b) showing the photographs of color reduction due to the scavenging effect of sericin

4. Conclusions

Sericin was efficiently extracted by soap-alkaline method with an appreciable degumming ratio of 24% and the lower yield can be improved by optimizing the extraction parameters. Presence of all characteristic functional groups with the random coils and β-sheets of amide I confirms the extraction of sericin.

The protein was found to be thermally stable with a specific degradation at 210 °C. This confirms that the extraction process did not affect the physico-chemical and thermal properties of sericin. Agar diffusion assay shows the antibacterial effect of sericin with more effect on gram positive bacteria. Sericin possesses anti-oxidant property which could be further improved by its fractionation.

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