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Research Article

Preparation and Evaluation of Sericin Extracted from Sericulture Waste Water for Pharmaceutical Applications.

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ABSTRACT

The present attempt has been made to characterize extracted sericin from the waste of sericulture industry and explore its pharmaceutical applications. Sericin was extracted by alkali degumming techniques (0.5 % sodium carbonate) and evaluated for chemical composition (including protein, ash, and amino acids), physical properties, surface topography, P-XRD, thermal study (DSC), and circular dichroism (CD) analysis. The molecular weight distribution of sericin was also investigated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). The results suggested that a preliminary investigation showed that presence of proteins and amino acids in sericin and confirmed from UV, FTIR, and ^{1}H-NMR study. Interestingly, sericin particles showed slightly corrugated hollow spheres with several wrinkles. From P-XRD and DSC data, sericin is completely amorphous in nature and it has thermal stability. Sericin protein was random coil confirmation indicating no change on secondary structure while extraction process and confirmed from CD. SDS-PAGE revealed that a major fraction of sericin was distributed around 25 kDa and the minor fraction was distributed around 220 kDa. Results suggested that sericin can be a valuable excipients in the pharmaceutical industry.

KEYWORDS

Sericin, protein, molecular weight, circular dichroism.

1. INTRODUCTION

Oral drug delivery is preferred route of Silk cocoon derived by Bombyx mori silkworm constitutes chiefly two proteins, fibroin which is water-insoluble and sericin is water soluble. In cocoons, fibroin content is approximately 75% whereas sericin is 25 % of the total silk protein. Further, sericin binds the two strands of fibroin fiber and helps to form the cocoons[1]. Sericin has molecular masses ranging from 10-300 kDa[2,3]. Sericin is made up of 18 amino acids, most of which have strongly polar groups such as carboxyl, hydroxyl, and amino groups. Sericin contains high aspartic acid and serine as compared with other polar amino acids[1]. The annual production of cocoons (dry) is about 400,000 tons worldwide, from that approximately 50,000 tons of sericin is produced at the reeling mill and discarded in the wastewater[4,5] which causes environmental pollution.

Sericin has been widely used in food, cosmetics, and pharmaceutical products making, as well as manufacturing of biomaterials[6-9]. It holds unique properties such as anti-bacterial, anti-oxidant and resistant to UV light[4,10,11].

From an environmental perspective, if waste sericin is recovered and recycled, it would not only reduce the environmental pollution load but will get the value-added product for various pharmaceutical applications. In the present attempt, sericin was recovered from silk industry wastewater and further evaluated for percent yield, isoelectric pH (PI), infrared spectroscopy, surface topography, X-ray diffractogram, thermal stability by DSC, circular dichroism, and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE).

2. MATERIALS AND METHODS

Silk industry wastewater containing sericin was produced at laboratory level by exploding the silk cocoons of *Bombyx mori* with different extraction techniques. The cocoons were collected from the sericulture farm Islampur (Sangli district) Maharashtra and stored in air tight bag prior to use. All other chemicals/reagents were obtained commercially.

2.1. Extraction of sericin (Degumming) by alkali method

For alkali degraded method, chopped pieces of cocoons were immersed in a 0.5% sodium carbonate solution and boiled for 30 min. The degummed sericin solution was concentrated at 60 ^{o}C by heat vaporization (Fig.1). An aqueous sericin solution so obtained was centrifuged at 5000 rpm for 30 min and the supernatant was separated. The supernatant was filtered and purified by dialysis against distilled water for 12 hrs using 10kD molecular cutoff cellulose tubing to remove any remaining traces of salt. Purified sericin solution was converted to the powder by a spray drying (LU-222 Advanced spray dryer, Labultima, Mumbai, India) under the following set conditions: inlet temperature 100 °C, outlet temperature 50 °C, feed rate 10 mL/min and aspiration speed 60 mmHg and stored in desiccator for further use[12,13].

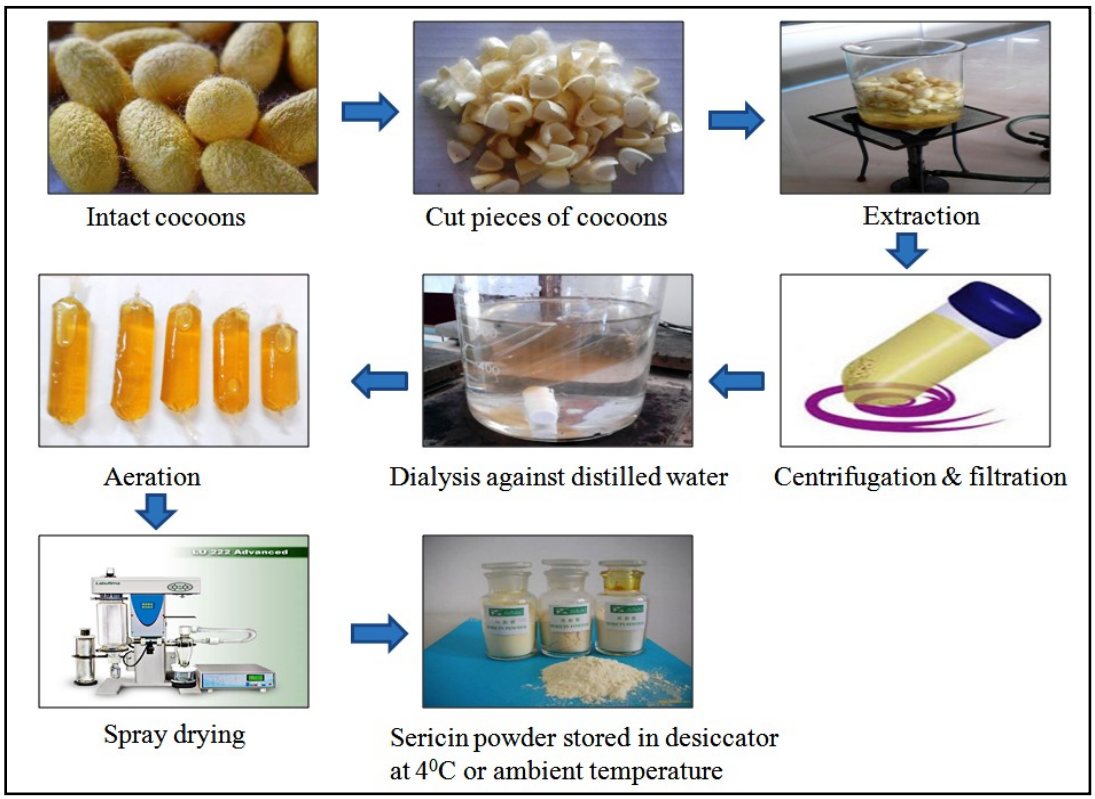

Fig.1. Overview of the extraction method for sericin.

2.2. Determination of the Percentage yield of sericin.

The percent yield of the silk sericin after degumming was calculated from Eq. 8.1,

$$\% \text{ Weight loss} = \left[\frac{W0-Wt}{W0}\right] \times 100 \text{ ----------(1)}$$ E

Where W_0 and W_t are the weight of the silk fiber samples before and after degumming, respectively.

2.3. Preliminary Chemical Tests

Separated silk sericin was subjected for preliminary tests like identification for the presence of different amino acid, protein, carbohydrates, etc. The tests like Biuret test, Ninhydrin test, Millon's test, Xanthoprotein test, Molisch test and like was carried out[14].

2.4. Solubility Studies

The solubility of sericin was carried out by a gravimetric method in which 1 gm of sericin was dispersed in different solvents like water, ethanol, acetone, chloroform, and methanol also in 0.1N solution of hydrochloric acid[13,15].

2.5. Isoelectric pH

Isoelectric pH of sericin was determined by using 1% solution of sericin in water; to this slowly added ethanol till precipitate was formed and pH of the solution was determined which is the isoelectric pH[16].

2.6. Moisture Content

Moisture content is an important parameter deciding the stability of the sericin. Methods like IR moisture balance was used for moisture determination, which gives a direct display of the weight loss related to the percentage of moisture[17].

2.7. Determination of Loss on Drying

Accurately weighed 1.5 gm of the sericin powder and transferred into a weighed flat and thin porcelain dish. Porcelain dish was dried in the oven at 100 ^{o}C and observed. The loss in weight is usually recorded as total moisture contains (bound and free water and volatile solvents17.

2.8. Moisture regains

Moisture regain is defined as the percentage of water present in a powder material of oven dry weight. Moisture regains of silk sericin, was determined by AACC method[17]. Oven drying procedure, using ambient air heated to 105 ^{o}C was adopted to record the moisture regain percentage for the given samples according to the following formula.

$$\text{Moisture regain } \% = \frac{\text{Wa} - \text{Wb}}{\text{Wb}} \times 100 - - - (2)$$

Where, Wa = weight (g) of the sericin powder before drying,

Wb = weight (g) of oven dried sericin powder.

2.9. Surface topography and Particle Size Determination

The size, shape and surface topography of the sericin was examined using Scanning Electron Microscopy (SEM) (JSM–6360; JEOL Ltd., Tokyo, Japan) at magnifications of 2000x and 5000x. Particle size was also determined by optical microscopy method using motic microscope (Motic BA210 Digital).

2.10. Nitrogen Estimation

The nitrogen content of sericin powder was determined by the Kjeldahl method[17]. Then it was converted to protein content by multiplying it by a factor of 6.25.

2.11. Nitrogen solubility Index

Nitrogen solubility index (NSI) was determined according to Adler-Nissen (18). Four gram sericin powder was dispersed in distilled water and stirred at 25^{o}C for 30 min, followed by centrifugation at 3500 rpm/min for 10 min. The supernatant was analyzed for soluble nitrogen content by the Kjeldahl procedure, and NSI was calculated according to Eq.4.

$$\text{NSI } (\%) = \left(\frac{\text{Nsoluble}}{\text{Ntotal}}\right) \times 100 \text{ ----------(3)}$$

Where, NSI is the nitrogen solubility index, $N_{soluble}$ is the soluble nitrogen, and N_{total} is the total nitrogen.

2.12. Protein determination

The concentration of proteins was determined by Lowry's method[19].

2.13. UV absorption spectra of sericin

The ultra-violet absorption spectra were obtained from UV/Vis spectrometer. About 10µg/ ml solution of sericin was prepared in water and scanned from 200- 800 nm to determine the wavelength of maximum absorbance.

2.14. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) Infrared spectroscopy of sericin was recorded using ATR-FTIR spectrophotometer (MIRACLE 10 Shimadzu IR Affinity-1 FTIR) in the region of 4000–400 cm^{-1}.

2.15. ^{1}H-Nuclear magnetic resonance (NMR)

^{1}H-NMR experiment was carried out of sericin using NMR spectrometer (Bruker) operated at 300 MHz, equipped with a 5 mm probe. Sufficient powder sample was dissolved in solvent DMSO and then used for analysis. Tetramethylsilane (TMS) was used as an internal standard. The experiment was recorded at 300 K.

*2.16. Powder X-ray diffraction (XRD***)**

Powder X-ray diffraction pattern of the sericin was obtained by using Philips PW1700 X-ray diffractometer with Cu k-a (λ = 1.540 Å) radiation and a crystal monochromator, with a voltage of 40 mV and a current of 30 A. The diffraction patterns run at 5-10° C min^{-1} terms of 2θ angles.

2.17. Differential scanning calorimetry (DSC)

Differential scanning calorimetry was conducted using a Mettler-Toledo DSC 821e instrument equipped with an intracooler (Mettler- Toledo, Greifensee, Switzerland). Indium/zinc standards were used to calibrate the DSC temperature and enthalpy scale. The samples were hermetically sealed into pierced aluminum pans and heated at a constant rate of 10 ^{o}C/min over a temperature range of 35 to 300^{o}C. Inert atmosphere was maintained by purging nitrogen gas at a flow rate of 50 mL/min.

2.18. Circular dichroism (CD) spectroscopy

Circular dichroism measurements of purified sericin were performed on a Jasco J-715 spectropolarimeter (Jasco Inc., Japan) using 1 cm path length quartz cuvette, thermostated at 25 °C. Spectra were measured from 250 to 185 nm, with five scans (at 100 nm/min), response time (0.25 s) was constant, bandwidth and slit width was 1 nm and 500 µl respectively.

Aliquots of each protein (200 µl) were added to a cuvette (1 cm path length). Spectra were background corrected, smoothed, and data were expressed in terms of θ using analysis function built into the Jasco software[20].

2.19. SDS–PAGE analysis

The SDS–PAGE analysis of sericin was performed according to Laemmli (21) with slight modification. The resolving gel was 8 % while the stacking gel was 4%, respectively. The system was run at 100 V for 2 hrs or till the die comes out of the gel in the tank buffer. Prechilled Tank buffer (SDS PAGE Lameli buffer) was used.

3. RESULTS AND DISCUSSION

Recovery of sericin was successfully and easily carried out from the silk cocoons of Bombyx mori by various techniques. The literature reported that the silk cocoons contain about 20 to 30 % of sericin protein and percentage yield was calculated on the basis of the composition of sericin in silk cocoons.

3.1. Percentage Yield

The percent yield of sericin using alkali degumming method was found to be 22.50± 2.51 %. It indicated that sericin is completely removed from silk fibroin in reeling process. It means that 75 % (w/w) yield was obtained and the powder was used for the further process.

3.2. Preliminary Chemical Investigation

Preliminary chemical investigation of sericin showed the presence of proteins and amino acids as given in Table 1. In amino acids predominantly serine, tyrosine, phenylalanine, arginine, cystine, aspartic acid, and methionine have been reported.

Table 1 Preliminary chemical tests for sericin.

Name of test	Result	Name of test	Result
1. Test for amino acids		2. Test for proteins	
a) Ninhydrin test	+	a) Biuret test	+
b) Millons test	+	b) Molisch test	+
c) Xanthoprotein test	+	c) Coagulation test	-
d) Hopkins- cole reaction	+	d) Libermanbuchardstest	-
e) Sakaguchi reaction	-	e) Salkowaski reaction	-
f) Sulphur reaction	+	f) Precipition test	+
		g) Glyoxalic acid test	-

3.3. Solubility Studies

The sericin is a protein of marked acid character so that it dissociates in water as an acid and becomes soluble. Since it is usually assumed that a protein, as an ampholyte, is least soluble at its isoelectric point, we can conclude that this point lies near pH 3.9 in the case of sericin. The solubility of sericin in various solvents is shown in Table 2. The sericin protein was found to be highly soluble in water as compared with other solvents.

Table 2 The solubility of sericin in various solvents.

Solvent	Solubility (mg/ml)
Water	112
Phosphate buffer (9-10 pH)	85
0.1N HCl pH 1.2	78
Ethanol	35
Methanol	25
Acetone	28

3.4. Isoelectric pH

The isoelectric pH of sericin was found to be 4. This matches with the finding of Oh et al., who reported isoelectric pH in the range of 3.8 - 4.4.[16].

3.6. Moisture Content

The moisture content of purified sericin powder was found to be in the range of 3-5%.

3.7. Determination of Loss on Drying

The % weight loss was found to be 2-3.5 % which indicates that the spray dried sericin absorbs the water but in a small amount.

3.8. Moisture regains

The moisture regains was found to be 9%. It indicates that sericin should be stored in airtight container.

3.9. Nitrogen Estimation

The total nitrogen content of sericin by Kjeldhal method was found to be 16.49±0.95 %. However; the pure sericin has 15-16% nitrogen content.

3.10. Nitrogen Solubility Index (NSI)

The NSI of spray dried sericin was found to be 92.69± 1.28 %. The standard NSI reported is 95.3 %. The NSI was determined to character the protein solubility of spray-dried sericin because fibroin is a water-insoluble fibrous protein, while sericin is a water-soluble globular protein. The protein composition of the spray-dried sericin recovered from the silk, accounting for about 103.06±0.95 %, with little or no contamination by fibroin. Next, the spray-dried sericin was used as materials for recovering pure sericin by removing the non protein components. This protein is determined by multiplying the Nitrogen content by 6.25.

3.11. Protein determination

The protein content of silk sericin by lowery method was determined and was found to be 1.468±0.09 mg/ml. The silk cocoon contains 30.10 % of sericin. It can be seen from these results that the main composition of sericin powder was protein, with a concentration of 97.88±0.09%.

3.12. Surface topography and Particle Size Determination

The sericin particles showed slightly corrugated hollow spheres with several wrinkles on its surface (Fig.3). The different shape produced might be due to solvent effect, especially evaporation rates. The bowl shape or corrugated hollow particles with wrinkles have a higher surface-to-volume ratio than smooth round surfaced particles and this will be significant in the enhancement of solubility and rate of dissolution. The particle size of spray dried sericin was found to be in the range of 1µm- 20µm. So the nature of particles is fine.

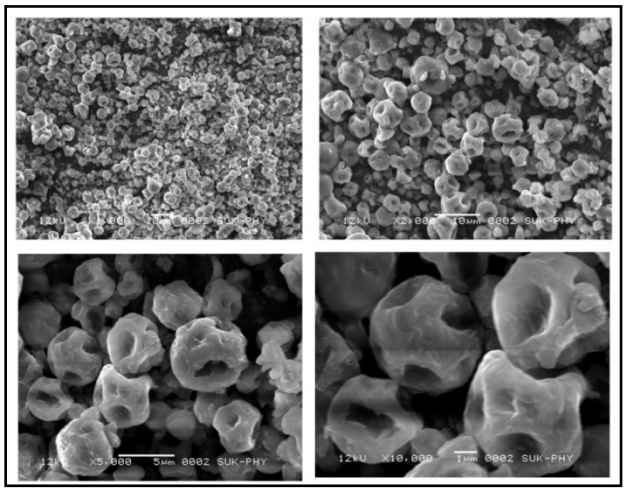

Fig. 3. Scanning electron microphotographs of sericin.

3.13. UV spectra of sericin

The sericin showed maximum absorption band at around 274.5 nm of wavelength (Fig.4.). Generally, proteins absorb near-ultraviolet region due to the electron transfer of aromatic amino acid, tryptophan, tyrosine, and phenylalanine. On the other hand, histidine absorbs at 210 nm, far-ultraviolet region (14,22). Sericin can be expected also the absorption at 280 nm, near ultraviolent region.

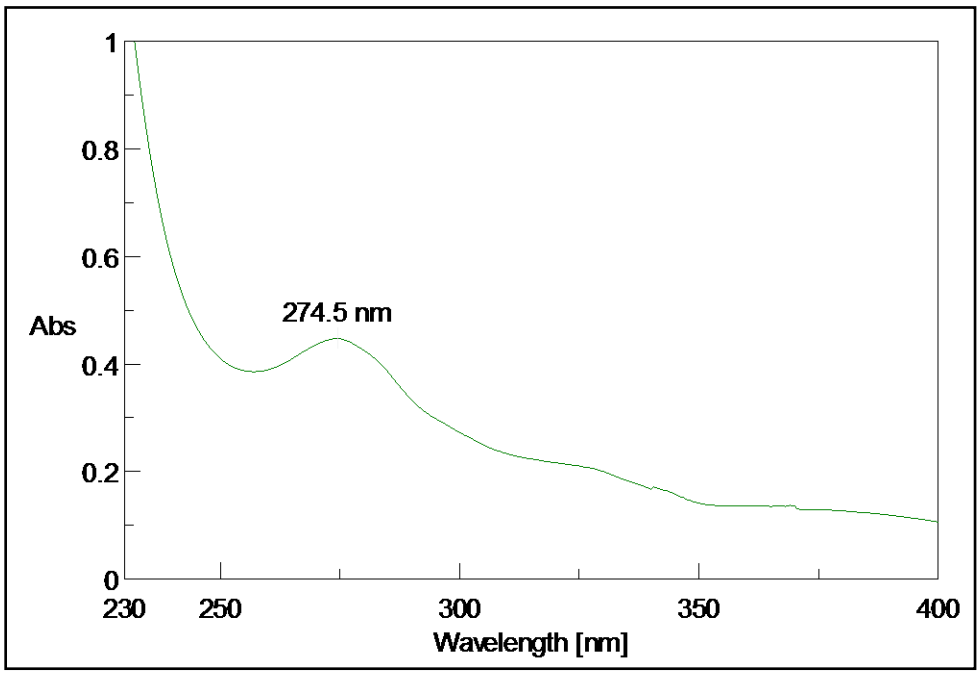

Fig. 4. UV spectra of sericin.

3.14. ATR-FTIR spectroscopy study

Silk sericin showed the characteristics absorption bands of protein, at 3360.00 cm^{-1} (N-H stretching), 2926.01 cm^{-1} (C-H stretching), 1647.21 cm^{-1} (primary amide; C=O stretching), 1523.76 cm^{-1}(secondary amide; N–H deformation and C–N stretching), 1392.61 cm^{-1}(tertiary amide; C–N stretching and N–H bending), 1247.94 cm^{-1} (C-N stretching) and 1070.70 cm^{-1} (S=O stretching) respectively and shown in Fig.5. Primary Amide absorption usually represents the C=O stretching vibration of the amide group.

Secondary amide absorption arises mainly from N-H bending and C-N stretching vibrations and tertiary amide arise due to the C-N stretching vibration coupled to the N-H in-plane bending vibration. The appearance of above peaks confirms the presence of amino acids like aspartic acid, serine, tyrosine, phenylalanine, tryptophan, arginine, and cystine etc. The absorption bands represented to C-H and O-H bending vibrations and C-OH stretching vibrations, which are also attributable to the abundance of hydroxyl amino acid side chains.

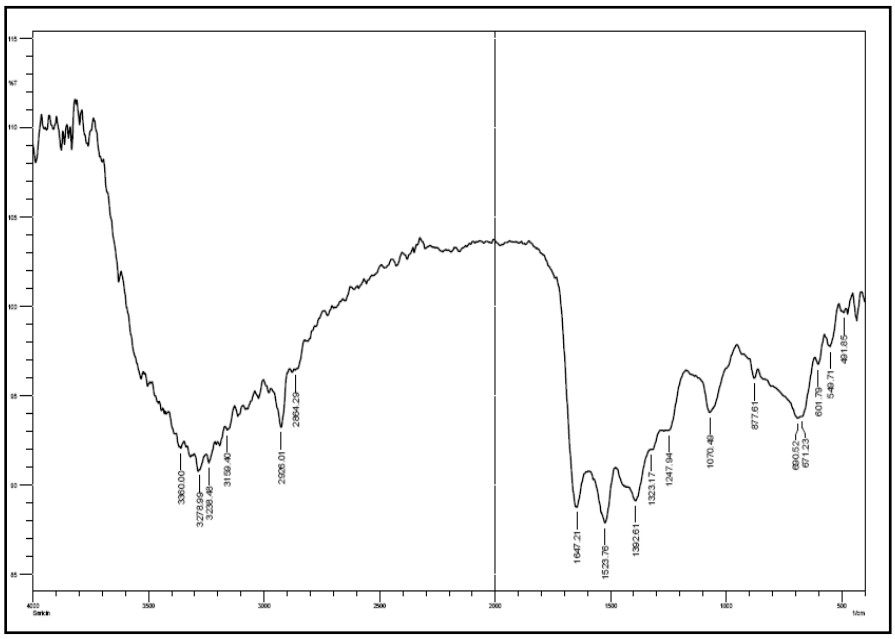

Fig. 5. ATR-FTIR Spectra of sericin

3.15. Nuclear Magnetic Resonance Study (^1H-NMR)

^{1}H-NMR spectra of sericin showed complex structural characteristics, the ^{1}H-NMR spectra, Fig. 6 showed major peaks in different regions. The peaks show the resemblance of the presence of protons of amino acids. From the ^{1}H-NMR peaks, it is predicted that there is hydrogen α to amino group which is slightly deshielded due to the presence of electronegative nitrogen atom, which appeared between 2- 2.9 ppm. Carboxylic acids are insoluble in $CDCl_3$ and thus to determine their spectra D2O is used with the small amount of sodium salt of the acid. Thus D2O proton exchange will convert the group to –COOD and the – COOH absorption near 12.0 will disappear. Thus H on the carbon next to – COOH appear in the same range that all H next to carbonyl i.e. 2.1-2.5. This confirms that sericin contains amines, carboxyl, and aromatic group protons.

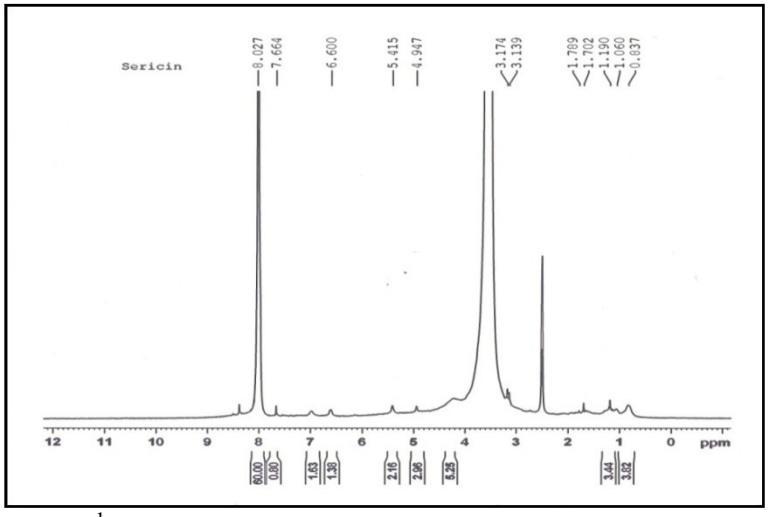

Fig. 6. ^{1}H-NMR spectra of sericin.

3.16. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry was performed to study the thermal behavior of sericin and shown in Fig.7. The first endothermic peak was found at 90 ^{o}C (lower temperature), due to evaporation of water and another broad exothermic peak was found at 280^{o}C, attributed to the thermal decomposition of sericin. This was associated with the cleavage of peptide bonds and the degradation of the side chain of amino acid residues. Former peaks signify the molecular mobility and melting attributed to decomposition induced by thermal.

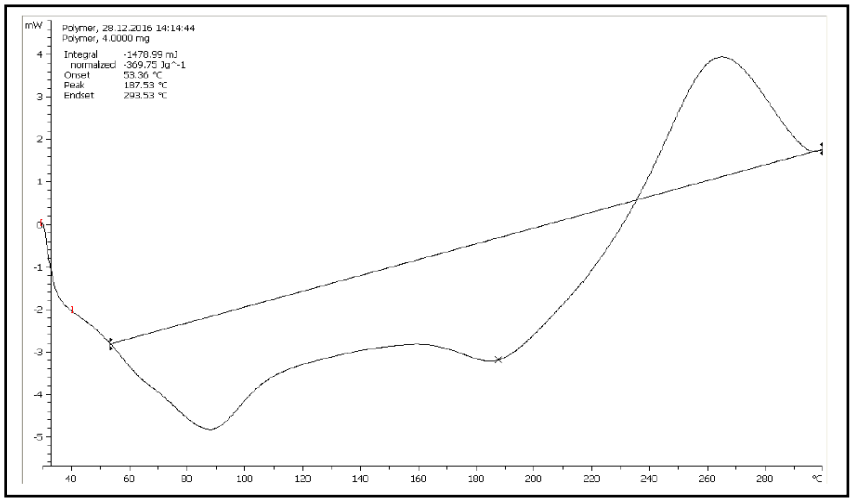

Fig. 7. DSC thermogram for sericin

3.17. Powder X-ray diffraction (PXRD) Study

PXRD spectra of sericin are depicted in Fig.8. The X-ray diffraction patterns of the sericin exhibited no peaks, it can be considered as fully amorphous in nature.

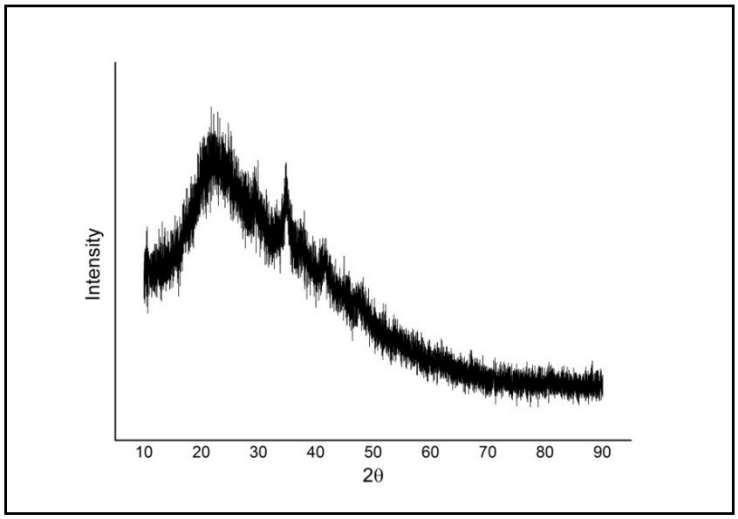

Fig. 8. PXRD diffractogram for sericin

3.18. Circular dichroism (CD) spectroscopy

The CD spectrum of sericin was interpreted in order to determine the conformation change during degumming process. The CD curve of sericin (Fig.9) showed the strong negative bands at

199 nm indicating the random coil conformation and weak negative bands at 218 nm indicating the β-structure. The possible conformations of sericin (molar %) were calculated using analysis function built into the Jasco-810 spectropolarimeter and the results showed 56.8% random coils, 43.2% β- sheets, but sericin powder had no β-turn and α-helix conformation. Hence the major confirmation of sericin protein was random coil confirmation indicating no change on secondary structure of sericin while extraction process.

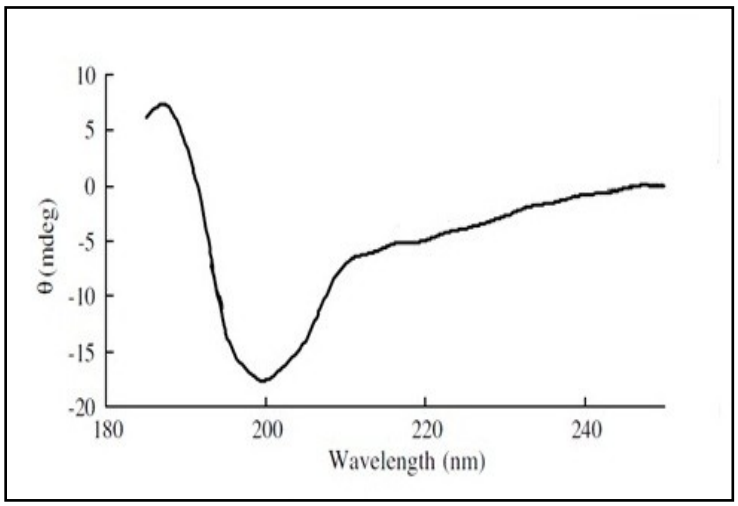

Fig. 9. CD spectra of sericin

3.19. SDS–PAGE analysis

The result indicates that the major fraction of sericin was distributed around 25 kDa (low molecular weight fraction) and the minor fraction was distributed around 220 kDa. SDS-PAGE analysis revealed a smear pattern (Fig.10) which strongly suggests that the polypeptides in sericin were degraded during the extraction process.

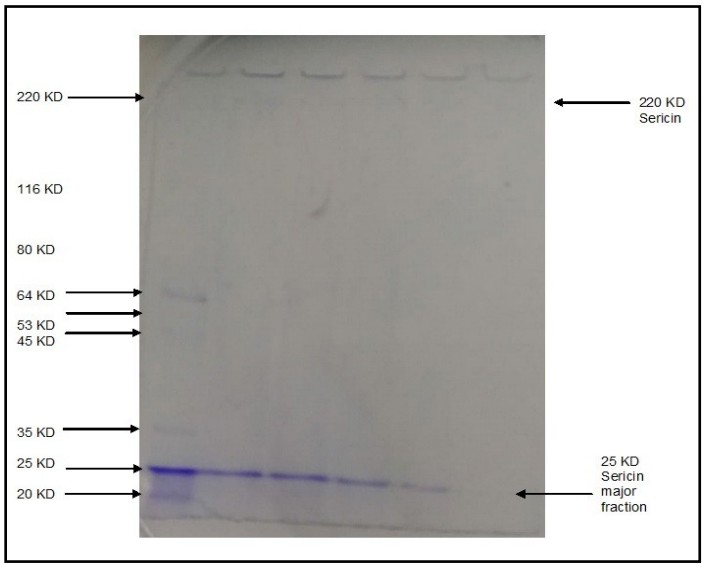

Fig.10. SDS PAGE analysis of sericin.

4. CONCLUSION

Sericin was successfully extracted from waste of sericulture industry using alkali degumming method. The UV, FTIR and ^{1}H-NMR data suggested that sericin contains proteins and amino acids mostly serine, aspartic acid, tyrosine, phenylalanine etc. The sericin was completely amorphous in nature and it has thermal stability. Further, random coil confirmation was observed in sericin protein indicating no change in the secondary structure during extraction. SDS-PAGE revealed that major fraction of sericin was distributed around 25 kDa whereas minor fraction was distributed around 220 kDa. Conclusively, sericin can be used in various pharmaceutical formulations.

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