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Extraction & characterization of sericin and its immobilization on hydroxylapatite nanoparticles for tissue engineering applications

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Abstract: Silk sericin is a natural macromolecular protein derived from silkworm, Bombyxmori. It is produced as waste from silk processing industry and has a wide range of applicationsin medicine, cosmetics, textiles and food industry. Due to wound healing property, anti-oxidant & and anti-microbial activity and due to gelling nature, sericin has been explored for tissue engineering applications.Hydroxyapatite (HA), a synthetic calcium phosphate, is chemically similar to bone Calcium Apatite.Due to its bioactive and biosorbable nature with long resident time, HA is the most suitable material for bone tissue engineering. Hydroxylapatite functionalized with collagen like proteins mimic the invivo environment conducive for bone formation. Hydroxylapatite-sericin conjugates are proven to have more controlled degradation and extended resident time in vivo and hence are suitable for in vivo applications.

In the present study different methods of extraction of sericin from silk fiber have been carried out (high temperature and high pressure, sodium hydroxide and sodium carbonate). The extracted protein was recovered by salting out using ammonium sulphate/ calcium chloride and the yield was assessed.The molecular weight range of the extracted protein was determined in SDS PAGE. Anti-bacterial (Bacillus subtilis, Pseudomonas, Bacillus licheniforms, Bacillus cereus, Aeromonasschubertii, Aeromonashydrophila,) and anti-fungal (Aspergillusflavus and Trichodermaharyanum) activities of all the preparations were tested.Nano particles of hydroxyapatite prepared by solution combustion method were used for adsorption of sericin.Hydroxylapatite-sericinnanoconjugates were prepared in Simulated Body Fluid (SBF) solution and were characterized. Sericin–hydroxyapatite conjugates may have potential tissue engineering applications.

Key words: Sericin, Sericin – hydroxyapatite conjugate,Biomaterial, anti-microbial activity.

1.Introduction

Silk worm silk is composed majorly of two proteins, Fibroin and Sericin. Constituting about 20-30% of the cocoon, sericinis adhesive in nature and helps in formation of cocoon by binding fibroin strands together. Sericin is a waste generated by silk industry with worldwideyield ranging up to 50,000 tones. It is a globular protein with high content of aspartic acid and serine, has high moisture retaining capacity and can gel under controlled conditions. It is has anti-oxidant[1],anti-microbial, wound healing properties,UV resistant[2] biocompatible and biodegradable. Due to these properties sericin has wide range of applications in textile, pharmaceutical and cosmetic industries [3]. Sericin can act as bio-adsorbent for removal of dyes from polluted water.

Hydroxyapatite is naturally occurring mineral form of calcium apatite found in bone, teeth and tendons. It can be synthesized in several forms including matrices with varying porosity[4]. HA is biocompatible, osteoconductive, osteogenic and does not elicit immune response. It has good mechanical properties and has low biodegradability. So far several clinical and experimental studies have proved the utility of HA in bone tissue engineering and dental repair.As the natural bone is made of collagen and hydroxyapatite, HA functionalized with proteins / peptides has been proven to be valuable due to improved biological response in terms of cell adhesion, cellular infiltration and vascularization[5], HA-sericinstimulates cell migration[6].

2.Materials and Methods:

2.1 Method of extraction of sericin:

2.1.1. Degumming of raw silk fibers: 5 g of silk was taken 100 ml of distilled water and autoclaved for 30 min/ 45 min/ 60 min at 121°C, 13 lb. alternately, degumming was carried out by boiling for 1 hr with or without 0.5% sodium carbonate/ sodium hydroxide. The degummed water, thus obtained, was filtered and taken for precipitation of sericin. Loss of weight of the raw silk fibers was calculated. The percentage yield was calculated using the formula, (initial wt- final wt)/(initial wt) *100.

2.1.2 Lyophilization: The degummed water was sealed in dialysis tubing and volume was reduced by keeping overnight in dextrose at room temperature. The solution was then dialysed against 10mM Tris Buffer (pH7.4) with several changes at interval of 2hrs. The protein solution was lyophilized and stored at -20°C.

2.2. Precipitation of sericin:

2.2a Ammonium sulfate precipitation: 1.5gm of Ammonium sulfate was added to10 ml of degummed water with continuous stirring. The mixture was left on ice for 30 minutes followed by centrifugation at 8,000 g at 4°C for 10mins. The pellet formed was washed with 95% ethanol, dried and stored at -20°C.

2.2b. TCA precipitation: The degummed water was mixed with TCA stock (500g of TCA in 350 ml distilled water) in 1:4 ratio (TCA stock: protein sample) and centrifuged at 8000 g for 10 min. The pellet was washed thrice with ice cold acetone and dried.

2.2c. Calcium chloride Precipitation: 10 ml of degummed water was addedto varying volumes of 1M calcium chloride (0.5 ml, 1.0 ml, 1.5 ml and **2.0** ml) and the total volume was adjusted to12 ml with distilled water. The solution was left on ice for 30 min and was centrifuged at 8000 g for 10 min.

2.3 Protein estimation: To calculate the total yield of all the performed by Lowry method [8]. For standard curve, various amounts of BSA was taken (10, 20, 40, 60, 80, 100µg) in a total volume of 250 µl of distilled water, incubated for 10 minutes with alkaline copper sulphate Solution and 0.125ml of 1:1 diluted Folin-Ciocalteu reagent was added and incubated for 30 min in dark at room temperature. OD was read at 750nm and graph was plotted.

2.4. SDS PAGE: all the protein preparations were analyzed in SDS PAGE by the method of laemmelli (Laemmlietal, 1970). Each well was loaded with 1 µg protein and electrophoresis was conducted at 100 volts. The gels were stained overnight with 0.5 % coomassie brilliant blue dissolved in methanol: acetic acid: water in 45:10:45 ratio. Destaining was done using staining solution minus the stain.

2.5 Anti-bacterial/anti-fungal activity of sericin was tested against the MTCC cultures, IMTECH and pseudomonas.

2413 Bacillus subtilis
429 Bacillus licheniforms
6629 Bacillus cereus
7153 Aeromonasschubertii
1739 Aeromonashydrophila
9952 Aspergillusflavus
3928 Trichodermaharyanum

Antibacterial activity of sericin: Bacterial lawn was made on 1.5% nutrient agar by spread plate method (log cultures developed from single colony) of each specific strain. Autoclaved filter paper discs were placed on the lawn and 10 µl of each of the sample was loaded. The cultures were incubated over night.

For testing the antifungal activity, MRBA agar plates with varying concentrations of sericin (1-2%) were prepared and fungus was inoculated at the center. The diameter of the fungal grown was monitored.

2.6. Preparation of hydroxyapatite

Hydroxyapatite powder was prepared by solution combustion method using calcium nitrate [Ca(NO3)2.4H2O] and diammonium hydrogen phosphate [(NH4)2HPO4] as the starting precursors for Ca and P, where calcium nitrate acts as the oxidizer. Glycine was used as organic fuel which, act as the reducer. All the chemicals used were from Merck specialities Pvt. Ltd. Mumbai, India. Calcium nitrate and diammonium hydrogen phosphate were taken in the proportion such that to give Ca to P ratio of 1.67 whereas glycine was used in stoicheometric proportion with respect to the oxidizer. The white powder mass resulted from combustion reaction was characterized by X-ray diffraction (XRD) and scanning electron microscope (SEM).

2.7.Preparation of Sericin-Hydroxyl apatite nanoconjugates:

Sericin was prepared in simulated body fluid (SBF) in varying amounts (62.5 µg in 150 µl) and was reacted with 10 mg of hydroxyl apatite (at pH 6.5, 7.25 and 8.0) for seven daysunder aseptic conditions,onplatform shaker at room temperature. The HA-sericin particles were washed three times with SBF and stored at ice cold temperature. These conjugates were tested for presence of protein as follows: 10 µl of the HA-sericin particle suspension was taken for protein estimation by Lowry method[7]. The contents of the assay were centrifuged and supernatant was used for reading OD at 660 nm**.**

3. Results and Discussion:

3. 1. Extraction of sericin:Extraction by boiling in presence of 0.5% of sodium carbonate (13.4%) and sodium hydroxide (6.6%) yielded more protein than boiling distilled water alone (4.4%), as calculated by loss of weight of the raw silk.

3.1.1 Effect of dialysis on the sample yield as estimated by Lowry method: Dialysis reduced the yield of extracted sample. This reduction is more in case of NaOH extraction. This may be due to generation of lower molecular weight sericinwhich is lost during dialysis(table 1).

Table 1

Sl.No	**Method of extraction of sample**	**Concentrationof protein estimated by Lowry method**
1	Lyophilized without NaOH	0.24mg/25µl
2	Lyophilized with NaOH	0.36 mg /25µl
3	Dialyzed without NaOH	0.152 mg /25µl
4	Dialyzed with NaOH	0.084 mg /25µl

3.1.2: Effect of time and extraction method on yield: The yield of high temperature extraction in the presence or absence Na_2CO_3 (0.5%). The sample was dialyzed and protein was estimated by Lowry method (figure 1).As can be deduced from the figure 1, Presence of 0.5 % Na_2CO_3 enhanced yield.

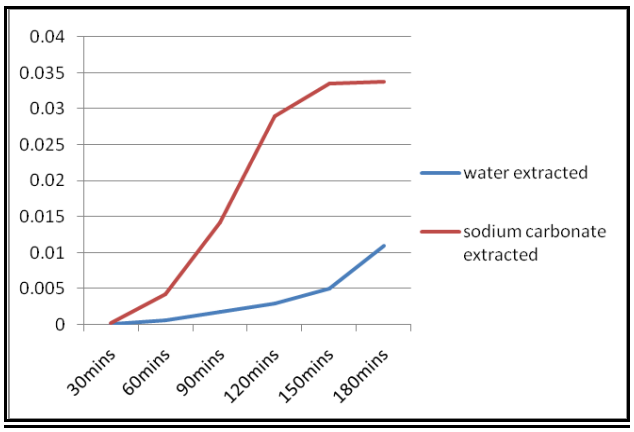


Figure 1.Extraction of sericin:Effect of time and extraction method on yield

3.1.3: Effect of time of autoclaving on yield: Increased time resulted higher yield.With prolongation of time enhanced yield was obtained (fig 2).

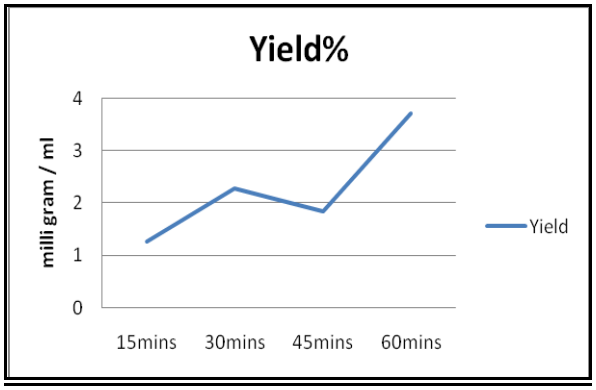


Figure 2 Effect of prolonged time on extraction by autoclving

3.2 analysis of molecular weight of Extracted sericin in SDS PAGE: The band appeared as a smear mostly ranging from 16 to 44 kda (figure 3). In the extraction process sericin does not come out as monomer. Hence smear of protein is seen in SDS PAGE.

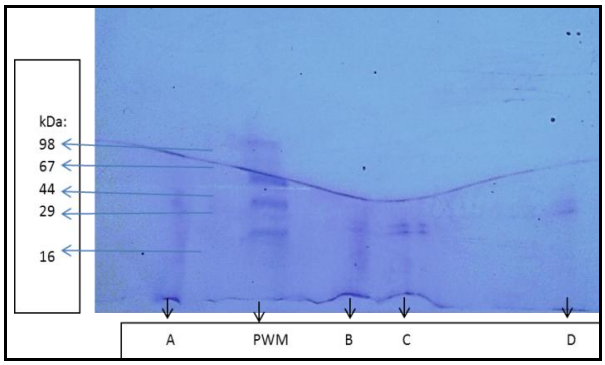


Figure 3: SDS PAGE analysis of the sericin extracted

Sample A- Extracted by boiling in distilled water, Mol wt range 16-67kDa
Sample B- Extracted by autoclaving, Mol wt range 16-44kDa
Sample C & D- Extracted in NaOH and ammonium sulphateppt, Molwt range-16-44kDa

3.3 Effect of sericin on microbial growth: Growth inhibitory property of sericin was tested on three Bacillussps**(subtilis/ cereus/ licheniforms)**and two Aeromonassps **(A. hydrophila (MTCC 1739) andA. schubertii),**as well as on two fungal sps (AspergillusflavusandTrichodermaharyanum).

3.3.1Effect of sericin on growth of Bacillussps (subtilis/ cereus/ licheniforms): The results show that sericin inhibits all three sps of bacillus. However, samples extracted in 0.5% NaOH and TCA precipitated samples have not shown any inhibition.

Figure 4: Bacillus subtilis Bacillus cereus Bacillus licheniforms

Sample Ammonium Sulfate Precipitated Water Boiled Samples	**Zone of Inhibition**
A- (15 min water boiled sample)	Positive
B- (30 min water boiled sample)	Positive
C- (45 min water boiled sample)	Positive
D- (60 min water boiled sample)	Positive
E-Dialyzed sample of the high temperature, 5%NaOH extracted	Negative
F-TCA precipitated from degummed water	Negative

3.3.2Effect of sericin on the growth ofAeromonashydrophila(MTCC 1739) andAeromonasschubertii, (MTCC 7153): The results show that sericin inhibits both sps. However, as in the case of Bacillus sps, samples extracted in 0.5% NaOH and TCA precipitated samples have not shown any inhibition

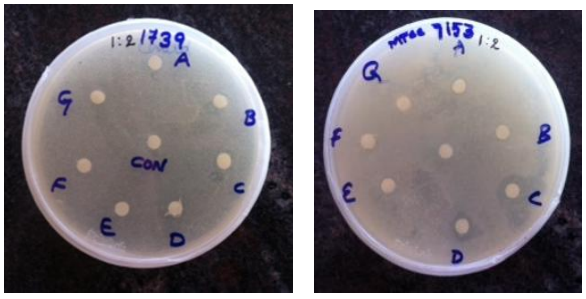


Fig 5 AeromonashydrophilaAeromonasschubertii

Sample Ammonium Sulfate Precipitated Water Boiled Samples	**Zone of Inhibition**
A- (15 min water boiled sample)	Positive
B- (30 min water boiled sample)	Positive
C- (45 min water boiled sample)	Positive
D- (60 min water boiled sample)	Positive
E-Dialyzed sample of the high temperature, 5%NaOH extracted	Negative
F-TCA precipitated from degummed water	Negative

3.4.6 Effect of sericin on the growth offungal sps:

MRBA medium was incorporated with sericin in varying dilutions (1%,to 2%)and fungus was inoculated in the center of the petriplate. Diameter of the growth was calculated on daily basis (Aspergillus flavus and Trichoderma haryanum).

No inhibition of growth observed for both the species tested (fig. 6 & 8). In contrast, growth was slightly promoted as represented in fig.7 and fig. 9. This may due to proteinaceous nature of sericin which might have been digested and utilized by the fungus.

Figure 6: Aspergillusflavus: No growth inhibition in presence of Sericin

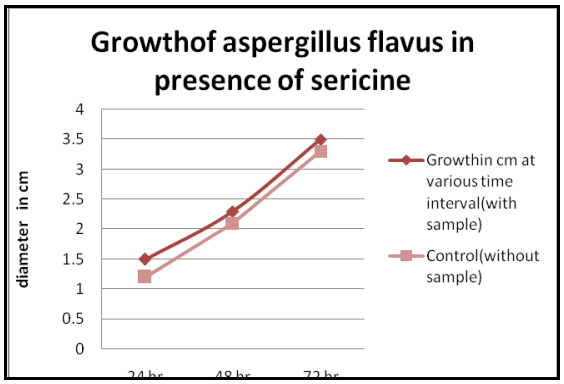


Figure 7: Effect of sericin (1%) on the Growth of A. flavus

Figure 8: Trichodermaharyanum grown in presence of sericin: No growth inhibition seen

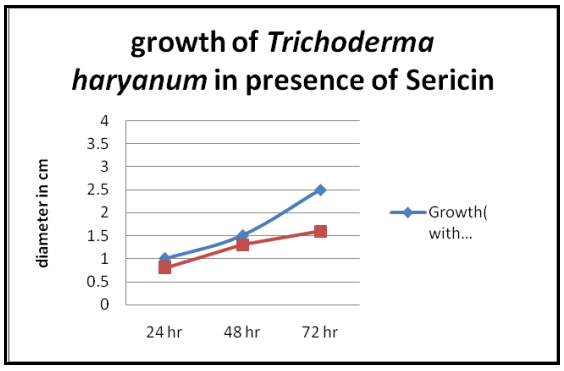


Figure 9:Trichodermaharyanum showing slightly higher growth in presence of 1 % sericin

3.5 The X-ray diffraction pattern of the Hydroxyl apatite nanoparticles:

The X-ray diffraction pattern of the solution combustion synthesized powder is shown in Fig.10. From the XRD spectra it is found that as prepared powder is basically pure HA with all the major peaks matching to the standard JCPDS (9-432) values corresponding to HA.

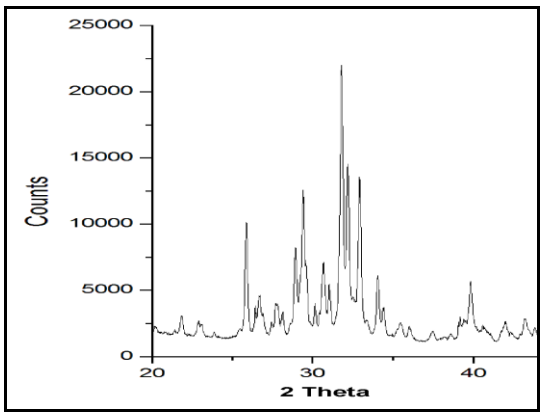


Fig.10 XRD spectra of HA synthesized by solution combustion method

The particle /agglomerate size analysis of these powders show that the particle size is in the range of 1µm to about 10µm. On the other hand the scanning electron microscope analysis of the as prepared HA powder shown in Fig. 11 show that the size of the primary particles is in the range of 100 to 150nm.

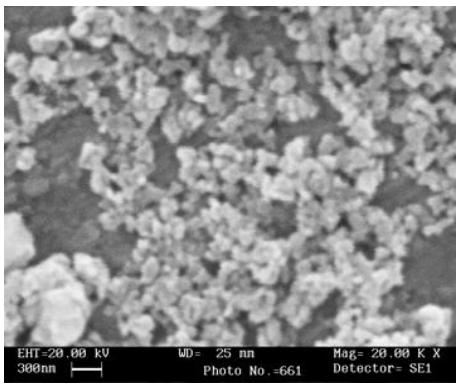
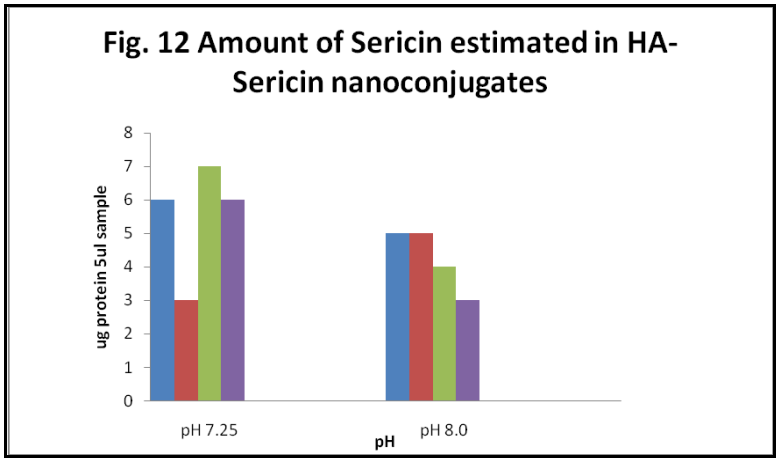


Fig. 11 Scanning Electron micrograph of HA powder at 20000X magnification



3.6. Hydroxyl apatite- sericinnano-conjugates were prepared in simulated body fluid. The conjugates were tested for the presence of protein in Lowry method. The amount of protein present in 5 µl of the conjugates was as given in the fig 12. Increasing the pH to 8.0 has slightly decreased the amount of protein bound to HA.Further characterization of these conjugates need to be carried out.

Conclusion:

Sericin is produced in large quantities worldwide as industrial waste and utilization of this protein is highly recommended for preventing environmental pollution. Due to its cell adhesion and growth promoting activity, sericin conjugated to Hydroxyl apatite is expected to enhance osteoconductive property of Hydroxyapatite and increase its resident time.

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