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Extraction of sericin protein from *Bombyx mori* L. cocoon (Race Nistari)

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Abstract
Sericin is a glue protein synthesized in the middle silk gland of the silkworm *Bombyx mori* L. Sericin exhibits several valuable biological and physicochemical properties which may vary according to the method extraction. The aim of the present study is to find out suitable extraction protocol for extraction of sericin from Nistari cocoon (Traditional race) and to analyze the molecular mass of the same by SDS-PAGE. In the present study, sericin is extracted employing different concentrations of urea, NaOH, Na_2CO_3, Triton X-100, LiBr, $CaCl_2$ and with different buffers. Among these, the highest yield was obtained when sericin was extracted with Sodium hydroxide solution followed by Sodium carbonate solution. However, the extract when run on a SDS-PAGE exhibited smears, indicated degradation of the sericin. Treatment with urea and Triton X-100 were found to be most effective in comparison in terms of both quality and quantity. SDS-PAGE profile of sericin extracted with 6 M urea and Triton X-100 exhibited clear and distinguishable bands within the ranges of 10 to > 225 kDa and 25-75 kDa respectively.

Keywords: Sericin protein, *Bombyx mori* L., Nistari

Introduction
Silk obtained from *Bombyx mori* L. is made of two proteins *i.e* Fibroin and Sericin. Fibroin is fibrous in nature which is secreted as delicate twin thread (Aramwit *et al.*, 2010) [1] that binds together to sericin protein ensuring the cohesion of the cocoon. Sericin is highly hydrophilic in nature and its molecular size ranges from 20 to 400 kDa (Kunz *et al.*, 2016) [3]. Sericin is insoluble in cold water and soluble in hot water. During the silk processing and manufacturing, silk sericin is removed by treating with different salts and chemicals which often lead to loss of valuable properties of sericin (Zhang *et al.*, 2002) [13]. The successful recovery of sericin from the waste effluents of silk industry without losing its biochemical properties is onerous task.

In the recent year, the physiochemical properties of sericin has been explored extensively, which makes it attractive across varied disciplines in the field of biomedicines, cosmetics, polymers and also in food industry. Sericin was also effectively used in tissue engineering as it own cryoprotectant properties that could replace the Fetal Bovine Serum albumin (FBS) (Kunz *et al.*, 2016; Liu *et al.*, 2022) [3, 4]. Apart from that high molecular weight sericin was successfully used as natural source for the development of biological membranes and biofilms (Silva *et al.*, 2012) [8]. In addition to that sericin exhibits numerous valuable properties such as antioxidation, UV resistance, moisture absorption and biocompatibility which make it worthy for cosmetic industries (Zhang *et al.*, 2002) [13].

The valuable aspect of silk sericin is molecular weight, which is highly influenced by the process employed during its extraction *i.e* chemicals, buffers and temperature and treatment duration. In traditional degumming process, sericin is removed by soap and soda solution by heating at 95 °C for about an hour which reduces the natural molecular weight of sericin (Silva *et al.*, 2012) [8]. The enzyme assisted extraction could be an alternative way to extract sericin from silk cocoon but again it is an expensive process and promotes the specific proteolytic hydrolysis of the primary sericin chain that leads to yield of low molecular weight sericin (Gimenes *et al.*, 2014) [2].

In the present investigation, Nistari cocoons are selected as a source of extraction of sericin. Nistari is an Indian traditional multivoltine silkworm race, which is very popular and reared all along the Indo-Gangetic plains of Bengal for more than 100 years. This race can be reared throughout the year as it is tolerant to adverse climatic conditions such as high temperature and humidity.

Till date there was no report of extraction and characterization of sericin from cocoons of Nistari race. Thus, in the present investigation an effort was made by using various protocols to isolate sericin from *Bombyx mori* L. (Nistari cocoons).

Materials and Methods

Sericin was extracted through different concentrations of six different chemicals (Urea, Triton X100, NaOH, Na_2CO_3, $CaCl_2$ and LiBr). Initially, fresh and deflossed cocoons of Nistari race were collected and cocoon shells were cut into small square sized pieces (1 cm^2). Then 5 ml of different concentrations of chemicals [Urea (1 M, 2 M, 4 M, 6 M and 8 M), Triton X 100 (0.5%, 1.0%, 1.5%, 2.0% and 2.5%), Sodium hydroxide (25 mM, 50 mM, 100 mM, 200 mM, 300 mM, 500 mM), Sodium carbonate (25 mM, 50 mM, 75mM, 100mM, 150mM, 200mM), Calcium chloride (25 mM, 50 mM, 100 mM, 200 mM, 300 mM, 500 mM), Lithium bromide (0.25 M, 0.50 M, 1.00 M, 2.00 M, 4.00 M, 6.00 M)] were added separately to 0.5 g of cocoon pieces and grinded grind in a mortar and pestle.

Sericin was also extracted with distilled water, where 0.5 g of cocoon pieces were dispensed and heated at different temperatures *i.e.* room temperature, 40 °C, 60 °C, 80 °C and 100 °C and incubated for 30 minutes. For precipitation of sericin, three volumes of ice cold acetone were added to the supernatants of each treatment separately and incubated at 4 °C for 2 hours to precipitate sericin. After incubation the mixture was centrifuged at 10000 rpm for 10 min at 4°C and the pellet were collected. The protein extracts obtained across different treatments was quantified by Bradford assay. All the sericin extracts were analyzed under 10% resolving gel with 5% stacking gel.

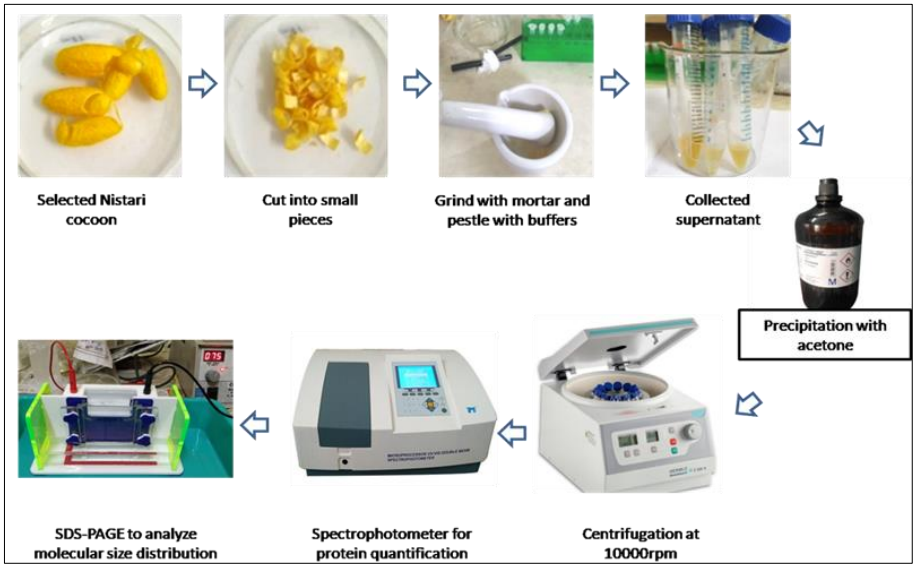

Fig 1: Diagrammatic representation of whole experimental setup for sericin extraction

Results and Discussion

Sericin extracted with water exposed to different temperatures shows an increase in concentration with a surge in temperature, which indicates that the solubility of sericin increases with an increment in temperature (Table 1). SDS page analysis of the water extracted sericin with different treatments exhibited a multiple banding patterns with five major common bands (200 kD, ~70 kD, ~30 kD and 15 kD). Where a smear was observed in the protein extracted in water at 100 °C indicating the degradation of the protein sericin (figure 2A). This shows that the solubility and degradation of the sericin in water increases with the increase in temperature. The results obtained in the present study corroborates with the findings that were reported in the previous study (Gimenes *et al.*, 2014) [2], who also stated that multiple banding pattern and sizes ranging from 20 to 200 kD on a 10% SDS PAGE.

Sericin extracted with different concentration of urea depicted increase yield of sericin with the increase in concentration of the urea. Urea is an organic compound known to be protein denaturant that disrupts the non-covalent interactions in the proteins, thereby increasing the solubility of the same. The SDS PAGE analysis of the proteins extracted with different concentrations of urea exhibited a multiple banding pattern (figure 2B). Proteins extracted with 6 M and 8 M of urea produced 4 major bands ranging from 15 kD to 200 kD (200 kD, ~70 kD, ~50 kD and ~25 kD). Where the sericin extracted with 4M, 2M & 1M shows 4 major bands above 25 kDa (*viz*., 70 kDa, 35 kDa, 30 kDa, 25 kDa) respectively. Where similar type of report was also observed in the previous study (Aramwit *et al.*, 2010) [1] in the extracted sericin with different concentration of urea and found to be in the range of 10 kDa to 225 kDa.

The yield of sericin extracted with 1%, 1.5%, 2%, 2.5% Triton X100 have no significant differences. Therefore it can be ascertained that 1% triton X100 solution is efficient enough to extract maximum amount of sericin protein from Nistari cocoon. Triton X100 is a non-ionic detergent generally used for protein extraction in the laboratories. SDS PAGE profile of Sericin obtained through different concentrations of triton X100 displayed a similar kind of banding pattern of sizes around ~75 kDa, 65 kDa and 25 kDa in concentration 1%, 1.5%, 2% and 2.5% (Figure 2C). This shows that the triton X 100 produces better result in terms of both quality and quantity.

The quantity of sericin extracted with different concentration of NaOH and Na_2CO_3 shows that the yield of extracted sericin increases with the increase of salt concentrations. Sodium hydroxide and Sodium carbonate both are strong base (alkali metal) generally used as a denaturing agent as it hydrolyses proteins. Sodium salts were mostly used in the silk industry

for the purpose of degumming of the silk. SDS-PAGE analysis shows the degradation of sericin with smear under both the sodium salt solution (Figure 3A and 3B). Similar kind of results were observed in the previous study (Srinivas *et al.*, 2015) [9] which reveals that sericin protein gets easily degraded in presence of Sodium salts or alkali solution. However, Sodium carbonate was found to be effective for extraction of 200 kDa sericin from *Anthereae mylitta* (Yun *et al.*, 2013) [12]. Extraction of sericin with calcium chloride also shows similar kind of result and_SDS-PAGE profile with an smear smear for all the concentrations (figure 4A) indicating that the sericin is highly degradable in Calcium chloride solution. However, less than 5mM concentration was not performed in the present investigation. There is possibility to obtain quality sericin protein with lower concentration less than the 5 mM. Similar kind of reports was also observed in the previous study as higher amount of silk sericin recovered with 10% $CaCl_2$ solution but the quality of the protein was found degraded (Yang *et al.*, 2014) [11]. The quantification of sericin extracted with different concentration of LiBr showed no significant differences (Table 1). SDS-PAGE analysis of Sericin extracted with different concentrations of LiBr shows multiple banding patterns ranges from 23 kDa to 48 kDa (Figure 4B).

Till date a number of protocols have been described by research enthusiasts across the world to extract and characterize the sericin protein from the cocoons of different silkworm races. In the present investigation, an attempt was made to extract sericin from Nistari cocoons using different chemicals, salts and detergents.

Previous studies also shows that the molecular size distribution of sericin depends primarily on the source of raw material (cocoons) and the extraction procedure employed (Takasu *et al.*, 2002; Aramwit *et al.*, 2010; Yun *et al.*,2013) [10, 1, 12]. Previously it was reported that sericin extracted with saturated aqueous lithium thiocyanate and beta mercaptoethanol solution with ethanol precipitation comprises three major polypeptides, with molecular weights of 400, 250 and 150 kDa as determined through SDS-PAGE (Takasu *et al.*, 2002) [10]. Crude Sericin powder extracted employing high temperature through autoclaving, followed by the lyopholization exhibits wide range of banding pattern ranges from 10 kDa to 100 kDa under SDS-PAGE, which affirms that sericin degrades at higher temperature (Rocha *et al.*, 2017; Manjubala *et al.*, 2020) [6, 5]. In the previous study, sericin extracted by employing high temperature and high pressure treatment with sodium hydroxide and sodium carbonate appeared smears under SDS PAGE (Srinivas *et al.*, 2015) [9]. In the previous works, sericin was extracted predominantly with sodium carbonate and through autoclaving (Silva *et al.*, 2014; Gimenes *et al.*, 2014) [7, 2] which results in production of degraded proteins.

Table 1: Quantity of protein extracted from 500 mg of cocoon by different chemical treatments

Chemicals used for sericin extraction	Concentration of the chemicals used	Protein/0.5g of cocoon
Urea	1 M	0.710 mg
	2 M	0.750 mg
	4 M	0.870 mg
	6 M	0.950 mg
	8 M	1.050 mg
Triton X 100	0.5%	3.901 mg
	1.0%	4.821 mg
	1.5%	4.753 mg
	2.0%	4.755 mg
	2.5%	5.120 mg
Sodium hydroxide	25 mM	1.550 mg
	50 mM	2.000 mg
	100 mM	2.625 mg
	200 mM	2.825 mg
	300 mM	2.875 mg
	500 mM	3.350 mg
Sodium carbonate	25 mM	1.126 mg
	50 mM	1.851 mg
	75 mM	1.870 mg
	100 mM	1.925 mg
	150 mM	2.370 mg
	200 mM	2.251 mg
Calcium chloride	25 mM	0.950 mg
	50 mM	1.001 mg
	100 mM	1.201 mg
	200 mM	1.250 mg
	300 mM	1.102 mg
	500 mM	1.351 mg
Lithium bromide	0.25 M	1.051 mg
	0.50 M	1.050 mg
	1.00 M	1.253 mg
	2.00 M	1.201 mg
	4.00 M	1.350 mg
	6.00 M	1.457 mg

M- Molar, mM- Milli Molar, mg- Milligram, %- percent, g- gram

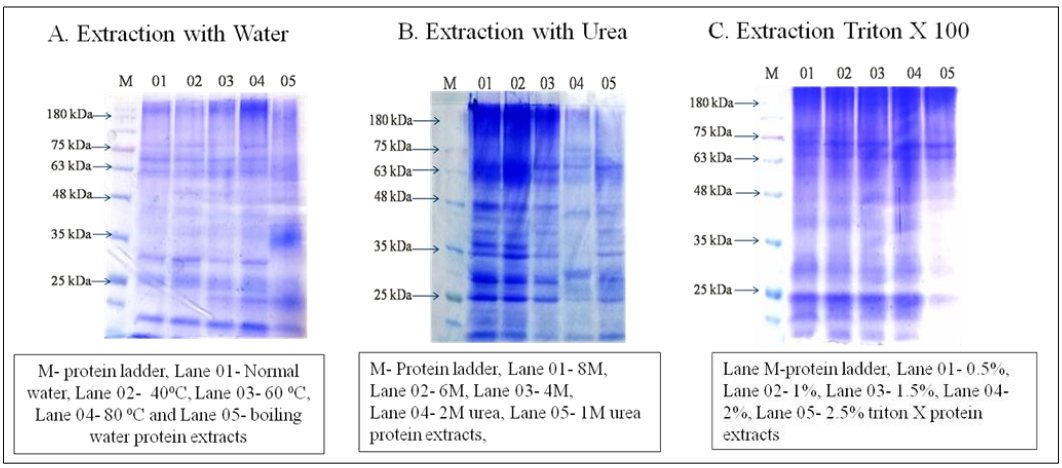

Fig 2: SDS PAGE analysis of sericin extracted with water at different temperatures, Urea and TritonX100 with different concentration from Nistari cocoons

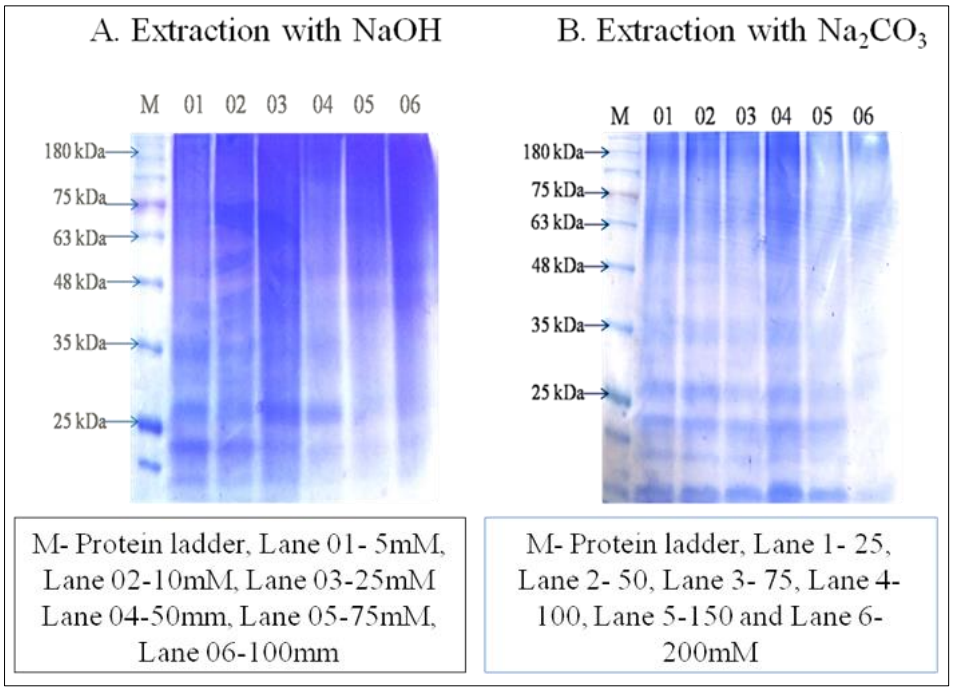

Fig 3: SDS PAGE analysis of sericin extracted with different concentration of Sodium hydroxide (NaOH) and Sodium Carbonate (Na_2CO_3)

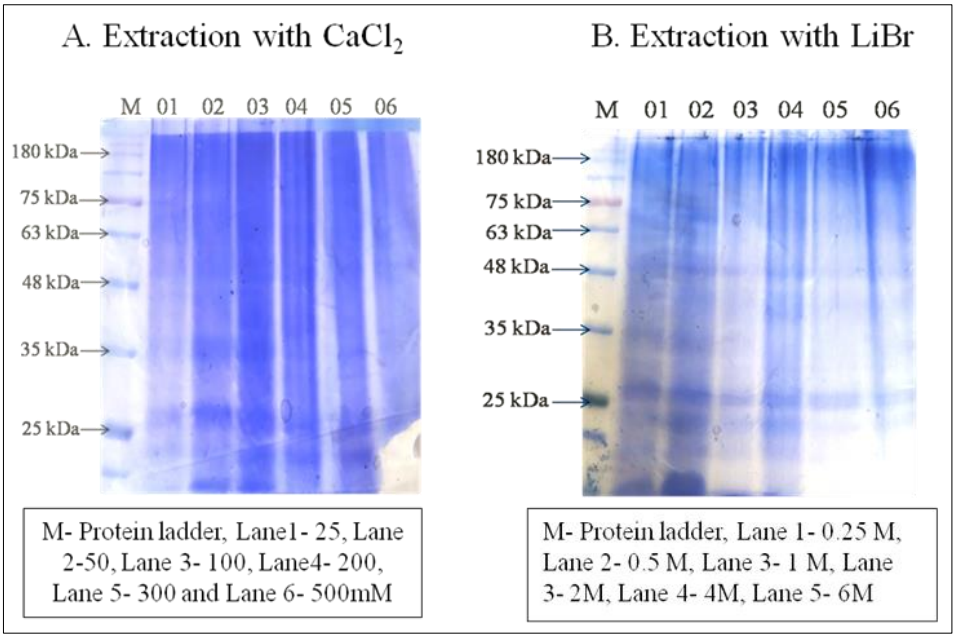

Fig 4: SDA PAGE analysis of sericin extracted with different concentration of Cacium Chloride ($CaCl_2$) and Lithium Bromide (LiBr)

Conclusion

Seven different extraction procedures were employed to extract sericin from Nistari cocoons and the molecular mass of the same was determined using SDS-PAGE gel electrophoresis. The yield of sericin was found to be more when extracted with sodium salts, but when the extracts run on SDS-PAGE exhibits smears, indicating the degradation of sericin. Considering the yield, molecular mass distribution and minimal degradation, treatment with urea and Triton X-100 were found to be most efficient in comparison with other treatments that were used in the present study. Sequencing of major bands of sericin from Nistari cocoons will help for details and further characterization for its biological properties which could be taken into consideration for further study.

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