International Journal of Biological Macromolecules 48 (2011) 32–37

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Refining hot-water extracted silk sericin by ethanol-induced precipitation

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ARTICLE INFO

Article history:
Received 9 August 2010
Received in revised form
20 September 2010
Accepted 20 September 2010
Available online 25 September 2010

Keywords:
Silk
Sericin
Bombyx mori
Protein-based material

ABSTRACT

In order to improve some inherent disadvantages in the mechanical properties of silk sericin (SS), we prepared ethanol-precipitated sericin (EpSS) by adding ethanol into hot-water extracted sericin (HS) solution. EpSS had higher viscosity compared to HS and it was due to the differences in their molecular weight distribution (MWD). The different MWD of EpSS was due to the different solubility of sericin molecules at various concentrations of ethanol, and the amino acid composition of EpSS showed that the more hydrophobic sericin is precipitated more readily in the presence of ethanol. The secondary structure of sericin is also changed from a random coil to β-sheet structure when the amount of ethanol added is high enough. The DSC analysis also revealed that EpSSs has a more compact structure. Finally, when beads were prepared from EpSS, they had enhanced compressive strength compared to those from HS.

1. Introduction

The silkworm, *Bombyx mori*, secretes two proteins, fibroin and sericin. Fibroin (SF) is the major protein and its content is about 75% of the total silk protein. It has been used as a textile but new applications can be found in biomedical fields as well [1,2]. On the other hand, sericin (SS) is the minor protein which envelops two strands of SF and it constitutes about 25% of the total silk protein. In order to get the unique luster and touch in silk textiles or to purify SF for biomedical applications, SS should be removed by a degumming process. Recently, there is much interest in natural polymers not only due to the environmental concerns but also due to the exhaustion of petroleum sources in the future. Considering that the annual production of silk cocoons is about 600,000 tons, nearly 150,000 tons of SS are abandoned every year, which could be a good natural resource if recycled. There are continuous efforts to reuse SS in various fields [3–6] but still their application is limited due to some unsuitable properties.

SS is not a single protein rather it is a group of proteins that is secreted from the middle gland of the silkworm and envelops SF. Therefore, it is important to know the exact composition and molecular weight of each protein because that can affect various properties of SS. However, there is no consensus on the exact composition or molecular weight of each protein. Sprague [7] collected SS directly from silkworm glands and found three large proteins (130, 210, and 220 kDa) in a mixture of proteins ranging from about 20 to 220 kDa. Gamo et al. [8] have isolated five different SS from various positions and maturation of the silkworm gland. The molecular weight of each protein was 309, 177, 145, 134, and 80 kDa. Recently, Takasu et al. [9] have fractionated SS proteins based on the solubility of each protein in the presence of various concentrations of ethanol and designated them as Sericin A (250 kDa), Sericin M (400 kDa), and Sericin P (150 kDa). At the genetic level, three sericin genes, *Ser1*, *Ser2* and *Ser3*, were identified and it has been reported that these genes produce different sizes of mRNA by alternative splicing [10–12]. Garel et al. [13] characterized four different *Ser1* mRNAs and calculated the molecular weight of the corresponding proteins as 76,425, 123,436, 283,617, and 330,767 Da. It has been reported that the *Ser1* transcript is differentially spliced via a tissue- and development-regulated process that is responsible for the diversity of SS molecules [14].

However, from a practical viewpoint, the molecular weights of SS from previous studies become meaningless because of the degradation of SS during the extraction procedure. Currently, SS can be removed or extracted from a cocoon by several methods. First, SS can be obtained directly from the silkworm gland and the aforementioned studies on the molecular weights of SS proteins adopted this method [7–9]. However, it is inapplicable in commercial levels where mass production is needed. Meanwhile, a conventional degumming process using soap in alkaline condi-

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doi:10.1016/j.ijbiomac.2010.09.008

tions can be effective for the removal of SS. But separation of soap from SS is difficult and the remaining soap could cause problems in further applications of sericin. Alternatively, using a urea solution with 2-mercaptoethanol could be useful for SS extraction because the degradation of SS can be minimized and more than 95% of the total SS can be extracted [15]. However, a further time- and cost-consuming dialysis procedure is needed before application. For these reasons, the most widely adopted method for SS extraction is the hot-water extraction method which uses only water without any chemicals. In our experience, SS can be extracted by boiling the cocoons in hot distilled water of 100–120 °C and the final degumming rate would be in the range of 18–25% which depends on the boiling time and temperature. It is simple and SS can be used directly after extraction without any further purification steps. However, this method causes the degradation of SS and recently it has been found that the main cleavage site is next to the aspartic acid residue [16]. Due to the degradation, the molecular weight distribution of hot-water extracted SS (HS) is altered significantly compared to the original SS in the silkworm gland. The molecular weight of HS has a broad distribution between 17 and 250 kDa [17–19]. However, it is still high enough to use as a natural polymer and many researchers continue to use this hot-water extraction due to the convenience. However, there is only limited information about the composition of HS.

Ethanol has been used for the fractionation of SS because it precipitates in the presence of ethanol [9,20]. Takasu et al. [9] isolated three main SS components by the addition of ethanol into the SS solution. According to their results SS molecules with different molecular weights could be separated by varying the amount of added ethanol. In other studies, ethanol was also used as a precipitant in order to separate sericin from the degumming solution [21]. Recently [22], we adopted the same method to hot-water extracted sericin (HS) solution while Takasu et al. used urea–mercaptoethanol solution for the extraction of SS. We found that ethanol-precipitated sericin (EpSS) had a narrower molecular weight distribution compared to the original HS. The beads made from EpSS had better formability and compressive stress than those from HS. However, little has been identified on the differences between EpSS and HS.

In this study, we proceeded to elucidate the differences between EpSS and HS in more detail. Various analyses were performed in order to determine differences in their molecular weight distribution (MWD), amino acid composition, viscosity, and secondary structure.

2. Experimental

2.1. Materials

Silkworm cocoons without the chrysalis were purchased from Heungjin Co. Ltd. All chemicals were purchased from Sigma–Aldrich (Yongin, Korea).

2.2. Preparation of ethanol-precipitated sericin (EpSS)

Sericin was extracted by boiling the silkworm cocoons with distilled water using an autoclave at 120 °C for 1 h. The extracted sericin solution was lyophilized in order to get the HS powder. EpSS was prepared by mixing a predetermined amount of ethanol to the HS solution. Three samples of HS solution were mixed with ethanol at different volume ratios (HS solution: ethanol = 75:25 (EpSS25), 50:50 (EpSS50), and 25:75 (EpSS75)) and the mixture was stirred at room temperature for 1 h. The mixed solution was centrifuged at 10,000 × *g* for 30 min and EpSSs were obtained as precipitates. After decanting the supernatant, the EpSSs were lyophilized into a powder.

Table 1
Yields of EpSS according to the amount of ethanol added ($n = 3$).

	EpSS25	EpSS50	EpSS75
Yield (%)	12.5 ± 0.2	36.7 ± 0.8	74.5 ± 0.6

2.3. Viscosity of HS and EpSSs

Each sample was dissolved in 1 M LiCl/DMSO solvent at the same concentration (20 wt.%). Dissolution was performed at 50 °C with shaking. After cooling to 25 °C, the viscosity of each solution was measured using rotational viscometer (DV-E Viscometer, Brookfield, USA) in a temperature-controlled chamber (25 °C). The viscosity of each solution was compared after it had reached a plateau.

2.4. Preparation of HS and EpSSs beads

HS and EpSSs were dissolved in 1 M LiCl/DMSO solvent at 50 °C with shaking. The dope solution (20 wt.%) was dropped into an ethanol coagulant bath through a 26G syringe using a syringe pump (KDscientific, USA). The dropped sericin beads were coagulated for another 1 h. Finally the coagulated sericin beads were filtered using a non-woven filter and washed with ethanol to remove LiCl and DMSO.

2.5. Characterization of HS and EpSSs

To measure the molecular weight distribution (MWD) of each sample, HS and EpSSs were dissolved in a 4 M urea solution at room temperature and filtered using cellulose acetate membrane with 0.2 μm pore size. MWD of HS and EpSSs was measured by gel filtration chromatography (GFC) (ÄKTA purifier, GE Healthcare, USA) using a Superdex column (Superdex 200 10/300GL, GE Healthcare, Sweden) at a flow rate of 0.5 ml/min. 4 M urea solution was used as an eluent. The standard molecular weight marker for GFC consisted of β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa).

The amino acid compositions of HS and EpSSs were analyzed using HPLC (HP1100, USA). Each sample was hydrolyzed by 6 N hydrochloric acid with 0.02 vol.% 2-mercaptoethanol at 110 °C for 24 h in a nitrogen filled vial. The final hydrolysates were dissolved in pH 2.2 sodium citrate buffers and analyzed by HPLC using an Inno-C18 column. The column temperature was 40 °C and the flow rate was 1 ml/min.

The changes in secondary structure of HS during ethanol addition were measured using a circular dichroism (CD) detector (Jasco J-715, Japan). The CD spectra were collected from 190 nm to 250 nm at a speed of 100 nm/min with a resolution of 1 nm. In order to get a clean spectrum the HS solution (1 wt.%) was diluted 30 times and a proper amount of ethanol was added.

The thermal properties of HS and EpSSs were measured by differential scanning calorimetry (DSC) (DSC-Q100, UK). The samples were heated from 50 °C to 400 °C at a heating rate of 10 °C/min.

The compressive strength of sericin beads was measured at a rate of 10 mm/min using a Minimat (Rheometric Scientific, USA). The compressive strength of each bead at 60% compressive strain was averaged.

3. Results and discussion

3.1. Yield of EpSSs

The yield of EpSS depended on the amount of ethanol added to the HS solution (Table 1). When the concentration of ethanol was adjusted to 25 vol.%, only 12.5% of total SS was precipitated.