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The effects of *Bombyx mori* silk strain and extraction time on the molecular and biological characteristics of sericin

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Sericin was extracted from three strains of Thai *Bombyx mori* silk cocoons (white shell Chul1/1, greenish shell Chul3/2, and yellow shell Chul4/2) by a high-pressure and high-temperature technique. The characteristics of sericin extracted from different fractions (15, 45, and 60 min extraction process) were compared. No differences in amino acid composition were observed among the three fractions. For all silk strains, sericin extracted from a 15-min process presented the highest molecular weight. The biological potential of the different sericin samples as a bioadditive for 3T3 fibroblast cells was assessed. When comparing sericin extracted from three silk strains, sericin fractions extracted from Chul4/2 improved cell proliferation, while sericin from Chul 1/1 activated Type I collagen production to the highest extent. This study allows the natural variability of sericin obtained from different sources and extraction conditions to be addressed and provides clues for the selection of sericin sources.

Key words: sericin; extraction; amino acid; molecular weight; biological properties

Silk is a natural protein composed of two main components. The core structural protein is called "fibroin" and the glue-like protein that holds these fibroin fibers together is "sericin." Silk fibroin accounts for about 75–80 wt% of raw silk. The amount of sericin which surrounds the two fibroin filaments is about 20%, depending on the species of raw silk. Waxes and fats (ca. 1%), as well as colorants and mineral components (ca. 1%), occur exclusively in the sericin layer. The colorants include xanthophyll, carotene, and flavones. Small amounts of amino sugars have also been detected.[1)]

Fibroin is a water-insoluble protein, consisting of layers of antiparallel β-sheets in the natural fibers, while sericin is a water-soluble hydrophilic protein containing both random coil and β-sheet structures. On the cocoon thread, sericin forms three layers from outside to inside covering the fibroin, which are known as I, II, and III. These three fractions of sericin are different in their solubility in hot water and their ratios of solubility for layers I, II, and III are 40:40:20, respectively.[2)] Sericin has been found to activate the proliferation of several cell lines and promote collagen production.[3–5)] It has also shown various biological activities including antibacterial, antioxidant and antityrosinase activities, UV protective properties, and coagulant, cryo-protector and moisturizing capabilities.[6)] Due to the intrinsic biological properties of sericin, several applications in different fields have been proposed using sericin alone or as a component.[7–9)] Kundu et al.[10)] published a detailed review in 2008 on the proposed use of sericins in pharmaceuticals, cosmetics, drug-delivery administration, foods, wound dressing, and as an additive for cell culture media.

Silk sericin-based materials are sensibly affected by the processing used to fabricate constructs, starting from the silk source. Among the families of silk, the *Bombyx mori* silkworm is one of the most widely studied strains in the biomedical field. However, different sources of *B. mori* silkworm exist, varying by the color and shape of cocoons produced to the amino acid composition. In order to provide a better "insight" into the subspecies of *B. mori* silk, a comparison of the characteristics of sericin extracted from three Thai native silk strains, Chul 1/1 (bivoltine, white shell), Chul 3/2 (bivoltine, greenish shell), and Chul 4/2 (bivoltine, yellow shell), as shown in Fig. 1, was performed in our previous study.[8)] We found that the heat-extracted sericin from these three strains showed a molecular mass in a range of 20–200 kDa. Sericin from all strains promoted type I

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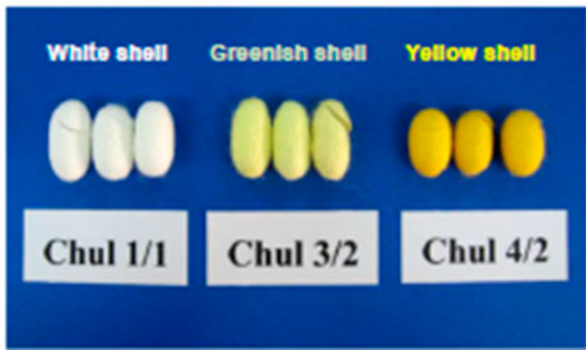

Fig. 1. Physical appearance of three different strains of *Bombyx mori* silk cocoons.

Note: White shell Chul 1/1 cocoons (left), greenish shell Chul 3/2 cocoons (middle), and yellow shell Chul 4/2 cocoons (right).

collagen production in a concentration-dependent manner, while sericin from the Chul 1/1 strain induced the highest synthesis of collagen when compared to sericin from other strains. Furthermore, sericin from all strains was not toxic to fibroblast cells.[11]

In addition to the difference in silk strains, the characteristics of the purified sericin were found to be affected by the extraction process. Sericin can be extracted by various methods, for example, high-pressure and high-temperature techniques or acid, alkaline, or enzyme techniques.[12] It was shown that various extraction techniques significantly influenced the biochemical activities of sericin. Kurioka et al.[13] reported that the acid-degraded, alkali-degraded, and hot water-degraded sericin exhibited different trypsin inhibitory activities and isoelectric points. The extraction method could also alter the amino acid composition and biological performances of sericin. We previously reported that sericin extracted by heat, urea, acid, or alkaline methods had different molecular weight, zeta potential, particle size, amino acid content, and antityrosinase activities.[12,14] Sericin extracted using urea exhibited the highest antityrosinase activity, whereas alkali-degraded sericin showed no inhibition of mushroom tyrosinase. Furthermore, heat-degraded sericin showed the least toxicity to mouse fibroblast cells and activated collagen production to the largest extent. On the other hand, urea-extracted sericin showed the lowest cell viability and collagen production.[14]

The goal of our study was to characterize and biologically evaluate fractions of sericin extracted from different silk strains by autoclaving for different treatment times. Sericin was extracted by heat (a high-pressure and high-temperature) technique due to the simplicity of the process and the highest purity of the obtained proteins. Extracted sericin fractions were investigated in terms of amino acid composition by high-performance liquid chromatography (HPLC) and molecular weights by gel filtration chromatography (GFC). The biological activity of sericin proteins was preliminarily assessed by culturing 3T3 mouse embryonic fibroblast cells in the presence of sericin-enriched culture media. The effect of different sericin samples on cell adhesion, morphology, proliferation, and collagen production was evaluated. The findings of this study can be useful for the selection of silk sources and sericin extraction conditions.

Materials and methods

Silkworm cocoons. Fresh *B. mori* cocoons were kindly supplied by Chul Thai Silk (Petchaboon Province, Thailand). Silkworm cocoons were produced in a controlled environment from three Thai native silk strains including Chul 1/1 (bivoltine, white shell), Chul 3/2 (bivoltine, greenish shell), and Chul 4/2 (bivoltine, yellow shell).

Extraction of sericin. Cocoons of *B. mori* silkworms were cut into small pieces. The greenish (Chul 3/2) and yellow shell (Chul4/2) cocoons were subjected to a depigmentation process with 70% ethanol at room temperature for 24 h with mild agitation (1 g of silk cocoon per 30 mL of ethanol). Sericin was extracted from the air-dried cocoons by heat (a high-pressure and high-temperature) technique.[8] Briefly, the cocoons were immersed in deionized water (DI water) and sericin was extracted by autoclaving (Tuttnauer 5075EL, Bern, Swiss) at 121 °C and 1 bar either in one single step for 60 min (total fractions—hereinafter WT, GT, and YT) or in two separate stages to obtain Fraction 1 (W1, G1, and Y1—extracted in the first 15 min) and Fraction 2 (W2, G2, and Y2—extracted in the following 45 min). The obtained aqueous solutions were then frozen and freeze-dried using a Lio-5P lyophilizer (5Pascal, Milan, Italy) to obtain dry sericin powder. In all cases, the amount of dry sericin powder was quantified and compared with the starting silk amount to determine the yield of the extraction process. The yield was reported in terms of mg of sericin powder per mg of silk cocoons, in percentage. Sericin batch solutions were prepared by dissolving sericin powder in DI water to obtain a concentration of 1 mg/mL. The sericin solutions were filtered using a sterile filter (0.22 μm) to remove impurities. The different sericin samples tested in this study are summarized in Table 1.

Determination of amino acid composition by RP-HPLC. The amino acid content of sericin samples was determined with the Waters AccQ-Fluor™ Reagent Kit using the AccQ-Tag™ amino acid analysis method (Waters Corp., Milford, MA, USA). Briefly, sericin powder (~5 mg) was hydrolyzed with 6-N HCl at 114 ± 2 °C in a silicone oil bath for 24 h.

The air-dried hydrolysates were reconstituted with 20-mM HCl to obtain a solution at a concentration in the range of 4–200 pmol and then derivatized with Water AccQ-Fluor Reagent to obtain stable amino acids. The amino acid content was determined by reverse-phase high-performance liquid chromatography (RP-HPLC) using an AccQ-Tag™ column (3.9 × 150 mm, Waters Corp., Milford, MA, USA) with a gradient of Waters AccQ-Tag™ Eluent A, Milli-Q water, and Acetonitrile (HPLC grade) at a flow rate of 1 mL/min. The amino acids were detected with the Jasco UV-1570 detector set (Jasco, Bouguenais, France) at 254 nm. The chromatograms obtained were compared with Waters Amino Acid Hydrolysate Standards.

Table 1. Nomenclatures of sericin samples extracted from different strains of *B. mori* silk cocoons at different extraction times and the respective extraction yield.

Silk strain	Sample code	Extraction time	Extraction yield [%wt]	Sample description
Chul 1/1	W1	0–15 min	25.4 ± 0.3	White shell Chul 1/1 cocoons-fraction 1
	W2	16–60 min	3.0 ± 0.1	White shell Chul 1/1 cocoons-fraction 2
	WT	0–60 min	25.9 ± 0.1	White shell Chul 1/1 cocoons-single step
Chul 3/2	G1	0–15 min	25.3 ± 1.0	Green shell Chul 3/2 cocoons-fraction 1
	G2	16–60 min	3.1 ± 0.1	Green shell Chul 3/2 cocoons-fraction 2
	GT	0–60 min	27.0 ± 0.5	Green shell Chul 3/2 cocoons-single step
Chul 4/2	Y1	0–15 min	25.1 ± 0.9	Yellow shell Chul 4/2 cocoons-fraction 1
	Y2	16–60 min	3.2 ± 0.1	Yellow shell Chul 4/2 cocoons-fraction 2
	YT	0–60 min	27.7 ± 1.5	Yellow shell Chul 4/2 cocoons-single step

Molecular weight measurement by GFC. The GFC analysis of the sericin samples was conducted with a Shodex SB-805 HQ column (Shodex OH pak®, Length 300 mm, Diameter 8.0 mm from Showa Denko, Munich, Germany). The silk sericin fractions were diluted with eluent solution (4 M Urea, 0.02 M Tris HCl, 0.15 M NaCl, pH 7.5) to obtain a concentration in the range of 0.5–0.8 mg/mL. The chromatography was operated with a flow rate of 1 mL/min at 27 ± 1 °C and was detected with a Jasco UV-1570 detector set (Jasco, Bouguenais, France) at 224 nm. The calibration curve was obtained with low/high molecular weight gel filtration calibration kit (GE Healthcare Europe, Freiburg, Germany).

Cell culture. Fibroblast 3T3 cells were cultured in Dulbecco's modified Eagle's medium with high glucose (Euroclone, Milan, Italy) containing 10% fetal bovine serum, supplemented with 1-mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL Fungizone® amphotericin B (Gibco®, Life Technologies, Monza, Italy). The culture medium was changed every other day, until cells reached confluence. Then, cells were harvested with 0.25% trypsin in PBS and resuspended in fresh medium. The cells were seeded on polystyrene 48-well plates at a density of 2×10^4 cells per well with 500 µL of culture medium and incubated under standard culture conditions (5% CO_2, 37 °C). After 4 h, the medium was removed and replaced with 450 µL of medium and 50 µL of sericin sample, obtaining a sericin-enriched culture medium with a sericin concentration of 100 µg/mL. Culture medium was replaced with fresh sericin-enriched medium every other day.

Cell morphological characterization by CLSM. After 24 h of culture in the presence of sericin in the medium, the cells were fixed and permeabilized with 4% paraformaldehyde solution in PBS with 0.2% Triton X-100. Cells were labeled with Oregon Green® 488 Phalloidin (Molecular Probes®, Life Technologies, Monza, Italy) for the visualization of filamentous actin (F-actin) and 4′,6-diamidino-2-phenylindole, dilactate (DAPI, Molecular probes®) to stain cell nuclei. The morphology of adhered cells was observed by Nikon A1 confocal laser microscope (Nikon Instruments, Florence, Italy).

Cell proliferation. Cells number and proliferation were assessed using PicoGreen® dsDNA quantitation assay (Quant-iT PicoGreen® dsDNA Assay, Invitrogen™, Grand Island, NY, USA), following the manufacturer's instructions. At predetermined time points (day 1, day 3, and day 7), culture medium was removed and cells were washed with PBS. Cells were covered with 500 µL of 0.05% Triton-X in PBS, and stored at −20 °C until analysis. Later, samples were thawed at room temperature and the supernatant was transferred to a 1.5-mL tube and sonicated for 10 s with a Hielscher ultrasonic homogenizer (UP400S, 400 watt—24 kHz, amplitude 50%, from Hielscher Ultrasonics, Teltow, Germany). Extracts of 100 µL were then placed in a black 96-well plate, and mixed with 100 µL of PicoGreen® working solution. Five independent samples were analyzed for each experimental condition. Fluorescence intensity was measured with a Tecan Infinite 200 microplate reader (Tecan Group, Männedorf, Switzerland) (excitation wavelength: 485 nm, emission wavelength: 535 nm). Fluorescence measurements were taken in triplicate. DNA content of each sample was determined on the basis of a calibration curve generated with a double-stranded DNA standard provided with the kit. According to the manufacturer's instructions, the approximate number of cells per sample was estimated from DNA content by the conversion factor of 7.7 pg DNA per cell.

Determination of soluble collagen production. Supernatants were collected from the cells cultured in the presence of sericin samples. The total amount of soluble type I collagen was assayed using the Sircol™ Collagen assay kit (Biocolor, Carrickfergus, UK). The test was conducted following the manufacturer's instructions and the collagen content was determined using a Tecan Infinite 200 microplate reader by measuring absorbance values at a wavelength of 500 nm. Four independent samples were analyzed for each experimental condition. Fluorescence measurements were performed in triplicate. The specific value of collagen content per cell was calculated by normalizing collagen amount with respect to the cell number obtained by PicoGreen® assay.

Statistical analysis. Data were expressed as mean ± standard deviation of the sample. Statistical differences were tested using an unpaired student's *t*-test.

Results

Sericin extraction

Silk cocoons produced by three different *B. mori* strains (Chul 1/1, Chul 3/2, and Chul 4/2) were treated with high temperature and high pressure in an autoclave to isolate water-soluble sericin proteins in a process that is commonly called *degumming*. Three different sericin fractions were extracted from each silk strain. Sericin fractions isolated from the cocoons in a conventional degumming process of 60 min (WT, GT, YT) were compared with sericin fractions extracted in an initial shorter process of 15 min (W1, G1, Y1) and the remaining proteins were extracted in a second process in the following 45 min (W2, G2, Y2). Table 1 summarizes all of the sericin samples analyzed in the study, along with their respective extraction yields.

In the case of the 60-min extraction process, the extraction yield was found to be 25.9 ± 0.1, 27.0 ± 0.5, and 27.7 ± 0.9%wt with respect to the initial silk amount for WT, GT, and YT, respectively. The short 15-min extraction process allowed sericin fractions (W1, G1, and Y1) to be isolated with a yield of about 25%wt in all cases. The W2, G2, and Y2 fractions isolated in a second 45-min long process were considerably smaller than W1, G1, and Y1, accounting for just about 3%wt of the initial silk weight.

Amino acid composition of sericin samples

The amino acid composition of the different sericin fractions was determined by HPLC. Results are reported in Table 2, in terms of amino acid residues for 1000 residues. In general, all protein samples showed very high content of serine, and relevant amounts of glycine, aspartic acid, and threonine. The composition was consistent with the typical sericin composition previously reported in the literature.[10] By comparing sericin fractions extracted in the 60-min process, we found that GT had a larger content of polar amino acids (due to a larger serine content) and a smaller amount of hydrophobic residues (due to a smaller content Glycine and Alanine) when compared with both WT and YT.

The amino acid residues were also functionally partitioned into three groups: (a) charged; (b) polar; and (c) hydrophobic amino acids. Intra-strain differences are visualized in Fig. 2. Total sericin fractions extracted in a single-step degumming process of 60 min (WT, GT, and YT) were directly compared with sericin fractions extracted in a shorter process of 15 min (W1, G1, and Y1) and with the remaining proteins extracted in the following 45 min (W2, G2, and Y2). Fractions extracted from Chul 4/2 (YT, Y1, Y2) did not show any relevant differences (Fig. 2(g)–(i)), while the amino acid compositions of fractions of sericin extracted from Chul 1/1 and Chul 3/2 showed larger variability. In general, fractions extracted in the first 15 min (W1 and G1) presented negligible differences with respect to total fractions (WT and GT) in terms of both amino acid composition (Table 2) and variations of grouped amino acid residues (Fig. 2(a)–(f)). On the contrary, fractions extracted in the second process (W2 and G2) exhibited marked deviations from WT and GT. In particular, fraction G2 showed 11% more charged amino acids (Fig. 2(c)), 11% less polar amino acids (Fig. 2(d)), and 11% more hydrophobic residues (Fig. 2(e)) than the respective total fraction GT.

Molecular weight of sericin samples

Molecular weight distributions of sericin fractions extracted from different strains of *B. mori* silk cocoons were analyzed by GFC. Molecular weight curves are presented in Fig. 3, while number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity (pdi) are summarized in Table 3. In general, fractions extracted in the short 15-min degumming process (W1, G1, and Y1) had the largest molecular weight when compared with both total fractions (WT, GT, and YT) and proteins extracted in the second degumming process (W2, G2, and Y2) (Fig. 3(a)–(c)). This tendency was also reflected by the estimated values of M_n and M_w presented in Table 3. The only partial exception was represented by fraction W1, which had a M_n (47 kDa) that was very similar to WT (53 kDa). In fact, W1 presented a marked bimodal distribution (Fig. 3(a)) due to the presence of a family of peptides with small molecular weight (2–5 kDa). On the other hand, fractions W2, G2, and Y2 had considerably smaller molecular weights, approximately equal to half the value of the respective fractions W1, G1, and Y1 (Table 3).

When comparing different silk strains, it is possible to note that sericins isolated from Chul 1/1 had a smaller molecular weight than sericins extracted from the other strains in terms of both M_n and M_w.

Cell attachment and proliferation

The morphology of cells cultured with sericin-enriched culture medium (100 μg/mL) was monitored by confocal microscopy. Both at day 1 and day 3, the morphology of attached cells did not seem to be affected by the presence of sericin fractions. In Fig. 4, we compared representative confocal images of cells cultured in the presence of Chul 1/1 sericin fractions (W1, W2, and WT) and untreated control cells. At day 1, cells appeared to be agglomerated in irregular clusters scattered onto the well surface. The morphology of cells varied from elongated to fully spread, with occasional round cells in all cases. At day 3, visual inspection showed that the culture well had a surface coverage of about 70–80% for all sericin samples and control wells. Cells cultured in the presence of Chul 3/2 and Chul 4/2 showed a similar behavior (data not shown). The number of cells per well at day 1, 3, and 7 is presented in Fig. 5(a). Overall, we found that cell proliferation was only slightly influenced by the presence of sericin at this concentration. However, cells cultured in the presence of sericin fractions W1, G2, GT, Y1, Y2, and YT showed significantly higher proliferation than control cells at day 3. The cells cultured in the presence of sericins Y1 and Y2 showed the highest number of cells, compared to cells cultured with other sericin samples and control cells at day 7. It is important to report, however, that visual inspection revealed that cells reached confluence in the wells

Table 2. Amino acid content of sericin samples extracted from different strains of *B. mori* silk cocoons at different extraction times expressed as residues per 1000 residues obtained by HPLC.

		Samples								
		Chul 1/1			Chul 3/2			Chul 4/2		
Amino acids*		W1	W2	WT	G1	G2	GT	Y1	Y2	YT
Charged	Asp	83	87	83	85	90	82	76	75	78
	Glu	23	27	23	24	33	20	22	25	19
	Arg	32	33	32	37	33	36	34	34	33
	Lys	50	49	52	41	52	50	51	53	53
	Total	188	196	190	187	208	188	182	187	183
Polar	Ser	332	308	334	335	294	340	338	337	332
	His	19	21	20	18	20	19	19	21	20
	Thr	91	93	91	96	91	96	96	93	96
	Cys	<1	<1	<1	1	<1	2	2	2	1
	Tyr	35	32	35	37	35	39	38	34	37
	Total	477	454	480	487	440	496	493	486	486
Hydrophobic	Gly	176	172	175	172	173	170	174	165	175
	Ala	72	79	72	75	79	69	71	74	77
	Pro	9	10	7	7	10	9	8	10	8
	Val	40	44	41	44	45	39	40	41	41
	Ile	19	19	15	10	18	11	11	15	12
	Leu	16	20	16	15	20	15	17	17	15
	Phe	4	8	5	4	7	4	5	5	4
	Total	335	351	330	327	352	317	325	327	331

*Charged amino acids. Asp: Aspartic acid; Glu: Glutamic acid; Arg: Arginine; Lys: Lysine; Polar amino acids. Ser: Serine; His: Histidine; Thr: Threonine; Cys: Cysteine; Tyr: Tyrosine; Hydrophobic amino acids. Gly: Glycine; Ala: Alanine; Pro: Proline; Val: Valine; Ile: Isoleucine; Leu: Leucine; Phe: Phenylalanine.

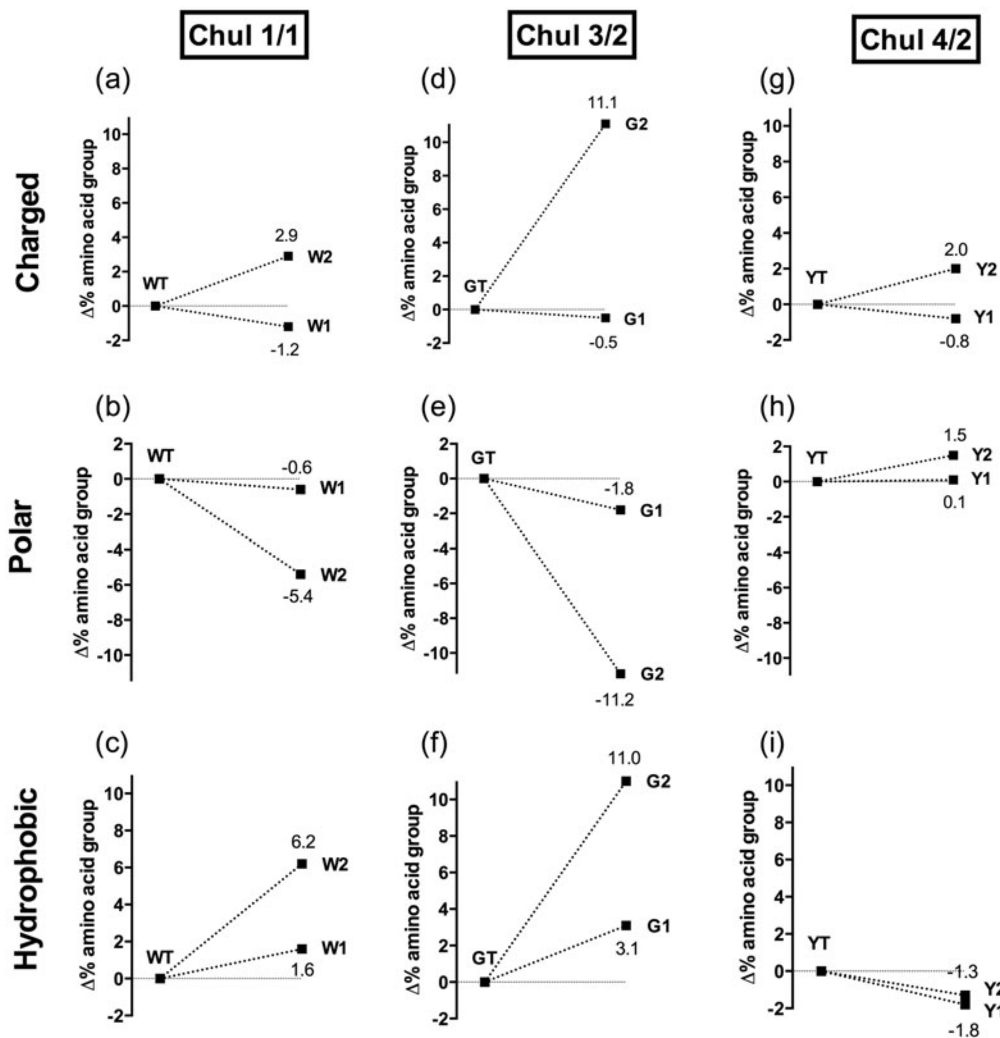

Fig. 2. Group variations between charged, polar, and hydrophobic amino acids for different sericin samples.
Notes: (a–c) Sericin fractions extracted from Chul 1/1 silk, (d–f) sericin fractions from Chul 3/2, and (g–i) sericin fractions from Chul 4/2.

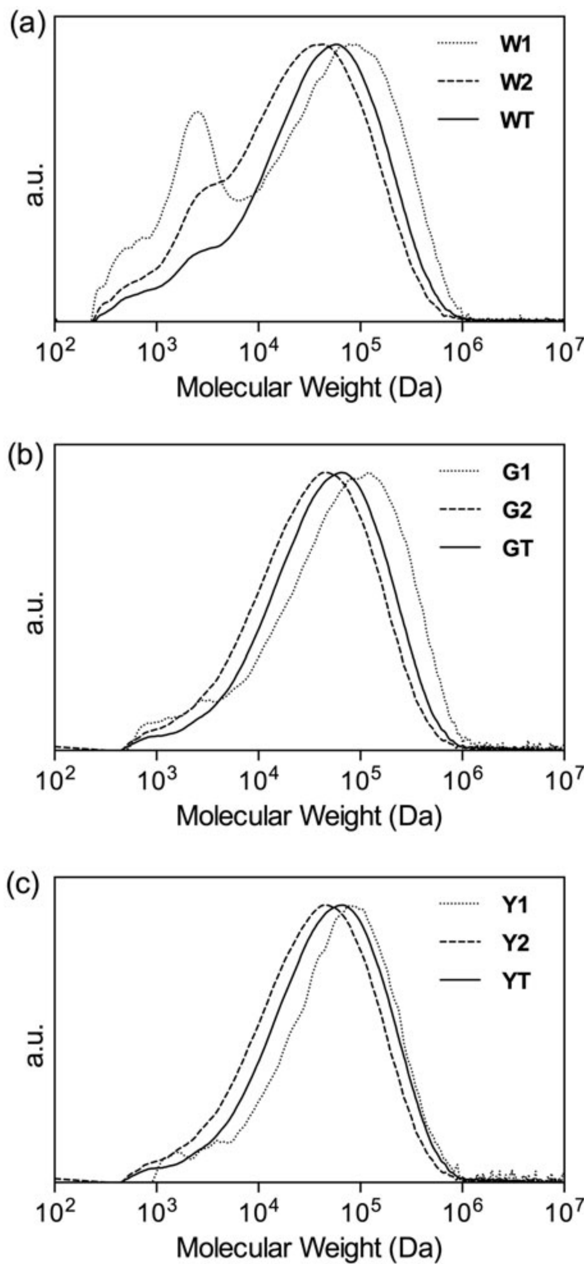

Fig. 3. Molecular weight distribution of sericin samples extracted from different strains of *Bombyx mori* silk cocoons at different extraction times.

Notes: (a) White shell Chul 1/1 cocoons: W1, W2, and WT fractions; (b) greenish shell Chul 3/2 cocoons: G1, G2, and GT fractions; and (c) yellow shell Chul 4/2 cocoons: Y1, Y2, and YT fractions.

Table 3. Molecular weights of sericin samples extracted from different strains of *B. mori* silk cocoons at different extraction times obtained by GFC.

Sericin samples	M_n [kDa]	M_w [kDa]	pdi [−]
W1	47	127	2.6
W2	31	72	2.3
WT	53	101	1.9
G1	116	179	1.6
G2	50	88	1.8
GT	75	119	1.6
Y1	100	143	1.4
Y2	58	95	1.6
YT	77	124	1.6

Note: M_n: number average molecular weight; M_w: weight average molecular weight; pdi: polydispersity.

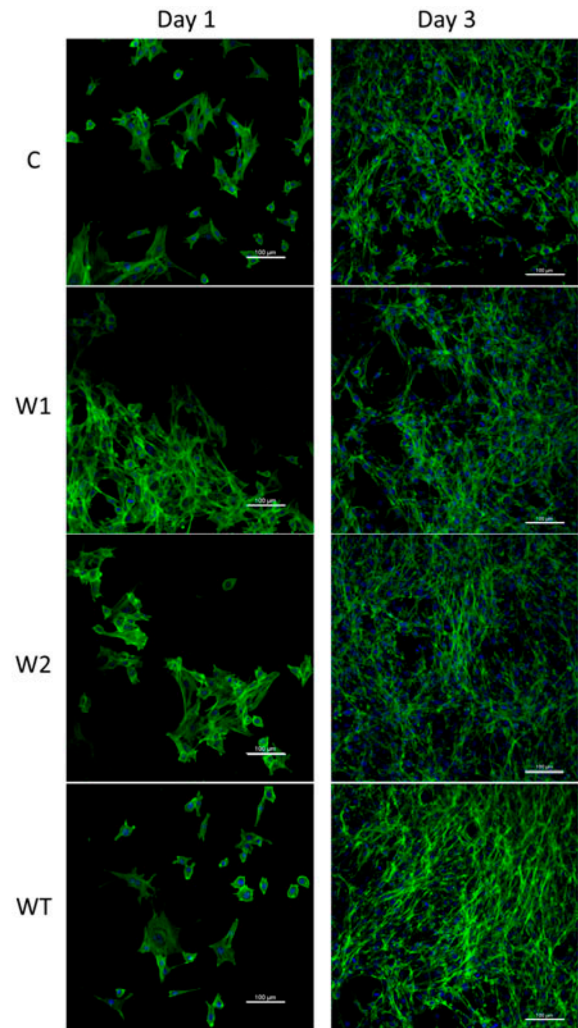

Fig. 4. Representative confocal images of fluorescently labeled of 3T3 fibroblast cells cultured in the presence of Chul 1/1 sericin samples (W1, W2, WT) at day 1 and day 3.

Note: Scale bar 100 μm.

before day 7 for all samples. Thus, proliferation results at day 7 could be influenced by the cell's reduced activity in a confluence state.

Collagen production

The effect of sericin-enriched medium on the production of soluble fractions of type I collagen from cells was also evaluated. Total collagen content was divided by cell number obtained by PicoGreen® assay to determine the specific value of collagen content per cell. The results are reported in Fig. 5(b). In general, collagen production was positively influenced by the presence of sericin. In particular, we found that cells cultured in the presence Chul 1/1 sericin fractions (W1, W2, and WT) produced collagen at a much faster pace in the first 3 days if compared to control cells at the corresponding time points. Also, Chul 4/2 sericins (Y1, Y2, and YT) allowed for a small but significant increase in collagen production at day 3. In contrast, fractions G1, G2, and GT had a little or no effect on collagen production per cell when compared with control both at day 3 and day 7. At day 7, just samples W1, G1, and YT presented a collagen content per cell that was significantly higher than that of the control.

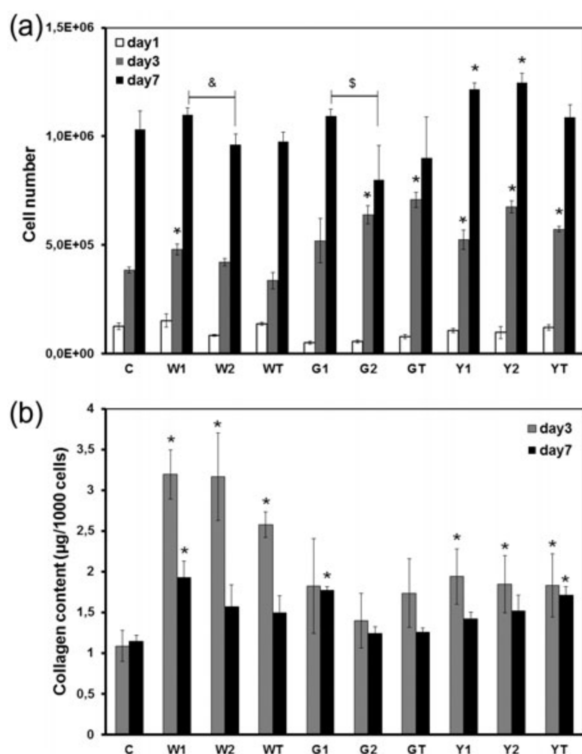

Fig. 5. Cell proliferation and collagen production for 3T3 fibroblasts cultured with sericin-enriched medium (100 μg/mL) and control cells (C) cultured on TCP in absence of sericin.

Notes: (a) Number of cells per well after 1, 3, and 7 days from seeding based on the total DNA content determined by Picogreen assay; (b) Soluble fraction of type I collagen produced by cells and determined by Sircol assay at day 3 and 7, reported values have been normalized with respect to the cell number. $*p < 0.05$ significant difference with respect to the values of untreated cells (C) at the corresponding time; $\&p < 0.05$ significant difference with respect to W1; $\$p < 0.05$ significant difference with respect to G1.

Discussion

In the past years, silk sericin has been investigated in terms of structure, function, and bioactivity. Based on the peculiar properties of sericin, several non-textile applications were proposed in the cosmetic, pharmaceutical, food additive, and biomedical fields.[6] However, recently, researchers have been taking into account the role of the processing method on the final material bioactivity, starting from the specific silk protein source. Sericins are a family of peptides with a molecular weight ranging from 24 to 400 kDa.[10] Sericin can be isolated by different extraction methods and from different silk sources; both factors can highly influence sericin properties and eventually the biological performance of the proteins. We have previously demonstrated that sericin extracted by a heat technique in an autoclave (121 °C, 1 bar, for 60 min) had lower levels of toxicity when compared to other extraction methods and could activate collagen production.[12] For this reason, the heat extraction technique was selected in this work to compare sericins extracted from three different strains of *B. mori* silk cocoons (Chul 1/1, Chul 3/2, and Chul 4/2). In addition, three separate fractions were extracted from each silk strain. In fact, total sericin fractions extracted in a standard degumming process of 60 min (namely WT, GT, and YT) were compared with sericin fractions extracted in a shorter process of 15 min (W1, G1, and Y1) and with the remaining proteins extracted in the following 45 min (W2, G2, and Y2). However, extraction yields reported in Table 1 show that most of the sericin proteins are actually extracted in the initial 15-min process. In fact, proteins extracted in the following 45 min account for just 3% of the dry silk weight.

In this study, the molecular characteristics of the different sericin fractions were also investigated in terms of amino acid composition and molecular weight distribution. We noticed that the amino acid composition of fractions extracted in the first 15 min were very similar to the total fractions extracted in a 60-min long process. On the contrary, the molecular weight of total fractions was considerably smaller than the respective sericin fraction extracted in 15 min (Fig. 3). Obviously, the high-pressure/high-temperature environment in an autoclave is quite aggressive for sericin proteins, resulting in protein chain cleavage and molecular weight reduction. On the other hand, proteins extracted in the 45-min process presented a significantly smaller level of serine residues (Table 2). This could suggest that in the second degumming stage, some fibroin fractions could be extracted from amorphous regions of the fiber together with the limited residual sericin. Also in this case, the long-treatment period in an autoclave contributes to the molecular weight reduction of Fraction 2.

These results are consistent with previous studies. For example, Silva et al.[15] demonstrated that the molecular weight of sericin strongly depended on the extraction method and conditions. Shelton and Johnson [16] suggested that sericin could be extracted with water at 50–60 °C for 25 days to avoid the decomposition of its structure. It was found that sericin extracted under this condition could have molecular weights as high as 100–200 kDa. On the other hand, the sericin extracted using sodium carbonate showed a molecular weight lower than 50 kDa.[15] Padamwar and Pawar[2] reported that the sericin extracted with hot water showed a molecular weight of 24 kDa whereas the spray-drying method produced sericin with a molecular weight in the range of 5–50 kDa. Extraction with enzymes and urea resulted in sericin with a molecular weight of 3–10 and 50 kDa, respectively. In the present work, we extracted sericin using a high-pressure and high-temperature process in an autoclave. In the case of total fractions extracted in the 60-min process, we obtained M_n ranging from 53 to 77 kDa, depending on the silk strain (M_w ranging from 101 to 124 kDa). In addition, molecular weight distribution appeared to be shifted to higher molecular weights in the case of Fraction 1, extracted in the first 15 min (Fig. 3 and Table 3). In this case, M_n was in the range of 47–116 kDa, depending on the silk strain (M_w ranging from 127 to 143 kDa). It is clear that the degumming time in an autoclave should be controlled carefully and that shorter times are preferable to preserve sericin structure. Interestingly, GFC curves showed that the W1 fraction (and W2 to a minor extent) extracted from Chul 1/1 cocoons showed a bimodal distribution of molecular weights due to the presence of a family of

small molecular weight proteins. The reason for this point is still unclear.

All sericin samples were tested as bioactive agents added into cell culture medium for 3T3 fibroblast cells. When comparing three cocoon strains, all fractions of sericin extracted from Chul 1/1 cocoons activated proliferation at a level lower than sericin fractions extracted from Chul 4/2 but was able to activate the collagen production of cells to a higher extent. We supposed that the difference in biological activity among fractions of sericins was due to their different amino acid compositions. The high percentage of polar amino acids in sericin fraction extracted from Chul4/2 improved cell proliferation, as reported elsewhere.[17,18] The high percentage of charged amino acids in sericin fraction extracted from Chul 1/1 would act as precursor to activate collagen synthesis because collagen peptide contains a lot of charged amino acids like aspartic acid, glutamic acid, and lysine.[19]

The findings of this study would help us to understand the variation in sericin obtained from different sources and extraction conditions. The data would be advantageous for the selection of silk stains and extraction conditions in order to obtain sericin that matches the application, for example, all fractions of sericin extracted from Chul 1/1 cocoons were appropriate for wound-healing applications due to its potential in the activation of collagen production.

Conclusions

Thai native silk cocoons belonging to different *B. mori* strains (Chul 1/1, Chul 3/2, and Chul 4/2) were treated by a high-pressure and high-temperature technique in an autoclave to extract sericin proteins. This study demonstrated that the amino acid composition, molecular weight, and biological activity of sericins extracted from different strains strongly depend on the extraction time and silk strain itself. For each strain, we found that the sericin samples extracted in a short 15-min process presented an extraction yield and amino acid composition that was similar to the samples extracted in a 60-min long process. However, longer treatment resulted in a marked reduction in sericin molecular weights. Murine 3T3 fibroblasts showed a higher degree of proliferation and collagen production *in vitro* when cultured in the presence of sericin fractions extracted from all three silk strains. In particular, sericins extracted from Chul 1/1 greatly activated the production of soluble type-I collagen fractions after three days of culture. These results suggest that sericin extracted from Chul 1/1 cocoons could support wound-healing processes via the activation of short-term collagen production.

Authors' contributions

PA had full access to all of the data in the study, accepts responsibility for the integrity of the data, and affirms that everyone who contributed significantly to the work has been listed. TS and WB developed the experimental design and performed all the tests. AM and CM did data analysis and data interpretation. All authors read the manuscript, provided critical input, and approved the final version.

Disclosure statement

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