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Formulation and characterization of silk sericin–PVA scaffold crosslinked with genipin

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ABSTRACT

A porous-three-dimensional scaffold shows several advantages in terms of tissue engineering since it can provide a framework for cells to attach, proliferate and form an extracellular matrix. Sericin, a by-product from the silk industry, can form a three-dimensional scaffold with PVA after freeze-drying but has a fragile structure. Glycerin (as a plasticizer) and genipin (a crosslinking agent) are necessary to make a strong and stable matrix. Our objective was to investigate the properties of a three-dimensional silk sericin and PVA scaffold with and without glycerin and genipin at various concentrations. SEM showed that adding glycerin into scaffold gave better uniformity and porosity. Smaller pore sizes and better uniformity were found as the concentration of genipin in the scaffold increased. The results of FTIR indicated that glycerin retained a high moisture content and had a major effect at 3286 cm^{-1}, indicating the presence of water molecule in the matrix structure. Adding genipin into the scaffold resulted in a higher degree of crosslinking or fewer free ϵ-amino groups, as shown by the decrease in the stretching (=C–H) peak and absorption peaks around 1370–1650 cm^{-1}, respectively. The sericin/PVA scaffold had a low water sorption capacity, but adding glycerin significantly increased this property. Genipin further enhanced the moisture absorption capacity of the scaffold and extended the time taken to reach equilibrium. After immersing the sericin/PVA scaffold into purified water, the scaffold completely dissolved within an hour, whereas the scaffolds containing glycerin or glycerin with 0.1% genipin swelled 8 and 11 times, respectively, compared with the initial stage after 6 h of immersion. In terms of mechanical properties, the sericin/PVA/glycerin scaffold exhibited a similar compressive strength to the scaffold with a high genipin concentration, whereas a low concentration of genipin softened and reduced the compressive strength of the scaffold. A small amount of sericin was released from the scaffold and a higher concentration of genipin, resulting in less protein leaching compared to non-crosslinked sericin/PVA. The fraction of protein released from the sericin/PVA/glycerin scaffold was about 4%, with values of about 1 and 0.04% in the case of scaffolds with 0.01 and 0.1% genipin, respectively. All results indicated that the composition of the scaffolds had a significant effect on their physical properties, and that can easily be tuned to obtain scaffolds suitable for biological applications.

1. Introduction

Accidental damage to the epidermis by ulcers, burns or other traumatic incidents may result in a series of morbid consequences that restrict epidermal regeneration. In the case of wounds that extend entirely through the dermis, skin substitutes such as xenografts, allografts and autografts need to be employed for wound healing. The challenge of designing substrates that will allow specific biological interactions is demanding, particularly in the case of tissue engineered skin substitutes. Natural biomaterials such as collagen, silk and chitosan have received increasing attention in the field of biomedical engineering due to their unique properties, including non-toxicity, biodegradability and biocompatibility [1,2]. Porous-three-dimensional scaffolds that can provide a framework for cells to attach, proliferate and form their extracellular matrix play an important role in manipulating cell functions in this approach [3]. Since a suitable scaffold should possess the specific structure of the tissue it replaces and must be capable in turn of being replaced in time via the ingress of new cells [4], the choice of material is of prime concern [5]. However, natural biomaterials themselves are normally unable to meet all the requirements of their applications. Polymer blending is a useful technique for modifying the properties of a single polymer.

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We have previously reported that silk sericin, a natural hydrophilic polymer extracted from silk cocoons during the degumming process, is non-toxic to fibroblast cells [6] and enhances wound healing by promoting collagen production in wounds [7]. Sericin is mainly comprised of serine and aspartic acid with strong polar side chains, thus enabling easy copolymerization and blending with other polymers to produce biocompatible materials with improved properties [8,9]. Earlier reports claimed that sericin was responsible for an immune response; however, few studies have investigated the use of sericin in the tissue engineering field. The immune response of sericin was subsequently proven to be dependent on the physical association with fibroin-silk fibers [10], but sericin itself generates very low immune responses [11]. Sericin forms fragile materials that are not suitable for use in medical applications, but Mandal et al. demonstrated that after blending with gelatin, silk sericin can form a scaffold and be a good candidate for tissue engineering applications [5]. In this study, polyvinyl alcohol (PVA) (a synthetic polymer with good biocompatibility, low toxicity and good mechanical properties) was blended with sericin. A crosslinking process is also believed to improve the permeability as well as the mechanical properties of proteins [12]. For this purpose, naturally occurring genipin was used to crosslink sericin. Genipin is found in traditional Chinese medicine and is extracted from gardenia fruit [13]. It is an effective naturally occurring crosslinking agent that can react with amino acids or proteins containing residues with primary amine groups such as lysine, hydroxylysine or arginine [14,15]. Sung et al. investigated the cytotoxicity, feasibility and biocompatibility of genipin for tissue fixation and found that genipin was 10,000 times less cytotoxic than the commonly used glutaraldehyde [16]. In addition, the treatment of animal wounds by genipin-crosslinked glue induced significantly lower inflammatory responses and more rapid recovery than those treated by aldehyde-crosslinked glues [16,17].

Glycerin, a commonly used plasticizer, has previously been mixed to improve silk film properties [18] and also helps to reduce phase separation between silk and PVA in the blend [19]. Glycerin content in blend films is important for the control of silk secondary structural transitions and influencing the mechanical properties of the films [20]. After mixing with silk, glycerin molecules interact with silk chains via intermolecular forces, most likely hydrogen bonds between hydroxyl groups of glycerin and amide groups of silk [19].

The purpose of this study was to investigate the properties of three-dimensional silk sericin and PVA scaffolds crosslinked with genipin at various concentrations that will have potential in tissue engineering due to their low toxicity.

2. Materials and methods

2.1. Preparation of three-dimensional silk sericin and PVA scaffold

The fresh, white-shell cocoons of *Bombyx mori* were kindly supplied by Chul Thai Silk Co., Ltd. (Petchaboon province, Thailand). Silkworm cocoons were produced in a controlled environment. After cutting the cocoons into pieces (about 5 mm^2), silk sericin was extracted using a high temperature and pressure degumming technique [21]. In brief, the silkworm cocoons were mixed with purified water (1 g of dry silk cocoon: 30 mL of water) and the samples were autoclaved (SS-320, Tomy Seiko Co., Ltd., Tokyo, Japan) at 120 °C for 60 min. After filtration through a membrane to remove fibroin, the sericin solution was concentrated until the desired concentration (approximately 7% (w/v)) measured by BCA Protein Assay Reagent, Pierce, Rockford, IL, USA) was achieved.

PVA (Ajax Finechem, New South Wales, Australia, molecular weight 77,000–82,000) was dissolved at 80 °C with constant stirring for about 4 h until it was completely dissolved to a concentration of 6% (w/v). Genipin was dissolved in ethyl alcohol to give a solution at a concentration of 20% (w/v). Sericin solution and PVA solution with and without glycerin were blended together at room temperature for at least 30 min to make a final wet composition of 3% (w/v) sericin, 2% (w/v) PVA and 1% (w/v) glycerin. Genipin solution at different concentrations (0.01–0.1% (w/v)) was added to the mixed solution of sericin, PVA and glycerin and stirred for 5 min, which was then poured into a petri-dish and frozen at −20 °C, followed by lyophilization (Heto LL 3000 lyophilizer, Allerod, Denmark) for 72 h. After drying, the scaffolds were accurately weighed to calculate the percentage of each component in dry weight basis.

2.2. Amino acid analysis

The amino acid composition of SS was measured by an amino acid analyzer (Hitachi L-8500A, Tokyo, Japan). Samples for amino acid analysis were hydrolyzed in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) at 100 °C for 24 h under vacuum. All experiments were done in triplicate.

2.3. Scanning electron microscopy (SEM) of scaffolds

Samples of silk sericin/PVA and silk sericin/PVA with glycerin and genipin at various concentrations were cut into pieces and mounted onto aluminum stubs and sputter coated with gold at 10–20 nm thickness. Observations were performed with a JSM-5800LV scanning electron microscope (JEOL, Tokyo, Japan) at 15 keV.

2.4. Pore size measurement of scaffolds

Pore size was measured using a modified method from Kang et al. [22]. The major and minor diameters of each scaffold were measured using a stereo microscope equipped with an optical micrometer. The pore size was calculated as the geometric mean of the major and minor diameters, respectively. At least 100 pores were assessed and the values presented indicate the mean ± standard deviation.

2.5. Fourier transform infrared spectroscopy (FTIR) of scaffolds

FTIR spectra were obtained using a Spectrum One (Perkin–Elmer, MA, USA) spectrophotometer. The sample was ground into appropriate size particles and a small amount of sample was sealed into a KBr pellet (thickness less than 0.5 mm) by a hydraulic press prior to measurement at ambient temperature. Spectra were obtained at 4 cm^{-1} resolution, under a dry air purge, with the accumulation of 16 scans. The IR spectra of all scaffolds were recorded within the range of 4000–400 cm^{-1}. The spectrum of the KBr disc was subtracted from each sample spectrum.

2.6. Degree of crosslinking

The degree of crosslinking was determined using a modified method from Bubnis et al. [23]. The free amino groups of sericin were reacted with 2,4,6-trinitrobenzene sulfonic acid (TNBS). The absorbance of the product from the TNBS reaction was detected by UV spectroscopy. The relative degree of crosslinking was obtained from the differences between the absorbance values of non-crosslinked and crosslinked scaffolds. Briefly, about 5 mg of the crosslinked scaffolds were weighed into a test tube into which 1 ml of 0.5% TNBS solution and 1 ml of 4% sodium hydrogen carbonate ($NaHCO_3$, pH 8.5) were added. This was then heated in a water bath maintained at 40 °C for 2 h. The non-crosslinked primary amino

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groups in the scaffolds reacted with TNBS and formed a soluble complex. This solution was further treated with 2 ml of 12.24N HCl at 70 °C for 2 h. The absorbance of the solutions was determined at 415 nm after suitable dilution spectrophotometrically. The degree of crosslinking was then calculated by the following equation, in comparison with the non-crosslinked samples:

$$\text{Degree of crosslinking}\,(\%) = \frac{1 - \text{Abs of crosslinked scaffold}}{\text{Abs of non-crosslinked scaffold}} \times 100$$

The experiment was performed in triplicate and values were expressed as the mean ± standard deviation.

2.7. Moisture absorption

The moisture absorption capacity of the silk sericin and PVA scaffolds was determined by placing the scaffolds into desiccators in which the relative humidity was controlled by salt solutions. Potassium chloride solution was used to obtain a relative humidity of 81.47 ± 1.48% at 25 °C [24]. Samples were removed from the desiccators after 0.5–120 h and carefully weighed. The moisture absorption capacity was calculated as the percentage of weight change compared to the initial weight. All experiments were performed in triplicate.

2.8. Swelling properties

Swelling studies were carried out according to Mandal et al. [5] with slight modifications. Briefly, the lyophilized scaffolds were accurately weighed in the dry state and then immersed in 10 mL of purified water. At various time intervals, the scaffolds were carefully removed and the amount of water contained in the scaffolds was determined precisely by weighing them in the swollen state. The experiments were performed in triplicate under the same conditions. The percentage swelling of the scaffolds at equilibrium was calculated using the following equation:

$$\%\,\text{swelling} = \frac{W_t - W_0}{W_0} \times 100$$

where W_0 is the weight of the dried test sample and W_t is the weight of the swollen test sample.

2.9. Mechanical properties of scaffolds

Compression tests were performed on flat samples of diameter 15 mm and thickness 8 mm with an Instron model 4301 instrument (Instron, Canton, MA), at a cross-head rate of 1 mm/min. Tangent modulus (E), stress and strain at the yield point (σ^* and ε^*), and the stress at 10% strain ($\sigma_{10\%}$) were obtained from the σ/ε compression curve. All experiments were performed in triplicate.

2.10. The release of sericin from sericin scaffolds

The release profile of sericin from scaffolds was plotted after placing the scaffold samples (diameter 35 mm) into PBS (pH 7.4) at room temperature with continuous stirring in a closed-container. The samples (1.5 mL) were taken out at different time points 0, 1, 15, 30 min, 1, 2 and 3 days, and the amount of sericin was measured using a BCA protein assay kit (Pierce, Rockford, IL). Briefly, the leached protein samples were collected, mixed with BCA reagents and vortexed. The absorbance was measured at 562 nm and the amount of protein released was compared with a bovine serum albumin standard curve. All experiments were performed in triplicate.

Table 1
Amino acid compositions of silk sericin.

Amino acid	Molar percent ± SD
Asp	15.64 ± 1.62
Thr	8.16 ± 0.62
Ser	33.63 ± 2.23
Glu	4.61 ± 0.35
Gly	15.03 ± 1.44
Ala	4.10 ± 0.22
Cys	0.44 ± 0.12
Val	2.88 ± 0.98
Met	3.39 ± 0.64
Ile	0.56 ± 0.18
Leu	1.00 ± 0.48
Tyr	3.45 ± 0.39
Phe	0.28 ± 0.09
Lys	2.35 ± 0.89
His	1.06 ± 0.21
Arg	2.87 ± 0.66
Pro	0.54 ± 0.11

Values are means by triplicate analysis.

2.11. Statistical analysis

Data was expressed as the mean ± SD. The statistical significance was determined by paired and unpaired Student's *t*-tests together with ANOVA. A value of $p < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Amino acid composition of silk sericin

Table 1 indicates the amino acid compositions of silk sericin. Serine, aspartic acid and glycine count for more than 50% of the whole amino acid compositions, similar to the results reported by others [25].

3.2. Morphology of silk sericin and PVA scaffold

Mixing sericin and PVA aqueous solution with or without glycerin can obtain a homogeneous mixture. It is expected that PVA only physically incorporated with sericin similar to the previous results of gelatin and PVA [26]. Liu et al. reported that gelatin had no significant effect on the thermal behavior of PVA, which indicated that no substantial change occurred in the PVA crystallite due to the presence of gelatin [26]. Genipin did not cause gel formation or significant increase in viscosity of sericin/PVA and glycerin solution (the viscosity of sericin/PVA/glycerin and sericin/PVA/glycerin with genipin solution were <0.3 dPa s). Scaffold composed of various concentrations of sericin or PVA, both ranging from 1 to 5% (w/v), were observed for their physical properties. The most suitable concentration of sericin and PVA which gave homogenous and stable matrix was sericin, PVA and glycerin at concentration 3, 2 and 1% (w/v) of wet weight basis, respectively (data not shown). It can easily form a scaffold after freeze-drying and appears as a smooth and homogenous material. After freeze-drying, final weight of the scaffold did not show significant difference compared with theoretical weight. From that result, various scaffolds composed of sericin (3% (w/v))/PVA (2% (w/v))/glycerin (1% (w/v)) and genipin at different concentrations were obtained. Without genipin, both sericin/PVA and sericin/PVA/glycerin scaffolds appeared off-white in color, which is the natural color of the silk cocoon. Genipin changed the color of the scaffold to pale blue (at a low concentration, 0.01%) and dark blue (at a high concentration, 0.1%) due to its own natural color. The sericin/PVA scaffold was rigid and less flexible compared to the scaffold composed of glycerin and genipin. Fig. 1A shows SEM images of a cross section of the sericin/PVA scaffold; the pores were not highly interconnected, both open and closed pore structures

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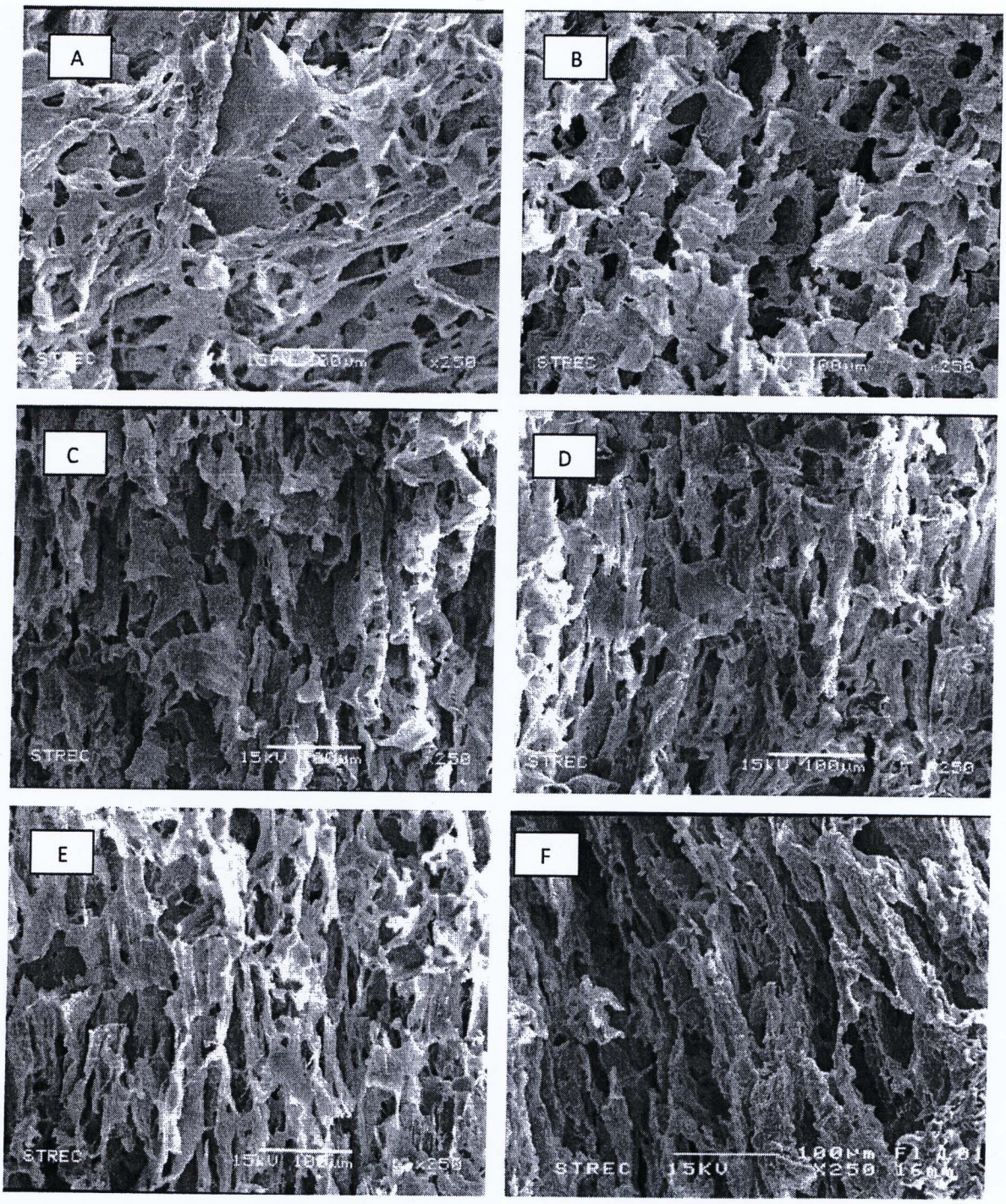

Fig. 1. SEM images of cross-section scaffold. A = sericin/PVA scaffold, B = sericin/PVA/glycerin scaffold, C = sericin/PVA/glycerin with 0.01% genipin scaffold, D = sericin/PVA/glycerin with 0.025% genipin scaffold, E = sericin/PVA/glycerin with 0.075% genipin scaffold, F = sericin/PVA/glycerin with 0.1% genipin scaffold.

were observed and the pore distribution was heterogeneous. However, better uniformity and porosity were observed in freeze-dried silk sericin/PVA/glycerin blended scaffolds (Fig. 1B). Different blend compositions ranging from 0.01 to 0.1% genipin were examined, with scaffolds showing smaller pore sizes and better uniformity as the genipin concentration increased (Fig. 1C–F). Table 2 exhibits the pore size distribution of sericin scaffolds. The sericin/PVA scaffold had a high pore size variation compared with the other types of scaffold while the sericin/PVA/glycerin scaffold exhibited smaller pore sizes and better uniformity compared with the sericin/PVA

Table 2
Pore size of sericin scaffolds ($n = 100$).

Scaffold compositions	Mean pore size ± SD (μm)
Sericin/PVA	39.13 ± 23.73
Sericin/PVA/glycerin	29.40 ± 12.60
Sericin/PVA/glycerin/0.01% genipin	48.19 ± 17.89
Sericin/PVA/glycerin/0.025% genipin	45.69 ± 15.38
Sericin/PVA/glycerin/0.05% genipin	40.87 ± 13.95
Sericin/PVA/glycerin/0.075% genipin	34.69 ± 13.87
Sericin/PVA/glycerin/0.1% genipin	33.63 ± 12.88

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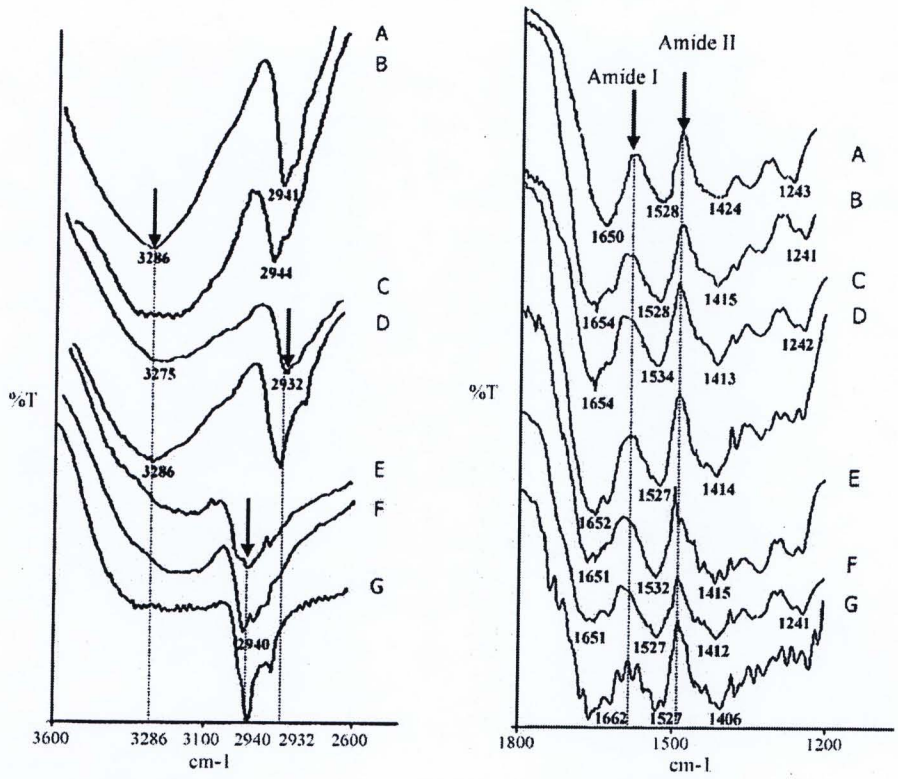

Fig. 2. FTIR spectra of sericin/PVA scaffold with and without glycerin and different concentrations of genipin. A represents sericin/PVA scaffold, B represents sericin/PVA/glycerin scaffold, C–G represent sericin/PVA/glycerin scaffold with 0.01, 0.025, 0.05, 0.075, 0.1% genipin, respectively.

scaffold. Adding genipin into the scaffolds resulted in an increase in the mean pore size which is an unexpected result. However, the size of the porous diameter decreased and uniformity increased following an increase in genipin concentration similar to the results of collagen crosslinking reported by Castaneda et al. [27]. All scaffolds were highly porous, which is quite suitable in terms of their use as tissue engineering material [3,28].

3.3. FTIR spectra

FTIR was used to confirm the secondary structural transition of dry sericin films. Fig. 2 represents the FTIR spectra of sericin/PVA scaffold with or without glycerin and various concentrations of genipin. The protein conformation was determined by identifying the peak positions of amide I, II and III corresponding to C=O, N–H and C–N stretching, respectively. As shown in Fig. 2, all scaffolds exhibited amide I and II peaks at around 1630 and 1520 cm^{-1}, and an amide III peak at 1230 cm^{-1}. The amide II band in Fig. 2 (B–G) in samples containing glycerin has a reduced intensity compared with that of the sericin/PVA scaffold, similar to the results reported by Gillgren et al. [29]. Generally, the amide II band responds to differences in the hydrogen bonding environment [30]. Thus, glycerin presumably has an effect on the hydrogen bonding of the amide groups such that amide–amide interactions are reduced by an increase in amide–plasticizer interactions.

The changes in the amide I band were similar in size to those of the amide II band. In the presence of glycerin, the intensity of the amide I band decreased. These results indicate that glycerin enhances the formation of α helical forms [29]. Furthermore, it can be assumed that the effect of glycerin is equally strong on the amide I band structure and the amide II band, similar to the results obtained with glycerin and zein, a cereal storage protein [29]. When 1% glycerin was added to the sericin/PVA scaffold as a plasticizer, the main change was the appearance of a broad peak at 3286 cm^{-1}, indicating that water was present in the sample. This was due to the moisture sorption properties of the glycerin plasticizer.

After adding different concentrations of genipin as a crosslinking agent to the sericin/PVA scaffold in the presence of glycerin, it was evident that the stretching (=C–H) peak and absorption peaks around 1370–1650 cm^{-1} diminished accordingly. This may be explained by the crosslinking effect to double bond and amides. These data indicated that adding the plasticizer and crosslinking agent drastically changed the molecular structures of sericin, providing it with its scaffold properties.

3.4. Degree of crosslinking

TNBS has been used as a UV chromophore in various procedures to determine primary amino groups in peptides and proteins [23]. Fig. 3 shows the percentage of crosslinks in the sericin/PVA/glycerin scaffolds with various compositions of genipin from 0.01 to 0.1% compared with that of the sericin/PVA and sericin/PVA/glycerin scaffolds. Higher concentrations of genipin in the scaffold resulted in a higher degree of crosslinking or fewer free ϵ-amino groups. The addition of 0.1% genipin to the scaffold increased the degree of crosslinking by approximately 30% compared with the sericin/PVA/glycerin scaffold, and up to 80% when compared with the sericin/PVA scaffold alone. Genipin at 0.01% concentration showed significant difference in degree of crosslinking when compared with the scaffold composed of 0.075 and 0.1% genipin. The crosslinking mechanism of genipin and sericin containing amine is not well understood. It is expected that the reaction occurs with amino acid lysine, hydroxylysine and arginine which contain in the primary amine side chain of sericin [14]. Touyama et al. proposed mechanism of a genipin with a methylamine [31]. The reaction occurred through a nucleophilic attack of the primary

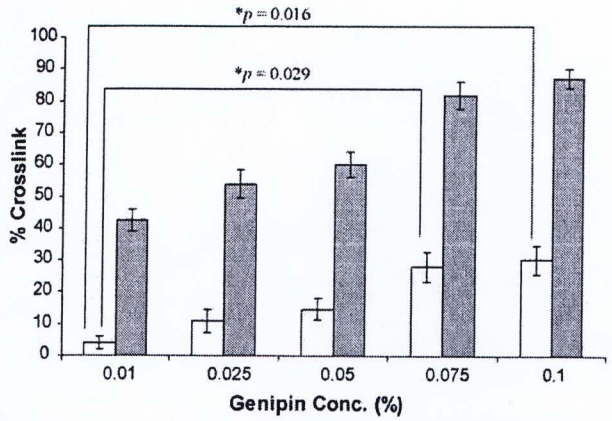

Fig. 3. Percentage of crosslinks in sericin/PVA/glycerin scaffold with various concentrations of genipin from 0.01 to 0.1% compared with crosslink that of the sericin/PVA and sericin/PVA/glycerin scaffolds, respectively. (□) indicates difference of percentage of crosslinks of sericin/PVA/glycerin + genipin with sericin/PVA/glycerin scaffold, (▩) indicates difference of percentage of crosslinks of sericin/PVA/glycerin + genipin with sericin/PVA scaffold. * indicates significant differences at $p < 0.05$.

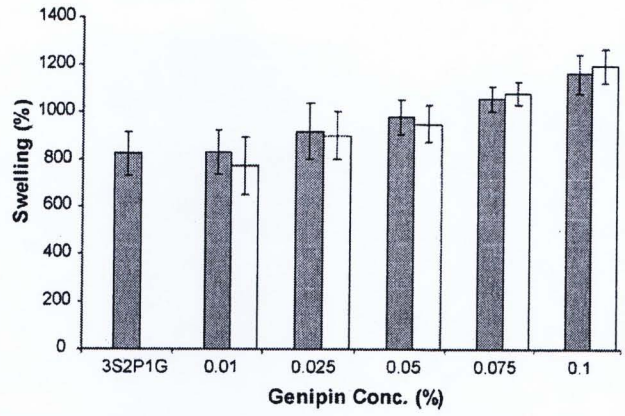

Fig. 5. The swelling of sericin/PVA scaffold with and without glycerin and various concentrations of genipin after immersion in water for 6 and 24 h. 3S2P1G = sericin/PVA/glycerin scaffold, 0.01–0.1% = sericin/PVA/glycerin with 0.01–0.1% genipin scaffold, respectively. (▩) indicates percentage of swelling after 6 h of immersion, (□) indicates percentage of swelling after 24 h of immersion.

amine on the C_3 carbon of genipin. This caused an opening of the dihydropyran ring. An attack on the resulting aldehyde group by the secondary amine then followed. The final step in the formation of crosslinking is believed to be the dimerization produced by radical reactions. This indicates that genipin can form both intramolecular and intermolecular crosslinks. Glycerin can enhance the crosslinking in the sericin/PVA scaffold, similar to the result reported by Brault et al., which indicated that plasticizers such as glycerin can significantly enhance the formation of crosslinks within caseinates (milk proteins chains) [32]. This effect was explained by the preferential binding concept described by Gekko and Timasheff [33]. Similar behaviors were observed with other plasticizer such as propylene glycol and triethylene glycol [34]. Our results further indicated that genipin can effectively crosslink sericin, as in other proteins such as collagen, gelatin and casein [35–37].

3.5. Moisture absorption

Fig. 4 shows the percentage weight change of the scaffolds after placing them in a high humidity environment. The results indicated that the sericin/PVA scaffold had the lowest ability to absorb moisture, but that adding glycerin significantly increased this ability. This may partly be due to the moisture absorption capacity of glycerin itself. After 24 h, sericin/PVA scaffold absorbed moisture significantly less compared with scaffolds composed of genipin ($p = 0.003$, 0.002, 0.002, 0.022 and 0.000 for the case of 0.01–0.1% genipin, respectively). Genipin also enhanced the moisture absorption capacity of the sericin scaffold and extended the time taken to reach equilibrium. The time required to attain equilibrium swelling was higher for the sericin/PVA/glycerin scaffold with genipin at a concentration between 0.01 and 0.075% compared with the sericin/PVA scaffold with and without glycerin. Without genipin, the moisture absorption capacity of the sericin/PVA and sericin/PVA/glycerin scaffold reached the equilibrium within 3 days while those containing genipin had not reached equilibrium even after 5 days. Genipin concentration of the scaffolds between 0.01 and 0.1% produced an approximately 10% difference in weight change from moisture absorption.

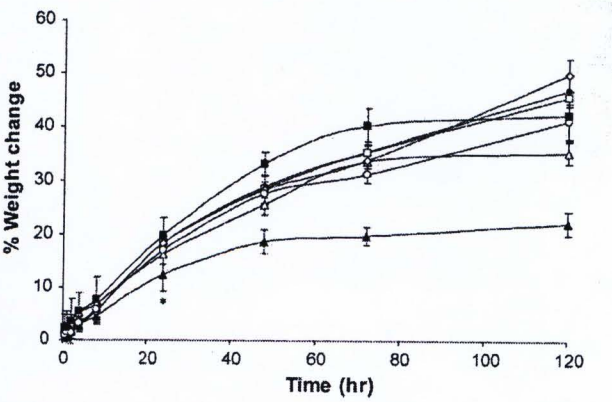

Fig. 4. The percentage weight change of sericin/PVA scaffold with and without glycerin and different concentrations of genipin after placing into high humidity (~80%) environment. (▲) represents sericin/PVA scaffold, (△) represents sericin/PVA/glycerin scaffold, (●) (□) (◊) (○) and (■) represent sericin/PVA/glycerin with 0.01, 0.025, 0.05, 0.075, 0.1% genipin scaffold, respectively. * indicates significant differences compared with sericin/PVA scaffold at $p < 0.05$.

Our results regarding the moisture absorption capacity of the sericin/PVA/glycerin scaffold with genipin as a crosslinking agent are similar to previously reported results [38]. Liu et al. found that increasing the degree of crosslinking by using genipin decreased the hydrophilicity of chitosan films which indicated that our scaffold composed of sericin/PVA/glycerin with 0.1% genipin (the highest degree of crosslink) should exhibit the lowest hydrophilicity. Since scaffold contained 0.1% genipin absorbed the highest amount of moisture (Fig. 4), it can be expected that the degree of hydrophilicity of the sericin scaffolds decreased with increasing moisture sorption capacity.

3.6. Swelling properties

The swelling of the sericin/PVA scaffold with and without glycerin and various concentrations of genipin after immersion in water for 6 and 24 h is shown in Fig. 5. The sericin/PVA scaffold was completely dissolved within 1 h. There was an 8-fold swelling of the sericin/PVA/glycerin scaffold compared with the initial weight after 6 h immersion and this scaffold was completely dissolved within 24 h. The swelling of sericin/PVA/glycerin with genipin increased over a period of time and was directly related to the percentage weight of genipin added to the scaffold base. At 0.1% genipin, the swelling after 6 and 24 h immersion was about 11 and 12 times that of the initial stage, respectively. However, no significant difference in swelling properties of sericin/PVA and sericin/PVA/glycerin scaf-

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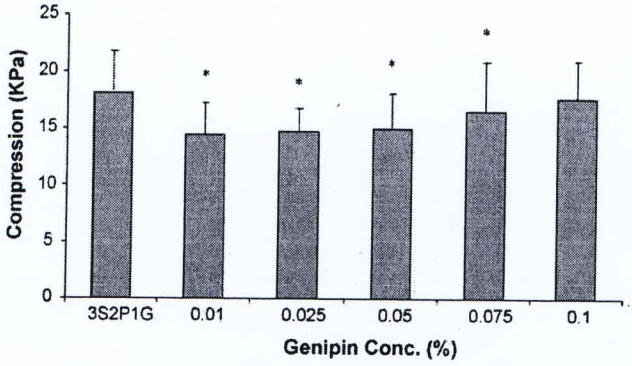

Fig. 6. The mechanical integrity of sericin/PVA/glycerin scaffold with different concentrations of genipin. Data represents mean ± SD ($n = 3$). 3S2P1G = sericin/PVA/glycerin scaffold, 0.01–0.1% = sericin/PVA/glycerin with 0.01–0.1% genipin scaffold, respectively. * indicates significant differences compared with 3S2P1G at $p < 0.05$.

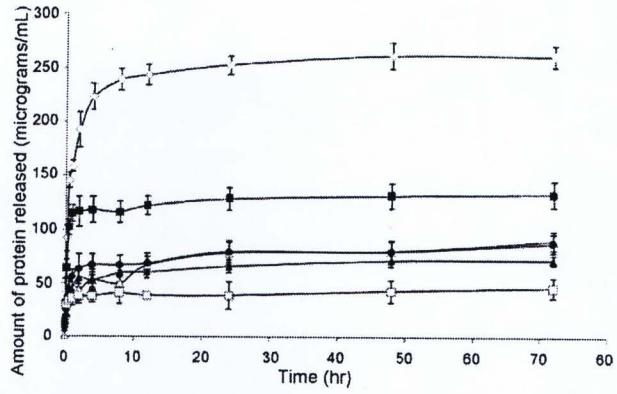

Fig. 7. The amount of protein released from the scaffolds. (◊) represents sericin/PVA/glycerin scaffold, (■)(△)(●)(▲)(□) represent sericin/PVA/glycerin with 0.01, 0.025, 0.05, 0.075 and 0.1% genipin scaffolds, respectively.

fold compared with scaffolds composed of genipin. Our finding is similar to the result reported by Chiono et al., which indicated that increasing the concentration of genipin led to greater swelling and degrees of dissolution of chitosan films [39]. A higher degree of genipin oligomerization resulted in a porous network with higher swelling properties [40]. The longer equilibrated moisture absorption time (Fig. 4) resulted in the higher swelling ratio (Fig. 5). This may be due to the flexible structure of the scaffold containing genipin, which was characterized by slow water sorption but a high water holding capacity, similar to that observed for mucin discs by Builders et al. [41]. The swelling properties at 6 and 24 h were not significantly different, because the three-dimensional scaffold allows its total surface area to interact with the water molecules during the initial swelling.

Our swelling results indicated that adding glycerin alone to the sericin/PVA scaffold is not enough to make scaffolds that are stable in an aqueous solution for 24 h. Genipin or other crosslinking agents are necessary in order to provide solid material suitable for biological applications.

3.7. Mechanical properties of scaffolds

Fig. 6 demonstrates the mechanical integrity of the sericin/PVA/glycerin scaffold with different concentrations of genipin. Without genipin, the scaffold exhibited greater compressive strength, similar to the scaffold with a high genipin concentration (0.1%). At a low concentration, genipin may soften and reduce the compressive strength of the scaffold but not to a significant level, while higher concentrations of genipin enhanced mechanical strength. Since silk sericin is amorphous in nature, the sericin scaffold normally has low mechanical strength. However, a plasticizer such as glycerin can increase the moisture content in the scaffold, it enhances the formation of crosslinks as also shown in other biopolymers [42]. Genipin can also increase the compressive strength of the scaffold due to its impact and hard structures. Our results showed that both plasticizer and crosslinking agent greatly improved the physical properties of the sericin/PVA scaffold. The concentration of genipin had a significant effect on the scaffold's characteristics. With regards to the physical appearance, glycerin greatly improved the flexibility of the sericin/PVA scaffold. Sericin/PVA/glycerin scaffold showed significant greater compressive strength compared with the scaffolds composed of 0.01–0.075% genipin ($p = 0.003$, 0.012, 0.016 and 0.012 for the case of 0.01–0.075% genipin, respectively). However, no significant difference in compressive strength of sericin/PVA/glycerin scaffold compared with the scaffolds composed of 0.1% genipin. Our findings suggested that glycerin or other plasticizers as well as the optimum concentration of genipin are required to provide the integrity and mechanical strength for the use of sericin in future applications.

According to the extremely high swelling property and very low compressive modulus of sericin/PVA/glycerin with genipin scaffold in the dry state, it indicates that this scaffold has high porosity with low density. This type of material is highly elastic and flexible which should be useful in fabrication of wound dressing. However, in this case the compressive modulus may still be even lower in the wet state, similar to the results found in others [43,44]. The low compressive modulus of sericin/PVA/glycerin with genipin scaffold may limit its use in tissue engineering, this similar mechanical property has been found in other scaffolds such as fibroin/hyaluronic acid composite or chitosan scaffold which could be used for neural tissue engineering or dermal substitution [45,46], but not suitable for bone tissue engineering.

3.8. Release of sericin from scaffolds

Fig. 7 demonstrates the amount of protein released from the scaffolds. The sericin/PVA scaffold completely dissolved and released all sericin in less than 30 min (data not shown). Fig. 7 shows that the sericin/PVA/glycerin scaffold without genipin released the highest amount of sericin, while higher genipin concentration led to the release of a lower amount of protein. Maximum protein leaching from all scaffolds was observed within 48 h. The fraction of protein released from the sericin/PVA/glycerin scaffold was approximately 4%, with values of about 1.03 and 0.04% in the case of scaffolds with 0.01 and 0.1% genipin, respectively.

As sericin can activate collagen production in wounds, low levels of sericin released from the scaffold will be beneficial and, at the same time, the matrix should also be stable. Even though the sericin/PVA scaffold released large amounts of sericin, the structure was completely degraded after immersion for a few hours. Since free sericin molecules that remain non-crosslinked contribute to the leached-out protein fraction, the sericin/PVA/glycerin scaffold that had the lowest degree of crosslinking compared to the scaffold with genipin exhibited higher sericin release, resulting in structural collapsed, which makes it unsuitable for further application. Adding genipin to the scaffold led to lower sericin release and a more intact structure. However, the fraction of protein released from the scaffold was quite low, with a maximum of about 4% in the scaffold without the crosslinking agent, while scaffolds with genipin released an even smaller amount of protein. Mandal et al. reported that these scaffolds can easily be tuned by varying their compositions to obtain the desired level of sericin release, which may be

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significant in terms of wound healing and tissue engineering [5]. Due to its hydrophilic structure, PVA was also released from scaffolds, as monitored by thin layer chromatography–densitometric method after immersion for 24 and 48 h, respectively. Briefly, silica gel was used as a stationary phase and methanol–water mixture (50%) was used as a mobile phase. The R_f value of 0.8 was a spot of PVA and detected by densitometric method. The result indicated that 66–70% of PVA (mean 68.3 ± 1.5%, $n = 3$) was released from sericin/PVA/glycerin with 0.05% genipin scaffold after 24 h water immersion. The amount of PVA released was constant even monitoring at a longer period of immersion (48 h) which indicates that the scaffold cannot release more PVA. While lower amount of PVA, approximately 33–40% (mean 36.7 ± 2.6%, $n = 3$), was released from sericin/PVA/glycerin with 0.10% genipin scaffold under the same condition. The significant lower amount of PVA released from scaffold containing high concentration of genipin (higher degree of crosslink) may be due to the higher entrapment of PVA between sericin chain, resulting in less available amount of this polymer to be released ($p < 0.01$). Taking into account the high swelling and the amount of protein as well as PVA released, erosion might be the degradation behavior of sericin/PVA/glycerin scaffolds. Since small amount of sericin and some portions of PVA were released from scaffold, part of the scaffold structure still maintained and stable even after 48 h immersion.

4. Conclusion

Porous-three-dimensional sericin and PVA scaffolds can be formed but are fragile. Adding a plasticizer such as glycerin along with genipin as a crosslinking agent, markedly improved their properties. Uniform pore distribution, stable structures with good compressive strength, high swellability and the desired level of sericin release can be achieved by varying the concentration of the crosslinking agent. Sericin, which is considered as a waste product from the silk industry, can thus be modified to form a good candidate for biomaterial products. In term of tissue engineering application, sericin/PVA/glycerin scaffold with 0.1% genipin seems to be a good candidate since it shows good moisture absorption, high swelling degree and good mechanical strength. However, the amount of sericin released from scaffold with 0.1% genipin is much lower than others, biological tests needs to be performed in order to confirm its beneficial in tissue engineering applications.

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Article

In Vitro Evaluation of the Antimicrobial Effectiveness and Moisture Binding Properties of Wound Dressings

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Abstract: A variety of silver-coated dressings and some impregnated with other chemicals are now available in the market; however, there have been few studies analyzing their comparative efficacies as antimicrobial agents. Moreover, their properties for retaining an appropriate level of moisture that is critical for effective wound healing have never been reported. Five commercially available silver-containing and chlorhexidine dressings, Urgotul SSD®, Bactigras®, Acticoat®, Askina Calgitrol Ag® and Aquacel Ag®, were tested to determine their comparative antimicrobial effectiveness *in vitro* against five common wound pathogens, namely methicillin-sensitive and -resistant *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Mepitel®, a flexible polyamide net coated with soft silicone, was used as a control. The zones of inhibition and both the rapidity and the extent of killing of these pathogens were evaluated. All five antimicrobial dressings investigated exerted some bactericidal activity, particularly against *E. coli*. The spectrum and rapidity of action ranged widely for the different dressings. Acticoat® had a broad spectrum of action against both Gram-positive and -negative bacteria. Other dressings demonstrated a narrower range of bactericidal activities.

Regarding the absorption and release of moisture, Askina Calgitrol Ag® absorbed and released the most moisture from the environment. Aquacel Ag® also exhibited good moisture absorption and moisture release, but to a lower degree. The other tested dressings absorbed or released very little moisture. Askina Calgitrol Ag® and Aquacel Ag® are good alternative dressings for treating wounds with high exudates and pus. An understanding of the characteristics of these dressings will be useful for utilizing them for specific requirements under specified conditions.

Keywords: antimicrobial; moisture absorption; wound dressing

1. Introduction

The skin is the largest human organ and acts as an extremely effective biological barrier. Cutaneous wounds are normally open to the environment and wound beds are favorable environments for bacterial growth. Burn wounds are open wounds and present a critical threat to burns victims, especially those with large areas of burns. This is the primary reason for dehydration, systemic infection and other complications suffered by burns victims [1-3]. One key factor for the effective treatment of burns patients is to close the wound as soon as possible [4,5]. Infections, either by bacteria or fungi, can lead to deterioration of the wound healing process [6] and severe systemic complications. The use of antibacterial agents locally and/or systemically can contribute to wound healing, especially for burn wounds. Their use inhibits microbial growth on or around the wounds and provides a suitable microenvironment for healing [1,2,4].

Wound management can be facilitated with dressings designed both to act as a temporary barrier and to promote wound healing [7]. Several topical antimicrobial agents are widely applied to wound dressings. Silver, in particular, has been a preferred additive to medicated wound dressings because it has broad antimicrobial and antifungal activity [8]. However, there are various forms and formulations of silver dressings available on the market, and little is known about their comparative effectiveness as antimicrobial agents and the spectrum of microbial killing that each provides. Other antimicrobials used in dressings are chlorhexidine, polyhexamethylene biguanide (PHMB) and iodine, but none of these have provided any evidence for promoting resistance. Silver has the advantage of having broad antimicrobial activities against Gram-negative and Gram-positive bacteria and there is also minimal development of bacterial resistance. However, there have been several reports that silver-impregnated dressings used on burns patients can induce hepatotoxicity and argyria-like symptoms [9,10]. It might soon be possible to meet the specific requirements for any particular circumstance, such as dressings that can adequately inhibit microbial growth, yet exhibit minimal silver toxicity and still enhance wound healing. Moreover, an appropriate dressing should be applied to a sensitive patient with wounds infected by pathogens.

Besides antimicrobial properties, the ability to absorb moisture is also an important factor for healing. Bolton *et al.* suggested that the use of more moisture-retentive dressings generally supports faster healing compared with less moisture-retentive dressings [11]. However, comparisons of

moisture absorption by dressings have never been studied even though the presence of moisture is known to accelerate the healing response compared with wounds that have been allowed to dry [12].

The objective of this study is to evaluate the antimicrobial effectiveness of five commercially available antimicrobial dressings *in vitro*. The moisture penetration of each dressing will also be investigated.

2. Results and Discussion

Table 1 shows the compositions of the studied coated dressings. All dressings contain either silver or chlorhexidine as an antimicrobial agent except for Mepitel® (which has been used as a control).

Table 1. Compositions of the studied coated dressings [13-15].

Dressing	Compositions of the dressing materials	Formulation compositions of the coated
Urgotul SSD®	Lipido-colloid dressing made of a polyester mesh impregnated with hydrocolloid particles (carboxymethylcellulose), vaseline particles and silver sulfadiazine	Silver sulfadiazine content 3.75%
Bactigras®	Chlorhexidine acetate 0.5% in white soft paraffin tulle dressing	Chlorhexidine acetate BP 0.5%
Acticoat®	Nanocrystalline silver being applied to high-density polyethylene mesh which is covered to either side of a rayon-polyester core	Nanocrystalline silver 105 mg/100 cm^2
Askina Calgitrol Ag®	Silver alginate wound dressing consists of an absorbent foam sheet. one surface of which is coated with an alginate matrix containing ionic silver together with a cleanser, moisturizer and a superabsorbent starch co-polymer	Silver 141 mg/100 cm^2
Aquacel Ag®	Sodium carboxymethylcellulose fibers containing 1.2% ionic silver. In the presence of exudate, the dressing absorbs liquid to form a gel, binding sodium ions and releasing silver ions	Silver 8.3 mg/100 cm^2
Mepitel®	Porous, semi-transparent, low-adherent wound contact layer, consisting of a flexible polyamide net coated with soft silicone	None

2.1. Corrected Zone of Inhibition Test

The result for the zones of inhibition generated by antimicrobial agents and dressings are presented in Table 2. The data show that all products generated an inhibitory zone against most individual microorganisms. All dressings inhibited bacterial populations to some extent except Bactigras®, which had no activity against *P. aeruginosa*. Acticoat® and Askina Calgitrol Ag® produced the largest zones of inhibition, which may due to the high concentration of silvercontained in these dressings (105 mg/100 cm^2 and 141 mg/100 cm^2, respectively) compared with 3.75% of silver sulfadiazine in

Urgotul SSD® and 0.5% chlorhexidine in Bactigras®. It is clear that different organisms produced differently sized zones of inhibition against the same dressing. Methicillin resistant *Staphylococcus aureus* (MRSA) and *B. subtilis* were less sensitive to the tested antimicrobial dressings, as shown by a smaller zone of inhibition compared with other organisms.

Table 2. Corrected zone of inhibitions (mm) generated by topical antimicrobial dressings.

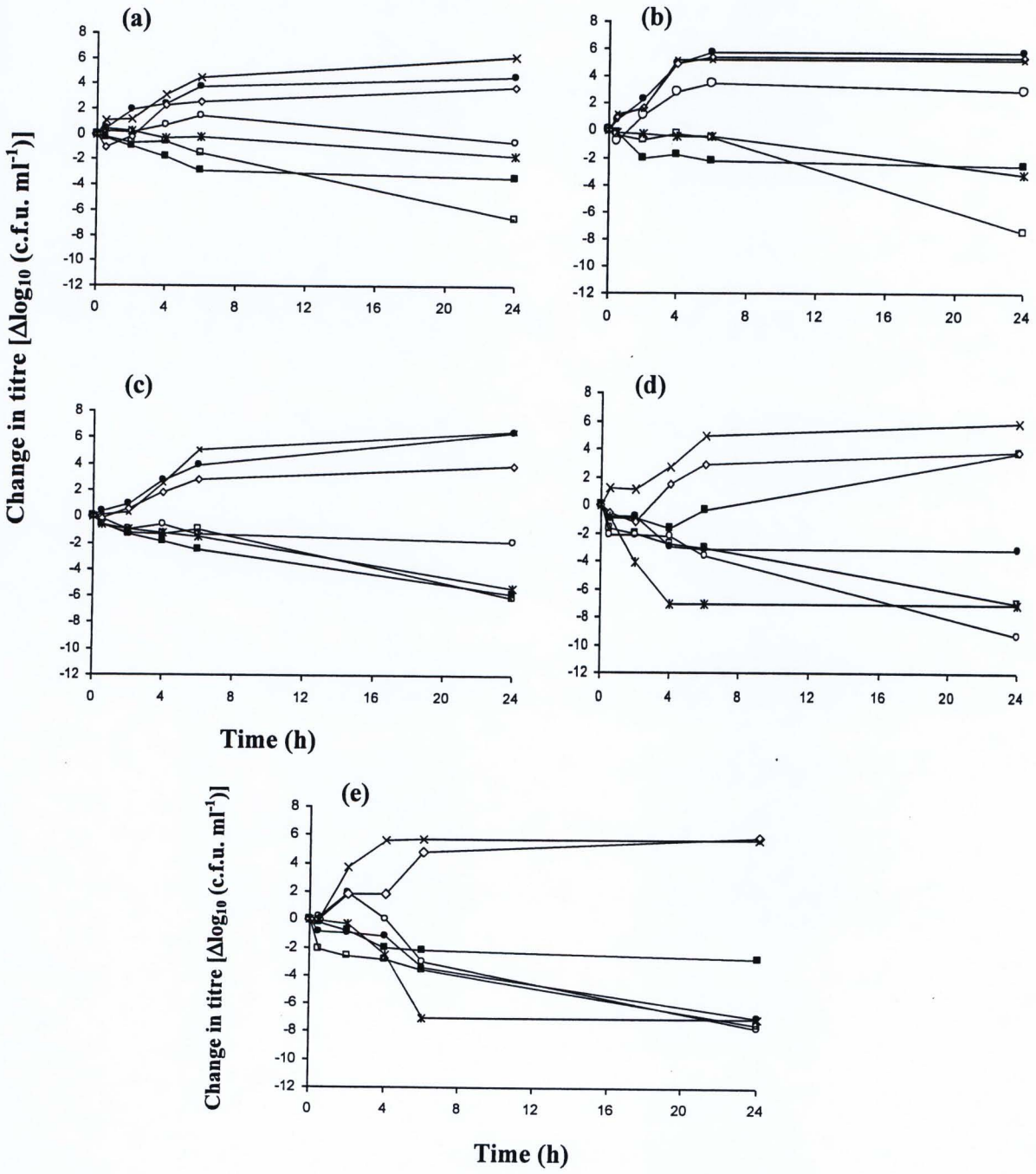
Microorganism	Urgotul SSD®	Bactigras®	Acticoat®	Askina Calgitrol Ag®	Aquacel Ag®	Mepitel®
S. aureus	1.41 ± 0.86	1.13 ± 0.42	13.30 ± 0.78	24.33 ± 3.12	12.97 ± 0.85	0.00
MRSA	0.19 ± 0.11	0.36 ± 0.33	6.69 ± 0.14	8.11 ± 4.33	1.84 ± 0.95	0.00
B. subtilis	2.39 ± 2.11	7.12 ± 1.24	10.98 ± 0.49	5.62 ± 1.48	6.69 ± 1.39	0.00
P. aeruginosa	9.05 ± 3.34	0	17.62 ± 4.82	21.08 ± 0.89	22.56 ± 1.77	0.00
E. coli	6.44 ± 1.22	0.78 ± 0.16	15.98 ± 0.84	12.42 ± 0.69	10.58 ± 0.47	0.00

Infection is a significant cause of delayed or prolonged wound healing, and high bacteria levels interfere with the progression of wound healing [16,17]. The broad antibacterial properties of silver and its derivatives have made it a good candidate and practical choice for creating silver dressings for wound care [13,14]. The results of this study show that all tested dressings investigated exerted some bactericidal activity, particularly on *E. coli.*

2.2. Bactericidal Activities of Antimicrobial Dressings

The spectrum and onset of action ranged widely for the various dressings. The bactericidal activities of the antimicrobial dressings against the five microorganisms are shown in Figure 1. Bactericidal activity was indicated by a reduction in bacterial counts presented as $\log_{10}$c.f.u. (colony forming units) mL^{-1} over time. These curves also indicated the rate of bacterial killing and provided an additional index of efficacy against the described isolate [18]. The normal growth rate of each organism was represented by the growth control and that of the Mepitel® dressing, which contained no antimicrobials. Overall, Acticoat® seemed to be the most effective dressing against these five tested organisms, especially with Gram-positive bacteria, whereas Urgotul SSD® and Bactigras® seemed to have a lower antimicrobial effect compared with the other dressings. For the Gram-positive bacteria, *S. aureus* (Figure 1a,b) and *B. subtilis* (Figure 1c), the Acticoat® dressing exerted maximal bactericidal activity, achieving more than a 4 log reduction of bacterial growth after 24 h. The killing patterns of *S. aureus* and *B. subtilis* by silver dressings were similar to MRSA, except for Aquacel Ag®, which slightly reduced both *S. aureus* and *B. subtilis* counts but had no effect on MRSA. With *P. aeruginosa* (Figure 1d), Acticoat®, Askina Calgitrol Ag® and Aquacel Ag® exhibited a good bactericidal effect. The maximal killing of *P. aeruginosa* was achieved at 4 h with Askina Calgitrol Ag® and the reduction in bacterial counts was sustained. The killing pattern for *E. coli* (Figure 1e) by Askina Calgitrol Ag® was similar to that for *P. aeruginosa* except for the maximal killing, which was found at 6 h. All dressings exhibited bactericidal activity and achieved more than a 4 log reduction of *E. coli* (Figure 1e) except for Bactigras®, which had a less pronounced effect.

Figure 1. The bactericidal activities of the antimicrobial dressings against five microorganisms. Values are the means of three experiments performed in triplicate. $\Delta\log_{10}$ c.f.u. ml^{-1} is the difference in $\Delta\log_{10}$ c.f.u. ml^{-1} at the time of bacterial inoculation, starting from $t = 0$. Strains: (**a**) Methicillin-sensitive *Staphylococcus aureus* (ATCC 6338P); (**b**) Methicillin-resistance *Staphylococcus aureus* (ATCC 25923); (**c**) *Bacillus subtilis* (ATCC 6633); (**d**) *Pseudomonas aeruginosa* (ATCC 27853); (**e**) *Escherichia coli* (ATCC 25922) and □ represents Acticoat®; ○ represents Aquacel Ag®; * represents Askina Calgitrol Ag®; ■ represents Bactigras®; ● represents Urgotul SSD®; ◊ represents Mepitel® and × represents growth control.

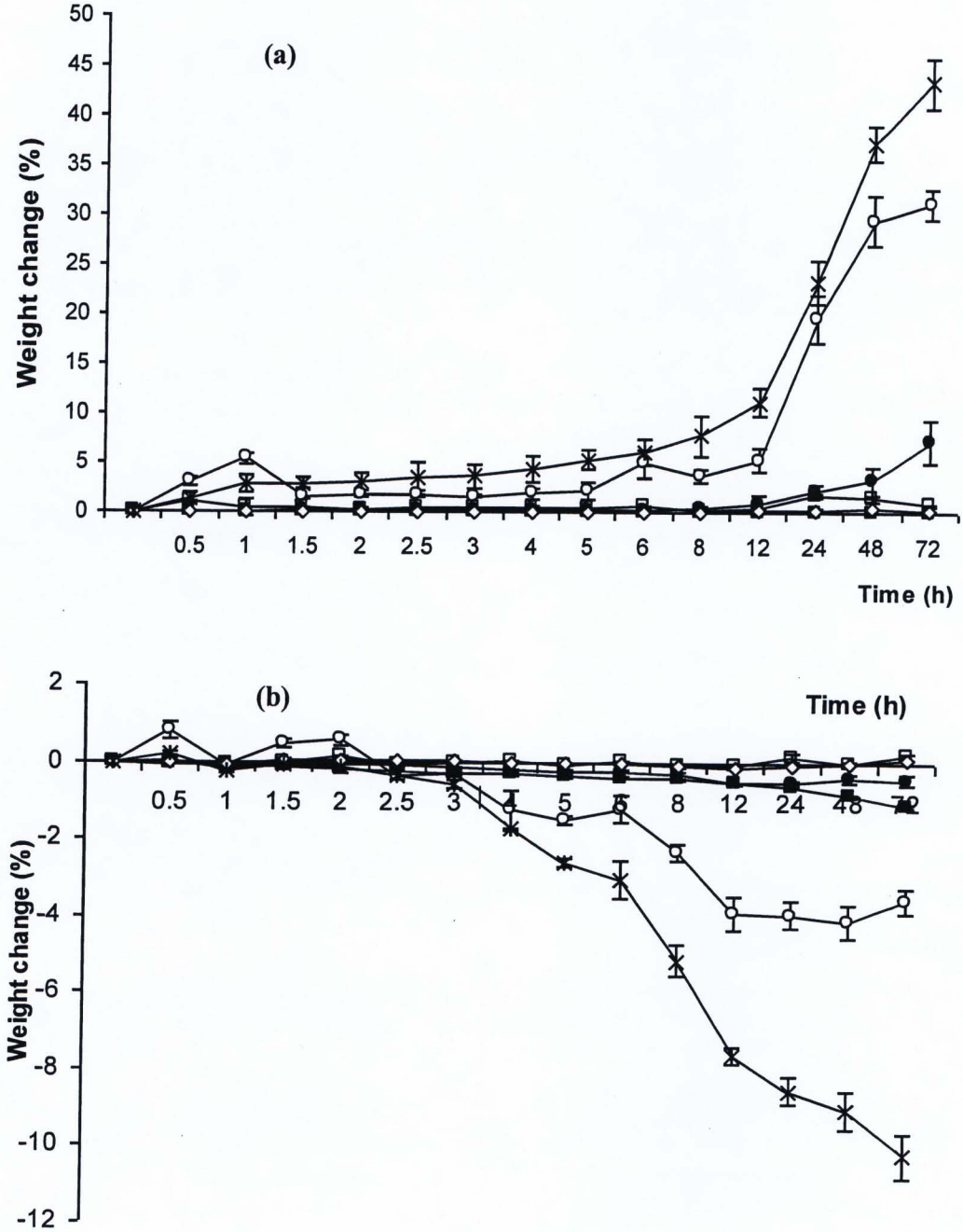


Acticoat® was effective and showed a broad spectrum of bactericidal activities on the bacteria tested with a long duration of action; these results are similar to those reported by Castellano *et al.* [19]. On the other hand, Askina Calgitrol Ag®, which contains the highest silver concentration compared with all tested dressings [14], had the most equivalent efficacy to Acticoat®. Since the silver form in Acticoat® is nanocrystalline, the molecular size and concentration of silver are higher than those in other dressings [20,21]. Because of its nanocrystalline form, Acticoat® also exhibits sustained-release of silver molecule resulting in longer duration of action. Urgotul SSD® showed bactericidal effects only with Gram-negative bacteria, similar to the results reported by Ip *et al.* [18]. The antimicrobial activity of Urgotul SSD® only generated from silver sulfadiazine, which shows the main activity on Gram-negative bacteria. Most dressings showed their bactericidal activity after the first hour of testing and the activity generally lasted for at least 24 h. One advantage of this rapid antibacterial action is that it allows wound healing to proceed without bacterial interference and reduces the likelihood that resistance will develop [18]. Since some dressings did not show their maximal bactericidal activity after 24 h, the limitation of this study is that it should have been extended for longer than 24 h, since some of the dressings might have a sustained effect for many days. Our study confirmed the effectiveness of silver and chlorhexidine dressings against a broad range of bacterial pathogens. With the enhanced bacterial killing effects, clinicians should be concerned that a too high level of silver could be delivered into the tissues and cause an adverse effect such as keratinocytes and fibroblasts toxicity that might affect on the recovery of wounds [18,22]. Suitable dressings for each use should be considered from the patient's sensitivity and the possible side effects from the level of silver in the dressings.

2.3. Wound Dressing Water Vapor Absorption

At steady state, potassium sulfate and potassium acetate in desiccators provided a relative humidity of 96.1 ± 1.5% and 22.4 ± 1.3% at 30 °C, respectively. Figure 2 shows the percentage weight change of each dressing after being placed into the desiccators at a relative humidity of 96.1% (Figure 2a) and 22.4% (Figure 2b), respectively for 0.5 to 72 h. Acticoat®, Bactigras®, Mepitel® and Urgotul SSD® absorbed or released very little moisture from the dressing at any humidity, whereas Askina Calgitrol Ag® absorbed and released the most moisture in humid conditions. After being placed in high humidity, Askina Calgitrol Ag® started to absorb moisture within 30 min and showed a significant weight change after 12 h. It also absorbed moisture close to 50% of its initial weight after being placed in a high humidity environment for 72 h and still did not reach a saturated condition. Nevertheless, it started to release moisture after placing it in a low humidity environment for 3 h and released approximately 10% of its weight after 72 h. In addition to Askina Calgitrol Ag®, Aquacel Ag® also showed a good absorption of moisture and had moisture release properties but to lower degree. Aquacel Ag® absorbed moisture up to 30% of its weight after 72 h in a high humidity environment after which it started to reach its saturated condition, whereas it showed approximately 4% moisture release after placing it in a low humidity environment for 72 h.

Figure 2. The percentage weight change of each dressing after placing into the desiccators with relative humidity at (**a**) 96.1% and (**b**) 22.4% for 0.5 to 72 h. □ represents Acticoat®; ○ represents Aquacel Ag®; * represents Askina Calgitrol Ag®; ■ represents Bactigras®; • represents Urgotul SSD® and ◊ represents Mepitel®.



Since moisture is also an important factor for wound healing, the presence of moisture avoids the delays in healing response, which occur when wounds are allowed to dry out [12]. Excessive fluid retention at the wound surface, however, can result in poor healing and the maceration of the surrounding tissue [23]. Our results indicated that Askina Calgitrol Ag® absorbed and released the most moisture of the dressings tested. This was most evident with the foam dressings since there are

great variations in the location of the surface moisture [24]. The greatest percentage weight change occurred with Askina Calgitrol Ag® at high humidity since the foam dressings have a tendency to expand or shrink easily. Moreover, it contains superabsorbent starch co-polymer, which also increases the absorption capacity. These data indicated that Askina Calgitrol Ag® is a good alternative for treating wounds with high exudates and infection. Our result is the first report on the moisture absorption property of various antimicrobial dressings that will be beneficial for clinical application.

3. Experimental Section

3.1. Corrected Zone of Inhibition Test

The antimicrobial effect of each dressing, Urgotul SSD®, Bactigras®, Acticoat®, Askina Calgitrol Ag®, Aquacel Ag® and Mepitel®, was tested using corrected zone of inhibition method. This test was performed according to the method reported by Gallant-Behm with some modifications [25]. Briefly, the bacterial isolates were grown in broth for 4 to 6 h, and the broth was used to inoculate Muller-Hinton agar plates to form a confluent lawn. The various wound dressings (about 1 cm^2) were applied to the center of each lawn, and all plates were incubated for 24 h at 37 °C. The inhibition zone surrounding the tested dressing was then determined. No plate dehydration was observed around the dressings and all tests were performed in triplicate with results expressed as a mean with standard deviations.

3.2. Bactericidal Activities of Antimicrobial Dressings

In order to determine the onset and duration of antimicrobial activity of each dressing, bactericidal activities at different time points were determined by bacterial broth culture method which was adopted from Fraser *et al.* with some modifications [26]. Dressings (about 1 cm^2) were prepared in an aseptic manner. Each square was placed in a sterile vial and the dressing subjected to a pretreatment with 800 μL of distilled water for 10 min (according to a previously established protocol for an absorbancy test for the volume required and duration required for pretreatment). Tryptone soy broth (2.2 mL) was then added to each vial to make up to a total volume of 3 mL.

A suspension of each organism was prepared in broth from fresh colonies after overnight incubation and the turbidity was adjusted to the 0.5 McFarland standard (~1×10^8 c.f.u./mL). An aliquot (10 μL) of the bacterial suspension was added to each vial containing the dressing. Control broths with and without bacterial inoculation were also included. The vials were then incubated with agitation at 35 °C in a water bath. Aliquots of 10 μL of the bacterial broth were sampled from each vial at specific time intervals (0, 30 min and 2, 4, 6 and 24 h) and serial 10-fold dilutions for each aliquot were prepared in broth. Duplicate aliquots (25 μL) of each of the serially diluted samples were spread on plates. The plates were then incubated overnight at 35 °C and colonies counted (c.f.u./mL). The dilutions that allowed quantification (10-150 colonies) were counted and the mean counts calculated. Nine vials, containing the five antimicrobial dressings as well as the control dressing (Mepitel®) together with the culture and the broth controls, were included in each experiment for each organism. Plate counts were measured in triplicate and each experiment was repeated three times to obtain a mean value of c.f.u. counts.

3.3. Wound Dressing Water Vapor Absorption

Dressings (about 9 inch^2) were prepared in an aseptic manner and precisely weighed. Each dressing was placed in a desiccator pre-equilibrated with salts to make the relative humidity a desired value. Potassium sulfate or potassium acetate powder was placed in a desiccator to achieve a percentage relative humidity of about 90% and 20% at 30 °C, respectively, as reported by Greenspan [27]. After 30 min, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 24, 48 and 72 h, each dressing was taken from the desiccator using sterile forceps and again precisely weighed. The equilibrium moisture absorption was determined by the percentage weight change [28]. The experiments were performed in triplicate.

4. Conclusions

From five-tested antimicrobial dressings, Acticoat® was the most effective dressing against the tested organisms, whereas Urgotul SSD® and Bactigras® showed a lower antimicrobial effect compared with other dressings. Regarding the water vapor absorption activity, Askina Calgitrol Ag® absorbed and released the most moisture in humid conditions. Aquacel Ag® also showed good moisture absorption and release properties but to lower degree, while the other dressings hardly absorbed or released any moisture.

Acknowledgements

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Effectiveness of Inflammatory Cytokines Induced by Sericin Compared to Sericin in Combination With Silver Sulfadiazine Cream on Wound Healing

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Abstract: Silk sericin (SS) has been shown to promote collagen synthesis during wound healing, but it lacks antimicrobial activity. We investigated the effectiveness and the induction of the inflammatory mediators IL-1β and TNF-α by SS, silver sulfadiazine (SSD) cream, and SS in combination with SSD cream on wound healing in rats. The results show that SS at 8% w/w partially inhibits SSD antibacterial activity. Treating wounds with a combination of SS and SSD did not induce significant wound size reduction when compared to other treatments. However, SS can promote collagen production in wounds even in the presence of SSD. Wounds treated with the combination of SS and SSD cream showed higher levels of IL-1β and TNF-α when compared to wounds treated by SS alone, but the differences were not significant. Although SS may decrease the antimicrobial effect of SSD, SS in combination with SSD cream has the benefit of promoting collagen production without generating significant levels of inflammatory cytokines.

Silk sericin (SS), a group of glue proteins produced in the middle silk gland of the silkworm, is an essential component of the cocoon filament. It is comprised of granular and high molecular weight proteins with adhesive and gelatin-like characteristics.[1] Our previous study demonstrated that SS can promote reduction in wound size and increase collagen production in rats without causing any allergic reactions.[2] Several studies also demonstrated the advantages of using SS in wound healing by enhancing the attachment and growth of mouse fibroblasts, human skin fibroblasts, and human and mouse hybridoma in culture media.[3-5] However, there are several reports on immune response that argue against silk sutures containing SS proteins[6-8] and assert that the SS proteins are responsible for skin irritation and allergies.[9] The fact that SS shows no antibacterial activity may also make it less attractive for wound regeneration.

Wound healing is a dynamic process that involves the integrated action of many cell types, the extracellular matrix, and chemical mediators. Wound repair can be divided into three overlapping stages: inflammation, formation

of new tissue, and tissue remodeling.[10] The initial inflammatory phase is characterized by localized activation of innate immune mechanisms. This results in an initial influx of neutrophilic granulocytes into damaged tissue followed by an accumulation of macrophages.[11] An infection that occurs during this stage will impede the regeneration process. The impact of local inflammatory responses on the wound healing process has been debated for decades. Recently, Eming et al[12] demonstrated that the number of macrophages infiltrating the wound tissue mediates accelerated tissue repair and thus is significant. The healing process is characterized by macrophages and fibroblasts initiating repair and deposition of new fibrous tissue above and below the dermal substitutes, which envelopes them under an epidermal layer.[13] Collagen production, an important factor for wound healing, is subsequently stimulated.[14] New blood vessels and capillaries are normally observed within the dermal substitutes and new fibrous tissue is generated beneath the dermal substitutes.

Severe injury of the skin can lead to sepsis, a major cause of high mortality in patients with burns. Various treatments have been used to prevent and control sepsis, but the most successful is the use of topical silver sulfadiazine cream (SSD).[15-17] Advantages of silver are its broad antimicrobial activities against gram-negative and gram-positive bacteria and minimal development of bacterial resistance.[18] Microscopic evaluation of SSD-treated wounds shows that SSD has the potential to preserve viable dermal tissue.[19] However, epidermal regeneration takes place at a rather slow rate under an easily disintegrating crust, and the newly formed epidermis temporarily has an "irritated" aspect characterized by spongiosis, parakeratosis, and pseudocarcinomatosis.[19,20] SSD can also induce hemolytic anemia in glucose-6-phosphate dehydrogenase-deficient burn patients, transient leukopenia, which occurs in 3%–60% of patients, and severe hypersensitivity reactions.[21-25]

Sericin, in combination with SSD cream, may combine the beneficial effect of collagen promotion with antimicrobial properties. However, the silver ion can bind to proteins, including human serum albumin[26] or other substances such as bilirubin,[27] which may inhibit its activity. During the acute inflammatory response, normally there is an increase in the acute-phase proteins, which is usually mediated by proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) or interleukin (eg, IL-1β).[28] An increase in the amount of these proteins is usually associated with the induction of anorexia, weight loss, sepsis-induced proteolysis, etc.[29,30] Since both SS and SSD can activate cytokine production, which in turn may affect the inflammatory reaction, it is important to monitor the inflammatory mediators induced by SS cream and compare the levels of these mediators to the levels induced by SS in combination with SSD cream during the healing process.

The aim of this study was to investigate the effectiveness of SS cream when compared to SS in combination with SSD cream by monitoring wound size reduction and the levels of the inflammatory mediators (TNF-α and IL-1β) induced during wound healing in rats.

Materials and Methods

Preparation of silk sericin and silk sericin cream. Fresh cocoons of Bombyx mori were kindly supplied by Chul Thai Silk (Petchaboon province, Thailand). Silkworm cocoons were produced in a controlled environment. After cutting cocoons into pieces (about 5 mm^2), SS was extracted with purified water (1-g of dry silk cocoon: 30-mL of water) by autoclaving (SS-320, Tomy Seiko, Tokyo, Japan) at 120°C for 60 minutes using a high temperature and pressure degumming technique.[31] After the membrane was filtered to remove fibroin (a fibrous protein from silkworm), SS powder was obtained by freezing and lyophilizing the SS solution using a Heto LL 3000 lyophilizer (Allrod, Denmark). SS cream was formulated using the combination of white petrolatum, mineral oil, lanolin, glycerin, bisabolol, propylparaben, and methylparaben. Due to the limited solubility of SS, the final concentration of SS in cream was 8% w/w. All chemicals were purchased from Sigma (Singapore) and used without further purification. SSD powder is a commercial product and was purchased from Chemie Trade (Mumbai, India). It was formulated using the same cream base as the SS cream. Sericin with SSD cream was prepared by adding SS and SSD into the cream base using the same formula as other preparations.

Animals. Eight-week-old male Sprague-Dawley rats, purchased from National Laboratory Animal Center (Mahidol University, Thailand) weighing 250 g ± 5 g, were used for these experiments. Each rat was caged alone at 25°C ± 2°C and subjected to a 12:12-hour light-dark cycle (standard fluorescent light) and had access to food and water *ad libitum*. Animals were acclimatized for 1 week before experimental use. The animals were maintained according to the *Guide for the Care and Use of Laboratory Animals* established by the National

Laboratory Animal Center (Mahidol University, Thailand).

Inhibitory effect of silk sericin on silver sulfadiazine. Antibacterial assays of SS solution were performed using a filter disk method.[32] Filter disks (diameter 6 mm) made from AA grade filter paper (Whatman, Maidstone, England) were placed on nutrient agar plates seeded with various strains of bacteria, including *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Micrococcus luteus*. SS solutions, 2-1 of 1%, 5%, and 8% were pipetted onto the center of each of the test disks. The organisms were incubated at 37°C ± 0.2°C for 24 hours.

The antibacterial activities of various concentrations of SS cream, 1% w/w SSD cream, and various concentrations of SS in combination with 1% SSD cream were evaluated against the previously mentioned five strains of aerobic bacteria using the standard cup-plate method.[33] Nutrient agar medium was used for culturing and the bacteria were incubated for 24 hours at 37°C ± 0.2°C. Inhibition zone diameters were measured using a zone reader.

***In-vivo* animal tests.** Forty-five rats were divided into three groups of 15. The skin over the dorsal area was shaved completely; application fields were outlined with a marking pen. Two full-thickness skin wounds were prepared by excision (1.5 cm x 1.5 cm) on the left and right side of the dorsum of each rat at least 3 cm apart to ensure no interference between each wound. Then, each wound was exposed to water at 90°C for 7 seconds in order to produce a full-thickness burn.[34,35] Group 1 animals were topically treated with the cream base on the left-side wounds (formula control wounds) and 8% SS cream on the right-side wounds (treated wounds). In Group 2, 1% SSD cream and 8% SS cream were topically applied to the left- and right-side wounds, respectively. In Group 3, 8% SS cream and 1% SSD with 8% SS cream were topically applied to the right- and left-side wounds, respectively. During surgery, the animals were anesthetized with 30-mg/kg intramuscular injection of Zoletil® 100 (Virbac Laboratories, Carros, France) and enrofloxacin (Baytril®, Bayer, Shawnee Mission, KS). Carprofen (Rimadyle®, Pfizer, New York, NY) at 5 mg/kg was injected subcutaneously every 24 hours for 5 days for pain reduction. Cream base, SS cream, SSD cream, or SSD in combination with SS cream was applied evenly to wounds daily after cleaning with normal sterile saline solution. The wound surface, body weight, and skin irritation were monitored. Five rats in each group were sacrificed on days 3, 7, and 15 after cream application. Tissue samples needed for cytokine and collagen determination were collected from all wounds from the area that was demarcated during initial wound creation. The Mahidol University Animal Care and Use Committee (MU-ACUC) approved the study protocol.

Wound measurement. Wound area measurements were taken using a stereomicroscope (Carl Zeiss, NY, Primo Star model, 0.3 x 0.65 objective lens) and photographed with MoticCam 2300® (1024 x 768 pixels). Motic Images Plus 2.0 ML was used for analyzing data.

Morphometric analysis for collagen content in rat tissues. The collagen content in rat tissues was analyzed using image analysis according to the method reported by Kaczmarek et al.[36] From each specimen, color images of 640 x 480 pixel resolution (at 400X) were acquired with a light microscope (BX51, Olympus®), stereoscope (Stemi 2000-C, ZEISS®) and a digital camera (MoticCam 1000, MoticCam®) running under an imaging analysis program (ImageJ, National Institutes of Health, Bethesda, MD). The organization and maturation of collagen bundles was assessed on paraffin sections of wounds stained by Masson's Trichrome. All morphometric analyses were blinded.

For collagen content analysis, color images of each wound were first adjusted by altering the color of an area of non-interest to white, and then that area was replaced with color mode. Adjusted images were then converted to grayscale images and enhanced with the median filter. The area of positive reaction was estimated by the number of black pixels and was determined to be the percentage of black pixels/field in the binary image.[36] At least 16 fields for each tissue sample were randomly selected for collagen content analysis.

Tissue preparation for cytokine determination. The excised rat tissue (100 mg) was placed in 1-mL of homogenate buffer (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1% Triton X-100 and 1% protease inhibitor) and homogenized for 2 x 30-second bursts under ice-cold conditions, then were centrifuged at 11,000*g* for 10 minutes at 4°C. The supernatants were collected and the levels of TNF-α and IL-1β were determined using a rat ELISA assay kit (R&D Systems, Minneapolis, MN). Briefly, either TNF-α or IL-1β diluent (50 µL) was added to each well plate. Then 50-L of homogenated tissue was added to the wells and the plate was incubated for 2 hours at room temperature. Each well was washed five times with a buffer solution followed by the addition of either 100-µL of TNF-α or IL-1β conjugate to each appropriate well. After the 2-hour incu-

bation, each well was again washed five times with buffer (400 µL) and 100-µL of substrate solution was added. The plates were incubated for 30 minutes at room temperature, and then 100-µL of stop solution was added. The reaction was measured quantitatively at 570 nm based on a standard curve of TNF-α and IL-1β.

Statistical Analysis

All data were expressed as mean ± SD. Evaluation of statistical significance was determined by paired and unpaired Student's *t*-test; $P < 0.05$ was considered significant.

Results

Inhibitory effect of silk sericin on silver sulfadiazine. Sericin solution extracted by the high temperature and pressure technique showed no inhibitory effect against any of the bacteria tested, while SSD cream inhibited all strains of bacteria even at the low concentration of 0.25% w/w (data not shown). SS in combination with SSD cream showed an antimicrobial effect but at a lower degree of inhibition than SSD cream alone, as shown by the smaller inhibition zone (Table 1). This indicates that the microbial inhibition effect of SS in combination with SSD cream came only from SSD. The cream containing 1% SSD in combination with 1% SS inhibited the test bacteria better than cream containing 1% SSD in combination with 8% SS, as indicated by larger clear zones in inhibition disk assays (Table 1). There was a significant difference in the size of the clear zone when comparing 1% SSD cream, 1% SSD in combination with 1% SS cream, and 1% SSD in combination with 8% SS cream for all of the test organisms.

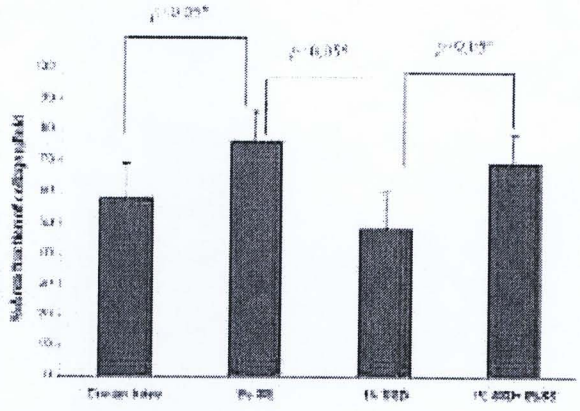

Figure 1. The percent area fraction of collagen per field from all wounds treated with different creams on day 15.

Wound measurement. Size reductions of the rat wounds treated with cream base, SS cream, SSD cream, and SSD in combination with SS cream are shown in Table 2. Compared to the cream base-treated wounds, the SS-treated wounds showed a significant difference in wound size on days 3 through 15. Wounds treated with 8% SS cream also showed a significant difference in size reduction when compared with wounds treated with 1% SSD for 7 days of treatment. However, 1% SSD in combination with 8% SS-treated wounds showed a significant difference in the size reduction only at the initial stage (day 3) when compared to wounds treated only with 8% SS cream. All wounds except the one treated with the cream

Table 1. Inhibition zone (mm) on agar plates treated with 1% SSD, 1% SSD + 1% SS, and 1% SSD + 8% SS cream (n = 6).

Microorganisms	1% SSD cream	1% SSD cream + 1% SS cream	1% SSD + 8% SS cream
B subtilis	18.7 ± 1.1*	13.1 ± 1.3*	11.9 ± 0.1*
E coli	12.3 ± 0.3*	11.8 ± 0.2*	10.8 ± 0.1*
M luteus	25.3 ± 0.6*	20.1 ± 0.5*	19.3 ± 0.2*
P aeruginosa	14.0 ± 0.4*	12.6 ± 0.1*	11.6 ± 0.1*
S aureus	11.8 ± 0.3*	11.7 ± 0.2*	10.7 ± 0.2*

*Indicates significant difference ($P < 0.05$).

Table 2. Percent reduction in size of rat wounds treated with cream base, SS cream, SSD cream, and SSD in combination with SS cream.

% wound size reduction	Treatment					
	Group 1		Group 2		Group 3	
	Cream base	8% SS	1% SSD	8% SS	8 % SS	1% SSD + 8% SS
Day 3	6.04 ± 3.12	19.49 ± 5.75*	11.40 ± 10.06	21.21 ± 6.06*	18.38 ± 8.47	11.43 ± 7.36*
Day 7	53.77 ± 5.49	73.17 ± 8.70*	60.11 ± 11.48	78.31 ± 6.65*	70.70 ± 5.12	74.43 ± 8.31
Day 15	76.13 ± 5.20	100 ± 0*	100 ± 0	100 ± 0	100 ± 0	100 ± 0

*Indicates significant difference when compared within the group ($P < 0.05$).

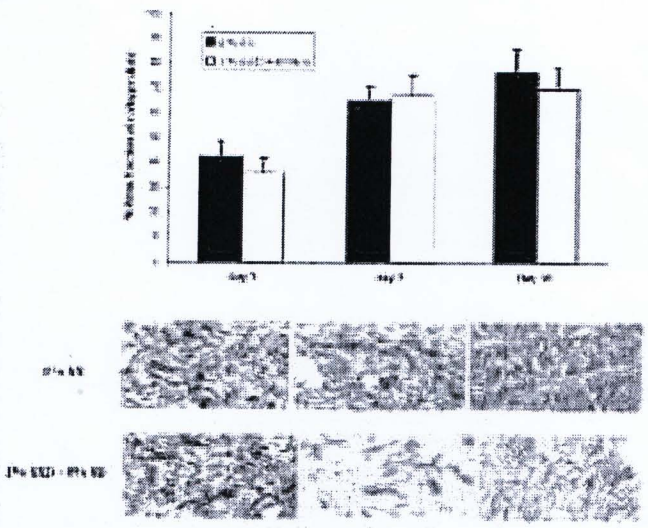

Figure 2. The percent area fraction of collagen per field and histological appearance of group 3 wounds treated with 8% SS and 1% SSD + 8% SS on day 3, 7, and 15, respectively.

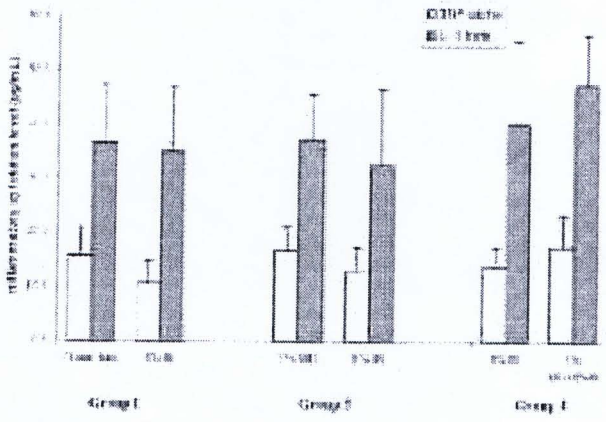

Figure 3. The amount of inflammatory cytokines generated from rat tissues after 7 days of treatment.

base had completely epithelized by day 15.

Collagen content in wounds. Collagen content in wounds was assessed by the percent area fraction of collagen per field. Figure 1 shows the percent area fraction of collagen per field from all rats' wounds treated with different creams on day 15. There was a significant difference in percent area fraction of collagen per field on day 15 between the cream base and the 8% SS-treated wounds, between the 8% SS-treated and the 1% SSD-treated wounds, and between the 1% SSD-treated and the 1% SSD in combination with 8% SS-treated wounds (Figure 1; $P < 0.05$). However, the fraction of collagen per field showed no significant difference between the cream base and the 1% SSD treated wounds or between the 8% SS-treated wounds and the 1% SSD in combination with 8% SS-treated wounds. This indicates that mainly SS, as reported by the fraction of collagen per field, generates the amount of collagen in wounds.

The percent area fraction of collagen per field and the histological appearance from group 3 wounds treated with 8% SS and 1% SSD in combination with 8% SS on days 3, 7, and 15 are compared in Figure 2. The results indicate that there is no significant statistical difference in area fraction of collagen in wounds treated with SS cream when compared to SSD in combination with SS cream on either days 3, 7, or 15.

TNF-α, IL-1β production from rat tissues. Figure 3 shows the amount of inflammatory cytokines generated from rat tissues after 7 days of treatment. There was no significant difference in IL-1β levels generated from wounds treated with either cream base, 8% SS cream, 1% SSD cream, or 1% SSD in combination with 8% SS cream. Within the same group, the 8% SS cream-treated wounds generated slightly lower levels of IL-1β compared to wounds treated with the other treatments. Regarding TNF-α levels, again there was no significant difference in the TNF-α levels generated from wounds treated with either cream base, 8% SS cream, 1% SSD cream, or 1% SSD in combination with 8% SS cream. Within the same group, the 8% SS cream-treated wounds generated lower TNF-α levels compared to wounds treated with other treatments, but the level of change was not significant.

Discussion

Our previous study demonstrated that SS, a globular protein from silkworm, has biological activities associated with wound healing and good biocompatibility and degradability.[2] Some of these biological activities may be due to the fact that most amino acids in SS have strongly polar side groups such as hydroxyl, carboxyl, and amino groups, which can covalently bind with each other or with active groups of other compounds.[37] Zhang[38] reported that SS also has antibacterial activity. However, the results of the present study indicate that this is not the

case and that SS solution at high concentrations (up to 8%) has a negative effect on the antibacterial activity across the five bacterial strains tested. This may be due to the protein extraction method used, as the extracted portion may not contain the fraction that confers antibacterial activity. Other researchers have reported that the antimicrobial property of SS was derived from a low molecular weight protein, seroin, from *B mori*.[39,40] Seroin is not involved in silk fiber construction and/or coating, and may play a role in protecting silk against microbial degradation.[40] Zurovec et al[39] reported that seroin polypeptides are present in silk as 22.5 kDa and 23 kDa molecules, and that these polypeptides are liberated from other silk proteins when silk components are dissolved. The procedure in the present study to prepare SS used high temperatures and pressures to dissolve the silk proteins may have caused the release of seroins and the loss of antimicrobial activity in the SS preparations. The molecular weight of SS extracted by high temperature and pressure methods similar to those employed here is in the range of 35 kDa–150 kDa,[41] which is consistent with seroin not being included in our SS preparations. Other SS extraction methods that contain seroin together with other lower molecular weight fractions should be used in future studies to better understand the effects of SS on wound healing, and may show dramatically different results, especially in its antimicrobial activity.

Silver has a long and intriguing history as an antibiotic, especially for burn wounds. The antimicrobial effect of silver and silver compounds is proportional to the bioactive silver ion (Ag+) released and its availability to interact with bacterial or fungal cell membranes.[42] Silver ion is biologically active and readily interacts with proteins, amino acid residues, free anions, and receptors. However, upon binding with other biological molecules, silver no longer retains its antimicrobial activity. Topical antimicrobial agents are thought to promote normal healing by protecting the wound from infection. Since SS can promote wound healing but lacks antimicrobial activity, combining SS with SSD cream may benefit the healing process. However, if silver molecules dominantly bind with SS, the silver compounds may be inactivated. The authors speculate that the decrease in antimicrobial activity at higher concentrations of SS in cream is due to SSD partially binding with SS. Zhao et al[43] reported similar results showing that silver can interact with other proteins such as collagen and DNA, resulting in a controlled release property.[43] The binding of a bioactive silver ion with SS is possible because SS has a strong negative molecular charge. At low SS concentrations, more unbound and bioactive silver ions are available compared to what is available at high SS concentrations. Nevertheless, we found that SS (as high as 8% w/w) did not totally refrain the activity of SSD. Since the present study was conducted *in vitro*, similar studies should be performed *in vivo* to validate these findings.

SSD in combination with SS cream did not show better healing properties with respect to wound size reduction when compared to SS cream only. This may be due to the uninfected conditions of the wounds tested, as there was no need for wound healing improvement via the antimicrobial action of silver. A study, with a bacterial challenge to the wounds, should be attempted to determine the effects of SS with and without SSD in the presence of infection, and without SSD in the presence of infection. However, SS cream still reduced wound size when compared with the cream base and SSD cream alone. Although the cream-containing silver did not accelerate the healing process in the present study, antimicrobial agents will remain a necessity for daily burn wound care under non-sterile conditions.

The present results further indicate that SS promotes collagen production even in the presence of SSD. Lee et al[44] reported a similar outcome; SSD did not inhibit collagen synthesis but improved dermal wound healing when coated onto a collagen membrane. SS in combination with SSD accelerated collagen deposition; however, there was not a significant reduction in wound size when compared to the other treatments. These data indicate that collagen is not the most important factor for wound healing, especially at the initial stage, which corresponds to the healing mechanism that collagen production normally occurs during formation of new tissue and tissue remodeling—the last stage of wound repair. Moreover, histological samples from wounds treated with 8% SS cream and 1% SSD in combination with 8% SS cream revealed closely packed, thick fibrils of collagen fibers and a collagen fibril organization with fibril bundles that were parallel to each other, which may indicate wound contraction and hypertrophic scar formation.[45,46] Further development for other forms of SS delivery, such as in a scaffold, may be beneficial to reducing wound contraction and result in a lower level of scar formation.

IL-1β and TNF-α are proinflammatory cytokines that are involved in a variety of immunological functions. Their levels are shown to be strongly upregulated during the inflammatory phase of healing and normally reach their peak levels on day 7 after injury.[47,48] IL-1β is a very

important inflammatory mediator in the skin and a key factor in initiation of the inflammatory response,[49,50] while TNF-α induces the expression of cutaneous and endothelial adhesion molecules, causing the development of skin irritation and inflammatory responses.[51] Goldberg et al[52] showed that TNF-α also suppresses the tissue growth factor-β (TGF-β)-induced fibroproliferative phenotypic genes such as collagen type IA and fibronectin at the mRNA level. SS itself can also activate IL-1β and TNF-α production from fibroblast cells, but not at significant level.[53] The present study showed that wounds treated with SS cream did not yield significantly high levels of IL-1β and TNF-α on day 7, which suggests SS did not induce an inflammatory or immunological response. However, these proinflammatory cytokine levels may vary depending on the response of each individual subject. In order to avoid that variable, the formulas being compared need to be tested on the same subject. SSD itself causes significantly profound inflammatory responses when used in severely burned children.[54] From our results, wounds treated with SSD in combination with SS demonstrated elevated levels of both proinflammatory cytokines when compared to SS-treated wounds—similar to results reported by other researchers.[54-56]

Conclusion

Adding SS to an SSD cream formulation can partially inhibit SSD action, as was shown by the smaller microbial inhibition zones, but does not significantly reduce wound size when compared to other treatments. Sericin can promote collagen production in wounds—even in the presence of SSD—without generating significantly higher levels of inflammatory cytokines. Although wounds treated with SSD in combination with SS show elevated levels of both proinflammatory cytokines when compared to SS-treated wounds, the levels are not significant and should not affect its clinical use. However, since there was no untreated control to compare proinflammatory cytokines levels, it cannot be concluded that the SS-treated wounds alone decreased this response.

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Article

The Effect of Sericin from Various Extraction Methods on Cell Viability and Collagen Production

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Abstract: Silk sericin (SS) can accelerate cell proliferation and attachment; however, SS can be extracted by various methods, which result in SS exhibiting different physical and biological properties. We found that SS produced from various extraction methods has different molecular weights, zeta potential, particle size and amino acid content. The MTT assay indicated that SS from all extraction methods had no toxicity to mouse fibroblast cells at concentrations up to 40 μg/mL after 24 h incubation, but SS obtained from some extraction methods can be toxic at higher concentrations. Heat-degraded SS was the least toxic to cells and activated the highest collagen production, while urea-extracted SS showed the lowest cell viability and collagen production. SS from urea extraction was severely harmful to cells at concentrations higher than 100 μg/mL. SS from all extraction methods could still promote collagen production in a concentration-dependent manner, even at high concentrations that are toxic to cells.

Keywords: sericin; cell viability; collagen; extraction; concentration

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1. Introduction

Extracellular matrix proteins such as collagen, fibronectin, and gelatin are known to play important roles in the attachment and growth of mammalian cells. We recently showed that silk sericin (SS), a high molecular weight granular protein with adhesive and gelatin-like characteristics, can promote growth of the mouse fibroblast cell line L929, as well as activation of collagen production both *in vitro* and *in vivo* [1,2]. Many studies have also demonstrated that SS can accelerate the proliferation and attachment of several mammalian cell lines [3-5], and insect cell culture was also reported to be improved by SS [6]. Moreover, SS added to freezing media as an alternative to fetal bovine serum improved the survival of various cell lines during cryopreservation [7]. However, Terada *et al.* reported that culture supplemented with 1.0% SS resulted in no viable cells, which indicates that the presence of 1.0% SS is harmful to cells [3]. These data demonstrate that the concentration of SS supplemented to culture medium is also a significant factor for cell viability. Nevertheless, the optimal concentration of SS for promoting cell viability has never been reported.

SS can be extracted by various methods, such as high pressure and high temperature techniques, acid or alkaline solutions, or enzyme extraction. The method of extraction significantly affects the biochemical activities of silk proteins. Kurioka *et al.* reported that acid-degraded, alkali-degraded, and hot water-degraded SS powders exhibit different trypsin inhibitory activities and have different isoelectric points [8]. Furthermore, different SS extraction methods alter its amino acid composition, which may influence its cell-growth and collagen secretion in cells.

The objective of this study was to investigate the chemical properties of SS extracted from Thai silk strains via various extraction methods, which have never yet been investigated. In addition, we determined the effect of various concentrations of SS obtained from the different extraction methods on fibroblast cell viability and collagen production. These data yield important fundamental information for further development of SS as a serum-free medium supplement.

2. Results and Discussion

It has long been known that SS can accelerate the proliferation of several cell lines, including hybridoma cells [3,5,6,9]. Tsubouchi *et al.* also reported that SS can enhance the attachment of cultured human skin fibroblasts [4]. The attachment and subsequent proliferation of fibroblast cells are considered to play important roles in the healing process of skin lesions. In this study, the L929 mouse fibroblast cell line has been used as a model to investigate the roles of SS from various extraction methods on cell viability and collagen production.

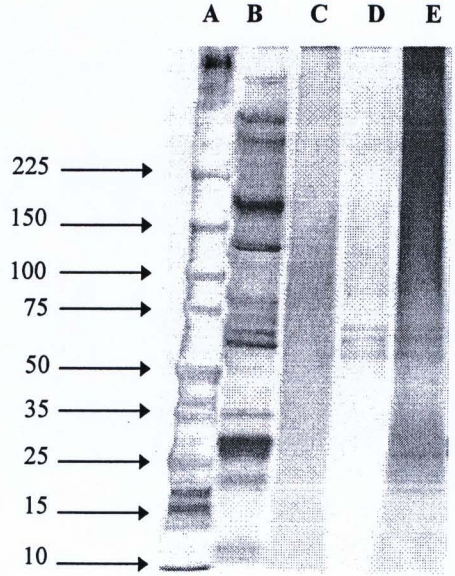
Recently, SS has been shown to have a protective effect against several toxicities, such as alcohol-induced gastric injury, in animal models [10]. However, the SS used in most previous reports [3,5,6,9,10] was extracted by heat or alkaline solution, even though SS can be extracted by various methods, which affect its physical and biological properties, as shown here. This is the first study to compare the enhancement of cell viability by SS at different concentrations derived by extraction procedures. We found that SS does not have only positive effects on cell viability, as at certain concentrations it starts to induce toxicity. This may be an explanation for the previously reported detrimental effects of SS in clinical uses [11,12]. Moreover, extraction methods also play an important role in SS activity. Many studies have used SS prepared by heat or alkaline extraction and

have reported the advantageous effects of this protein on cells. This is in agreement with our 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) result, which showed that SS extracted by heat, acid and alkaline solution is rarely toxic to cells at concentrations up to 100 μg/mL. Nevertheless, the extraction method has not previously been emphasized in relation to the use of SS, which may lead to confusion, as we have proven that SS derived by urea extraction method was severely toxic to cells. Since SS has been widely investigated for its use in biomedical applications, this point should be clarified in order to avoid misleading interpretation of results.

2.1. Molecular Weight of SS

SS extracted by various methods has different molecular weights, as shown in Figure 1. Different extraction methods provided different molecular weight SS, which may result in different chemical and biological properties. SS extracted with urea showed clear bands with molecular weights ranging from 10 to >225 kDa. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of acid-degraded and alkali-degraded SS showed distinguishable bands within the range of 50-150 kDa and 15-75 kDa, respectively. However, the number of bands from acid-degraded and alkali-degraded SS were much lower than that of SS extracted with urea. SDS-PAGE of SS prepared by the high temperature and high pressure degumming technique showed broad bands, with molecular weights ranging from 25-150 kDa. Our findings are in agreement with those reported by Sprague, which indicates that SS is a mixture of at least 15 different polypeptide chains, ranging in size from 20 to 220 kDa [13].

Figure 1. Molecular weight of SS extracted by various methods. (**A**) Marker (**B**) Urea-extracted sericin (**C**) Heat-degraded sericin (**D**) Acid-degraded sericin (**E**) Alkali-degraded sericin.



According to these studies, SS from heat, acid, alkaline and urea extraction methods show different molecular weights. This may seem insignificant, but it may in fact reflect biological properties of SS, such as its antimicrobial activity. Other researchers have reported that the antimicrobial property of SS

is derived from a low molecular weight protein, seroin, from *B. mori* [14,15]. Seroin is not involved in silk fiber construction and may play a role in protecting silk against microbial degradation [15]. Zurovec *et al.* reported that seroin polypeptides are present in silk as 22.5 and 23 kDa molecules, and that these polypeptides are liberated from other proteins when silk components are dissolved [14]. The implication of these findings is that urea extraction should be the only method that would provide SS with antimicrobial activity.

2.2. Particle Size and Zeta Potential Measurement

Table 1 shows the zeta potential and particle size of SS from different extraction methods. SS from all extraction methods exhibited negative zeta potential values. Zeta potential of SS from urea extraction yielded the highest negative charge, followed by acid-degraded SS, heat-degraded SS and finally alkali-degraded SS. Alkali-degraded SS had the largest particle size, followed by heat-degraded and acid-degraded SS, while SS extracted by urea solution had the smallest particle size. Since urea-extracted SS is present as a very small-sized compound in water, it may be in soluble form, while SS extracted by other methods may be present as hydrocolloids.

Table 1. Zeta potential and particle size of SS from different extraction methods.

Extraction method	Zeta potential (mV)	Mean size (nm)
Heat	-20.69 ± 2.14	110.42 ± 35.07
Acid	-32.12 ± 5.26	23.80 ± 16.07
Alkaline	-15.87 ± 2.89	824.42 ± 86.67
Urea	-68.36 ± 5.67	4.62 ± 2.44

Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. The magnitude of the zeta potential gives an indication of the potential stability of the system, where high zeta potentials (either negative or positive) indicates electrically stabilized particles, while colloids with low zeta potentials tend to coagulate or flocculate [16]. From our results, SS obtained from the alkaline extraction method had more of a tendency to coagulate in this solution, which corresponds to the largest particle size. However, SS obtained by heat, acid and urea extraction were stably dispersed with a lower degree of coagulation compared to alkali-degraded SS. Moreover, SS obtained from urea extraction was the most electrically stable and had the least tendency to coagulate, which was confirmed by the smallest mean particle size.

2.3. Amino Acid Analysis

Amino acid content in SS extracted from various methods is shown in Table 2. There were slight variations in the amino acid percentage in SS extracted by different methods; however, the main amino acid component in SS was still the same. Serine was the dominant amino acid in SS (~30%), while aspartic acid and glycine composed about 10-20%. The amount of methionine found in heat-degraded SS was significantly higher than in SS extracted by other methods, while the amount of tyrosine found in urea-extracted SS was significantly lower than in SS extracted by other methods. Moreover, heat-extracted SS, which contains the highest amount of methionine and cysteine, sulfur-containing

amino acids that can generate double-helical structures, can induce the highest levels of collagen production. This result corresponded with our previous report, which showed that methionine in SS relates to its collagen production activity [1].

Table 2. Amino acid composition of SS extracted using various methods (in mole%).

Amino acid	Extraction method of SS			
	Heat	**Urea**	**Acid**	**Alkaline**
Asp	15.64	18.31	15.93	19.88
Ser	33.63	31.27	31.86	30.01
Glu	4.61	5.27	5.75	5.93
Gly	15.03	11.23	10.49	11.01
His	1.06	3.26	2.47	1.72
Arg	2.87	5.41	4.92	4.92
Thr	8.16	8.36	8.51	6.49
Ala	4.10	4.33	3.72	4.21
Pro	0.54	1.46	0.78	1.24
Cys	0.54	0.39	0.53	0.23
Tyr	3.45	0.36	5.56	5.24
Val	2.88	2.96	2.95	2.94
Met	3.39	0.12	0.06	0.15
Lys	2.35	3.14	3.48	2.89
Ile	0.56	0.96	0.87	0.75
Leu	1.00	1.58	1.43	1.56
Phe	0.28	0.60	0.71	0.81

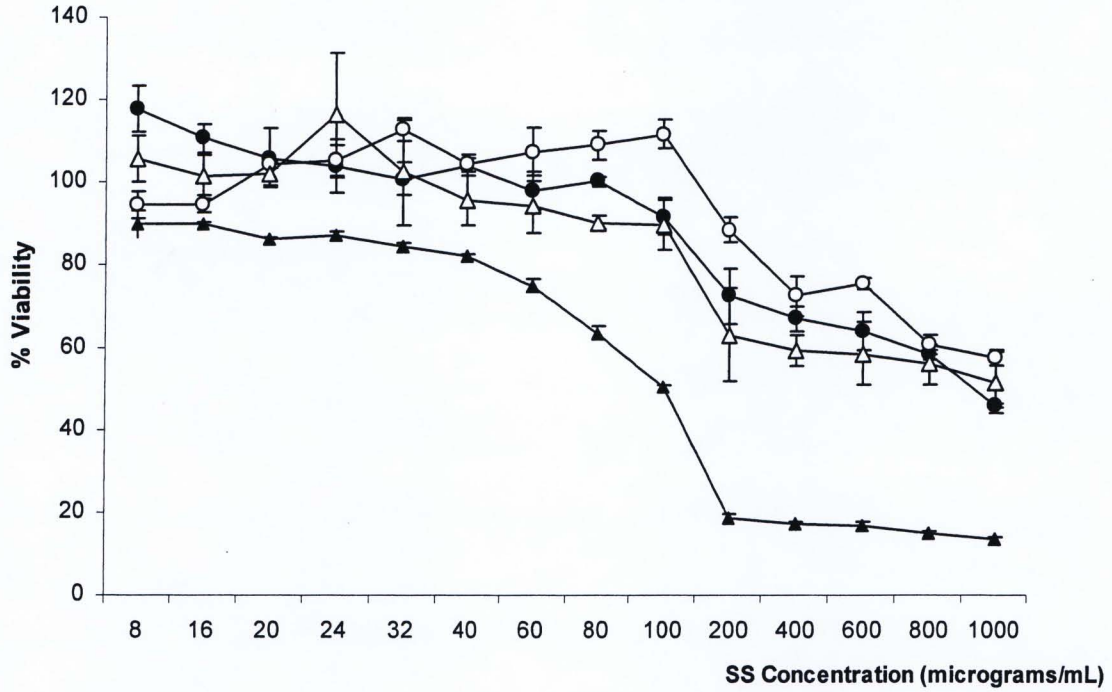
2.4. Cytotoxicity of SS Solution

The MTT assay indicated that SS solutions from all extraction methods had no toxicity to mouse fibroblast cells at concentrations up to 40 μg/mL after 24 h incubation (Figure 2). It also indicated that heat-degraded and alkali-degraded SS exhibit the least cell toxicity. SS derived from all extraction methods except urea extraction could promote cell viability at low concentrations. SS from urea extraction showed slight toxicity at concentration as low as 60 μg/mL and its toxicity became significant at concentrations higher than 100 μg/mL, while SS from other extraction methods showed toxicity to a lesser extent than urea-extracted SS, as shown by the percent viability of fibroblasts. These data indicate that extraction method and SS concentration have significant effects on growth and viability of fibroblast cells.

Heat-degraded SS showed the least toxicity to L929 cells at concentrations up to 100 μg/mL, while acid-degraded and alkali-degraded SS showed similar results, but at lower levels of activation, which is consistent with results of other reports [3,5,9]. However, at concentrations higher than 100 μg/mL, viability of L929 cells decreased. Similarly, Terada *et al.* reported that SS from alkaline extraction at low concentrations increased the population in HeLa (human epithelial cell) cultures, while higher concentration of SS (0.3%) did not [3]. This study also reported that SS at 1.0% was severely harmful

to the murine hybridoma (2E3-O) cell line [3]. From these data, we can conclude that concentration and the extraction method of SS, as well as the particular cell line, can affect the cell viability.

Figure 2. Viability study of L929 cells incubated with SS solutions via the MTT assay after incubation for 24 h. Error bars represent the standard error of the mean (n = 3). (Δ) Acid-degraded sericin, (○) Alkali-degraded sericin, (●) Heat-degraded sericin, (▲) Urea-extracted sericin.

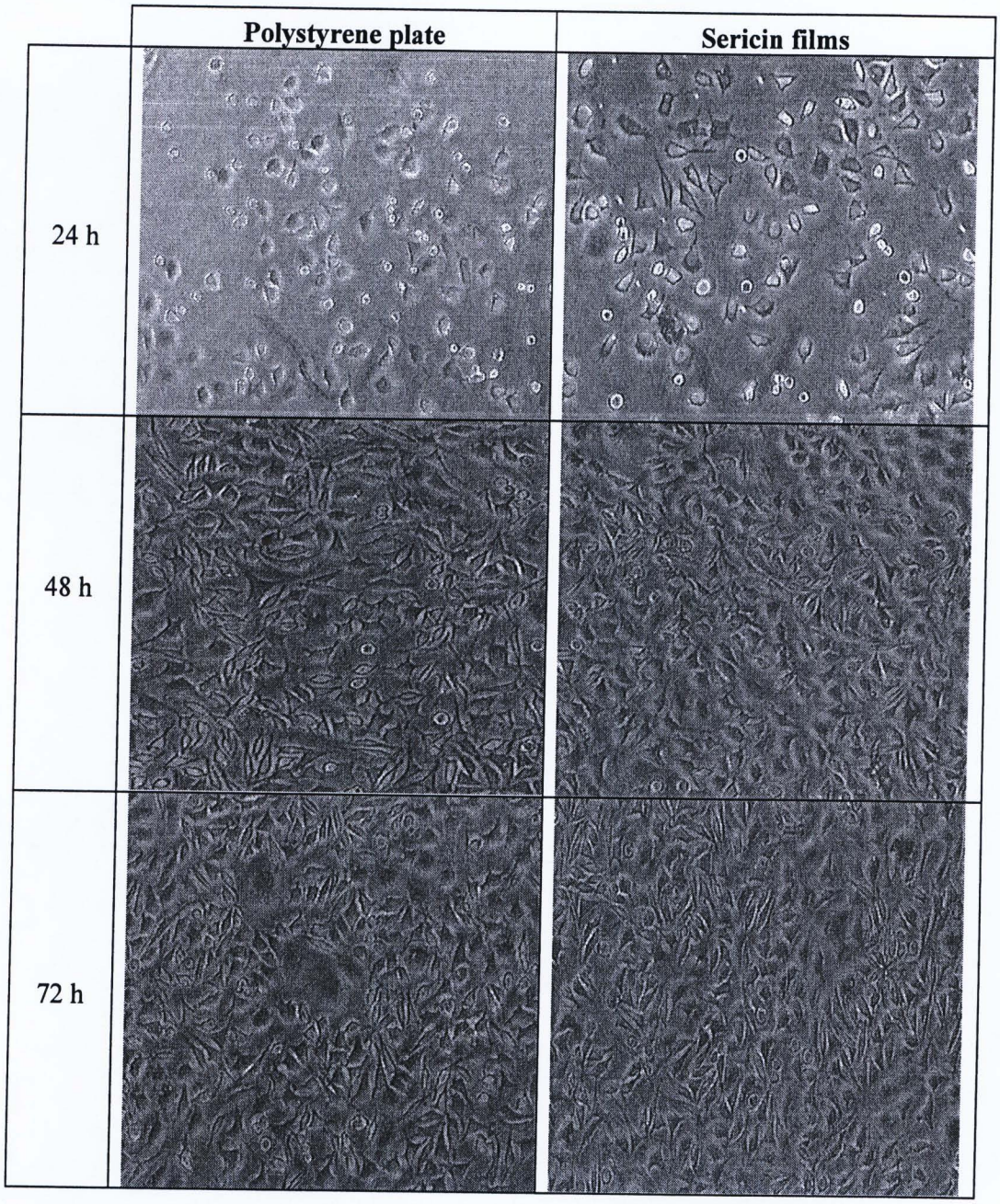


According to our results, the optimum concentration of SS for promoting cell viability depends on the SS extraction method. SS obtained by urea extraction should not be used as a supplement for serum-free medium, since it is toxic to cells. Heat-degraded and alkali-degraded SS are the most suitable for cell culture with the optimum concentration at 100 μg/mL. Low concentrations of acid-degraded SS were beneficial to cells. Concentrations as low as 8 μg/mL lead to the greatest cell viability.

2.5. Adherence of L929 Mouse Fibroblast Cell Line to SS Films

Since heat-degraded SS significantly promoted cell growth compared to other extraction techniques, and because of its chemical-free property, heat-degraded SS was used to study the adherence of fibroblast cells on SS films. The morphology of L929 mouse fibroblasts cultured on SS heat-extracted films observed at 24, 48, and 72 h are shown in Figure 3. Cells started attaching to SS films and began proliferating after 24 h similar to cells on styrene culture plates, which were used as a positive control. Approximately 70% of cells on both control and SS plate attached to the surface at 48 h and became confluent after 72 h. After 72 h, cells fully proliferated and formed complete pseudopodia like structures on styrene culture as well as SS plate. Moreover, the number of cells attached on SS plate at 72 h was slightly higher than number of cells on styrene culture plate.

Figure 3. Morphology of L929 mouse fibroblasts cultured on polystyrene culture plates and on SS films at 20X at 24 h, 48 h and 72 h after cell seeding at 20,000 cells/1,257 mm^2.

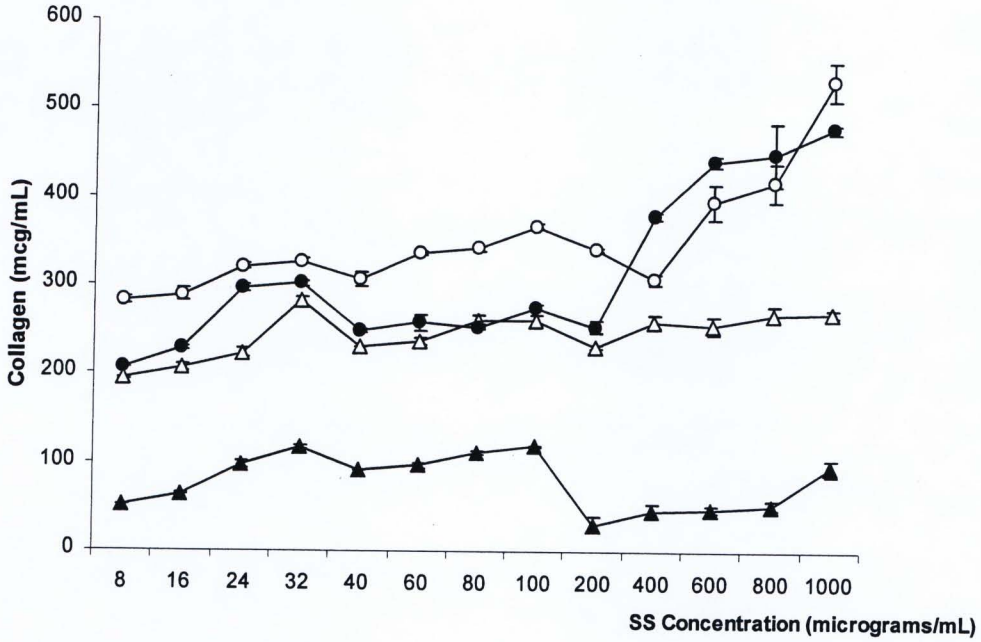


2.6. Determination of Soluble Collagen Production Induced by SS

SS extracted by all methods can induce collagen type 1 production from the fibroblast cell line L929 (Figure 4), while the negative control (fibroblast cells without SS as supplement in culture medium) did not produce any collagen (data not shown). However, urea-extracted SS induced the lowest amount of collagen production, compared to SS extracted by the other methods at all concentrations. Heat-degraded SS induced the highest collagen type 1 production at concentrations from 8-200 μg/mL. At concentrations higher than 200 μg/mL, alkali-degraded SS could activate the

highest collagen production. However, alkali-degraded and heat-degraded SS at concentrations higher than 200 μg/mL induced significant levels of collagen type 1, but resulted in fewer viable cells. These data indicate that SS at concentrations higher than 200 μg/mL can induce collagen production, even though it is toxic to cells.

Figure 4. Collagen type 1 production in fibroblast cell line L929 when various SS concentrations were added into the culture medium for 24 h to make the final concentration of SS in each well 8-1,000 μg/mL, respectively. Error bars represent the standard error of the mean (n = 3). (Δ) Acid-degrade sericin, (○) Alkali-degraded sericin, (●) Heat-degraded sericin, (▲) Urea-extracted sericin.



Enhancement of fibroblast collagen production in cells is normally related to transforming growth factor (TGF)-β [17], which is generally released only from surviving fibroblast cells 2 h after cells are activated by chemicals or trauma, and reaching peak levels after 12 h [18]. This supports our result that collagen content in cell culture still increased at SS concentrations higher than 100 μg/mL, even though the percentage of cell viability decreased. Collagen production may be generated from fibroblast cells, which are activated by silk protein at an early stage when most cells are still viable before SS becomes toxic to cells.

3. Experimental Section

3.1. Materials

3.1.1. Silkworm Cocoons

Fresh *Bombyx mori* cocoons were kindly supplied by Chul Thai Silk Co., Ltd. (Petchaboon province, Thailand). Native Thai silkworms, white cocoons, were produced in a controlled environment.

3.1.2. Fibroblast Cell Culture

The mouse fibroblast cell line L929 (Chinese Academy of Preventive Medical Sciences, Beijing, China) was cultured in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 U penicillin and 100 U streptomycin per mL) under 5% CO_2 at 37 °C. The medium was changed every 2 days. When cells reached confluence, they were harvested using 0.25% trypsin-EDTA (Gibco®, California, USA), followed by addition of fresh culture medium to create a new single cell suspension for further incubation.

3.1.3. Preparation of SS Powder Using a High Temperature and High Pressure Degumming Technique (Heat-Degraded SS Powder)

Cocoons of *B. mori* silkworms were cut into square pieces and extracted with purified water by autoclaving (SS-320, Tomy Seiko Co., Ltd., Tokyo, Japan) at 120 °C for 60 min. The aqueous solution obtained from autoclaving silk cocoons was collected and called heat-degraded SS. The aqueous solution was then filtered to remove insoluble material, which is fibroin. After that, the filtrate was frozen and lyophilized using a Heto LL 3000 lyophilizer (Allerod, Denmark) to obtain SS powder. The SS molecular weight from all strains was estimated by SDS-PAGE.

3.1.4. Preparation of SS by Citric Acid and Sodium Carbonate Solution (Acid-Degraded and Alkali-Degraded SS Powders)

Acid-degraded and alkali-degraded SS powders were extracted using a previously described method by Kurioka *et al.* with some modifications [8]. For acid-degraded SS powder preparations, cocoons were cut and added to a 1.25% citric acid solution, then boiled for 30 min. After removing insoluble fibers by paper filtration, the clear filtrate was immediately dialyzed in distilled water for three days using cellulose tubing (Cellusep T2, MWCO 6,000-8,000, Sequin, Texas, USA) and distilled water was changed regularly. The pH of the final solution was measured to verify complete removal of citric acid. The SS solution was then frozen and lyophilized. Alkali-degraded SS powder was prepared similarly, using 0.5% sodium carbonate solution instead of citric acid.

3.1.5. Preparation of SS by Urea Solution

SS extracted by urea solution was prepared using a previously described method with some modifications [4]. Freshly cut cocoon shells were soaked into 8 M urea aqueous solution for 30 min and then refluxed at 85 °C for 30 min. Centrifugation and filtration were performed to remove all insoluble residues. The solution was thoroughly dialyzed in distilled water using cellulose tubing (Cellusep T2, MWCO 6,000-8,000, Sequin, Texas, USA) for three days and distilled water was changed regularly. The pH of final solution was measured to verify complete removal of urea solution. The SS solution was frozen and then lyophilized.

3.2. Methods

3.2.1. Molecular Weight Determination

To determine the molecular weight of SS, polyacrylamide gel electrophoresis was performed as previously described with some modifications [19]. Briefly, sample solutions for SDS-PAGE were prepared by adding an equal volume of sample buffer (0.25 M Tris-HCl, pH 7.0 containing 4% SDS, 10% sucrose, 10% 2-mercaptoethanol, and 0.025% bromophenol blue) to each protein solution. Each sample solution was then incubated at 98 °C for 2-3 min and loaded onto a 5%-20% gradient gel (Atto Corporation, Tokyo, Japan). Electrophoresis was performed in 125 mM Tris base with 0.96 M glycine and 0.5% SDS, polypeptide bands were detected by silver staining.

3.2.2. Particle Size and Zeta Potential Measurement

The size of self-aggregates was measured by a dynamic light scattering method based on the particle size option in a Zetasizer Nano-ZS (ZEN 3600, Malvern Instruments Ltd., Worcestershire, UK). The scattered intensity was registered at a scattering angle of 90° at 25 °C. Zeta potentials were measured by a Zetasizer Nano-ZS instrument with palladium-coated electrodes. All samples were adjusted to pH 7.0 prior to particle size and zeta potential measurement. The zeta potential presented is the average value of analyses in triplicate.

3.2.3. Amino Acid Analysis

SS amino acid compositions were measured with an amino acid analyzer (Hitachi L-8500A, Tokyo, Japan). Samples were hydrolyzed in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) at 100 °C for 24 h under vacuum prior to amino acid analysis. All experiments were performed in triplicate.

3.2.4. Cytotoxicity of SS Solution

L929 mouse fibroblast cells at an initial concentration of 2×10^4 cells/well were seeded in a 96-well plate in DMEM containing 10% FBS. After 24 h, the culture medium was replaced with fresh medium. SS solutions of various concentrations in purified water were filter sterilized by 0.22 μm membrane filter (Sartorius Ltd., Epsom, UK) prior to adding to the culture medium to give final SS concentrations in each well at 8.0-1000 μg/mL. Cells without SS solution served as negative controls. Melittin, a peptide from bee venom toxin, from 0.125 to 1.0 mg/mL, was used as a positive control. After incubation for 24 h, MTT assay was performed to evaluate cell activity [20]. The absorbance was determined by a microplate reader (Biohit 830, Biohit®, Helsinki, Finland) at a wavelength of 570 nm. The percentage of viable cells was calculated and compared to the negative control. All experiments were done in triplicate.

3.2.5. Adherence of L929 Mouse Fibroblast Cell Line on SS Films

Heat-degraded SS films were cast from SS solution (0.1% w/v, in water pH 5) in polystyrene 12-wells cell culture plates (well diameter 20 mm). After air-drying, the films were crosslinked by

ultraviolet (UV) irradiation for 60 min and sterilized with 70% ethanol followed by phosphate-buffered saline (PBS, pH 7.5) before seeding cells. L929 mouse fibroblast cells were seeded onto the sterilized films (2×10^4 cells/well). Cells were cultured in DMEM containing 10% FBS and antibiotics (100 U penicillin and 100 U streptomycin per mL) under 5% CO_2 at 37 °C. Cells were harvested at 24, 48 and 72 h, and the morphology of cells on culture plates (control) and on films coated on culture plates were observed by light microscopy (Nikon, TS100, Melville, New York, USA). All experiments were done in triplicate.

3.2.6. Determination of Soluble Collagen Production Induced by SS

L929 mouse fibroblast cells were cultured at the same cell content and method as for the cytotoxicity study of SS solution. Cells without SS solution served as a negative control. Supernatants were collected after cell incubation for 24 h. The total amount of soluble collagen type 1 was assayed using the Sircol® collagen assay kit (Biocolor Ltd., Northern Ireland, UK). The results were determined by a microplate reader (Biohit 830, Biohit®, Helsinki, Finland) at a wavelength of 500 nm. All experiments were done in triplicate. The amount of collagen was calculated based on a standard curve of soluble collagen (standard bovine collagen type 1, produced from USA disease free animals).

4. Conclusions

SS can promote cell viability at certain concentrations, but it can be toxic to cells at higher concentrations. The method of extraction of SS also has significant effects on cell viability. Urea-extracted SS showed the lowest cell viability compared to SS extracted by heat, acid and alkaline methods. Urea-extracted SS was severely harmful to cells at concentrations higher than 100 μg/mL. Heat-degraded SS activated the highest collagen production, while urea-extracted SS showed the lowest level of collagen activation. SS from all extraction methods could still promote collagen production in a concentration-dependent manner, even at high concentrations that are toxic to cells, which indicate that collagen was generated before fibroblast cells departed.

Acknowledgements

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Properties and antityrosinase activity of sericin from various extraction methods

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The present study investigated the chemical properties and antityrosinase activities of SS (silk sericin) extracted from different Thai silk strains via various extraction methods. Different silk strains contain distinct SS with various amino acid compositions, which are significantly influenced by the extraction method used. Urea extraction of SS was the only method that provided clearly distinguishable bands and had the most significant impact on SS conformation as illustrated by FTIR (Fourier-transform infrared) spectra. The use of urea or either acidic or alkaline chemicals in the extraction process also influenced SS thermal behaviour. With regard to biological activity, SS extracted using urea exhibited the highest antityrosinase activity, whereas alkali-degraded SS showed no inhibition of mushroom tyrosinase. Pigments, primarily flavonoids and carotenoids from silk cocoons, were also found to enhance tyrosinase inhibition of SS.

Introduction

The silkworm, *Bombyx mori*, synthesizes and secretes two classes of silk proteins, SF (silk fibroin) and SS (silk sericin). SF is a fibrous protein that has been widely investigated for biomedical purposes owing to its physiochemical properties and relatively inert immune response [1]. SS, on the other hand, was long considered to simply be a waste product in the silk industry until it was found to have important biochemical functions such as antioxidant activity [2,3], antityrosinase activity [3] and effects on tumour progression [4–6] and can be used as serum-free freezing medium for mammalian cells [7]. Silks differ widely in composition, structure and biochemical properties depending on the specific source and strain. Thai silk, particularly the yellow Nangnoi silk, contains a significant amount of pigments, which are primarily associated with carotenoids and flavonoids [8,9]. These polyphenolic compounds are most commonly known for their antioxidant properties and other diverse biochemical functions, such as antityrosinase, antiallergy or anti-inflammatory activities [10,11].

Normally, pigments coexist and accumulate in the layers of cocoon sericin [12]. The components that give colour to silk cocoons are associated with phenolic compounds in mulberry leaves, the sole food for *B. mori* larvae, and the content of cocoon colour components varies depending on the silkworm strain [12]. There are several native strains of silkworm in Thailand with various colours of cocoon shells. The most common native Thai silk is Nangnoi (yellow cocoon shell), which has been found to contain several flavonoids such as c-prolinylquercetins, while other Thai silk cocoons have white and yellow–green shells. Hayashiya et al. [13] found that green cocoon shells contain at least nine fluorescent yellow compounds, five of which have been identified as flavonoid-related compounds. These flavonoid compounds, in addition to SS, are also responsible for the antioxidant properties of *B. mori* cocoons [3,12]. Although the types and amount of flavonoids in silk cocoons have been found to differ genetically, little is currently known about the antityrosinase activity generated from flavonoids or SS from different silkworm strains.

Tyrosinases are copper-containing enzymes that catalyse the ortho-hydroxylation of monophenols to catechols and their subsequent oxidation to ortho-quinones [14]. Tyrosinases are thought to play roles in cancer and neurodegenerative diseases such as Parkinson's disease [15]. In addition, tyrosinases represent a significant target in the fields of agriculture, food and medicine, which has led to widespread screening for compounds with potent antityrosinase activity.

Key words: amino acid, antityrosinase activity, extraction, pigment, sericin, silk strain.

Abbreviations used: DSC, differential scanning calorimetry; FTIR, Fourier-transform infrared; MWCO, molecular-mass cut-off; SF, silk fibroin; SS, silk sericin.

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Extraction methods significantly affect the biochemical activities of silk proteins. Kurioka et al. [16] reported that acid-, alkali- and hot water-degraded SS powders exhibit different trypsin inhibitory activities and pI values. Furthermore, different SS extraction methods may alter amino acid compositions, which are directly related to antityrosinase activity. Tyrosine-containing peptides are substrates for the tyrosinase enzyme and possibly contribute to its activation, whereas cysteine-containing peptides are known to effectively inhibit tyrosinase activity [14,17].

The objectives of the present study were to investigate the chemical properties of SS extracted from different Thai silk strains via various extraction methods. In addition, we determined the antityrosinase activity of SS and the effects of flavonoids from silkworms on tyrosinase inhibition.

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 [Chul 1/1 (bivoltine, white shell), Chul 3/2 (bivoltine, greenish shell) and Chul 4/2 (bivoltine, yellow shell)].

Preparation of SS powder using a high–temperature and high-pressure degumming technique (heat-degraded SS powder)

Cocoons of *B. mori* silkworms were cut into square pieces (approx. 5 mm^2). Coloured silkworm cocoons were extracted three times in 70% ethanol (1 g of silk cocoon and 30 ml of ethanol) for 24 h at room temperature (25 °C) to remove all flavonoids and carotenoids. After drying the remaining cocoon shells (~97% from initial cocoon weight), SS was extracted with purified water (1 g of dry silk cocoon and 30 ml of water) by autoclaving (SS-320; Tomy Seiko, Tokyo, Japan) at 120 °C and 15 lbf/in^2 (1 $lbf/in^2 = 6.9$ kPa) for 60 min. The aqueous solution obtained from autoclaving of silk cocoon was collected and called heat-degraded SS. The aqueous solution was then filtered to remove insoluble material, which is fibroin. After that, the filtrate was frozen and freeze-dried using a Heto LL 3000 lyophilizer (Heto-Holten A/S, Allerod, Denmark) to obtain SS powder. The SS molecular mass from all strains was estimated by SDS/PAGE.

Preparation of SS by citric acid and sodium carbonate solution (acid- and alkali-degraded SS powders)

Acid- and alkali-degraded SS powders were extracted by the same method as that described previously by Kurioka et al. [16] but with some modifications. For acid-degraded SS powder preparations, cocoons were cut and colours were extracted using the high-temperature and high-pressure degumming technique described above. The remaining cocoon shells were added into a 1.25% citric acid solution (1 g of dry silk cocoon and 18 ml of citric acid solution) and boiled for 30 min. After removing insoluble fibres by paper filtration, the clear filtrate was immediately dialysed in distilled water for 3 days using cellulose tubing [Cellusep T2; MWCO (molecular-mass cut-off) = 6000–8000; Sequin, TX, U.S.A.]. The SS solution was then frozen and freeze-dried.

Alkali-degraded SS powder was similarly prepared using 0.5% sodium carbonate solution instead of citric acid.

Preparation of SS by urea solution

Freshly cut cocoon shells (6 g) were soaked into aq. 8 M urea (150 ml) for 30 min and then refluxed at 85 °C for 30 min. Centrifugation and filtration were performed to remove all insoluble residues. The solution was thoroughly dialysed in distilled water using cellulose tubing (Cellusep T2; MWCO = 6000–8000; Sequin) for 3 days. The SS solution was frozen and freeze-dried using a Heto LL 3000 lyophilizer.

Measurement of SS powder colour

SS powder colour values were measured using a spectrocolorimeter tristimulus colour analyser (Model JS555; Color Techno System Corporation, Tokyo, Japan) calibrated with a white porcelain reference plate. Visible reflectance spectra (380–770 nm) were obtained using a silicone photocell and pulsed xenon lamp (illuminant D65, 0° view angle, illumination area diameter 8 nm). Colour parameters from spectra were calculated by the spectrocolorimeter. The colour coordinates of the uniform colour space CIELAB (CIE L*a*b* colour scale of the Commission Internationale d'Eclairage) were determined by reflectance (L^*) and chromaticity (a^* and b^*). The L^* value indicates brightness ranging from black ($L^* = 0$) to white ($L^* = 100$). The a^* value ranged from –60 (green) to 60 (red) and the b^* value ranged from –60 (blue) to 60 (yellow). All experiments were performed in triplicate.

Molecular mass determination

To determine the molecular mass of SS, PAGE was performed as previously described with some modifications [18]. Pigments from silk cocoons (Chul 3/2 and 4/2) were removed prior to SS extraction to confirm that carotenoids or flavonoids would not interfere with molecular mass determination. Briefly, sample solutions for SDS/PAGE were prepared by adding an equal volume of sample buffer [0.25 M Tris/HCl, pH 7.0, containing 4% (w/v) SDS, 10% sucrose, 10% (v/v) 2-mercaptoethanol and 0.025% Bromophenol Blue] to each protein solution. Each sample solution was then incubated at 98 °C for 2–3 min and loaded on to

a 5–20% gradient gel (Atto Corporation, Tokyo, Japan). Electrophoresis was performed in 125 mM Tris base with 0.96 M glycine and 0.5% SDS; polypeptide bands were detected by silver staining.

Amino acid analysis

SS amino acid compositions were measured with an amino acid analyser (Hitachi L-8500A; Hitachi, Tokyo, Japan). Samples for analysis were hydrolysed in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole (Wako Pure Chemical Industries, Tokyo, Japan) at 100 °C for 24 h under vacuum. All experiments were performed in triplicate.

DSC (differential scanning calorimetry)

The thermal properties of SS powders obtained from various extraction processes were examined using a differential scanning calorimeter (DSC 204 F1 Phoenix®; Netzsch, Selb, Germany). Measurements were performed at a heating rate of 10 °C/min and a nitrogen flow rate of 60 ml/min from room temperature to 400 °C.

FTIR (Fourier-transform infrared) measurements

FTIR spectra of SS powders were obtained with an FTIR spectroscope (PerkinElmer) using KBr pellets. For all measurements, the thickness of the specimen was fixed at 2 mm.

Measurement of antityrosinase activity

Pigments from silk cocoons (Chul 3/2 and 4/2) were removed prior to SS extraction to confirm that antityrosinase activities were purely generated from SS, and carotenoids or flavonoids had no effect on the measurements. Assays were performed as previously described with minor modifications [19]. Tyrosinase (1000 units/ml; Sigma, St Louis, MO, U.S.A.) from a mushroom solution was prepared at a concentration of 100 units/ml in 0.2 M phosphate buffer solution (pH 6.5). Tyrosinase mushroom solution (150 μl) and phosphate buffer solution at pH 6.5 (300 μl) were mixed with or without the SS sample (0.8 mg). The mixture was then pre-incubated at 25 °C for 5 min before adding 300 μl of 1.25 mM dopa (3,4-dihydroxyphenylalanine; Sigma) solution, and the reaction was monitored at 475 nm. The percentage of inhibition of tyrosinase activity was calculated as

$$\text{Inhibition, } (\%) = [(A - B)/A] \times 100$$

where A represents the difference in the absorbance of the control sample between incubation time periods of 0.5 and 1.0 min, and B represents the difference in the absorbance of the test sample between the same incubation times.

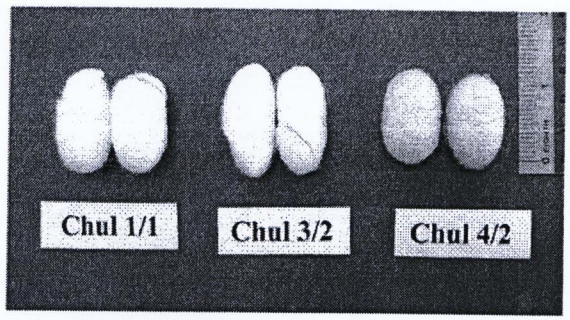

Figure 1 Physical appearance of Thai silk cocoons from three different strains: Chul 1/1 (white shell), Chul 3/2 (greenish shell) and Chul 4/2 (yellow shell)

Table 1 Percentage yield of SS extracted by different methods

Silk strain	Extraction method	Yield (%)
Chul 1/1	Heat	21.27 ± 3.83
	Urea	18.60 ± 4.08
	Acid	15.19 ± 2.36
	Base	12.18 ± 1.11
Chul 3/2	Heat	18.36 ± 0.29
	Urea	21.09 ± 7.19
	Acid	8.41 ± 1.14
	Base	6.93 ± 1.23
Chul 3/2	Heat[a]	17.00 ± 3.14
	Urea[a]	23.10 ± 4.12
	Acid[a]	8.33 ± 2.36
	Base[a]	5.93 ± 1.26
Chul 4/2	Heat	21.47 ± 0.62
	Urea	20.43 ± 1.06
	Acid	13.60 ± 2.13
	Base	12.69 ± 0.65
Chul 4/2	Heat[a]	19.58 ± 1.39
	Urea[a]	20.33 ± 3.26
	Acid[a]	11.86 ± 1.89
	Base[a]	11.60 ± 1.67

[a]SS yield from coloured silk cocoon after removal of pigment.

Each result is presented as the mean from three concurrent readings. Kojic acid was used as a positive control.

Statistical analyses

Results are expressed as means ± S.D. Statistical significance was determined by paired and unpaired Student's t tests together with ANOVA. $P < 0.05$ was considered statistically significant.

Results and discussion

Figure 1 displays the physical appearance of Thai silk cocoons from the three different Chul 1/1, Chul 3/2 and Chul 4/2 strains. SS yield using the various extraction methods are listed in Table 1. For all silk strains, SS extracted by the

Table 2 Colour values (reflectance measurements: L^*, a^* and b^*) in SS powder extracted by urea solution from Chul 3/2 and Chul 4/2 strains

Strain	L^* (mean ± S.D.)	a^* (mean ± S.D.)	b^* (mean ± S.D.)
Chul 3/2	93.41 ± 3.61	−4.02 ± 1.62[a]	15.53 ± 1.65[a]
Chul 3/2[b]	94.00 ± 4.56	−0.81 ± 0.22	6.29 ± 2.01
Chul 4/2	84.83 ± 3.89	−0.28 ± 0.14[a]	53.6 ± 2.23[a]
Chul 4/2[b]	94.43 ± 5.68	0.41 ± 0.29	3.34 ± 0.79

[a]Significant differences compared with same strain.
[b]Colour-extracted SS.

high-temperature and high-pressure method and by urea solution had higher yields compared with that extracted with citric acid and sodium carbonate solutions. Although ethanol can remove a significant amount of carotenoids and flavonoids from cocoon shells, minor amounts of both compounds were still present, as shown by chromaticity. SS extracted with urea appeared to give the most clearly distinguishable protein bands. SS yield in all silk strains was not affected by colour extraction from silk cocoons with ethanol, which indicated that no proteins had been dissolved by ethanol extraction.

Colour values (L^*, a^* and b^*) of SS powders from the Chul 3/2 and Chul 4/2 cocoons are shown in Table 2. SS powder from the Chul 3/2 strain was bright yellow and green in colour, as shown by an elevated negative value of a^* and high positive value of b^* respectively. However, SS powder generated from the Chul 3/2 cocoons after colour extraction showed much reduced a^* and b^* values, indicating that the green and yellow colour intensities decreased significantly, possibly from lower amounts of carotenoids and flavonoids in the cocoon shells. According to a^* and b^* values, the colour extraction process can remove approx. 80% of coloured pigments from the silk strain Chul 3/2. A similar result was also found in SS from the Chul 4/2 cocoons. Since silk cocoons from the Chul 4/2 strain have an intense yellow colour indicating higher pigment composition compared with the Chul 3/2 strain, the colour extraction process can successfully remove the pigment, as shown by the fact that b^* values were reduced by approx. 90%. The L^* and b^* values were used to determine the amount of coloured-pigment removal and we found a linear relationship with good correlation between changes in L^* and b^* values with the amount of coloured-pigment removal ($r^2 = 0.93$ for L^* and $r^2 = 0.94$ for b^*). It was also aligned with the studies by Peterson et al. [20]. From this correlation, more than 80% of pigments were removed from the Chul 3/2 and Chul 4/2 cocoons. These results show that carotenoids and flavonoids, along with other colour substances, are mostly removed from SS powders. Thus our molecular mass, amino acid content and antityrosinase activity results could be attributed to the effect of SS alone.

SS molecular masses from different strains and extraction methods are shown in Figure 2. SS extracted with

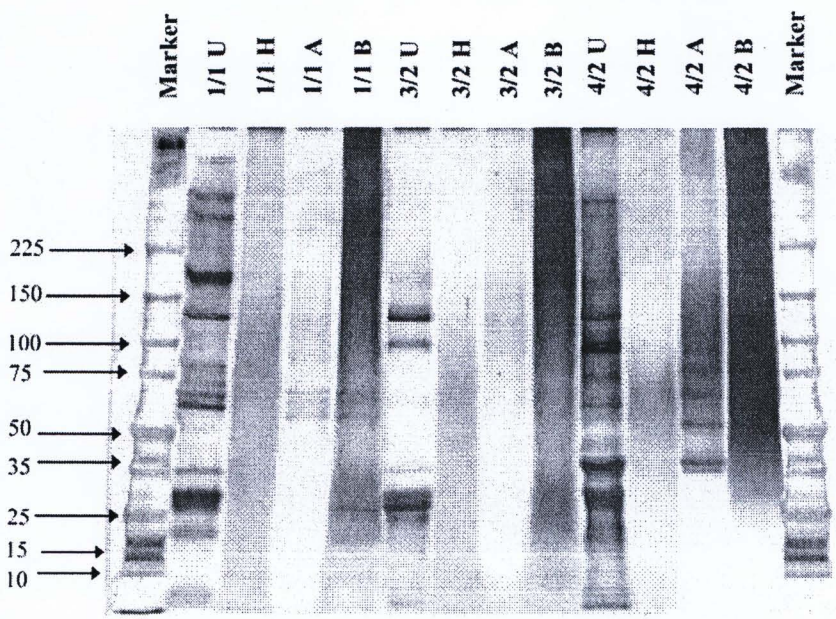

Figure 2 SDS/PAGE of SS extracted from Chul 1/1, Chul 3/2 and Chul 4/2 strains using the following methods: urea solution (U), high temperature and high pressure (H), citric acid solution (A) and sodium carbonate solution (B)

Different silk strains with various extraction methods show different molecular mass SS ranging from 10 to >225 kDa.

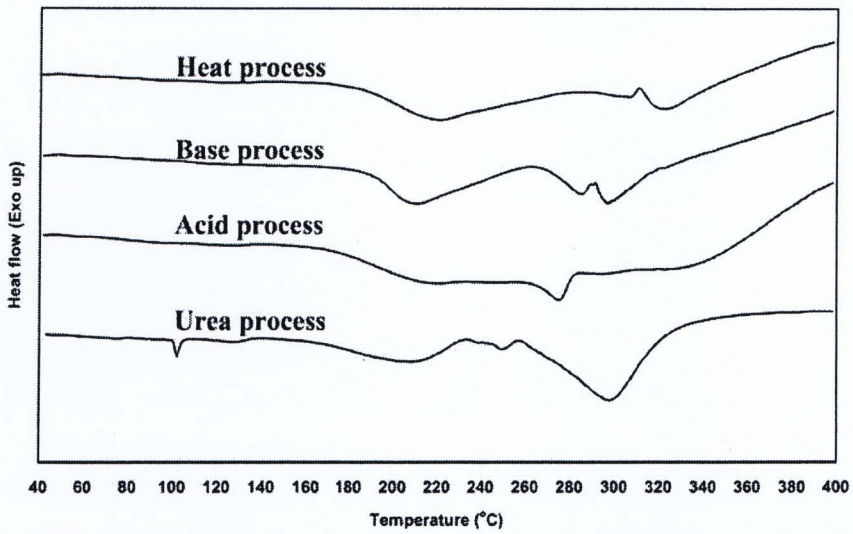

Figure 3 DSC of SS obtained by different extraction methods (acid, alkali, urea and heat processes), which showed an endothermic degradation peak at 210°C

Higher endothermic degradation temperatures of SS powder at 220°C were observed only from heat process SS.

urea from the Chul 1/1 and Chul 4/2 strains showed clear bands with molecular masses ranging from 10 to >225 kDa, whereas SS from the Chul 3/2 strain had a molecular mass in the range between 10 and 150 kDa. This result indicates that different silk strains contain distinct SS and other outer proteins, which may result in varying physical and biological activities. SDS/PAGE of acid- and alkali-degraded SS from all strains also displayed distinguishable bands within the range of 35–150 and 15–75 kDa respectively. However, the number of bands from acid- and alkali-degraded SS was much less than the number of bands from SS extracted with urea. SDS/PAGE of SS prepared by the high-temperature and high-pressure degumming technique showed continuous bands with molecular masses ranging from 50 to 150, 35 to 100 and 35 to 75 kDa for Chul 1/1, Chul 3/2 and Chul 4/2 respectively. Our findings are in agreement with those reported by Sprague [21], which indicated that SS is a mixture of at least 15 different polypeptide chains, ranging in size from 20 to 220 kDa.

The thermal behaviours of SS powders extracted from various processes are presented in Figure 3. We found that SS powder obtained from extraction methods using urea, acidic and alkaline solutions showed an endothermic degradation peak at 210°C. However, the endothermic degradation temperatures of SS powder at 220°C obtained from high-temperature and high-pressure degumming techniques were higher than those obtained from other processes. The observed degradation temperature corresponded to the 221°C reported by Lamoolphak et al. [22], implying that the use of chemicals during the extraction process influences the thermal stability of SS.

FTIR spectra of SS powders obtained from different extraction methods (heat, acid, alkali and urea) are shown in Figure 4. The peak positions of amide I (C=O stretching), amide II (N-H deformation and C-N stretching) and amide III (C-N stretching and N-H deformation) of SS powders derived from heat and alkali processes were located at 1650, 1530 and 1238 cm^{-1} respectively. These amide bands contribute to the primary random coil structure of SS [23,24]. Sericin powder obtained from acid extraction showed similar peak patterns except for the amide I characteristic peaks, which appeared at 1650 and 1624 cm^{-1}, indicating the presence of a random coil and β-sheet conformation respectively [25]. The SS powder prepared by urea extraction exhibited different characteristic peaks than those from SS extracted by other methods. The specific amide II peak was not clearly observed, while amide I occurred at 1650 and 1621 cm^{-1}, corresponding to a random coil and β-sheet respectively. In addition, the amide III characteristic peak shifted to 1225 cm^{-1} and a shoulder at 1250 cm^{-1}, indicating the presence of a β-sheet. Moreover, peaks at 1460 and 1150 cm^{-1} were found to correspond to urea in SS extracted using urea solutions [26]. However, the urea molecules presented in SS obtained by the urea extraction method might be incorporated as a part of SS molecules that cannot be removed by dialysis. This was supported by stable pH at a slightly acidic range after being thoroughly dialysed. The FTIR results revealed that the extraction process of SS could affect the chemical structure of SS. Among the four extraction methods used here, extraction with urea had the most significant impact on SS conformation.

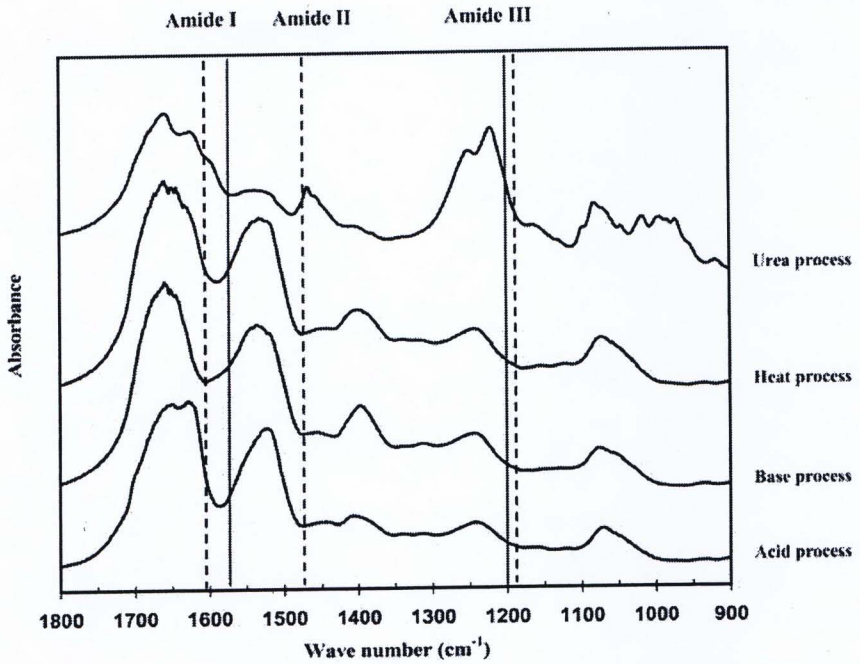

Figure 4 FTIR spectra of SS obtained from various extraction methods: acid, alkali, urea and heat processes

The solid vertical line indicates a β-sheet; the broken vertical line indicates a random coil. The peak positions of amide I (C=O stretching), amide II (N-H deformation and C-N stretching) and amide III (C-N stretching and N-H deformation) of SS from heat and alkali extractions were located at 1650, 1530 and 1238 cm^{-1} respectively. SS from acid extraction showed similar peak patterns except for the amide I characteristic peaks, which appeared at 1650 and 1624 cm^{-1}. The SS powder prepared by urea extraction exhibited different characteristic peaks than those from SS extracted by other methods, indicating that urea had the most significant impact on SS conformation.

Amino acid compositions of SS extracted by different methods are listed in Table 3 for all three strains. The results indicated that the Chul 1/1 strain contained the highest amount of methionine and cysteine residues. Whereas the methionine content in Chul 1/1 showed a significant difference ($P < 0.05$) when compared with both the Chul 3/2 and Chul 4/2 strains, the cysteine content in Chul 1/1 was significantly different ($P < 0.05$) when compared with the Chul 4/2 strain only. Moreover, the SS amino acid compositions from the same strain varied according to the method of SS extraction.

All SS had high amounts of serine and glycine with no significant differences between strains or extraction methods ($P > 0.05$). However, all strains did show a significant difference in aspartic acid content among the urea, heat and alkaline extraction methods, although heat and acid extraction showed no significant difference in aspartic acid levels. For all strains, alkaline extraction provided SS with the highest levels of aspartic acid. This may be due to the fact that aspartic acid normally has a negative charge at physiological pH, as the carboxylic side chain can easily be deprotonated in alkaline solutions to produce water-soluble $COO^{-}NH_4^{+}$ species. All extraction methods employed water-based solvents, thereby generating high amounts of water-soluble serine and glycine, whereas very few hydrophobic amino acids such as proline, leucine, isoleucine and phenylalanine were found. High arginine content in SS extracted from urea may be because of hydrogen-bonding between the guanidine group on arginine and the amino group on urea. We found the highest tyrosine content by acid extraction, which may due to protonation of tyrosine side chains.

We assessed direct inhibitory effects of SS on semi-purified tyrosinase and listed the results from our tyrosinase assays in Figure 5. Kojic acid was used as a positive control, as it significantly inhibits the catalytic activity of mushroom tyrosinase. SS, particularly from the Chul 3/2 strain, was found to inhibit mushroom tyrosinase. Overall, SS obtained from different extraction methods exhibited different degrees of tyrosinase inhibition, even within the same silk strain. SS from the Chul 1/1 strain showed significant differences in antityrosinase activity when the various extraction methods were compared. Our results indicate that SS extracted with urea had the highest degree of antityrosinase activity when compared with other extraction methods for all strains, whereas alkali-degraded SS showed no inhibition of mushroom tyrosinase. Only acid-degraded SS from coloured silk cocoons exhibited

Table 3 Composition of amino acids of SS from Chul 1/1, Chul 3/2 and Chul 4/2 strains extracted by different methods

Values are the means from triplicate analysis. H, heat; U, urea; A, acid; B, alkali.

Amino acid	Chul 1/1				Chul 3/2				Chul 4/2			
	H	U	A	B	H	U	A	B	H	U	A	B
Asp	15.64	18.31	15.93	19.88	15.62	17.93	16.00	21.58	15.97	17.69	16.61	19.92
Ser	33.63	31.27	31.86	30.01	34.50	32.24	32.01	28.41	33.84	30.69	31.95	27.59
Glu	4.61	5.27	5.75	5.93	4.76	6.02	5.40	7.66	4.86	5.97	5.88	7.03
Gly	15.03	11.23	10.49	11.01	15.09	10.75	10.38	11.16	15.14	10.96	10.69	12.58
His	1.06	3.26	2.47	1.72	1.22	2.82	2.83	2.38	1.37	2.50	2.29	2.15
Arg	2.87	5.41	4.92	4.92	2.95	5.21	4.87	3.79	3.09	5.71	5.24	4.83
Thr	8.16	8.36	8.51	6.49	8.43	8.78	8.78	6.09	8.34	9.04	8.30	5.56
Ala	4.10	4.33	3.72	4.21	4.45	3.80	3.57	3.96	4.98	4.63	3.56	4.40
Pro	0.54	1.46	0.78	1.24	0.62	0.79	0.73	0.92	0.71	1.16	0.79	1.01
Cys	0.44	0.39	0.53	0.23	0.43	0.33	0.50	0.19	0.27	0.42	0.52	0.16
Tyr	3.45	0.36	5.56	5.24	3.64	1.24	5.81	4.92	3.47	2.67	5.59	4.90
Val	2.88	2.96	2.95	2.94	3.04	3.28	3.03	3.03	2.92	2.98	2.76	2.99
Met	3.39	0.12	0.06	0.15	0.57	0.08	0.06	0.13	0.18	0.06	0.05	0.15
Lys	2.35	3.14	3.48	2.89	2.51	3.55	3.03	2.71	2.78	2.50	3.16	3.08
Ile	0.56	0.96	0.87	0.75	0.65	0.95	0.90	0.87	0.61	0.74	0.66	1.03
Leu	1.00	1.58	1.43	1.56	1.15	1.58	1.44	1.51	1.11	1.63	1.37	1.81
Phe	0.28	0.60	0.71	0.81	0.39	0.66	0.67	0.72	0.36	0.63	0.57	0.81

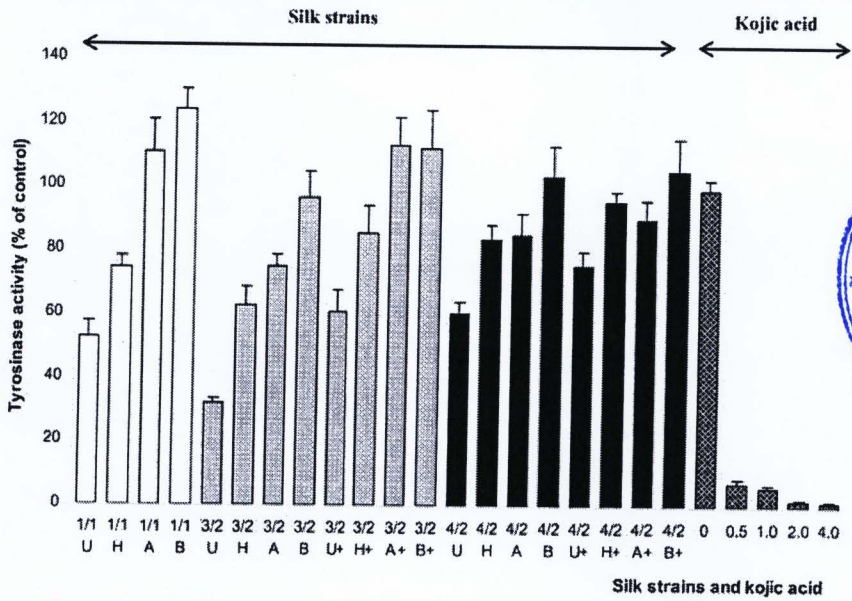

Figure 5 Effect of SS on mushroom tyrosinase activity compared with that of kojic acid

SS obtained by different extraction methods exhibited different degrees of tyrosinase inhibition, even within the same silk strain. SS, particularly from the Chul 3/2 strain, was found to inhibit mushroom tyrosinase. The '+' sign indicates SS from coloured silk cocoons after the removal of pigment. H, heat; U, urea; A, acid; B, alkali.

antityrosinase activity, but at a very low degree of inhibition. As shown in Table 2, the pigment extraction process was sufficiently effective so that it is reasonable to conclude from Figure 5 that SS itself has a significant antityrosinase activity. Pigments from silk cocoons were shown to also affect antityrosinase activity, as shown by the stronger tyrosinase inhibition of SS from coloured silk cocoons with or without pigment extraction. Comparing all strains and extraction methods, SS extracted from the Chul 3/2 strain by urea without pigment removal showed the highest degree of tyrosinase inhibition.

With regard to individual amino acids, Kahn [17] reported that cysteine appeared to be the best tyrosinase inhibitor. However, the observed inhibition was due to conjugation of cysteine with the enzymatically produced quinine rather than from direct enzyme inhibition [27]. This may also explain our result that acid-degraded SS showed the highest amount of cysteine but had less

antityrosinase activity than SS extracted with urea. As peptides that interact with tyrosinase can act as potential inhibitors, arginine-containing peptides are considered to be the most tyrosinase-binding peptides especially from the shorter peptides, whereas valine-containing peptide is one of the most tyrosinase-inhibiting peptides [14]. Sericin extracted with urea from all strains showed significantly high amounts of arginine and valine compared with SS extracted by other methods. Tyrosine-containing peptides are substrates for tyrosinases and thus may contribute to tyrosinase activation. Acid-degraded SS from all strains exhibited high tyrosine levels with a rather low degree of tyrosinase inhibition. Furthermore, Kahn [17] also indicated that peptides containing a negatively charged aspartic acid or glutamic acid residue are highly unfavourable for the tyrosinase–peptide interaction and therefore show lower antityrosinase activity [17]. This correlates to our alkali-degraded SS, which exhibited significantly high levels of aspartic acid and glutamic acid and low antityrosinase activity. However, all of the results presented here were from *in vitro* studies, and further *in vivo* studies are needed to determine biologically relevant antityrosinase activities.

In conclusion, we demonstrated that silk strains and extraction methods affect both physical and biological properties of SS. The urea extraction method appears to be the most efficient way to obtain clearly distinguishable bands with a broad range of molecular masses. Different extraction methods significantly affected the amino acid content of SS from all strains. With regard to the biological effects of SS, urea-extracted SS showed the highest antityrosinase activity, while alkali-degraded SS exhibited the lowest tyrosinase inhibition when compared with other extraction methods. Pigments from coloured cocoons also exhibited an antityrosinase effect, as shown by a lower degree of tyrosinase inhibition after pigment removal.

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The Effect of Sericin with Variable Amino-Acid Content from Different Silk Strains on the Production of Collagen and Nitric Oxide

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Abstract

Although silk sericin (SS) enhances the growth and attachment of fibroblast cells, its toxicity remains questionable. We investigated the effect of SS extracted by heat with variable amino-acid content on *in vitro* collagen promotion and nitric oxide synthesis. After 24 h of incubation, SS, especially from the Chul 1/1 strain which has the most methionine and cysteine content, enhanced fibroblast growth. The molecular mass of heat-extracted SS from these three strains showed a slightly different range, but within 20–200 kDa, which were all identified as sericin. SS from all strains promoted type-I collagen production in a concentration-dependent manner, while SS from Chul 1/1 strain could induce the highest amount of collagen synthesis when compared to SS from other strains. Nitric oxide was found in the culture medium after activation by SS from the Chul 1/1 strain but reached a level that was not toxic to the cells. We conclude that SS is not toxic to fibroblast cells. Moreover, methionine and cysteine content in SS are important factors to promote cell growth and collagen synthesis.

Keywords

Sericin, methionine, cysteine, collagen, nitric oxide

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1. Introduction

It has been well established that proteins such as collagen, fibronectin, etc., play an important role in the attachment and growth of mammalian cells [1]. Recently, silk proteins, fibroin (SF) and sericin (SS), have been widely investigated in the biotechnological field. SF, the fibrous protein produced by silkworms, promotes cell attachment and growth and has positive effects on cell adhesion, viability and differentiated functions [2–7]. On the other hand, SS, a glue protein from silk, enhances the attachment and growth of mouse fibroblasts, human skin fibroblast together with human and mouse hybridomas when added into culture media [4, 8, 9]. These findings have implicated the use of silk proteins as wound healing materials. Although silk fabric was shown to be effective for atopic dermatitis [10], there have been several studies demonstrating immune responses against silk sutures containing SS proteins [11–14] which may cause skin irritation and allergic reactions [15].

Methionine and cysteine, the sulfur amino acids, are known for their capability of accelerating the healing rate of wounds in injured rats [16]. Methionine, a key amino acid for wound regeneration, is normally converted to cysteine before deposition in the tissue protein [17]. Recently, Sen *et al.* demonstrated that methionine supplement increases homocysteine (Hcy) accumulation, which is associated with inflammatory response and matrix remodeling such as collagen type-1 synthesis and matrix metalloproteinases (MMP)-9 activity [18]. We hypothesize that SS extracted from various silk strains may contain different amounts of sulfur amino acids, which may result in the production of other wound healing factors such as collagen production and other inflammatory mediators, such as nitric oxide (NO), secreted from cells.

Wound repair is a complex cellular and acellular response to achieve tissue restoration following injury [19]. A coordinated balance of promoting and compromising factors is a prerequisite for normal healing. Part of cell-to-cell and cell-to-matrix interaction is mediated through soluble mediators such as inflammatory cytokines, NO and others [20]. Nitric oxide, a short-lived radical and biological mediator, is an important immune mediator with profound both cytotoxic and regulatory roles. It has been shown to be synthesized in wounds during normal healing [21]. Nitric oxide level is a good indicator for cell inflammation and should be monitored during the wound healing process.

An increase in collagen production is also an important factor for wound healing [22]. Type-I collagen is the main collagen of bone, skin, tendon and newly healed wounds [23]. Dal Pra *et al.* showed that procollagen type-1 C-peptide is released into the medium when SF in the β-sheet form is co-cultured with human epidermal keratinocytes and dermal fibroblasts [24]. Our previous study showed that SS promotes collagen production in rat wounds resulting in much greater wound-size reduction and faster healing [25]. However, *in vitro* collagen promotion by SS, a condition which is more controllable than *in vivo* settings, remains to be demonstrated.

The main purpose of this study was to investigate the relationship between SS with variable amino-acid content extracted from different Thai silk strains and *in vitro* type-I collagen production. We also monitored NO production from fibroblast cells induced by SS.

2. Materials and Methods

2.1. Silkworm Cocoons

Fresh cocoons of *Bombyx mori* were kindly supplied by Chul Thai Silk (Petchaboon Province, Thailand). Silkworm cocoons were produced in a controlled environment. Three Thai native silk strains, Chul 1/1 (bivoltine, white shell), Chul 3/2 (bivoltine, greenish shell) and Chul 4/2 (bivoltine, yellow shell), were used in these experiments.

2.2. Preparation of SS

Cocoons of *B. mori* silkworms were cut into pieces (about 5 mm^2). Removal of flavonoids and carotenoids from colored-silkworm cocoons were carried out as reported previously with slightly modifications [26]. Briefly, 70% ethanol was added into silk cocoon (1 g of silk cocoon 30 ml of ethanol) for 24 h at room temperature three times and shook gently. After drying the left-over cocoon shells (approx. 97% from initial cocoon weight), SS was extracted with purified water (1 g dry silk cocoon 30 ml water) by autoclaving (SS-320, Tomy Seiko, Tokyo, Japan) at 120°C for 60 min using a high temperature and pressure degumming technique. After filtration with a membrane to remove fibroin, SS powder was obtained by freezing and lyophilizing the SS solution using a Heto LL 3000 lyophilizer (Allrod, Denmark). The molecular weight of SS from all strains was analyzed by SDS-PAGE. In order to confirm that most of SS were not extracted during removal of flavonoids and carotenoids, ethanol extract of colored-silk cocoons was analyzed also by SDS-PAGE.

Polyacrylamide-gel electrophoresis was performed as described previously with some modifications [27]. Briefly, sample solutions for SDS-PAGE were prepared by adding the same volume of sample buffer (0.25 M Tris-HCl of pH 7.0, containing 4% SDS, 10% sucrose, 10% 2-mercaptoethanol and 0.025% bromophenol blue) to each of the protein solutions. Each sample solution was incubated at 98°C for 2–3 min and put on a 5–20% gradient gel (Atto, Tokyo, Japan). Electrophoresis was done in 125 mM Tris base with 0.96 M glycine and 0.5% SDS. Silver-staining was then used to detect polypeptide bands.

2.3. Fibroblast Cell Line

The mouse fibroblast cell line L929 (Chinese Academy of Preventive Medical Sciences, Beijing, China) was cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 U penicillin and 100 U/ml streptomycin) under 5% CO_2 at 37°C. The media was changed

every 2 days. When cells reached confluence, they were harvested using 0.25% trypsin-EDTA (Gibco®, USA), followed by addition of fresh culture medium to create a new single cell suspension for further incubation.

2.4. Amino-Acid Analysis

The amino-acid composition of SS was measured by an amino-acid analyzer (Hitachi L-8500A, Tokyo, Japan). Samples for amino-acid analysis were hydrolyzed in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole (Wako, Tokyo, Japan) at 100°C for 24 h under vacuum. All experiments were done in triplicate.

2.5. Cytotoxicity of SS Solution

L929 mouse fibroblast cells at an initial concentration of 5×10^5 cells/well were seeded in a 96-well plate in DMEM containing 10% FBS. After 24 h, the culture medium was changed into fresh medium and SS solutions in purified water of various concentrations, after filtered sterilization using a 0.22 μm membrane filter (Sartorius, Epsom, UK), were added to the culture medium to make the final concentration of SS in each well at 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml, respectively. Cells without SS solution served as negative controls. Melittin, a peptide from bee venom toxin, of various concentrations from 0.125 to 1.0 mg/ml, was used as a positive control in fibroblasts. After incubation for 24 h, a 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate cell activity [28]. The absorbance was determined by a microplate reader (Biohit 830, Biohit®, Helsinki, Finland) at a wavelength of 570 nm. The percentage of viable cells was calculated and compared to the negative control. All experiments were done in triplicate. Differences between data were analyzed using a one-way ANOVA at $P < 0.05$.

2.6. Determination of Soluble Collagen Production Induced by SS

L929 mouse fibroblast cells were seeded at an initial concentration of 5×10^5 cells/well in a 96-well plate in DMEM containing 10% FBS. After 24 h, the culture medium was changed into fresh medium and SS solutions in purified water of various concentrations, after filtered sterilization using a 0.22 μm membrane filter (Sartorius), were added to the culture medium to make the final concentration of SS in each well at 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml, respectively. Cells without SS solution served as a negative control. After incubation for 24 h, the supernatants were collected. The total amount of soluble collagen type I was assayed using the Sircol® collagen assay kit (Biocolor, UK). The results were determined by a microplate reader (Biohit 830, Biohit®) at a wavelength of 500 nm. All experiments were done in triplicate. The amount of collagen was calculated based on a standard curve of soluble collagen (collagen type I standard from American disease-free animals). Differences between data were analyzed using a one-way ANOVA at $P < 0.05$.

2.7. NO Assay by the Griess Reaction

L929 mouse fibroblast cells at an initial concentration of 5×10^5 cells/well were seeded in a 96-well plate in DMEM containing 10% FBS. After 24 h, the culture medium was changed into fresh medium and SS solutions in purified water of various concentrations, after filtered sterilization using a 0.22 μm membrane filter (Sartorius), were added to the culture medium to make the final concentration of SS in each well at 0.2–1.0% (w/v), respectively. Cells without SS solution served as a control. After incubation for 24 h, NO levels were determined by the Griess reaction. In brief, 100 μl Griess reagent (1% N-(1-napthyl)-ethylenediamine dihydrochloride and 1% sulfanilamide in 2.5% phosphoric acid) was mixed with an equal volume of experimental cell supernatant. The resulting solution yields a pink solution for a positive result and a yellow solution for a negative result. The reaction was recorded quantitatively at 570 nm based on a standard curve of NaNO. All experiments were done in triplicate.

3. Results

Figure 1 represents the physical appearance of Thai silk cocoons from three different strains. The amino-acid compositions of SS extracted by heat method from three different strains are shown in Table 1. The results indicated that Chul 1/1 strain contains the highest amount of methionine and cysteine. Methionine content in Chul 1/1 shows significant difference ($p < 0.05$) when compared to Chul 3/2 and Chul 4/2 strain while cysteine content in Chul 1/1 shows significant difference ($P < 0.05$) only when compared to Chul 4/2 strain.

Figure 2 indicates the molecular mass of SS extracted by heat from Chul 1/1, Chul 3/2 and Chul 4/2 strain using SDS-PAGE. SS extracted by heat from Chul 1/1, Chul 3/2 and Chul 4/2 strain have a molecular mass of 35–150, 35–100 and 35–75 kDa, respectively, corresponding with the result reported by Sprague which indicated that sericin is a mixture of at least 15 different polypeptide chains, ranging in

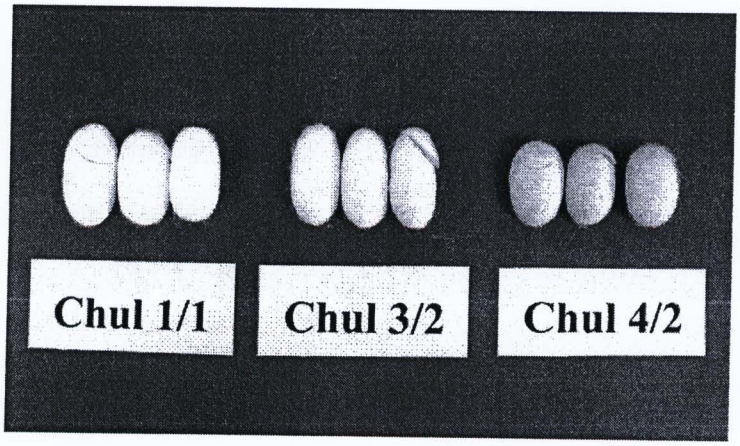

Figure 1. Physical appearance of Thai silk cocoons from three different strains. This figure is published in colour in the online edition that can be accessed *via* http://www.brill.nl/jbs

Table 1.
Composition of amino acids of SS from Chul 1/1, Chul 3/2 and Chul 4/2 strains

Amino acid	Sericin (mol%)		
	Chul 1/1	Chul 3/2	Chul 4/2
Asp	15.64	15.62	15.97
Thr	8.16	8.43	8.34
Ser	33.63	34.50	33.84
Glu	4.61	4.76	4.86
Gly	15.03	15.09	15.14
Ala	4.10	4.45	4.98
Cys	0.44*	0.43	0.27
Val	2.88	3.04	2.92
Met	3.39**	0.57	0.18
Ile	0.56	0.65	0.61
Leu	1.00	1.15	1.11
Tyr	3.45	3.64	3.47
Phe	0.28	0.39	0.36
Lys	2.35	2.51	2.78
His	1.06	1.22	1.37
Arg	2.87	2.95	3.09
Pro	0.54	0.62	0.71

Values are means from triplicate analysis; *significant difference when compared to Chul 4/2 strain ($P < 0.05$); **significant difference when compared to both Chul 3/2 and Chul 4/2 strains ($P < 0.05$).

size from 20 to 220 kDa [29]. It clearly shows that SS from Chul 1/1 strain contained higher-molecular-mass peptides when compared to SS from Chul 3/2 and Chul 4/2 strains. SDS-PAGE of ethanol extract from colored-silk cocoons indicates only low-molecular-mass peptides (<10 kDa) (data not shown). This result confirms that SS was not removed during flavonoids and carotenoids removal process.

3.1. Viability of L929 Fibroblast After Incubation with SS

Results of the MTT assay indicated that SS solutions from all types of silk used in this experiment did not cause cell death after incubation for 24 h (Fig. 3). SS extracted from the Chul 1/1 strain, which contained the highest amount of methionine and cysteine, even promoted the growth of fibroblast cells, especially at a concentration of 1.0 mg/ml. The percent viability of fibroblasts activated by the Chul 1/1 strain at 1.0 mg/ml showed a significant difference in cell growth compared to other concentrations ($P < 0.05$), while the Chul 3/2 strain showed a significant difference ($P < 0.05$) in percent viability of fibroblast cells at 0.4 mg/ml. SS extracted from the Chul 4/2 strain, which contained the lowest amount of methionine and cysteine,

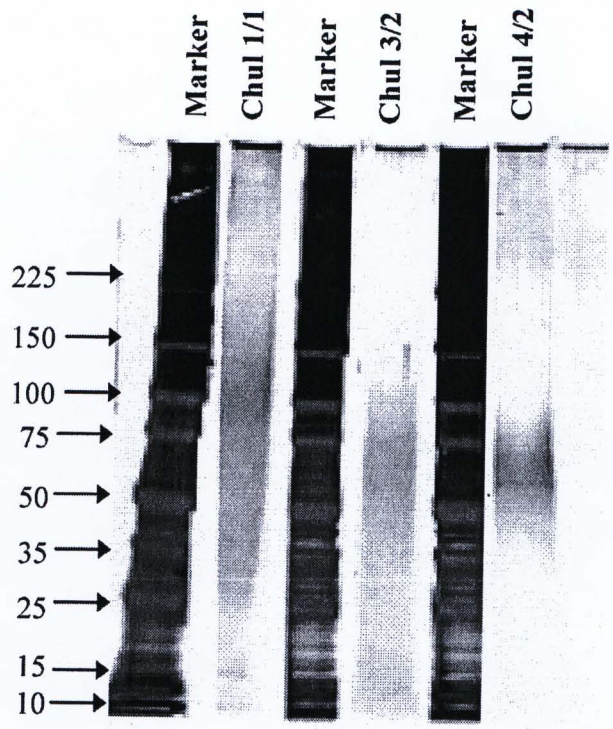

Figure 2. SDS-PAGE of SS extracted by heat from Chul 1/1, Chul 3/2 and Chul 4/2 strain. This figure is published in colour in the online edition that can be accessed *via* http://www.brill.nl/jbs

did not show a significant difference in percent viability of fibroblasts at any concentration.

3.2. NO Production Induced by SS

The results of SS-induced NO production in mouse fibroblasts are shown in Fig. 4. NO could not be detected in culture supernatants from nearly all SS solutions (limit of detection 0.10 μg/ml), except for the Chul 1/1 strain which generated NO at concentration-dependent manner. This indicated that SS from Chul 3/2 and 4/2 did not provoke cells to generate NO.

3.3. Collagen Production Induced by SS

The results showed that collagen type I production in L929 cells increased significantly after treatment with SS at various concentrations as compared to control. The amount of collagen produced by the cells was between 185 and 644 μg/ml. SS extracted from all three Thai cocoon strains promoted collagen production from fibroblasts, as shown in Fig. 5. The amount of collagen generated from fibroblasts after SS activation from the Chul 1/1 strain showed a significant difference at every concentration ($P < 0.05$) while SS from the Chul 3/2 strain induced significant differences in collagen production at every concentration, except between 0.4 and 0.6 mg/ml. However, SS extracted from the Chul 4/2 strain showed a significant difference in collagen production only at 1.0 mg/ml. This indicated that collagen production is intensively induced by SS from the Chul 1/1 strain, while SS from Chul 3/2 and Chul 4/2 promote collagen production at a lower level, respectively.

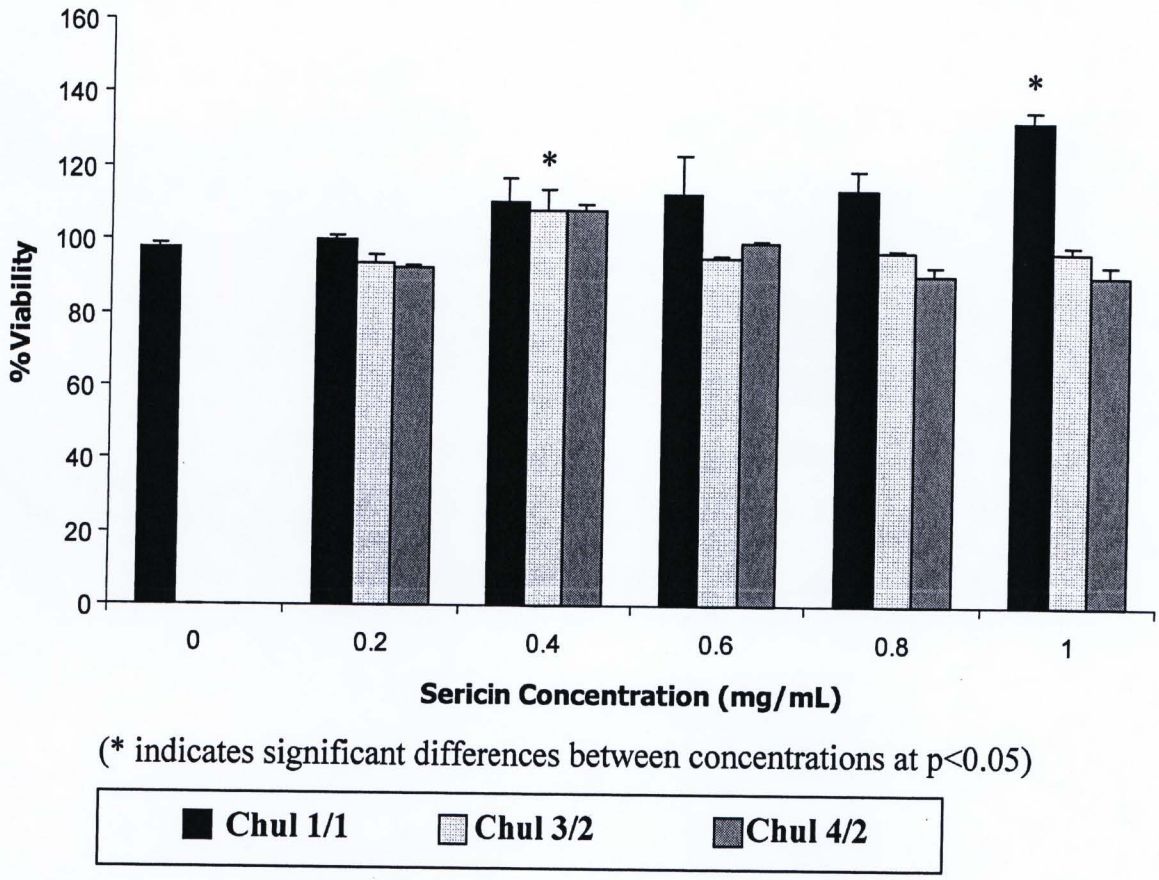

Figure 3. Viability study of L929 cells incubated with SS solutions *via* the MTT assay when various amounts of SS were added into the culture medium for 24 h to make the final concentration of SS in each well 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml, respectively. Error bars represent the standard error of the mean ($n = 3$).

Collagen production by fibroblast cells corresponded with SS concentration from all silk strains in a concentration-dependent manner. These results indicated that SS, especially with high methionine and cysteine content one, plays an important role in collagen synthesis in fibroblasts.

4. Discussion

Cocoon shells of silkworm compose of several components smaller than the structural silk proteins. Three of the low-molecular-mass proteins have been characterized in *B. mori* [30]. Seroin, which has a molecular mass of less than 13 kDa, is one of the small proteins with high content of polar amino acids and glycosylation [31]. This protein is hardly involved in silk fiber construction and coating [30]. Peptides of 6 kDa and 11 kDa, secreted exclusively in the middle silk gland section, are recognized as proteinase inhibitors of the Kunitz and Kazal family, respectively [30, 32]. Flavonoid components in the cocoon shell have molecular masses in the range 280–470 Da, while carotenoids have a molecular mass of less than 800 Da [33]. Flavonoids and carotenoids removal by ethanol seems to be efficient since the amount extracted is similar to that in the previous report, and cocoon shells have

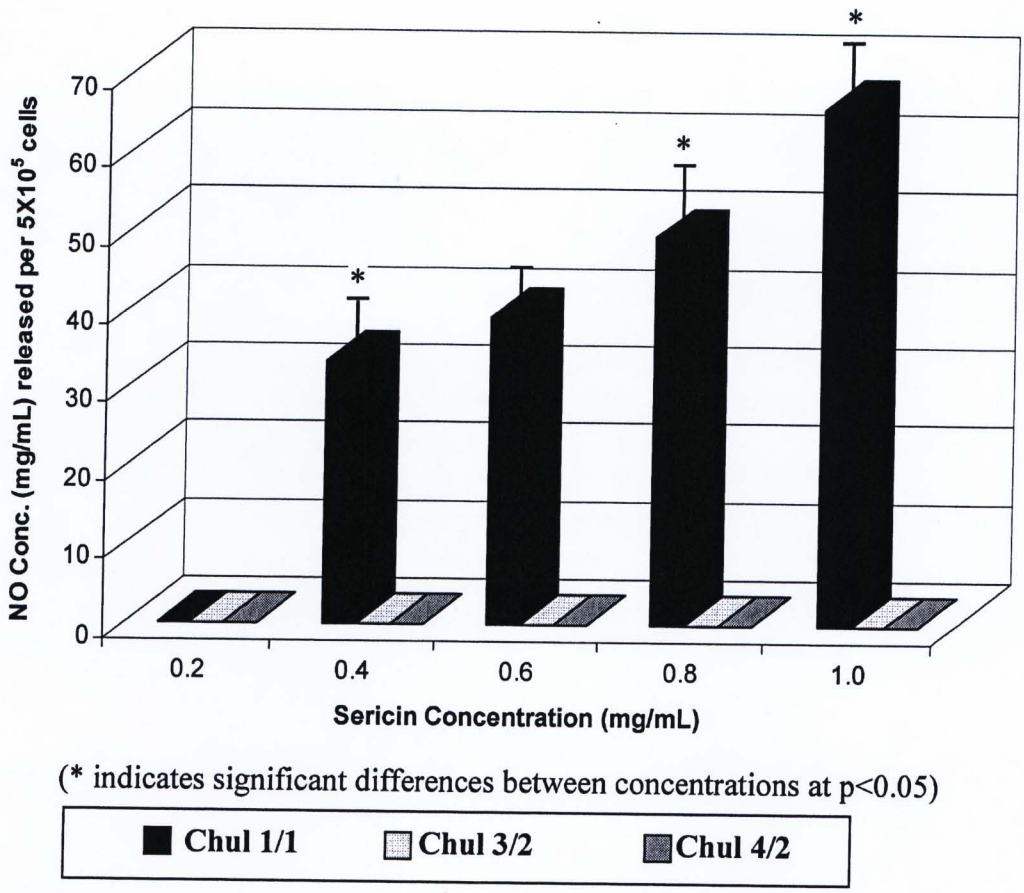

Figure 4. The amount of NO produced from L929 mouse fibroblasts when various amounts of SS from Chul 1/1, Chul 3/2 and Chul 4/2 strains were added into the culture medium for 24 h. Error bars represent the standard error of the mean ($n = 3$).

no colors left after 24 h [26]. Fractionation of silk proteins from ethanol extraction of colored-silk cocoons by SDS-PAGE shows a molecular mass of around 10 kDa which has been discarded. From their molecular mass range, these fractions might be proteinase inhibitors and possibly seroin.

According to amino-acid content analysis, there was no statistical significant difference in the main amino acid (aspartic acid, serine and glycine) content of SS extracted from these three silk cocoon strains. The amounts of serine, glycine and aspartic acid in the SS fraction extracted by heat method were similar to the amounts reported by others [27, 34]. However, the different amount of methionine and cysteine in the three different strains may affect their secondary structures; this needs further investigation. SS from Chul 1/1 strain had the highest amount of methionine and cysteine content. Moreover, the amount of methionine in Chul 1/1 strain was significantly different when compared to others ($P < 0.05$). Methionine plays an important role in wound healing and has already been proven to induce hyperhomocysteinemia (HHcy) which causes an inflammatory reaction, increases accumulation of extracellular matrix components, such as collagen type I, and activates MMP-9 which takes an active part in extracellular matrix modeling [18]. The highest methionine-content SS can promote the fastest growth rate of fibroblast

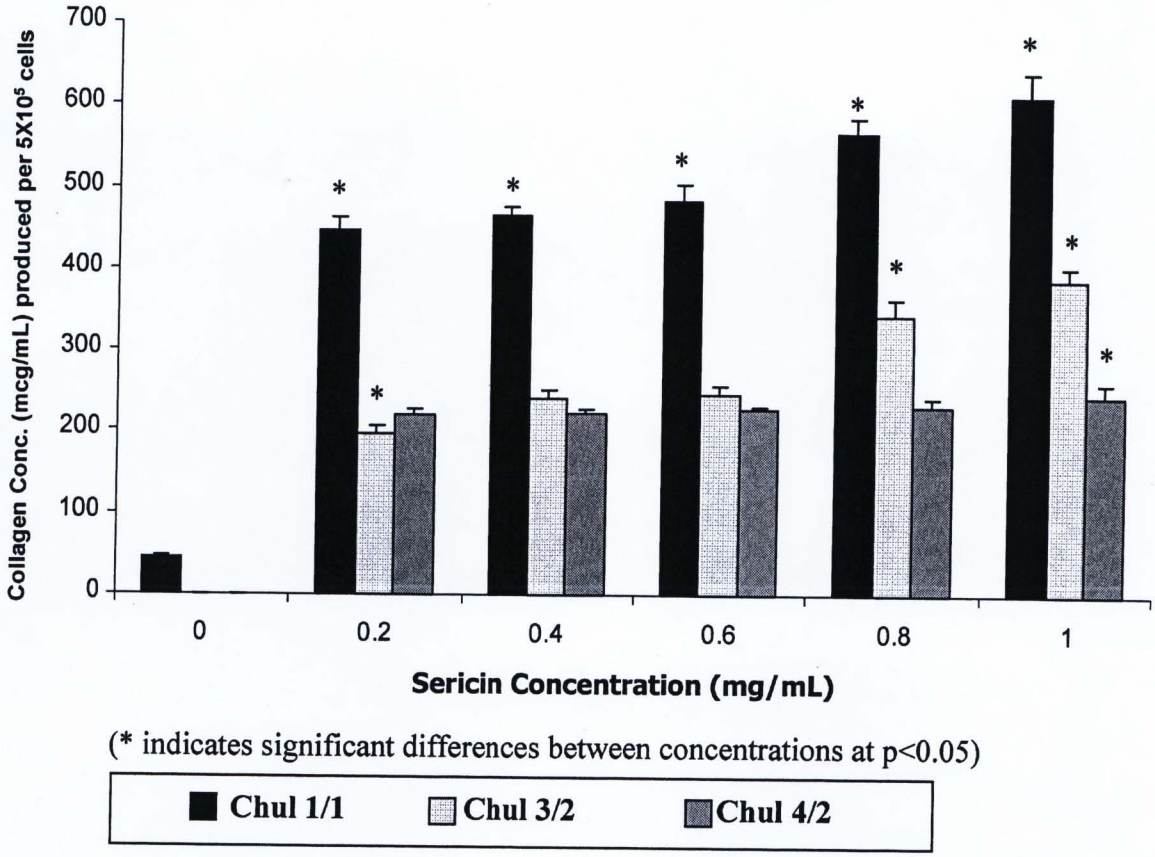

Figure 5. Collagen type-I production in fibroblast cell line L929 when various amounts of SS were added into the culture medium for 24 h to make the final concentration of SS in each well 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml, respectively. Error bars represent the standard error of the mean ($n = 3$).

cells. Even though there is no previous report on the concentration of sulfur amino acid required for promoting protein synthesis, Sen *et al.* demonstrated that concentration of methionine as low as 172 μg/ml can stimulate endothelial cells function [18]. Methionine and cysteine are well known for their ability of accelerating the healing rate of wounds in injured rats [16]. However, the underlying mechanisms that could explain the healing effects of methionine remain obscure.

SDS-PAGE indicates that the molecular mass of SS from Chul 1/1 strain is higher compared to others. According to biophysical characterization of macromolecules, molecular size and net surface charge density are the key parameters for their functions [35, 36]. Macromolecular crowding causes the excluded-volume effect that has been appreciated to have manifold effects in biology [37]. However, Lareu *et al.* demonstrate that anionic dextran, which has smaller molecular mass compared to neutral dextran, enables dramatically greater procollagen conversion *in vitro* [35]. According to this information, the net surface charge density of high-molecular-mass SS from Chul 1/1 strain needs to be further investigated to see its influence on the physiological phenomena.

Nitric oxide is usually produced during inflammatory conditions such as wound healing by the inducible isoform of the enzyme NO synthase [38]. Normally, large quantities of NO are generated during cell inflammation and have detrimental ef-

fects on various cellular functions that are linked to cGMP [39, 40]. However, smaller concentrations of NO can be potentially beneficial [39, 41–43]. Witte *et al.* reported that in the presence of low concentrations of NO, fibroblast collagen synthesis together with total protein synthesis are enhanced, and this increase is independent of the collagenase pathway [38, 41]. From our results, the Chul 1/1 strain induced NO production at a quantity of less than 70 mg/ml at 1.0 mg/ml of SS concentration. This NO concentration is much lower than the reported toxic concentration to fibroblasts which is 400 μM of NO-generating S-nitrosothiols, S-nitroso-N-acetylpenicillamine (SNAP) [39]. High methionine content in SS extracted from Chul 1/1 strain may also express cytoprotection, resulting in less toxicity of NO to cells, similar to the result reported by others [44]. Additionally, our results also indicated that SS extracted from the NO-generated (Chul 1/1) strain promotes the highest amount of collagen type I production from fibroblast cells compared to the non-NO-generated strain. This data is in good agreement with previous studies that reported an optimum level of NO together with high methionine can promote collagen synthesis.

5. Conclusion

Methionine content in SS shows a significant effect on the growth of fibroblast cells. Although SS from all silk strains can promote the growth and attachment of fibroblast cells, the highest methionine-content SS exhibits the fastest growth rate, and significant levels of collagen production and NO generation. All SS promote collagen type I production from cells in a concentration-dependent manner. Nitric oxide generated from cells can be found only when activated by SS with high methionine-content; however, this level is nontoxic to cells and even promotes collagen and protein synthesis. We conclude that SS should be safe for fibroblasts and is a good candidate for biological application at the range of its concentrations used in this study.

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Monitoring of inflammatory mediators induced by silk sericin

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Silk proteins have been shown to be good candidates for biomedical materials. However, there have been some reports regarding immunological and allergic responses to silk sericin. Our objective was to investigate the inflammatory mediators induced by sericin both *in vitro* and *in vivo*. Mouse monocyte and alveolar macrophage cell lines were used for monitoring levels of interleukin (IL)-1β and tumor necrosis factor (TNF)-α generated after activation by sericin at concentrations of 0.2–1.0 mg/mL. The amounts of TNF-α and IL-1β produced by both cell lines corresponded, in a dose-dependent manner, with the sericin concentration in the culture medium. The levels of TNF-α and IL-1β generated after sericin activation by macrophage cells were higher than those generated by monocytes. However, these cytokine levels would not cascade to other inflammatory effects. Inflammatory mediators were also monitored from sericin-treated, cream base-treated and normal saline-soaked full-thickness rat excisions. Using wound size measurements and ELISA assays, sericin-treated wounds were shown to heal faster and had lower levels of inflammatory mediators, as compared with the cream base-treated and normal saline-soaked wounds. It can be concluded that sericin promotes the wound healing process without causing inflammation.

[**Key words:** Inflammatory; Mediators; Sericin; Interleukin; Tumor necrosis factor]

Silk proteins, fibroin and sericin, have been widely investigated for their applications as biomaterials. Silk fibroin (SF) has been shown to be blood-compatible and binds lower levels of fibronectin than polystyrene or poly (2-hydroxyethylmethacrylate), which are both used as biomedical materials (1). SF can be used as a biomaterial coating of artificial trachea and may have the potential to be used in human tracheal defect reconstruction (2). Several studies have demonstrated a further advantage of using silk sericin (SS) in wound healing as it enhances the attachment and growth of mouse fibroblasts and human skin fibroblasts together with human and mouse hybridomas when added into culture media (3–5). SS can also promote wound healing in rats by inducing collagen production (6). However, there are several reports concerning immune responses to silk sutures containing SS proteins (7–9), which are assumed to be responsible for skin irritation and allergies (10). These reports have prevented the further development of SS in biomaterial applications.

Since an immune system reaction is a significant issue when considering the use of any biomaterial, a thorough investigation of the inflammatory response induced by SS is necessary before its development for medical use. The activation of the innate immune response, specifically macrophage activation, is a useful determinant of the biocompatibility of biomaterials (11). Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are frequently encountered proinflammatory cytokines. These cytokines are involved in a variety of immunological functions as well as interactions with a variety of target cells. It is widely accepted that IL-1, which is constitutively expressed in keratinocytes and accumulates in all epidermal layers (12), is a very important inflammatory mediator in the skin, and it is believed to be the main switch that initiates the inflammatory response (13). TNF-α is another proinflammatory cytokine that induces the expression of cutaneous and endothelial adhesion molecules to develop skin irritation and inflammatory responses (14). It is stored in epidermal mast cells and is also expressed by keratinocytes in response to irritation. The expression of TNF-α is transiently induced after treatment with various irritants and is independent of the release of IL-1 (15). Both IL-1 and TNF-α are first response cytokines, which initiate a cascade of events involving other second response cytokines, ultimately leading to inflammation.

The objective of this study was to examine the direct activation of inflammatory mediators by SS, both *in vitro* and *in vivo*, and therefore gain a better understanding of the relationship between SS and biological responses. To accomplish this goal, native SS from *Bombyx mori* was prepared and analyzed in an *in vitro* macrophage and monocyte assay to assess cytokine activation. Inflammatory mediators activated by SS were also investigated *in vivo*, using a rat wound-healing model.

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MATERIALS AND METHODS

Materials

Preparation of SS and SS cream The fresh, white-shell cocoons of *B. mori* were kindly supplied by Chul Thai Silk Co., Ltd. (Petchaboon province, Thailand). Silkworm cocoons were produced in a controlled environment. After cutting the cocoons into pieces (about 5 mm^2), SS was extracted using a high temperature and pressure degumming technique (16). In brief, the silkworm cocoons were mixed with purified water (1 g of dry silk cocoon: 30 mL of water) and the samples were autoclaved (SS-320, Tomy Seiko Co., Ltd., Tokyo, Japan) at 120 °C for 60 min. After filtration with a membrane to remove SF, SS powder was obtained by freezing and lyophilizing the SS solution using a Heto LL 3000 lyophilizer (Allrod, Denmark). SS cream was formulated using white petrolatum, mineral oil, lanolin, glycerin, bisabolol, propylparaben and methylparaben. All chemicals were purchased from Sigma (Singapore). Due to its limited solubility, the maximum concentration of SS in cream was 8% w/w, which was used in this experiment.

Fibroblast cell line The mouse fibroblast cell line L929 (Chinese Academy of Preventive Medical Sciences, Beijing, China) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco®, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco®, Grand Island, NY, USA) and antibiotics (100 U penicillin and 100 U/mL streptomycin, Gibco®, Grand Island, NY, USA) under 5% CO_2 at 37 °C. The media were changed every 2 days. When the cells reached confluence, they were harvested using 0.25% trypsin-EDTA (Gibco®, Grand Island, NY, USA), followed by the addition of fresh culture medium to create a new single cell suspension for further incubation.

Mouse monocyte cell line Mouse monocyte cell line J774.2 (ECACC, Sigma-Aldrich, St. Louis, MO, USA) was cultured in DMEM containing 10% FBS and antibiotics (100 U penicillin and 100 U/mL streptomycin) under 5% CO_2 at 37 °C. The media were changed every 2 days. When the cells reached confluence, they were harvested using 0.25% trypsin-EDTA, followed by the addition of fresh culture medium to create a new single cell suspension for further incubation.

Alveolar macrophage cell line Alveolar macrophage NR 8383 (ATCC CRL-2192, Rockville, MD), a rat cell line, was cultured in F-12 Kaighn's medium (Gibco®, Grand Island, NY, USA) supplemented with 15% FBS and antibiotics (100 U penicillin and 100 U/mL streptomycin) under 5% CO_2 at 37 °C. The media were changed every 2 days.

Animals Eight-week-old male Sprague-Dawley rats, weighing 250 ± 5 g, purchased from the National Laboratory Animal Centre, Mahidol University, Thailand, were used in these experiments. Each rat was caged individually at 25 ± 2 °C and was subjected to a 12:12 h light–dark cycle (standard fluorescent light) and allowed *ad libitum* access to chow and water. The animals were acclimatized for 1 week before use. The animals were maintained according to the "Guide for the Care and Use of Laboratory Animals" established by the National Laboratory Animal Centre, Mahidol University, Thailand.

In vivo animal tests Forty-eight rats were divided into two groups of twenty-four. The skin over the dorsal area of each rat was shaved completely, and application fields were outlined with a marking pen just prior to skin excision. In both groups, two full-thickness skin wounds were prepared by excision (1.5 × 1.5 cm) on the dorsum of each rat under aseptic surgery. Group 1 animals were topically treated with normal saline (NS) on left-sided wounds (control wounds) and 8% SS cream on right-sided wounds (treated wounds). Group 2 animals were topically treated with a cream base (formula control wounds) and 8% SS cream (treated wounds) on the left- and right-sided wounds, respectively. Surgery was performed under anesthesia using Zoletel 100, intramuscularly injected, as recommended by the manufacturer. Baytril® (enrofloxacin, Bayer, Leverkusen, Germany), an anti-bacterial agent, was also injected subcutaneously in the area at least 3 cm away from the wounds, in order to avoid complicating the local inflammatory response. Rimadyle® (carprofen, Pfizer, NY, USA) at 5 mg/kg was also subcutaneously injected into the area at least 3 cm away from the wounds every 24 h for 5 days as an analgesic. All wounds were cleaned daily using sterile NS solution. Cream base or SS cream was reapplied evenly to the wounds daily after cleaning, and chlorhexidine gluconate IV securement dressing (Tegaderm®, 3M, MN, USA) was used to protect the wounds from the rat's grooming. On day 7, twelve rats from each group were scrutinized, and tissue samples from all the wounds were collected at the same area at the initial dermatotomy up to subcutaneous levels for *in vivo* inflammatory mediator assays. The remaining rats were kept in order to monitor wound healing by measuring the wound size reduction for a total of 15 days after application, during which observations of the wound surface, body weight and skin irritation were noted. This protocol was approved by the Mahidol University Animal Care and Use Committee (MU-ACUC).

Methods

Effect of SS solution on cell proliferation L929 mouse fibroblast cells at an initial concentration of 5×10^5/well were seeded in a 96-well plate in DMEM containing 10% FBS. After 24 h, the culture medium was replaced by fresh medium, and SS solutions at various concentrations in purified water, after filtered sterilization by a 0.22 μm membrane filter (Sartorius Ltd., Epsom, UK), were added to the culture medium to achieve a final concentration of SS in each well of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. Cells without SS solution served as negative controls. Melittin, a peptide from bee venom toxin, at various concentrations ranging from 0.125 to 1.0 mg/mL was used as a positive control in the fibroblasts. After incubation for 24 h, a 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate cell activity (17). The absorbance was determined by a microplate reader (Biohit 830, Biohit®, Helsinki, Finland) at a wavelength of 570 nm. The percentage of cell proliferation was calculated and compared to the negative control. All experiments were done in triplicate. Differences between data were analyzed using a one-way ANOVA at $p<0.05$.

Determination of TNF-α, IL-1β production from NR8383 and J774.2 cells after induction by SS NR8383 and J774.2 cells in F-12 Kaighn's medium and DMEM containing 10% FBS, respectively, were seeded at an initial concentration of 5×10^5/well into a 96-well plate and were incubated at 37 °C with 5% CO_2. After 24 h, the culture medium was replaced with fresh medium, and various concentrations of filter-sterilized SS solutions in purified water were added to the culture medium to achieve final SS concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. Cells without SS solution served as the negative control. Cells were further incubated for 24 h, then supernatants were collected and assayed for TNF-α and IL-1β using either a rat or mouse ELISA assay kit (R&D systems, MN, USA). The minimal detectable doses of TNF-α and IL-1β were approximately 5.0 pg/mL and 3.0 pg/mL, respectively. All experiments were done in triplicate. The TNF-α reaction was quantitatively recorded at 570 nm, based on a standard curve of 12.5–800 pg/mL for the NR8383 cell line and 47–1500 pg/mL for the J774.2 cell line. The same wavelength was also used to record IL-1β levels, based on a standard curve of 31–2000 pg/mL for the NR8383 cell line and 15–500 pg/mL for the J774.2 cell line. Lipopolysaccharide (LPS) from *E. coli* was used as a positive control in both the monocyte and macrophage assays. Data was analyzed using a one-way ANOVA and a value of $p<0.05$ was considered to be statistically significant.

Wound size measurements Wound areas were measured by three veterinarians before the new treatments were applied, using a stereomicroscope (Carl Zeiss, NY, USA, Primo Star model, 0.3 × 0.65 objective lens). All veterinarian measurements were performed blind. Wound areas were photographed with a MoticCam 2300® at 1024 × 768 pixels. Motic Images Plus 2.0 ML was used for analyzing the data.

Histological evaluations After 15 days, the rats in each group were terminated and skin samples were taken. The central portion of the underlying tissue was taken and fixed in 10% buffered formalin. Each specimen was embedded in a paraffin block, and thin sections (3 μm) were prepared and stained with hematoxylin-eosin (H&E). Wound-healing effects were examined histologically under a light microscope, an Olympus BX 50 (Tokyo, Japan), using low-power magnification, at 200× and 100×.

Tissue preparation for cytokine determination Rat tissue samples (100 mg) were placed in 1 mL of homogenate buffer (50 mM Tris–HCl, pH 7.5, 150 mM sodium chloride, 1% Triton X-100, 1% protease inhibitor) (Sigma, MO, USA) and homogenized (2 × 30 s bursts) with a homogenizer (Sonics, CT, USA) under an ice-cold bath. Following centrifugation (11,000 g for 10 min at 4 °C), the supernatants were collected and TNF-α and IL-1β levels were determined.

Determination of TNF-α, IL-1β in rat tissues Cytokine (TNF-α and IL-1β) production was determined using rat ELISA assay kits (R&D systems, MN, USA). Either TNF-α or IL-1β diluent (50 μL) was added to each well plate. Then, 50 μL of homogenated tissue was added to the wells, and the plate incubated for 2 h at room temperature. Each well was washed five times with buffer solution (concentrated buffered solution containing surfactant and preservative, part 895024, R&D Systems, Minneapolis, MN, USA), followed by the addition of either 100 μL of TNF-α or IL-1β conjugate to each appropriate well. Following 2 h of incubation, each well was again washed five times with buffer (400 μL), then 100 μL of substrate solution was added. The plates were incubated for 30 min at room temperature, then 100 μL of stop solution (diluted hydrochloric acid solution, part 895714, R&D Systems, Minneapolis, MN, USA) was added. The reaction was quantitatively measured at 570 nm, based on a standard curve of TNF-α or IL-1β.

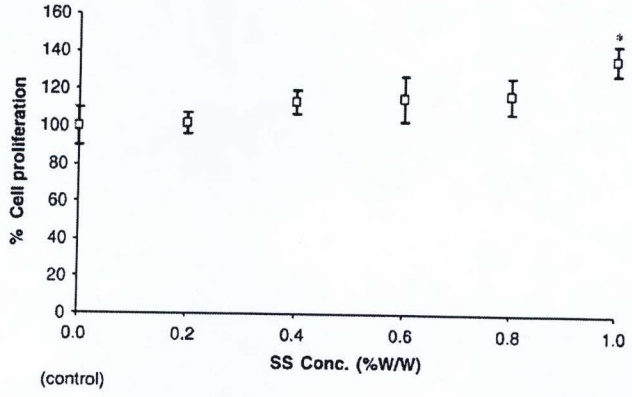

FIG. 1. Percentage of fibroblast cell (L929) proliferation after 72 h of incubation with SS solution at various concentrations. * indicates significant differences at $p<0.05$.

Statistical analysis Data was expressed as the mean ± SD. The statistical significance was determined by paired and unpaired Student's *t*-tests together with ANOVA. A value of $p<0.05$ was considered to be significant.

RESULTS

Effect of SS solution on cell proliferation Fig. 1 shows the percentage of cell proliferation after treatment with SS solution. Results from the MTT assay indicated that SS solutions at 0.2–1.0 mg/mL did not cause cell death after incubation for 24 h. The solution even promoted the growth of fibroblast cells, particularly at a concentration of 1.0 mg/mL. The fibroblast cells activated by SS solution at 1.0 mg/mL showed a significant difference in cell growth, as compared to that of other concentrations ($p<0.05$).

TNF-α, IL-1β production from NR8383 and J774.2 cells after induction by SS Fig. 2 shows the amounts of TNF-α and IL-1β released from the NR8383 and J774.2 cells after SS activation. The concentrations of TNF-α and IL-1β produced by both cell lines corresponded, in a dose-dependent manner, with the SS concentration added to the culture medium. The levels of IL-1β and TNF-α produced by both cell lines after exposure to SS at a concentration of 0.2–1.0 mg/mL were approximately 30–350 pg/mL and 30–500 pg/mL, respectively. We also found that the levels of TNF-α and IL-1β generated by macrophage cells after SS activation were higher than those generated by monocytes.

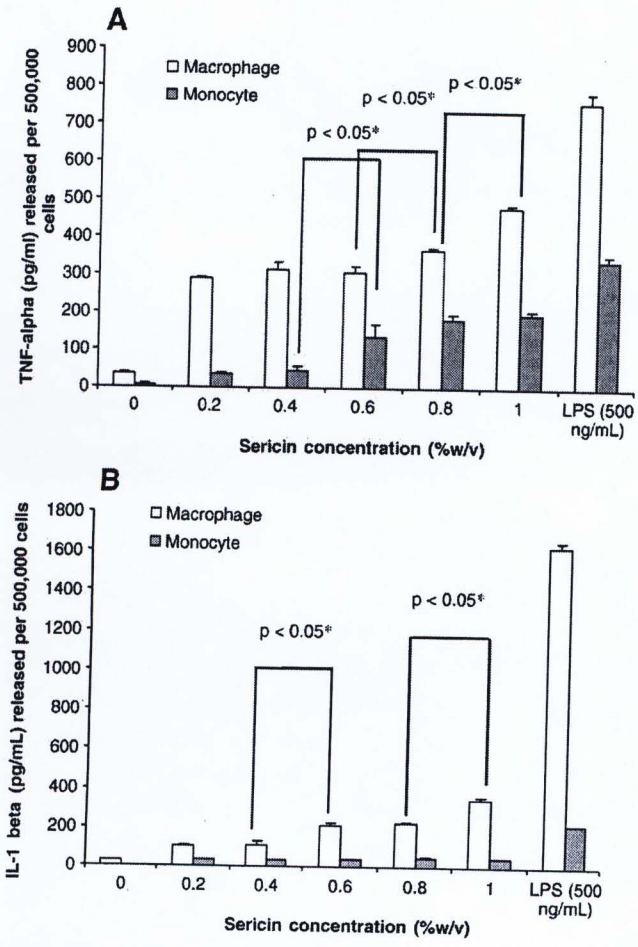

FIG. 2. The effect of various SS concentrations on TNF-α (A) and IL-1β (B) production in alveolar macrophage (NR8383) and monocyte (J774.2) cell lines after 24 h of culture. Error bars represent the standard error of the mean ($n=3$). Open bar represents IL-1β and TNF-α generated from the macrophage (NR8383) cell line, closed bar represents IL-1β and TNF-α generated from the monocyte (J774.2) cell line. * indicates significant difference when compared to a one-step lower concentration ($p<0.05$).

Appearance of wound surfaces treated with NS, cream base and SS cream Fifteen days after dermatotomy, the SS-treated wounds had almost completely epithelized (Fig. 3), while the control (NS, base cream) wounds still showed some open areas and inflammation. Fig. 4 shows the histological examinations of skin tissues by H&E staining. The cream base-treated and NS-soaked wounds were not fully epithelized and exhibited some ulcers, while the SS-treated wounds showed complete epithelization. There was also some unevenness of the epidermis near the ulcers in the cream base-treated and NS-soaked wounds.

Wound-size reduction The percentages of wound size reduction in full-thickness rat excisions in Groups 1 and 2 are shown in Figs. 5A and 5B, respectively. The SS-treated wounds had a higher percentage of wound size reduction, as compared with the cream base-treated and NS-soaked wounds. All the wounds showed signs of inflammation during the first week of dermatotomy, as shown by wound size enlargement. Our end-point was chosen as 90% healing. Completion of wound healing has been found to be generally variable and dependent upon other factors, such as animal interference. The time to reach 90% wound healing of the SS-treated wounds was much less than that of the cream base-treated and NS-soaked wounds (11, 16 and 17 days, respectively) (Fig. 6).

TNF-α, IL-1β production from rat tissues The mean levels of TNF-α and IL-1β generated from rat wound tissue after the application of cream base, 8% SS cream and NS to the wounds are shown in Fig. 7. The sericin-treated wounds showed much lower levels of both inflammatory mediators. The level of TNF-α generated from SS-treated wounds ranged from 6.87–25.20 pg/mL, while TNF-α levels generated from NS-soaked wounds and cream base-treated wounds ranged from 19.87–35.87 pg/mL and 10.17–26.53 pg/mL, respectively (data not shown). Interleukin-1β levels generated from SS-treated, NS-soaked and cream base-treated wounds ranged from 11.15–42.30 pg/mL, 13.75–89.30 pg/mL and 34.30–55.74 pg/mL, respectively (data not shown).

DISCUSSION

Cells release various cytokines as signaling molecules to control the tissue environment and to induce local reactive responses. Fibroblasts synthesize and secrete IL-1β and TNF-α, which are involved in the modulation of skin growth, repair, and scarring during skin inflammation (18–20). Previous studies have reported that TNF-α and IL-1β at concentrations less than 100 ng/mL are not toxic to keratinocytes (21, 22). Here, we examined the inflammatory potential of SS using TNF-α and IL-1β as inflammation indicators. Soluble SS at a concentration range of 0.2–1.0 mg/mL is non-toxic to fibroblast cells, as shown by the increasing rate of cell proliferation. Moreover, SS does not significantly activate TNF-α and IL-1β release from the cells into the growth medium during short-term incubation, as shown by our experiments. The maximum levels of TNF-α and IL-1β released from monocytes and macrophage cells after SS induction were 500 pg/mL and 350 pg/mL, respectively. According to previous reports (21, 23, 24), these levels of cytokines would not cause an inflammatory response or prevent cellular proliferation.

Immediately following injury, inflammatory factor signaling is initiated by platelet degranulation, which recruits a number of immune cells to the wound site (25). It is known that IL-1 and TNF-α are released from disrupted endothelial cells within the first 12 h, and their levels peak within the first day of wounding (26), shortly after the release of monocyte chemoattractant protein (MCP)-1. However, the level of TNF-α significantly decreases during healing (27). Seven days after injury, the TNF-α and IL-1β levels of SS-treated

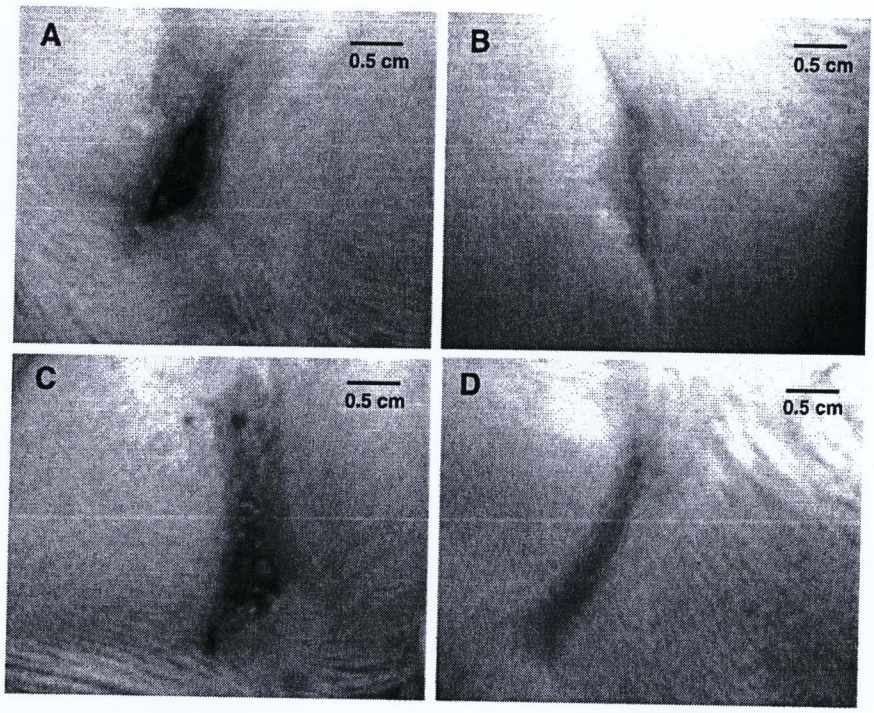

FIG. 3. Appearance of wounds on day 15 after full-thickness dermatotomy. (A, B) Appearance of wounds from the same rat, treated with NS and SS cream, respectively. (C, D) Appearance of wounds from the same rat, treated with cream base and SS cream, respectively.

wounds decreased significantly to levels of less than 25 pg/mL and 58 pg/mL, respectively. These low levels indicate that there is no significant activation of inflammatory cytokines by SS. Therefore, the application of SS to the wounds generated TNF-α and IL-1β at non-toxic levels. Although there appears to be a wide difference between the TNF-α levels of the SS-treated samples of Group 1 and Group 2, this may due to the individual responses from living subjects. Nevertheless, the TNF-α and IL-1β levels in rat tissues of SS-treated wounds 7 days after injury are still significantly lower than the levels found in NS-soaked wounds and cream base-treated wounds, which indirectly imply that SS-treated wounds have a better healing process. In contrast to the significant decrease in both IL-1β and TNF-α levels in

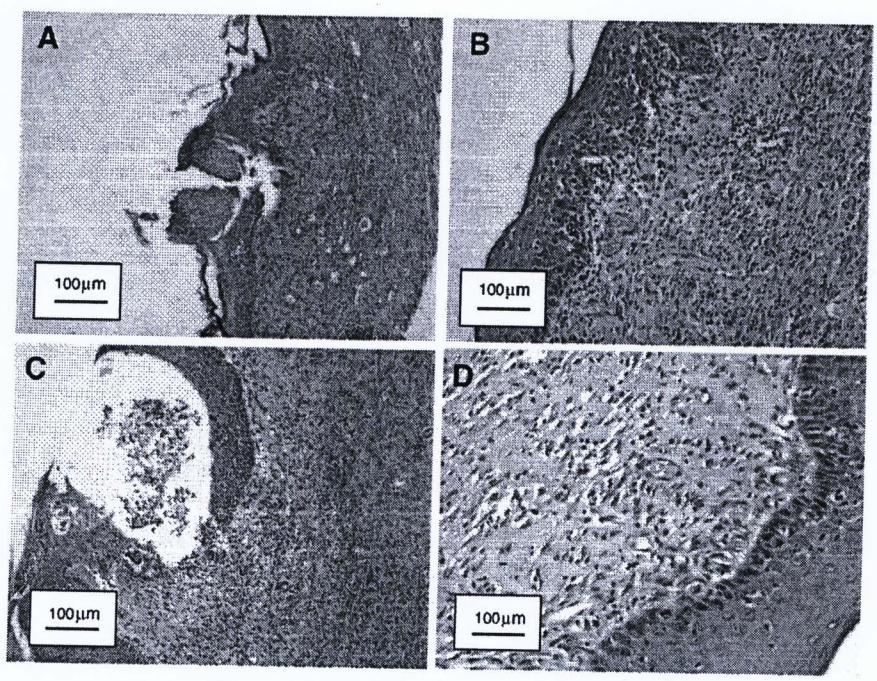

FIG. 4. Histological examinations of skin tissues on day 15 after full-thickness dermatotomy by hematoxylin-eosin (H&E) staining; 200×. (A, B) Appearance of wounds from the same rat, treated with NS and SS cream, respectively. (C, D) Appearance of wounds from the same rat, treated with cream base and SS cream, respectively.

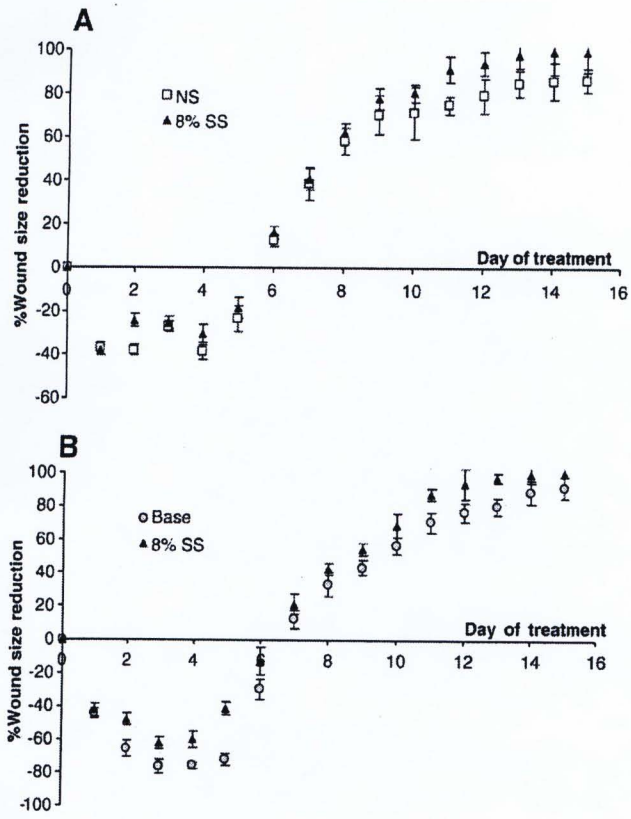

FIG. 5. Percentage of wound size reduction in full-thickness rat excisions in Group 1 (A) and Group 2 (B). Open square represents percentage of wound size reduction in NS-soaked wounds, closed circle represents percentage of wound size reduction in cream base-treated wounds, closed triangle represents percentage of wound size reduction in SS-treated wounds.

tissues, there is a significant elevation of both IL-1β and TNF-α levels in the macrophage cells. This may result from an increase of proinflammatory cytokine levels during the first 24 h after injury, which decline after that period. Panilaitis et al. demonstrated similar results for TNF-α release in a murine macrophage cell line and reported a decrease in TNF-α production after long-term culture for 7 days, as compared to the level measured after 24 h (11). Moreover,

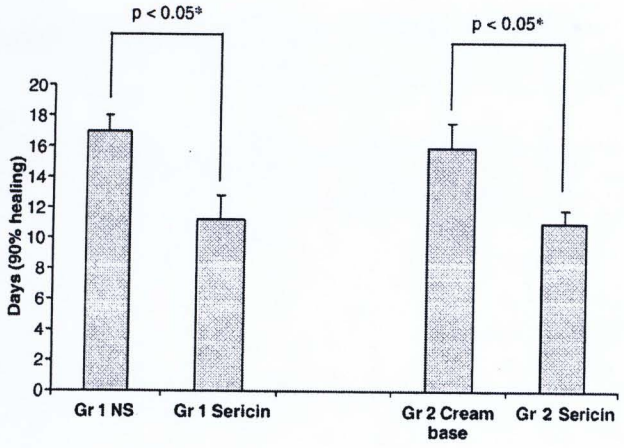

FIG. 6. Effect of SS cream over time to achieve 90% wound healing in rats.

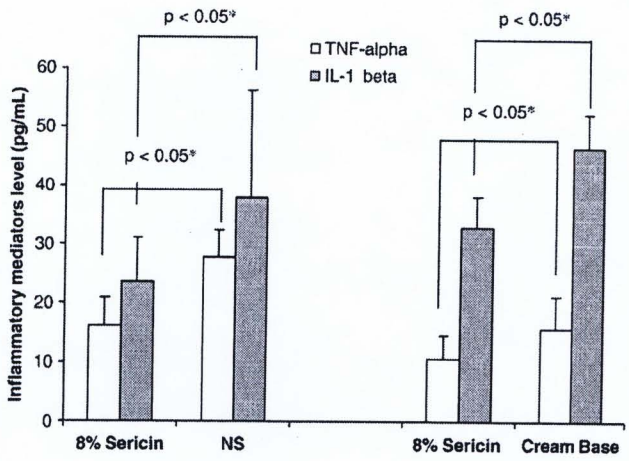

FIG. 7. The mean levels of IL-1β and TNF-α from rat tissue after 7 days of treatment. Error bars represent the standard error of the mean ($n = 3$). Open bar represents TNF-α generated from wounds, closed bar represents IL-1β generated from wounds. * indicates significant differences at $p<0.05$.

other biological factors in rats may also have an effect on inflammation, while only macrophage cells are active in the cell culture environment. The same result has also been demonstrated *in vivo* by other researchers (28). Since the amounts of TNF-α and IL-1β released from monocytes and macrophages in our experiments are negligible, this data indicates that SS does not cause any severe damage to cells and the levels of both cytokines tend to be lower several days after injury. Furthermore, minor irritability caused by SS to the cells, if any, should be considered as an acute rather than a chronic effect, since cytokine levels from macrophages (acute inflammatory response) are higher than the levels released from monocytes (chronic inflammatory response).

In conclusion, this study demonstrates that SS is non-toxic to fibroblast cells and can promote the wound healing process, as compared to NS-soaked and cream base-treated wounds. The levels of IL-1β and TNF-α released from monocytes and macrophages after the addition of SS into the culture media are low, which indicate that SS is not toxic to the cells. The levels of both inflammatory mediators, IL-1β and TNF-α, found in rat tissues 7 days after injury are significantly lower in SS-treated wounds, as compared with NS-soaked and cream base-treated wounds.

ACKNOWLEDGMENTS

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