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Cytocompatibility of silkworm cocoon layer extracts

You-Young Jo[1], Sung-Kuk Kim[2], Kwang-Gill Lee[1], Sung Min Bae[1], Jong-Ho Kim[3], Bong-Seob Shin[3], Jong-Young Jeon[3], and HaeYong Kweon[1*]

[1]*Sericultural and Apicultural Materials Division, National Institute of Agricultural Science, RDA, Wanju-gun, 55365, Republic of Korea*
[2]*Chonbuk National University School of Dentistry, Jeonju-si, Jellabuk-do 54896, Republic of Korea*
[3]*School of Textile Engineering and Fashion Design, Kyungpook National University, Sangju, Republic of Korea*

Abstract

Recently silk polymer produced by *Bombyx mori* silkworm has been considered as biological macromolecules. Silk polymer was extracted in PBS solution at 37°C for 72 h or 72°C for 24 h. The effect of EtOH treatment on the cocoon extraction was also examined. The extraction yield of cocoon was less than 1 wt% regardless of extraction conditions. UV spectroscopy showed that the experimental extracts have absorption bands at 280 nm. There is no cytotoxicity effect on the mouse fibroblast L929 cell. The phenotype of L929 cell was not changed under the experimental conditions. The proliferation behavior of L929 cell was not affected by the addition of cocoon extract. Therefore, cocoon extract might be cytocompatible and can be used as promising biomaterials.

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Introduction

A silkworm cocoon of *Bombyx mori* provides silk fiber for textile industries for more than 4,000 years. Recently, silk polymer has been revealed several advantages for biological materials and then applied various functional materials such as cosmetics, suture and artificial eardrum (Kundu *et al.,* 2013; Ju *et al.,* 2014; Vepari and Kaplan, 2007; Kim *et al.,* 2010), and so on.

In the view point of material, silk is a natural protein polymer composed of two proteins: one is a central protein called silk fibroin and another is gum-like protein called silk sericin. Silk fibroin is a natural fibrous protein with a semicrystalline structure and it consists of heavy chain (390 kDa), light chain (25 kDa) and glycoprotein. Each polypeptides linked by disulfide bond (Zhou *et al.,* 2001). Especially, fibroin as the main component of silk protein has found diverse applications in the biomedical field, which has a strong tensile strength, biodegradability and biocompatibility (Horan *et al.,* 2005; Meinel *et al.,* 2005). Hence, many researchers select silk fibroin as one of the materials for biomedical application.

Silk sericin is a protein polymer, which acts as an adhesive binder to maintain the structure of the fibers. Sericin is an albuminoid protein composed of serine (30%) and other amino acids (Teramoto and Miyazawa, 2005; Garel *et al.,* 1997). Sericin was removed easily from *Bombyx mori* cocoon using a thermo or chemical process called degumming (Mo *et al.,* 2009). Naturally, sericin is insoluble in cold water although it is degraded and soluble in hot water. After

***Corresponding author.**
HaeYong Kweon
Sericultural and Apicultural Materials Division, National Institute of Agricultural Science, RDA, Wanju-Gun, Republic of Korea
Tel: +82-63-238-2872 / FAX: +82-63-238-3832
E-mail: hykweon@korea.kr

degumming, sericin is also can be processed into various forms such as film, gel and particles (Teramoto *et al.,* 2008). Sericin has many functions for cell metabolism. Sericin was indicated to suppress lipid peroxidation and tyrosinase inhibitory activity (Kato *et al.,* 1998). In addition to anti-oxidant activity and chemopreventive effects of sericin was found (Fan *et al.,* 2009; Zhaorigetu *et al.,* 2003), Silk sericin from silkworm cocoons accelerates the proliferation of mammalian cell line (Terada *et al.,* 2002; Aramwit *et al.,* 2009; Aramwit and Sangcakul 2007). Another study reported anti-cancer effects of sericin in colon cancer (Kaewkom *et al.,* 2012). Like this, sericin was excellent cell growth supplement and cell signal regulation agents.

Recently one of the authors reported the effect of silkworm cocoon layer membrane for guided bone regeneration (Ha *et al.,* 2014). When silkworm cocoon is used as bioembedding materials, it contacts biological environment. Although, silk proteins have known as excellent biocompatible characteristics, it is necessary to clarify the safety of cocoon materials on the biological environment. Therefore, the author examined to know cytocompatibiliy of silkworm cocoon extracts for further application of silkworm cocoon. The extraction of silkworm cocoon was conducted under biological conditions and then examined the proliferation and cell viability of mouse fibroblast L929.

Materials and Methods

Materials

Bombyx mori silkworm cocoons produced by three Korean commercial varieties, Baekok-jam, Keumok-jam and Daesung-jam, were obtained from Uljin Farm, Kyeongsangbuk-do, Korea. Phosphate buffered saline (1X PBS, pH 7.4) was purchased from Life technology (California, U.S.A.). L929 cell line for cytotoxicity and proliferation evaluation was purchased from American Type Culture Collection (Virginiea, U.S.A.). For cell culture, L929 cell growth medium, fetal bovine serum (FBS) and penicillin-streptomycin antibiotics were purchased from LONZA (Basel, Switzerland). Ez-cytox was purchased from Daeil Lab service (Seoul, Korea). Acrylamide gel gradient 4-12% was purchased from Life technology from U.S.A. The ProsiBlue gel staining solution was purchased from GenDEPOT (Texas, U.S.A.) And all of cell culture related instrument were purchased from SPL Lifescience (Uijeongbu, Korea).

Preparation of cocoon extracts

Each cocoon was washed with tap water and then dried absolutely with room temperature in the dark. The cocoon was sliced into 5 mm x 5 mm pieces. Each cocoon slices were divided into two groups; one group was treated with absolute ethyl alcohol (EtOH) for 24 h at R.T. and another group was not treated EtOH. After treatment, cocoon was dried with R.T. for overnight. The extraction condition were followed; weigh 1 g of cocoon and add into 10 mL of 1X PBS buffer at 37°C for 72 h and/or 72°C for 24 h. Save the cocoon extracts and then filtered it with 0.22 um syringe filter (Sartorius Ltd., Epsom, UK) and protein concentration of all extracts was determined by bicinchronic acid (BCA) assay. Forty ug of each extracts used for cell cytotoxicity and proliferation assay.

UV vis spectrometry

Ultraviolet visible spectra of cocoon extracts were measured with a S-3100 UV-vis spectrophotometer (Sinco, Seoul, Korea). All of cocoon extracts were diluted to X50 in 1X PBS and UV spectrum was measured.

Fibroblast cell culture

Murine fibroblast cells L929 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) at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air. The medium was changed every 2 d. When cells reached confluence, they were harvested using 0.25% trypsin-EDTA (Gibco, California, USA), for further experiment.

Cytotoxicity assay of cocoon extracts

L929 mouse fibroblast cells were seeded into a 96-well culture plate at 2×10^4 cells/well in a 100 uL culture medium. After incubation for 24 h, the culture medium was

replaced with fresh medium containing 4 ug of each extracts. Cocoon extracts of various extraction conditions were filter-sterilized by 0.22 um membrane filter prior to adding to the culture medium. Cells without cocoon extracts served as negative controls. After incubation for 24 h and 48 h, Ez-cytox assay was performed to evaluate cell activity. The absorbance was determined by a Multiscan GO microplate reader (Thermo scientific, Massachusetts, U.S.A.) at a wavelength of 450 nm. After that, cells morphology observed under an EVOS XL Core optical microscope (Life technology, California, U.S.A.). The percentage of viable cells was calculated and compared to the negative control. All experiments were done in triplicate.

Proliferation assay of cocoon extracts

L929 mouse fibroblast cells were seeded into a 96-well culture plate at 2 x 10^3 cells/well in a 100 uL culture medium. After incubation for 1, 2, 4 and 7 d, Ez-cytox assay was performed. Another experimental condition except seeding cell number and incubation period is same with cytotoxicity assay.

Results

Extraction of silkworm cocoon

Silkworm cocoons were extracted in PBS solution for 72 h at 37°C and 24 h at 72°C. Extraction test was performed with 3 kinds of commercial Korean silkworm varieties, Baekok-jam, Keumok-jam and Daesung-jam cocoon. As shown in Table 1, the extraction ratio of each cocoon was less than 1 wt% regardless of extraction condition.

UV visible spectrum of cocoon extracts

Silkworm cocoon extracts were characterized with UV-VIS spectrometer and shown in Fig 1. As shown in Fig 1, all kinds of experimental cocoon extracts showed an absorption peak at 280 nm, a characteristic band of protein.

Cell viability of fibroblast cells

To examine the safety of cocoon extracts on mammal cell, the extract of each cocoon was added into cell culture medium for fibroblast cell L929. The cell viability was evaluated by Ez-cytox assay after 48 h incubation and shown in Fig 2. As shown in Fig. 2, L929 cell viability was shown over 80% regardless of extraction conditions and silkworm varieties extracted.

Cell morphology

L929 cell morphology was observed using optical microscope

Table 1. Extraction condition and extraction ratio of *Bombyx mori* silkworm cocoon

Sample name	Extraction Temp.	Extraction Time	EtOH Treatment	Initial weight (g)	Dried weight after extraction (g)	Extracts Ratio
Baekok-jam Cocoon	37°C	72 h	X	1.001	0.932	<1%
			O	1.005	0.93	<1%
	72°C	24 h	X	1.004	0.933	<1%
			O	1.003	0.93	<1%
Keumok-jam Cocoon	37°C	72 h	X	1	0.916	<1%
			O	1.003	0.932	<1%
	72°C	24 h	X	1.001	0.927	<1%
			O	1.002	0.924	<1%
Daesung-jam Cocoon	37°C	72 h	X	1.002	0.932	<1%
			O	1	0.933	<1%
	72°C	24 h	X	1	0.921	<1%
			O	1	0.926	<1%

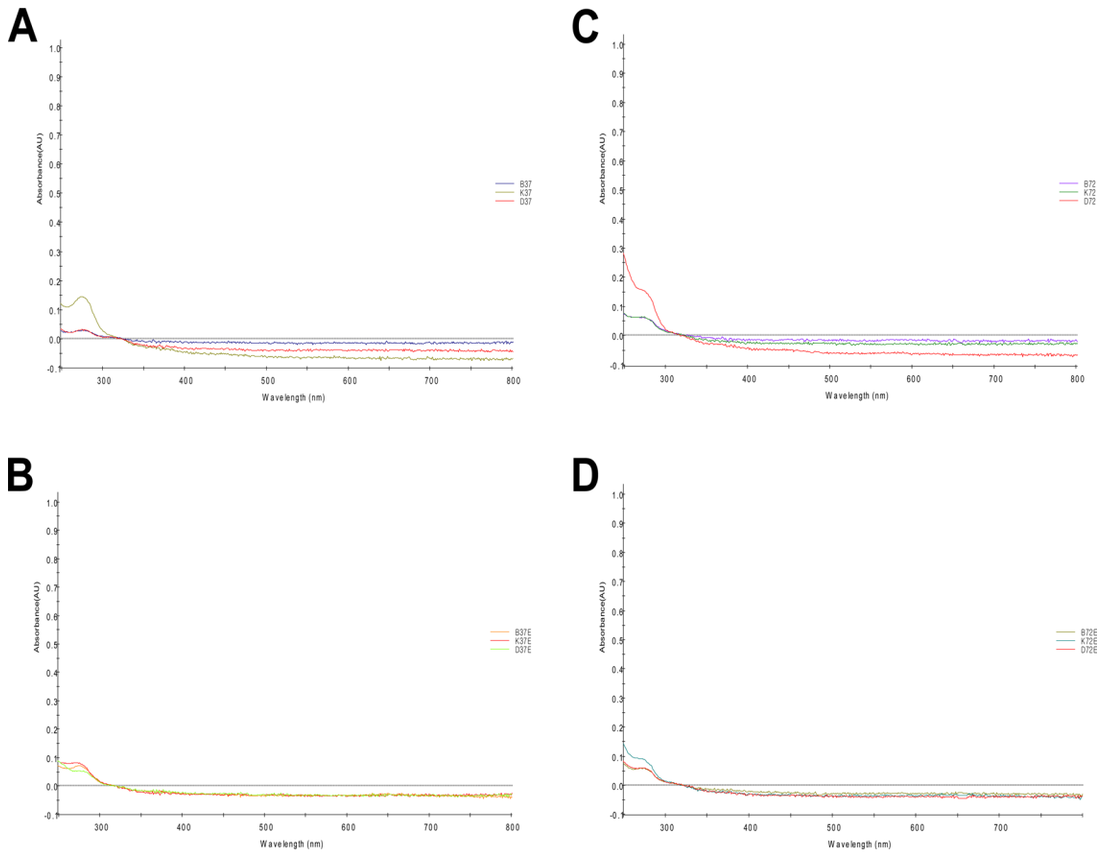

Fig. 1. UV spectra of cocoon extracts. 3-species cocoon extracts were diluted to X50 with 1X PBS (pH 7.4). The spectrum of each cocoon extracts was measured by a S-3100 UV-vis spectrophotometer. (A) UV spectra of cocoon extracts at 37°C, (B) UV spectra of cocoon extracts at 37°C treated with ethanol, (C) UV spectra of cocoon extracts at 72°C, (D) UV spectra of cocoon extracts at 72°C treated with ethanol. B is Baekok-jam, K is Keumok-jam and D is Daesung-jam.

and shown in Fig 2B and 3B. In this experiment, L929 cells showed typical spindle-shape phenotype. The cell shape of fibroblast cultured with cocoon extract is also same spindle-like morphology.

Proliferation of fibroblast cell

Cell proliferation assay is one of typical methodology for cytocompatibility. Cell proliferation curve of L929 cell was shown in Fig 3. As shown in Fig. 3, cell proliferation was increased with culture time. The mouse fibroblast L929 was growth more than 10 times for 7d. There are no significant differences among the varieties of silkworm. Moreover, EtOH treatment and extraction temperature condition showed no difference of OD values. These results indicated that the function of extracts was different according to the extraction condition.

Discussion

Silk has been used as textile fiber and surgical suture. Now a day silk has been considered as biomedical materials due to its cytocompatible and tissue compatible properties. Silk materials have been investigated as tissue scaffold, tympanic patch, guided bone regeneration matrix, and so on. So, the author examined the cytocompatible properties of silkworm cocoon extract. The cocoon was extracted in PBS solution at 37°C for 72 h or 72°C for 24 h. As shown in Table 1, the extraction ratio of Korean domestic silkworm cocoon was less than 1wt.% regardless of extraction conditions and silkworm source. UV spectrometer (Fig 1) showed that the extracts have an absorption band at 280 nm and it means that the extract was a protein material from silkworm cocoon. Silkworm cocoon is composed of hydrophobic fibroin and hydrophilic sericin. So, the extract is considered as easy soluble fraction of sericin. To know the

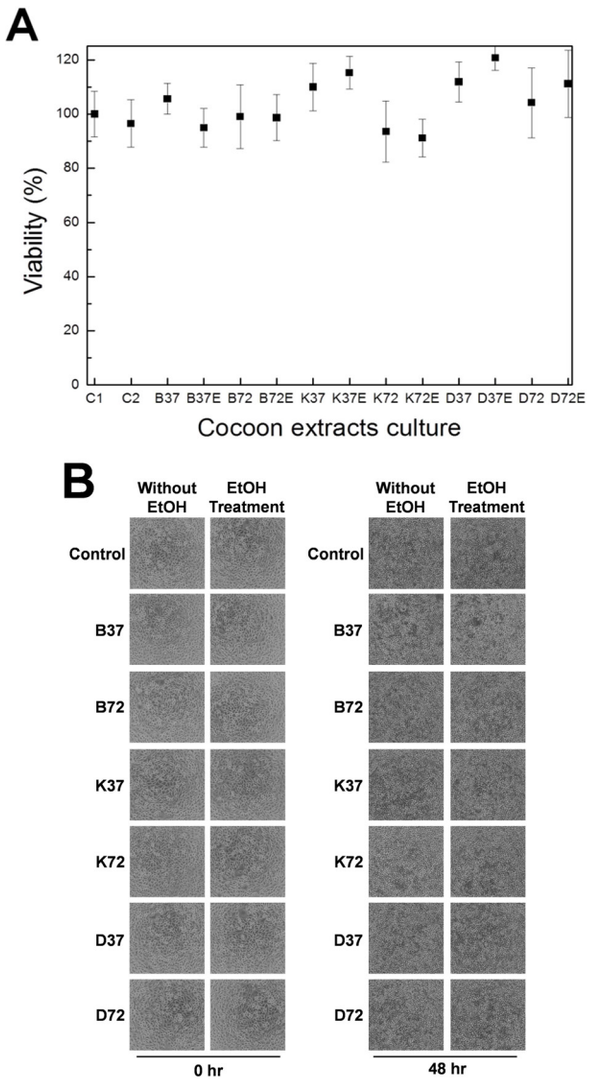

Fig. 2. The cytotoxicity L929 fibroblast cell with 3-species cocoon extracts. L929 fibroblast was seeded onto 96-well plate at 2 x 10^4 cells/well in 100 uL. 4 ug of each extracts were treated to cells for 48 h. (A) After treatment, cell viability was evaluated by Ez-cytox and O.D. value was analyzed at 450 nm by microplate reader. (B) The morphology of the extracts-treated cells compared with control. C is negative control, B is Baekok-jam, K is Keumok-jam and D is Daesung-jam extracts.

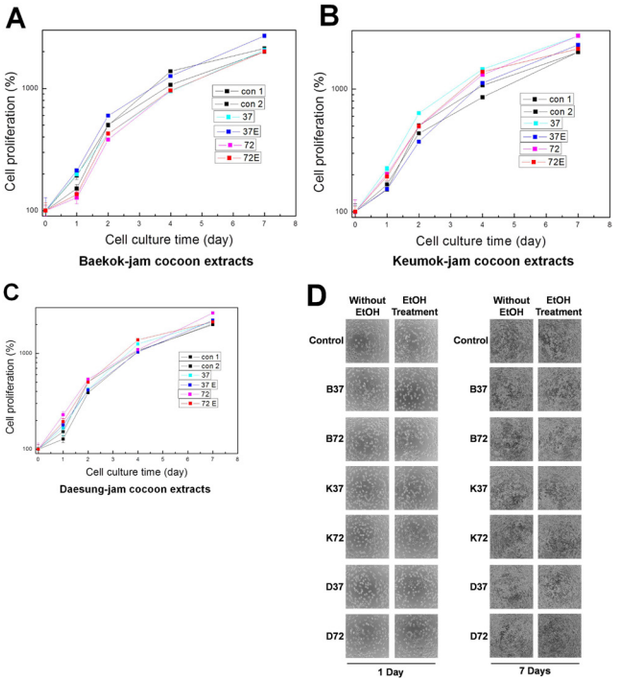

Fig. 3. The proliferation assay of L929 fibroblast cell with 3-species cocoon extracts. L929 fibroblast was seeded onto 96-well plate at 2 x 10^3 cells/well in 100 uL. 4 ug of each extracts were treated to cells for 7 d. After treatment, cell viability was evaluated by Ez-cytox and O.D. value was analyzed at 450 nm by microplate reader. (A) Baekok-jam extracts, (B) Keumok-jam extracts, (C) Daesung-jam extracts. (D) The morphology of the cells after 7 d.

feasibility of silkworm cocoon itself as biomaterials, the effect of PBS extracts on cell viability of mouse fibroblast cell L929 (Fig 2) was evaluated through Ez-cytox assay after 48 h cultivation. Cell viability was measured over 80% regardless of extraction conditions, EtOH treatment, and silkworm varieties extracted compared to control ones. Silk sericin is known to promote the growth of the mouse fibroblast cell line L929 by activation of collagen production (Aramwit *et al.,* 2009; Aramwit and Sangcakul 2007). These results indicated that all of samples of Korean domestic silkworm cocoon extract do not induce the cytotoxicity of fibroblast. The morphological observation (Fig 2B, Fig 3B) showed that the spindle-like shape is not changed during the cell culture by adding cocoon extracts. According to the proliferation results (Fig 3), the extracts do not interrupted the growth of fibroblast cells. From the above results, in the common biological conditions there is a little component of silkworm cocoon extracted. The extracts have no cytotoxicity and have good cell compatible property. Therefore, silkworm cocoon might be used as biomedical materials with further study for physicochemical and biological properties.

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