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# Cell Proliferation Inhibition by Sericin from the Wild Silkworm, *Cricula trifenestrata*

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## Abstract

**Sericin is a major silk protein, and recent studies have shown that sericin has numerous physiological activities against cultured cells. For example, it accelerates mammalian cell proliferation and protects insect cells against cell death. In this study, we examined the activities of sericin prepared from the wild silkworm, *Cricula trifenestrata*. The results presented here indicated that *C. trifenestrata* sericin has anti-proliferative activity against feline kidney cells, while sericin from *Bombyx mori* promoted cell proliferation. The components of the sericin preparation that played a role in inhibition of proliferation were analyzed by fractionation through partition chromatography (Folch method) and reversed-phase chromatography. Our analysis showed that at least two components in the *C. trifenestrata* sericin preparation promoted the anti-proliferative activity in an additive or even synergistic manner. Therefore, differences in components between insect species may contribute to different activities against cultured cells.**

## Keywords

**Sericin, Wild Silkworm, Cell Death, Anti-Proliferative Activity**

## 1. Introduction

Sericin, a major silk protein, is the sticky material surrounding silk fibroin. During the production of silk threads, sericin is removed from the cocoon using hot water and discarded. However, recent studies have demonstrated

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several biological and physiological activities in sericin preparations that can be applied for medicinal use and biological technologies. For example, sericin is known to accelerate the proliferation of several mammalian cell lines [1] [2] and protect insect cells from death caused by acute serum deprivation in culture [3], and has protective and healing activities on the skin [4]-[6]. As sericin has been shown to activate cell proliferation and protect against cell death, this protein may be a useful additive to serum-free media for culturing mammalian tissues and cell lines [1] [7]-[9].

The majority of these observations were derived from sericins from industrially used silkworms distributed worldwide from China (e.g., *Bombyx mori*). However, limited information is available regarding sericins from other species, including wild silkworms. To determine whether sericins from different silkworms or silk moths have similar activities, we examined sericin from *Cricula trifenestrata* with regard to its effects on cell proliferation. The wild silkworm, *C. trifenestrata*, which produces gold-colored cocoons, is native to Indonesia and Malaysia, but it has been removed from agriculture and forestry environments as it is considered a pest. In the present study, we examined the effects of sericin from *C. trifenestrata* (CS) on mammalian cell proliferation using feline kidney distal cells (FKD) that can be cultured in serum-free or serum-containing media. We showed that CS has anti-proliferative activity against mammalian cells, in contrast to previous reports showing that sericin from other silkworm species accelerates cell proliferation. We discuss the inverse activities of sericins against cultured cells between different silkworm species.

## 2. Materials and Methods

### 2.1. Preparation of Sericin

Samples of 10 g of CS cocoons were cut into pieces and washed at 65˚C for 3 minutes in 100 volumes (w/v) of water. The washed cocoon pieces were boiled in 50 volumes of 0.5% $Na_2CO_3$ for 40 minutes and then filtered through a 17 G glass filter. The filtrate was dialyzed against water for 4 days at room temperature with several changes of fresh water (Karimazawa *et al*., manuscript in preparation). The dialyzed solution was lyophilized, kept at −20˚C, and used as the CS preparation. Sericin from *Bombyx mori* (BS) was purchased from Wako Chemical industries, Osaka, Japan. The purity of the CS preparation was examined by SDS-PAGE and the amino acid composition of lyophilized proteins.

### 2.2. Cell Line, Culture Conditions, and Proliferation Assay

FKD cells established in our laboratory from distal kidney tubules were used for the cell proliferation assay. FKD cells were cultured in Eagle's minimum essential medium (MEM) Nissui (Nissui Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 0.1% sodium bicarbonate, and 2 mM glutamine. The cells were grown at 37˚C in humidified air containing 5% $CO_2$ and were maintained by passage every 4 days. For culture in serum-free medium, we used Opti pro-SFM (Life Technologies Japan, Tokyo) containing 100 U/ml penicillin G potassium. The cell proliferation rate in culture in the presence or absence of sericin preparations was analyzed by either trypan blue exclusion assay or by measuring the relative number of viable cells using a Cell Counting Kit (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions.

### 2.3. Analysis of Caspase-Dependent Cell Death

Caspase-dependent cell death was assayed as described in [10] using increasing concentrations of the pan-caspase inhibitor, Z-VAD fmk (benzyloxycarbonylvalyl-alanyl-aspartic acid (O-methyl)-fluoro-methylke-tone; Peptide Institute Inc., Osaka, Japan) [11]. As a control experiment, staurosporine was used as a typical apoptosis inducer [12]. For the proliferation assay, FKD cells were grown in 50 µl of MEM containing 10% FBS in 96-well plates ($1.5 \times 10^4$ cells per well) in the presence or absence of sericin or other materials. The relative number of viable cells was measured using a Cell Counting Kit (Dojindo). One-way ANOVA was used for statistical comparison of the results among multiple groups. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

### 2.4. Fractionation of Sericin Components

CS was first fractionated using partition chromatography according to the Folch method. CS (0.2 g) in 50 ml of

water was mixed with 62.5 ml of chloroform and 125 ml of methanol in a separating funnel, and agitated for 10 minutes at room temperature. After filtration through filter paper, the mixture was added to 62.5 ml of chloroform and 62.5 ml of water, stirred for 1 minute, and then incubated without agitation until the two layers separated. Materials in the upper (aqueous phase) and lower (organic phase) layers were collected, vacuum-dried, and dissolved in water. Fractions obtained using the Folch procedure were further separated by reversed-phase chromatography with a C18 cartridge (Sep-Pak Plus, Waters Co., Milford, MA). After loading the samples, fractions were eluted with 0.1% TFA and 60% acetonitrile. The fractions were dried under vacuum and dissolved in water to an appropriate concentration for use.

## 3. Results and Discussion

**Figure 1** shows the effects of CS on FKD cells cultured in MEM supplemented with 10% FBS. As shown in the figure, the proliferation of cells was inhibited to less than 50% in the presence of 0.1% (w/v) sericin from CS within 48 hours. Conversely, the same concentration of sericin prepared from a different silkworm species, *B. mori*, activated the proliferation of FKD cells by about twofold. This result was consistent with previous reports [1] [2]. Thus, the inhibitory effect of sericin on this cell line seemed to be specific to the wild silkworm, *C. trifenestrata*.

However, it remained possible that CS indirectly inhibits cell proliferation through an interaction with serum in the medium, which leads to the inactivation of growth factors. We also examined this hypothesis using serum-free Opti pro-SFM medium (**Figure 2**). Similar to the growth in MEM-FBS medium, *C. trifenestrata* sericin inhibited FKD cell proliferation under these culture conditions in a concentration-dependent manner (**Figure 2(a)**). Microscopic images also revealed the low density of cells in the presence of CS (**Figure 2(b)**). Therefore, CS directly inhibited FKD cell proliferation.

To determine whether the apparent inhibitory effect resulted from cell death, we examined apoptotic pathways using staurosporine as a typical apoptosis inducer. As shown in **Figure 3**, 3 μM staurosporine inhibited the proliferation of FKD cells to less than 50% after 16 hours in culture. The inhibition was rescued in the presence of the caspase inhibitor Z-VAD fmk in a dose-dependent manner (10, 50, and 100 μM). The results confirmed staurosporine-induced cell death in FKD cells, which was inhibited by Z-VAD fmk. Similar experiments were performed using CS (**Figure 3**, F-I). CS inhibited the proliferation of FKD cells, as shown in **Figure 1**. However, increasing concentrations of Z-VAD fmk did not interfere with the inhibitory effects of CS. These experiments indicated that the inhibitory effect on FKD cell proliferation was not due to typical caspase-dependent apoptosis.

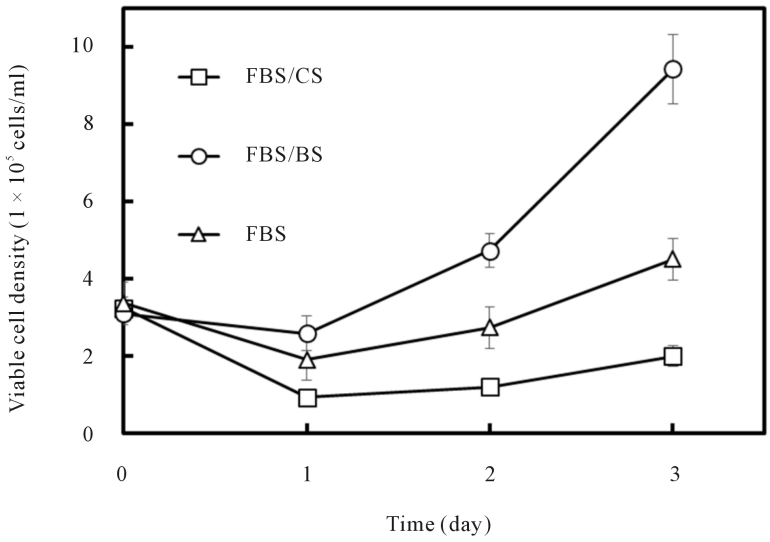


**Figure 1.** Effects of CS on the proliferation of FKD cells. FKD cells were cultured in MEM supplemented with 10% FBS in the presence (FBS/CS and FBS/BS) or absence (FBS) of 0.1% sericin. Cell density was monitored every 24 hours using the trypan blue exclusion method. Data represent the averages of three independent experiments and are shown as the means ± SD.

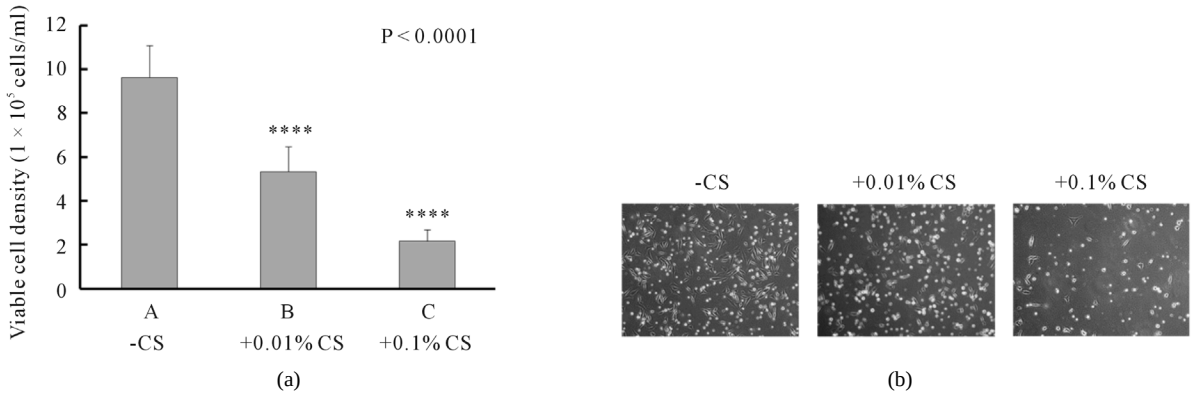


**Figure 2.** Effect of CS on the proliferation of FKD cells in serum-free Opti pro-SFM medium. (a) FKD cells were cultured for 48 hours in the presence (B, and C) or absence (A) of CS. Cell densities were measured as described in the legend to **Figure 1**. Statistical analysis was performed as described in the legend to **Figure 1**. Statistically significant compared with the control, ****P < 0.0001. (b) Microscopic images (×400) of FKD cells shown in (a).

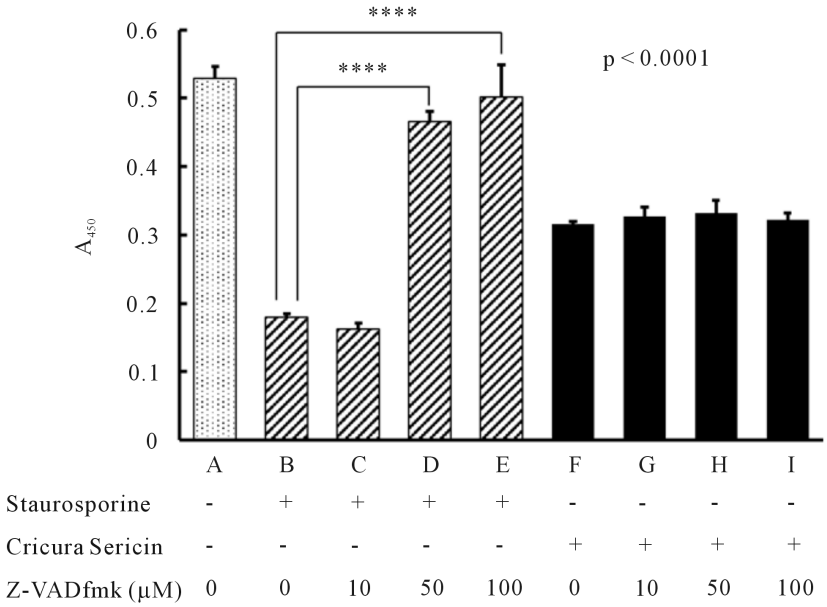


**Figure 3.** Pan-caspase inhibitor Z-VAD fmk does not affect FKD cell death induced by CS. FKD cells were cultured in MEM containing 10% FBS in the presence (B) or absence (A) of 3 μM staurosporine. (C)-(E) In the presence of 3 μM staurosporine together with the indicated concentrations of Z-VAD fmk for 16 hours. (F)-(I) Effects of Z-VAD fmk on CS-induced cell death. The relative numbers of viable cells were measured based on absorbance at 450 nm using a Cell Counting Kit. Statistically significant compared with the control, ****P < 0.0001.

To further explore the specific chemical and biochemical properties, CS was subjected to partial purification using the Folch method followed by reversed-phase chromatography. The sericin preparation was first separated using the Folch procedure into two layers, *i.e.*, the upper aqueous and lower organic phases, both of which were subjected to proliferation assay. Inhibitory activity against FKD cells was observed in the upper aqueous polar fraction (**Figure 4(a)**). This fraction was further fractionated using reversed-phase chromatography. Fractions obtained with 0.1% TFA and 60% acetonitrile were assayed for cell proliferation inhibition compared with the crude sericin preparation (**Figure 4(b)**). The activity was observed in both 0.1% TFA and 60% acetonitrile fractions, although the latter fraction showed significantly lower activity. However, when equal amounts of the two fractions were added simultaneously to the culture medium, they inhibited cell proliferation in an additive or even synergistic manner. These results suggested that at least two components in the CS preparation were responsible for the inhibition of FKD cell proliferation.

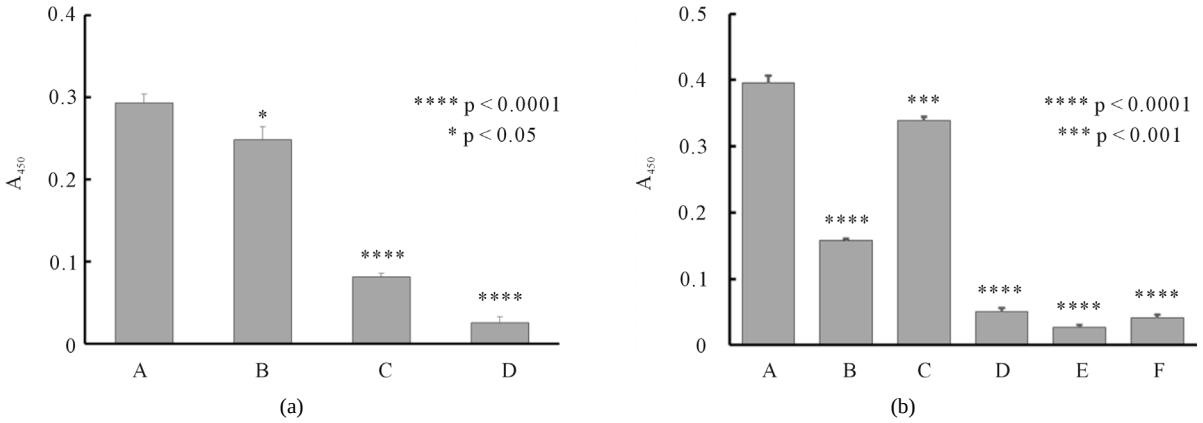


**Figure 4.** (a) Fractionation of CS using the Folch method. CS was fractionated as described in the Materials and Methods. After partitioning, two layers (lower and upper phases) were recovered and evaporated under vacuum. Samples were dissolved in water and equal amounts were assayed for anti-proliferative activities, as described in the legend to **Figure 3**. (A) Control. (B) Lower (organic) phase. (C) Upper (aqueous) phase. (D) CS before fractionation. Cells were cultured for 48 hours. Viable cells were measured using a Cell Counting Kit (absorbance at 450 nm). Statistical analysis was performed as described in the legend to **Figure 2**. Statistically significant compared with the control, $^{*}P < 0.05$, $^{****}P < 0.0001$. (b) Reversed-phase chromatography of the active fraction of CS. The upper phase fraction extracted using the Folch method (**Figure 4(a)**) was separated using reversed-phase chromatography using C18 cartridges. Fractions eluted with 0.1% TFA and 60% acetonitrile were recovered, lyophilized, and dissolved in water. Aliquots of the fractions were subjected to cell proliferation assays. (A) Control. (B) 0.1% TFA fraction. (C) 60% acetonitrile fraction. (D) 0.1% TFA fraction together with 60% acetonitrile fraction. (E) Upper phase fraction obtained using the Folch method. (F) CS before fractionation. Statistically significant compared with the control, $^{***}P < 0.01$, $^{****}P < 0.0001$.

In the present study, we showed that CS has direct anti-proliferative activity against FKD cells. This activity was caspase-independent, indicating that sericin-induced cell death does not involve typical apoptotic pathways. To examine its chemical and biochemical properties, the sericin preparation was fractionated by partition chromatography (Folch method) followed by reversed-phase chromatography. The results showed that a polar substance (s) and at least two components contributed to the activity.

However, there is a fundamental contradiction between our results and those reported by other groups showing that BS promotes the proliferation of mammalian cells [1] [2]. We did not observe proliferation-promoting activity in crude or fractionated CS, and at present we cannot explain these contradictory results. However, the difference may be due to the use of different cell lines, *i.e.*, variable susceptibilities to sericin between cell types. There may also be physicochemical differences between BS and CS. For example, BS is white while CS is gold. Some pigments or substances attached to the sericins may differ, and may not be entirely removed during extraction. As mentioned above, at least two different components synergistically contribute to the anti-proliferative activity. This observation suggests that different component(s) in CS are involved in the activity compared to BS.

## 4. Conclusion

Sericin prepared from cocoons of *Cricula trifenestrata* has anti-proliferative activity against mammalian culture cells. Sericin-induced cell death does not involved caspase-dependent typical apoptotic pathways, and at least two different components in the sericin preparation seem to be synergistically contribute to this activity.

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