



การใช้เซริซินในการยับยั้งการเจริญเติบโตของรา่น้ำ^๑ และการใช้ทดแทนซีรัมในการเพาะเลี้ยงเซลล์

Utilization of Sericin on Water Mold Growth Inhibition and Serum Substitution in cell culture

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บทคัดย่อ

เซริซินเป็นโปรตีนการที่พบทามธรรมชาติมีหน้าที่ยึดเส้นใยไฟเบอรินของรังไหม ซึ่งถูกกลอกออกในขั้นตอนการผลิตเส้นไหมดิบ ในการวิจัยครั้งนี้ได้ทำการสกัดโปรตีนเซริซินจากรังไหมของดักแด่ไหม (*Bombyx mori* L.) 2 สายพันธุ์ คือ นางน้อยศรีสะเกษ และญี่ปุ่น โดย 3 วิธี ได้แก่ 1) การต้มใน 1.2% กรดซิตริก 2) การต้มในน้ำกลัน และ 3) การต้มใน 8 M ญูเรีย ที่เติม 2% เปด้า-เมอร์แคปโตเอทานอล ที่อุณหภูมิ 80°C จากนั้นนำของเหลวจากวิธีการต้มใน 1.2% กรดซิตริกมาเตรียมผงซีริซิน ทำการวิเคราะห์แบบแพนโปรตีนด้วยเทคนิค sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) และทดสอบคุณสมบัติเซริซินในการยับยั้งการเจริญเติบโตของรา่น้ำ *Saprolegnia diclina* โดยการแข่งเส้นใยราในสารละลายน้ำเซริซินความเข้มข้นและเวลาต่างกัน ก่อนนำไปเลี้ยงบนอาหาร GY รวมทั้งทดสอบความสามารถในการใช้เซริซินทดแทนซีรัมในการเพาะเลี้ยงเซลล์ A549 human lung alveolar ผลการวิจัยพบว่าเซริซินที่สกัดได้มีน้ำหนักโมเลกุลอยู่ระหว่าง 5 ถึง 116 กิโลดالتัน และการแข่งเส้นใยรา *S. diclina* ในสารละลายน้ำเซริซินสามารถยับยั้งความหนาแน่นของเส้นใยราได้ โดยการแข่งเส้นใยในสารละลายน้ำเซริซินจากพันธุ์ญี่ปุ่น ที่ความเข้มข้นร้อยละ 3 เป็นเวลา 120 นาทีสามารถยับยั้งอัตราการเจริญเติบโตของรา่น้ำได้มากที่สุดและแตกต่างจากกลุ่มควบคุมอย่างมีนัยสำคัญ ($p<0.05$) นอกจากนี้พบว่าการเพาะเลี้ยงเซลล์ A549 human lung alveolar ในอาหาร Ham's ที่มีเซริซินเข้มข้น 0.5% จากพันธุ์นางน้อยศรีสะเกษและญี่ปุ่น เมื่อเวลาผ่านไป 72 ชม. มีเซลล์ที่มีชีวิตจำนวน 2.75×10^4 และ 2.5×10^4 เซลล์/มล. ตามลำดับ ซึ่งใกล้เคียงกับกลุ่มควบคุมที่ใช้ Fetal bovine serum ที่มีจำนวนเซลล์ 3×10^4 เซลล์/มล.ดังนั้น เซริซินจึงเป็นอีกทางเลือกหนึ่งในการใช้เป็นสารควบคุมการเจริญของเชื้อราและเป็นสารทดแทนซีรัมในการเพาะเลี้ยงเซลล์ได้

ABSTRACT

Sericin is a natural adhesive protein to attach fibroin fiber in silk cocoons which is removed during raw silk production. In this study, Sericin protein was extracted from cocoons of two varieties of silkworm (*Bombyx mori* L.); Nang Noi Srisaket and UB1, by three methods including 1) boiling in 1.2% citric acid, 2) boiling in distilled water and 3) heating in 8 M urea containing 2% β -mercaptoethanol at 80 °C. Sericin powder was then prepared from boiling in 1.2% citric acid method. The protein patterns from these methods were assayed by SDS-PAGE. The potential of sericin in inhibiting the growth of water mold (*Saprolegnia diclina*) was investigated by soaking water mold hyphae in various sericin concentrations and timings before transfer to GY agar. Furthermore, the ability of sericin in serum replacement in A549 human lung alveolar cell culture was investigated. The results on SDS-PAGE showed the molecular weight of sericin ranged from 5 to 116 kDa. The pretreatment *S. diclina* hyphae in sericin solution was able to decrease the density and/or the length of water mold mycelium. Those hyphae immersed in 3% of UB1 sericin solution for 120 minutes was significantly inhibited hyphae growth rate ($p<0.05$) comparing to the control group soaked in distilled water. Moreover, the results of A549 human lung alveolar cell culture indicated that after 72 hrs, cells grown on 0.5% sericin from Nang Noi Srisaket and UB1 provided the highest numbers of viable cells which were 2.75×10^4 and 2.5×10^4 cells/ml, respectively similar to those cultured on 10% FBS with $\times 10^4$ cells/ml. Therefore, sericin is an alternative potential antifungal substance and serum replacement agent.

คำสำคัญ: เซริซิน ราน้ำ การยับยั้งการเจริญของเชื้อรา การทดแทนซีรัม

Keywords: Sericin, SDS-PAGE, *Saprolegnia diclina* water mold, Antifungal activity, Serum substitution

INTRODUCTION

The two compositions of *Bombyx mori* silk cocoon are fibroin; a fiber protein, and sericin; an adhesive protein (Dash et al., 2007). Fibroin is the main protein contributing about 70% to 80% covering with sericin constituting about 20% to 30% to adhere fibroin for making cocoons. *B. mori* sericin is a family of proteins whose molecular mass

ranges from 10 to 310 kDa (Wei et al., 2005) combining from 18 strong polar functional grouped amino acids, such as serine (31 Molar%), glycine (19.1 Molar%), asparagin (17.8 Molar%) and threonine (8 Molar%) (Kato et al, 1998). During silk thread production, sericin is eliminated in wastewater contributing almost 30% of total cocoon production and can cause water pollution. Vaithanomsat and

Kitpreechavanich (2008) reported that sericin waste solution contains high BOD (4,840 mg/L), COD (8870 mg/L) and nitrogen content (11%). Since sericin degummed water from silk manufacturing contains high useful amino acids, it is considered as a valuable source of many modified products. Hence, there are several researches on recovery of sericin from degumming bath such as using micro, ultra and nano filtration methods (Capar et al., 2008) and infrared (IR) heating (Gupta et al., 2013). Sericin has been recorded with several important biological and pharma-cological properties including anticoagulant and antioxidant activities (Sarovart et al., 2003), antibiotic and antibacterial activities (Aramwit et al., 2012), antitumor effects (Kaewkorna et al., 2012), moisture absorption (Genç et al., 2009), inhibitory action of tyrosinase (Aramwit et al., 2010) and UV resistance (Patel and Modasiya, 2011).

Saprolegniasis is one of the main fungal diseases infecting in fish and fish eggs caused by water molds in the genus *Saprolegnia* (Bruno et al., 2011). *S. diclina* was reported as a pathogen on Nile tilapia (*Oreochromis niloticus*) eggs and able to grow in a wide range of pH (4.0-11.0), temperature (10-35°C) and NaCl concentration (0-30 ppt) (Panchai et al, 2007). To prevent *Saprolegnia* infections in fisheries and farms, fish eggs were

pretreated with clotrimazole or malachite green, a banned agent in many countries.

In mammalian cell culture, 10% (v/v) Fetal Bovine Serum (FBS) is the most widely required in culture media aiding in cell growth and proliferation as the source of nutrients, hormones and growth factors (Ozturk, 2004). However, there are some disadvantages of using FBS; for instance, viral contamination, unknown factors containing, genotypic and phenotypic cell stability interfering and expensive reagent. Therefore, serum-free medium has been developed. Sericin protein has been also considered as a culture medium supplement for various mammalian cell lines (Terada et al., 2002). Sericin was reported its ability to promote mammalian mitosis via many different signaling pathways making it a potent cell growth factor (Sato et al., 2011).

In this study, sericin was extracted from *B. mori* cocoons with three methods and investigated the antifungal properties of sericin powder in inhibiting water mold *Saprolegnia diclina* growth as an alternative biological reagent in aquarium treatment. Additionally, we observed capability of sericin in FBS replacement in culture media by investigation of cell viability and proliferation of A549 human lung alveolar cells in cell culture.

MATERIALS AND METHODS

Sericin extraction and preparation of sericin powder

Silk cocoons derived from two silk varieties, Thai yellow cocoon Nang Noi Srisaket and hybrid white cocoon UB1 were kindly supplied from National Queen Sirikit Silk Institute, Khon Kaen, Thailand (Figure 1).



Figure 1 Silk cocoons from *Bombyx mori*

- (A) Nang Noi Srisaket and
- (B) UB1 varieties

Three methods of cocoon sericin extraction were applied including 1) boiling in 1.2% v/v citric acid 2) boiling in distilled water and 3) heating in 8 M urea containing 2% β -mercaptoethanol at 80°C. Sericin powders were obtained from boiling in 1.2% citric acid method as following description. Ten grams of cocoons were boiled in 500 ml of 1.2% v/v citric acid for 20 mins. Then, the solution was filtered, three volumes of 95% ethanol were

then added and stored at 4°C overnight. The precipitated sericin was centrifuged at 4000 rmp for 10 mins, the gel-like precipitant was collected and incubated at 60°C for 12 hrs to evaporate remained ethanol. Finally, dried sericin was grounded to get sericin powder.

Protein pattern analysis

Sericin samples collected from three different methods and sericin powder were processed by Bio-Rad Protein Assay Kit (BIO-RAD, USA) based on Bradford method to determine protein concentrations. The absorbance at 595 nm of Bovine Serum Albumin (BSA) concentrations of 2, 4, 6, 8, 10 and 12 μ g/ml were individually measured and constructed as a standard curve. The absorbance values from samples were interpolated in the standard curve. Then, an equal concentration of 15 μ g sericin from each extraction method were diluted and loaded into the 12% acrylamide gel, Amersham Bioscience SDS-PAGE kit and run at room temperature using 25 mA, BIORAD power supplier for 90 mins. The standard protein ladder, PRO-STAIN™ Prestained Protein Marker II was used for protein molecular weight determination. The gel was stained with 1% Coomassie Brilliant Blue R250 overnight and then destained in destaining solution (10% methanol and 14% glacial acetic acid) for 2 hrs. The gel with clear bands was dried on gel dryer for 1 hour.

Antifungal activity assay

Water mold of *Saprolegnia diclina* was kindly identified according to Panchai et al., 2007 and supplied by Mr. Kwanprasert Panchai. *S. diclina* hyphae were subcultured in Glucose-Yeast Extract (GY) agar for two days at 25°C. To prepare sericin solutions, sericin powders from Nang Noi Srisaket and UB1 were dissolved in 5 ml sterile distilled water and heated at 70°C for 10 mins, then diluted to the final sericin concentrations of 1%, 2%, 3% and 4% (w/v) of *S. diclina* hyphae on GY agar were cut with a sterilized cork borer and immersed in each sericin solution and distilled water as a control (0% sericin) for 30, 60 and 120 mins. Finally, hyphae were placed at the center on GY agar plates and incubated at 25°C. Each experiment was carried out with five replicates. The length of radial extension and density of *S. diclina* colonies were measured every 24 hrs until the colonies of control group cover all plate area. The growth rates were calculated.

Serum substitution of sericin in animal cell culture

A549 human lung alveolar cells were provided by Assistant Professor Sorujsiri Chareonsudjai, Department of Microbiology, Faculty of Medicine, Khon Kaen University. Cells were trypsinized by trypsin-EDTA for three mins before centrifuged at 1800 rpm, 4°C in PBS pH 7.5 for 10 mins. The precipitant was collected and suspended in Ham's

culture media for the initial cell counting. Then, the same concentration of A549 human lung alveolar cells (9×10^4 cells) were cultured in standard Ham's media with 0%, 0.025%, 0.1%, 0.3%, 0.5% and 1% of Nang Noi Srisaket and UB1 sericin solutions comparing to Ham's media added with 10% Fetal Bovine Serum (FBS). The cell cultures were performed in 6-well plates and incubated at 37°C with 5% CO₂ for 72 hrs. The cells were stained with 0.4% Tryphan Blue for determining cell viability and observing in hemocytometer under light microscopy (100X). Each sample was repeatedly counted four times and the average number of viable cells was taken and then statistically compared by mean of ONE WAY ANOVA.

RESULTS AND DISCUSSION

Sericin protein patterns

The sericin powder yield obtained from boiling 10 g. of Nang Noi Srisaket and UB1 silk cocoons in 1.2% v/v citric acid following with precipitating in 95% ethanol and 60°C incubation were 1.73 g (17.3% w/w yield of raw cocoon) and 1.31 g (13.1% w/w yield of raw cocoon) respectively. The colors of sericin powder relate to the cocoon colors. The protein molecular weights of sericin from different extraction methods were analyzed using SDS-PAGE.

As shown in Figure 2, the results indicated that sericin solutions extracted from boiling in 1.2% citric acid, boiling in distilled water and sericin powder appeared in continuous distribution patterns with different molecular weights which were between 9-52 kDa, 11-116 kDa and 5-73 kDa respectively. Meanwhile, sericin solution extracted by heating in 8 M urea containing 2% β -mercaptoethanol was the only one method that presented separated bands with 15, 20,

28, 34, 35 and 68 kDa. The continuous patterns on SDS-PAGE were similar to previous study (Gupta et al, 2014) and due to sericin polypeptides breaking down into fractions under boiling procedure. The various sizes of sericin in the result related to previous studies that sericin molecular weight ranged widely from about 10 to over 300 kDa (Zhang, 2002) and represented a family of proteins with diverse molecular weight distribution (Wu et al, 2007).

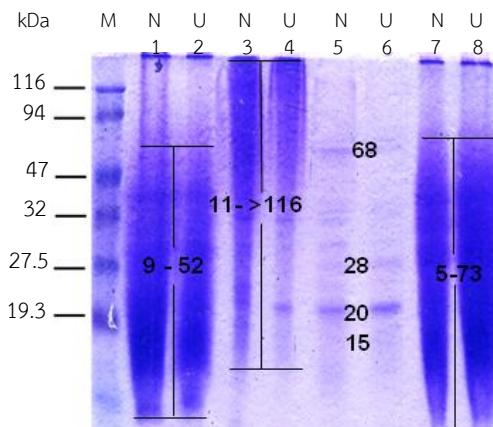


Figure 2 Sericin pattern on 12% SDS-PAGE. M = standard protein marker, N = Nang Noi Srisaket U = UB1, Lane 1-2 = boiling in 1.2% citric acid, 3-4 = boiling in distilled water, 5-6 = heating in 8 M urea with 2% β -mercaptoethanol and 7-8 = sericin powder

Antifungal activity of sericin powder

The present study tested the activity of sericin inhibiting the growth of water mold *S. diclina*. The results shown in Table 1 and Figure 3 demonstrated that *S. diclina* hyphal immersion in sericin solution was inhibited the growth indicated by the density and/or length of mycelium on the GY agar. In table 1, some sericin treatments presented longer radial

extension than the control group (0% sericin), however, the density of the mycelium was less (shown in Figure 3D). The hyphal immersion in 3% UB1 sericin solution for 120 mins was the most efficient to inhibit the growth of *S. diclina* as shown in Figure 3B.

The property in antimicrobial activity of sericin was reported that involving with silk proteinase inhibitors 1 and 2 (SPI 1 and 2)

inhibit bacterial and fungal proteinases the potent of antibacterial activity (Aramwit et al., 2014). Nirmala et al., 2001). Silver nanoparticles (AgNP) capped with silk sericin also showed

Table 1 The hyphal radial extensions and growth rates of *S. Diclina* immersed in various sericin concentrations and timings

Treatment	% sericin	Hyphal radial extension (mm.) (growth rate(mm/hr))		
		after 67 hr. on GY agar		
		exposure times		
		30 min	60 min	120 min
Distilled water (control)	0%	39.25 (0.586)	38.25 (0.571)	39.85 (0.595)
	1%	40.75 (0.608)	38.50 (0.575)	38.00 (0.567)
	2%	39.35 (0.587)	40.85 (0.610)	38.85 (0.580)
	3%	39.15 (0.584)	38.35 (0.572)	36.00 (0.537)
	4%	41.85 (0.624)	40.50 (0.604)	39.65 (0.592)
NNS Sericin	1%	34.85 (0.520)	38.50 (0.575)	36.35 (0.542)
	2%	35.00 (0.522)	39.50 (0.590)	40.25 (0.602)
	3%	36.50 (0.544)	38.75 (0.578)	34.15 (0.510)
	4%	39.15 (0.584)	39.15 (0.584)	38.65 (0.577)
UB1 Sericin	1%	34.85 (0.520)	38.50 (0.575)	36.35 (0.542)
	2%	35.00 (0.522)	39.50 (0.590)	40.25 (0.602)
	3%	36.50 (0.544)	38.75 (0.578)	34.15 (0.510)
	4%	39.15 (0.584)	39.15 (0.584)	38.65 (0.577)

NNS = Nang Noi Srisaket variety * The most inhibited treatment

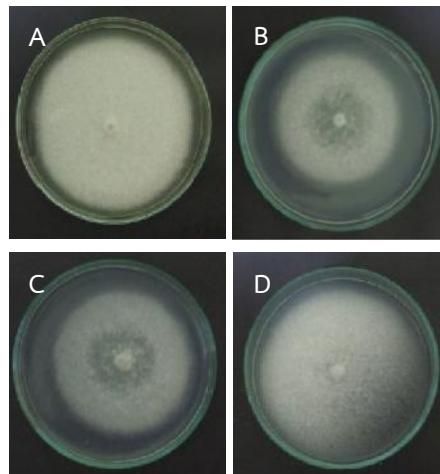


Figure 3 *S. diclina* hyphae grown for 67 hrs after immersed in (A) distilled water (B) 3% UB1 sericin (C) 3% Nang Noi Srisaket sericin and (D) 4% Nang Noi Srisaket sericin for 120 mins

Sericin as a supplement in animal cell culture

After culture A549 Human lung alveolar cells in Ham's media with 0%, 0.25%, 0.1%, 0.3%, 0.5% and 1% of sericin solution from both varieties and with 10% FBS, as the control for 72 hrs, the results indicated that addition of sericin in media could provide A549 Human lung alveolar cell viability. The numbers of average viable cell in each experimental media were shown in Figure 4.

The viability of A549 Human lung alveolar cells in Ham's media containing 0.5% Nang Noi Srisaket and UB1 sericins were 2.75×10^4 and 2.5×10^4 cells/ml, respectively which were slightly lower than in 10% FBS medium (3×10^4 cells/ml) with statistically different ($p < 0.05$). However, the results showed that the cell viability was decreased in 1% sericin added medium.

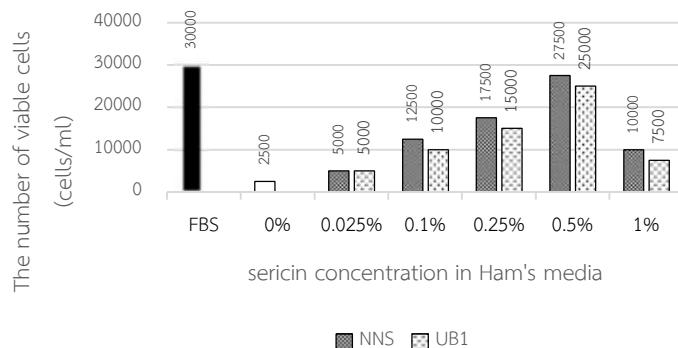


Figure 4 The average numbers of viable A549 human lung alveolar cells after 72 hrs. cultured in Ham's media containing FBS and various sericin concentrations.

The results proved that sericin could accelerate the proliferation of A549 Human lung alveolar cells similar to previous studies on other types of cell; such as, 2E3-O, HepG2, human cervical epithelium cells and human embryonic kidney 293 cells (Terada et al. 2002), human skin fibroblast (Tsubouchi et al. 2005) and L929, tumour and hybridoma cells (Cao and Zhang, 2015). The property of sericin powder in Fetal Bovine Serum substitution in this study corresponds to its high content of

amino acids. However, the appropriate concentration must be concerned for different types of cell lines.

CONCLUSIONS

We investigated different sericin molecular weights obtained from different extraction methods with ranging between 5 to 116 kDa. In inhibiting of water mold (*S. didelpha*) growth study, pretreatment of hyphae in 3% UB1 for 120 mins could decrease the density

and radial length of hyphae after grown on GY agar for 67 hrs. The sericin protein powder was also potential to use as a serum supplement in culture media for A549 Human lung alveolar cell culture. The suitable concentration of sericin was 0.5% which successfully accelerated cell proliferation in this study. These results indicated that sericin could be an alternative potent and widely effective agent in both antifungal activity and substitution of serum for human cell culture. In addition, Sericin protein is by product waste in silk manufacturing with high value in many purposes. This study also proved the benefit of sericin in antifungal activity and serum replacement in human cell culture.

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