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Antibacterial action of eri (*Samia ricini*) sericin against *Escherichia coli* and *Staphylococcus aureus*

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Abstract

Silk sericin is a family of water-soluble proteins extracted from the silkworm cocoon. Studies have been attempted to recycle the sericin waste from mulberry *Bombyx mori* sericulture to develop value-added products in the biomedical, pharmaceutical, cosmetic, and food industries. Accordingly, a wild species of the non-mulberry silkworm called eri (*Samia ricini*), has been promoted, especially in northeastern Thailand. Thus, increased production of sericin-containing wastewater from eri sericulture is predicted in the near future. Technical knowledge must be developed to support utilization of eri sericin in biotechnological industries. This work characterizes the antibacterial action of eri sericin in the representative Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria models. The potency of eri sericin as an antibacterial agent was demonstrated by the disc diffusion assay and critical-dilution micromethod, as well as by evaluating the effect of eri sericin on bacterial growth. The action of the potent eri sericin was found to be bactericidal. Scanning electron microscopic analysis determined significant morphological alterations at the cellular level in both *E. coli* and *S. aureus*.

Keywords : *Coriandrum sativum* L., hydropriming, deterioration

Introduction

Silk is well known as a product of special insects that belong to the Bombycidae and Saturniidae families in the order Lepidoptera. Silk proteins are composed of two protein families, designated as sericin and fibroin. The silk fibroin is fibrous in nature forming the main silk filament content, while sericin is an amorphous glue-like substance sticking and surrounding layers of fibroin (Mondal et al., 2007). The lustrous silk yarn is obtained after the removal of sericin is accomplished by a process called “degumming”, usually by boiling in water under various conditions, as well as by proteolytic enzymes (Yamada et al., 2001). In the textile industries, sericin is exclusively released as the unutilized byproduct from the process of making sericin-free fibroin fibers. Studies in the last decades have attempted to recycle the sericin waste to manufacture value-added products. (Zhang, 2002; Kundu et al., 2008).

Eri silkworm (*Samia ricini*) is currently a non-mulberry silkworm of importance for sericulture in northeastern Thailand where there is abundance in host plants (Sirimungkararat et al., 2005). Increased production of sericin containing wastewater from eri sericulture is predicted in the near future. If this sericin is recovered and recycled, it could provide significant economic and social benefits. To reach this goal, laboratory work is required to verify eri sericin biological activities. Recently, different biological activities have been reported for the eri sericins prepared by different degumming processes (Nuchadomrong et al., in press). We established herein the characterization of the antibacterial action of eri sericin in the representative Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria models.

Materials and Methods

Preparation of eri sericin

The eri silkworms were cultivated to produce cocoons at the Entomology Section, Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University. Cocoon shells were cut into pieces of ca. 1 x 1 cm and boiled in distilled water for 60 min or in 0.25% Na₂CO₃ for 90 min. The degumming solution obtained from either process was subjected to sericin salting out by 80% (NH₄)₂SO₄ (Nuchadomrong et al., in press). The precipitate was dissolved in distilled water, then underwent dialysis and freeze-drying to yield the 60-min water degummed eri sericin powder and the 90-min 0.25% Na₂CO₃ degummed eri sericin powder. Once the powder was dissolved in distilled water, it was sterilized by 0.22 μm pore-sized membrane filtration for further investigation of its antibacterial activities.

Bacterial strains

Escherichia coli TISTR 887 and *Staphylococcus aureus* TISTR 885 were obtained from the MIRCEN Culture Collection (Thailand Institute of Scientific and Technological Research).

Disc diffusion assay

One loop of bacterial colonies from a nutrient agar (NA) slant was grown for 18 h in 20 mL of nutrient broth (NB) at 37°C by shaking at 160 rpm. The cell suspension was diluted with fresh NB to a final density of 1.5x10⁸ colony forming units (CFU)/mL corresponding to a McFarland value of 0.5. Assay plates were prepared by pouring with warm melted NA medium (20 mL) containing the bacterial suspension (200 μL; 3x10⁷ CFU). Sterile filter discs 6 mm in diameter were impregnated with 20, 30, and 40 μg of eri sericin solution and placed on the inoculated agar surface. A negative control was included using a disc loaded with 40 μg BSA. At the end of the incubation period (20 h), antimicrobial activity was determined by the clear zones around the filter discs.

Critical-dilution micromethod assay

Diluted solutions of eri sericin were added to wells of a 96-well microculture plate containing bacterial inocula (1.5×10^7 CFU in 100 μ L NB medium), to make the final sericin concentrations in the range of 30 to 0.01 μ g/mL. After 24 h of shaking (160 rpm) at 37°C, OD_{600nm} was measured as the growth index.

Growth profile response

Bacterial inocula of 1.5×10^8 CFU/mL were cultured (160 rpm, 37°C) in NB composed of eri sericin at a specified concentration. The OD_{600nm} values were measured and plotted against the culture times at 5-h intervals, comparing them to the control growth without the eri sericin effect.

Sample preparation for scanning electron microscopy (SEM)

Bacterial cultures in NB with or without potent eri sericin were grown to a specified time. Cells were collected (1,000 \times g for 5 min), washed once, and resuspended with fresh NB. The cell suspension was dispersed on a 0.2 μ m pore size filter. The filter was successively soaked for 3 h in a fixative solution (2.5% glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.5), immersed in 1% osmium tetroxide for 3 h, and rinsed three times in distilled water. Stepwise ethanol dehydration was performed for 10 min each in 20, 30, 40, 50, 60, 70, 80, 90, 95 and 100%, and followed by 100% ethanol for an additional 24 h. After drying at 37°C, the sample was coated with gold vapor. SEM analysis was conducted by using a Hitachi Model S-3000N scanning electron microscope at the Biomaterial Research Laboratory Unit, Faculty of Dentistry, Khon Kaen University.

Results and Discussion

Disc diffusion assay

The antibacterial activity of eri sericin was preliminarily investigated by the disc diffusion assay. The Gram-negative *E. coli* was more susceptible to the 90-min Na₂CO₃ degummed eri sericin with an effective dose initially at 30 μ g (Fig. 1A), while the effective dose for the 60-min water degummed eri sericin was at 40 μ g (data not shown). The Gram-positive *S. aureus* was strikingly restricted to inhibition by the 60-min water degummed eri sericin (Fig. 1B). The species' differential susceptibility to different eri sericin is attributed to involve evolutionary distinct cell wall and/or cell membrane characteristics.



Figure 1: Clear zones of growth inhibition in *E. coli* tested with the 90-min Na₂CO₃ degummed eri sericin (A), and in *S. aureus* tested with the 60-min water degummed eri sericin (B). BSA was used as the negative control. The amount in micrograms (μ g) was numerically labeled.

Critical-dilution micromethod assay

In this assay, bacterial cells were exposed to eri sericin more violently than in the disc diffusion assay. *E. coli* growth was not detectable spectrophotometrically when treated with the 90-min Na_2CO_3 degummed eri sericin at the critical concentration ($0.2 \mu\text{g/mL}$). However, the *S. aureus* growth was completely inhibited by the 60-min water degummed eri sericin at a relatively high dose ($30 \mu\text{g/mL}$). The mode of action of the potent eri sericin is possibly affected by the physiological function of bacterial cells.

Growth profile response

An eri sericin dose lower than the critical lethal value was included in the medium (the 90-min Na_2CO_3 degummed eri sericin at $0.1 \mu\text{g/mL}$ for *E. coli* or the 60-min water degummed eri sericin at $20 \mu\text{g/mL}$ for *S. aureus*). As shown in Figure 2, a bactericidal effect was noticeable over the growth period. A more serious effect was observed in the stationary phase, thereby, it induced a rapid declining phase.

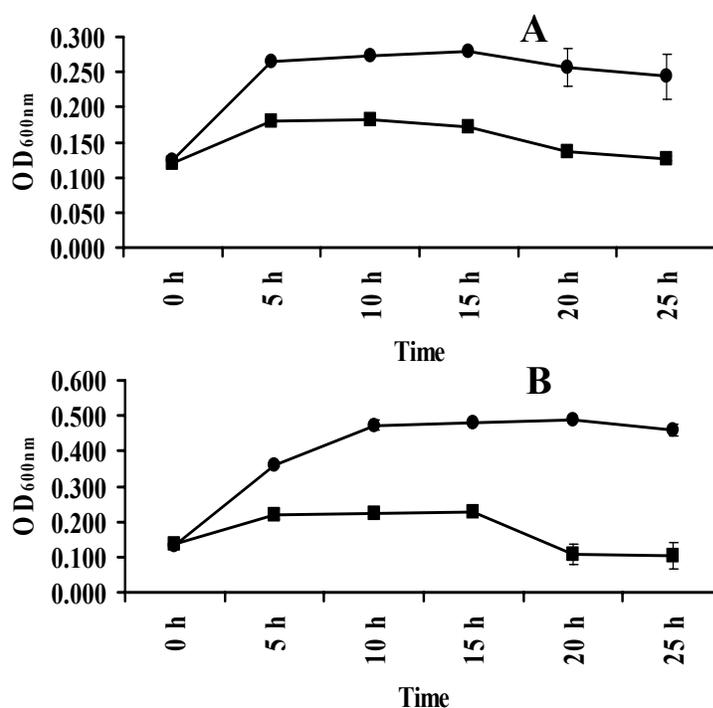


Figure 2: Growth profiles of *E. coli* (A) and *S. aureus* (B). Circle symbols represent the control growth while rectangular symbols are for the eri sericin treatment experiments with 90-min 0.25% Na_2CO_3 degummed eri sericin ($0.1 \mu\text{g/mL}$) for *E. coli*, and with the 60-min water degummed eri sericin ($20 \mu\text{g/mL}$) for *S. aureus*.

SEM analysis of *E. coli* and *S. aureus*

The effect of eri sericin on bacterial cells was studied in *E. coli* and *S. aureus* as the representatives for Gram-negative and Gram-positive cells, respectively. Morphological investigations were performed with 15-h *E. coli* culture in the treatment of 90-min 0.25% Na_2CO_3 degummed eri sericin ($0.1 \mu\text{g/mL}$), and with 15-h *S. aureus* culture in the treatment of 60-min water degummed eri sericin ($20 \mu\text{g/mL}$). Control cells at 15-h culture were analyzed in parallel, which exhibited typical rod-like morphology of *E. coli* (Fig. 3A, 4A) and coccus morphology of *S. aureus* (Fig. 3C, 4C).

The eri sericin treatment in *E. coli* caused prominent morphological alterations (Fig. 3B, 4B). Slim elongated cells were obvious with signs of membrane damage, i.e. shrunk and deep roughening of the cell surface. Therefore, it is proposed that the eri sericin from 90-min 0.25% Na₂CO₃ degumming causes membrane dysfunction in *E. coli*.

In *S. aureus* cells treated with 20 µg/mL of the 60-min water degummed eri sericin, it was remarkable that *S. aureus* culture formed large cell aggregations (Fig. 3D). SEM at higher magnification (20000x) also gave a number of striking characteristics on the cell surface of eri sericin-exposed cells (Fig. 4D). The most striking morphological trait was the concave structure on the cell surface illustrating the cell segregation step in cytoplasmic division. This observation was accompanied by cell deformation, cells with reduced sizes, and cell shrinkage. More than one mechanism is supposed to take part in the action of the eri sericin from the 60-min water degumming on *S. aureus*. It is likely that the eri sericin elicits failure in cell division and cell growth, which is expected as the cause of the early declining phase, together with the induction of membrane dysfunction.

Antibacterial chemotherapeutic agents have been commonly used to control infectious diseases. However, the use of antibiotics is now being discouraged due to the emergence of drug resistance. There has been an increasing interest in the exploration of antibacterial proteins having mechanisms of action different from those of the conventional drug compounds. Diverse mechanisms have been established other than for the known action of lysozyme, for examples, the human antibacterial psoriasin protein which causes pore formation on bacterial membranes (Michalek et al., 2009) and the antibacterial RNase 7 enzyme (Harder and Schroder, 2002)

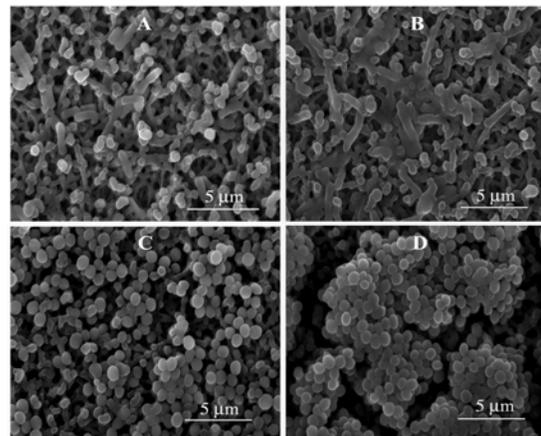


Figure 3: SEM micrographs at 7000x magnification of control *E. coli* (A), *E. coli* treated with 0.1 µg/mL of the 90-min 0.25% Na₂CO₃ degummed eri sericin (B), control *S. aureus* (C), and *S. aureus* treated with 20 µg/mL of the 60-min water degummed eri sericin (D).

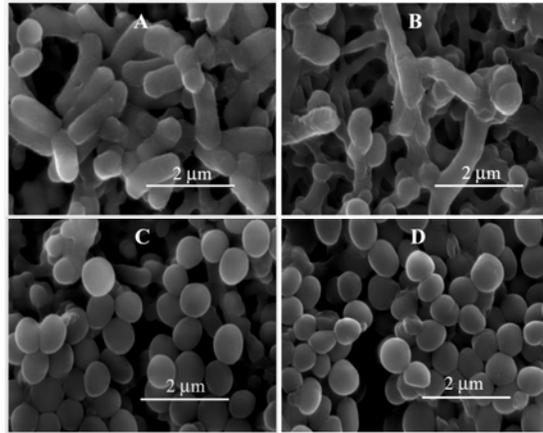


Figure 4: SEM micrographs at 20000x magnification of control (A) and eri sericin treated *E. coli* (B); control (C) and eri sericin treated *S. aureus* (D). Details of the eri sericin treatments were as described in Figure 3.

Conclusions

We described here the antibacterial action of eri sericin prepared by two different degumming methods in the representative bacteria model of Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) by disc diffusion assay, critical-dilution micromethod, and by the effect of eri sericin on bacterial growth. The 90-min 0.25% Na₂CO₃ degummed eri sericin was potent on *E. coli*, while the 60-min water degummed eri sericin was potent on *S. aureus*. The action of potent eri sericin was found to be bactericidal against growing cells. SEM micrographs illustrated aberrant membrane morphology showing membrane dysfunction in both *E. coli* and *S. aureus*. The potent eri sericin on *S. aureus* was especially shown to cause failures in the process of cell division. This is the first reported evidence for the antibacterial action of eri sericin at the cellular level.

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