Brief Report

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Induction of acute silkworm hemolymph melanization by *Staphylococcus aureus* treated with peptidoglycan-degrading enzymes

Yasuhiko Matsumoto*, Eri Sato, Takashi Sugita

Department of Microbiology, Meiji Pharmaceutical University, Tokyo, 204-8588, Japan.

SUMMARY *Staphylococcus aureus*, a Gram-positive bacterium, causes inflammatory skin diseases, such as atopic dermatitis, and serious systemic diseases, such as sepsis. In the skin and nasal environment, peptidoglycan (PGN)-degrading enzymes, including lysozyme and lysostaphin, affects *S. aureus* PGN. However, the effects of PGN-degrading enzymes on the acute innate immune-inducing activity of *S. aureus* have not yet been investigated. In this study, we demonstrated that PGN-degrading enzymes induce acute silkworm hemolymph melanization by *S. aureus*. Insoluble fractions of *S. aureus* treated with lysozyme, lysostaphin, or both enzymes, were prepared. Melanization of the silkworm hemolymph caused by the injection of these insoluble fractions was higher than that of *S. aureus* without enzyme treatment. These results suggest that structural changes in *S. aureus* PGN caused by PGN-degrading enzymes affect the acute innate immune response in silkworms.

Keywords *Staphylococcus aureus*, silkworm, innate immunity, melanization

1. Introduction

Staphylococcus aureus, a Gram-positive bacterium found on human skin and in the nasal cavity, is a causative agent of inflammatory skin diseases such as atopic dermatitis and serious systemic diseases such as sepsis (*1,2*). Atopic dermatitis is caused by the immune activation of human skin cells by *S. aureus* (*3*). In the human skin, *S. aureus* components such as peptidoglycan (PGN) and acyl lipopeptides are recognized by Toll-like receptors, which trigger innate immune activation of the skin (*4,5*). Therefore, elucidating the molecular mechanisms of immune induction mechanism by *S. aureus* is important for understanding the risk of the onset of inflammatory skin diseases.

In the epidermis and nasal environment, *S. aureus* is affected by a variety of PGN-degrading enzymes such as lysozyme and lysostaphin (*6–10*). Lysozyme cleaves the β-1,4 linkage between *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) in PGN (*11,12*). Lysozyme is present in the granular layer of the human epidermis, eccrine sweat glands, sebaceous glands of hair follicles, and body fluids, such as human tear fluid and nasal secretions (*6,7*). Lysostaphin is an enzyme that cleaves the pentaglycine crosslinker in the PGN of *S. aureus* (*13*). Lysostaphin is secreted by *Staphylococcus simulans*, a coagulase-negative staphylococcus on human skin (*10*). Therefore, these PGN-degrading enzymes may affect *S. aureus* and alter its immune-inducing activity against host immune cells. However, the effects of PGN-degrading enzymes on the immune-inducing activity of *S. aureus* remain unknown.

Silkworms are useful experimental animals for assessing the virulence of pathogenic microorganisms and activation of innate immunity (*14–16*). The melanization reaction of silkworm hemolymph is one of the innate immune mechanisms (*14,17,18*). When bacteria or fungi enter the silkworm, the infected silkworm produces melanin in the hemolymph to repair the wound and coagulate pathogens. In insects, including silkworms, the immune response *via* hemolymph melanization and the Toll pathway is mediated by the same signaling pathway (*17,19*). Therefore, the melanization response in silkworms is an indicator of innate immune induction (*20,21*). Melanization in silkworm hemolymph by pathogens such as *Porphyromonas gingivalis*, *Cutibacterium acnes*, and *Candida albicans* is useful as an indicator of innate immunity (*22–24*).

In this study, the effect of PGN-degrading enzymes on the induction of innate immunity by *S. aureus* was investigated using melanization of the silkworm hemolymph as an indicator. We found that the PGN-

degrading enzymes lysozyme and lysostaphin increase *S. aureus*-induced acute innate immunity in silkworms.

2. Materials and Methods

2.1. Reagents

Tryptic soy broth was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Protease K was purchased from QIAGEN (Hilden, Germany). Lysozyme and lysostaphin were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

2.2. Bacterial strain and culture condition

S. aureus Newman strain was used in this study. The *S. aureus* Newman strain was spread on tryptic soy broth agar and incubated under aerobic conditions at 37°C for 1 day (*24*).

2.3. Preparation of enzyme-treated *S. aureus* cells

Enzyme-treated *S. aureus* cells were prepared as previously described (*24*). Autoclaved *S. aureus* cells were diluted with phosphate-buffered saline (PBS) to an absorbance at 600 nm (A_{600}) = 3 in 1 mL, and protease K (0.75 AU/mL) (50 µL) was added. After incubation for 1 h at 50°C, samples were centrifuged at 15,000 rpm for 10 min at room temperature. The precipitate was suspended in physiological saline solution (0.9% NaCl: PSS) (1 mL) and the remaining enzymes were inactivated by incubation at 80°C for 30 min. The samples were centrifuged at 15,000 rpm for 10 min at room temperature, and the precipitate was diluted with PSS to A_{600} = 1 to obtain the precipitate sample (Protease-treated *S. aureus* precipitate: Pro-ppt).

Lysozyme (100 mg/mL) (10 µL) and lysostaphin (10 mg/mL) (5 µL) were added alone or in combination with 300 µL of the precipitate sample (Pro-ppt), adjusted to 1 mL with PBS, and the samples were incubated at 37°C for 2 h. The incubated samples were centrifuged at 15,000 rpm for 15 min at room temperature and the supernatant was removed. The precipitate fraction was prepared by adding PSS (1 mL). Lysozyme-treated *S. aureus* precipitates, lysostaphin-treated *S. aureus* precipitates, and both lysozyme and lysostaphin-treated *S. aureus* precipitates, were named Lz-ppt, Ls-ppt, and Lz+Ls-ppt, respectively.

2.4. Silkworm rearing

The silkworm-rearing procedures have been previously described (*25*). Silkworm eggs (Hu Yo × Tukuba Ne) were purchased from the Ehime-Sanshu Co. Ltd. (Ehime, Japan), disinfected, and hatched at 25°C-27°C. Silkworms were fed an artificial diet, Silkmate 2S (Ehime-Sanshu Co., Ltd.). Silkworm injection experiments were performed as previously described (*25*). Fifth-instar silkworm larvae were fed Silkmate 2S (1.5 g/silkworm) overnight.

2.5. Hemolymph melanization assay

The *in vivo* melanization assay was performed as described previously (*24*). Bacterial samples (50 µL) were injected into the silkworm hemolymph using a 1-ml tuberculin syringe (Terumo Medical Corporation, Tokyo, Japan). Silkworms were maintained at 37°C for 3 h. The hemolymph was collected from the larvae through a cut on the first proleg. Silkworm hemolymph (50 µL) was mixed with 50 µL of PSS. The absorbance at 490 nm was measured using an iMark™ microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.6. Statistical analysis

Statistical differences between groups were analyzed using Tukey's test or the Tukey-Kramer test. Each experiment was performed at least twice. Statistical significance was set than 0.05 ($P < 0.05$).

3. Results and Discussion

Lysozyme and lysostaphin are PGN-degrading enzymes that cleave at different sites (Figure 1A). *C. acnes* PGN exhibits melanization-inducing activity in the silkworm hemolymph, and that activity is reduced by treatment with PGN-degrading enzymes (*24*). In the present study, an insoluble fraction containing *S. aureus* PGN was prepared (Figure 1B). Autoclave-treated *S. aureus* cells were washed to remove free DNA, RNA, amino acids, and proteins (Figure 1B). The insoluble fraction containing *S. aureus* PGN was prepared as Pro-ppt after protease treatment to remove insoluble and membrane-anchored proteins (Figure 1B). Subsequently, insoluble fraction after lysozyme treatment (Lz-ppt), insoluble fraction after lysostaphin treatment (Ls-ppt), and insoluble fraction treated with both enzymes (Lz+Ls-ppt) were prepared (Figure 1B). Administration of the Lz-ppt fraction enhanced melanization of the silkworm hemolymph compared with that of the Pro-ppt fraction (Figure 2A, B). The result suggests that the activity of *S. aureus* against silkworm hemolymph to induce melanization was increased by lysozyme treatment.

The lysozyme treatment experiment revealed that the structure of the sugar chain of *S. aureus* PGN affected its innate immune-inducing activity. Next, to determine the importance of the amino acid chain linker of PGN for its innate immune-inducing activity, we examined the effect of lysostaphin, which cleaves the pentaglycine cross-linker of *S. aureus* PGN. Melanization of silkworm hemolymph by injection of the Pro-ppt fraction was increased by lysostaphin pretreatment (Figure 2C, D). The result suggests that the induction of silkworm

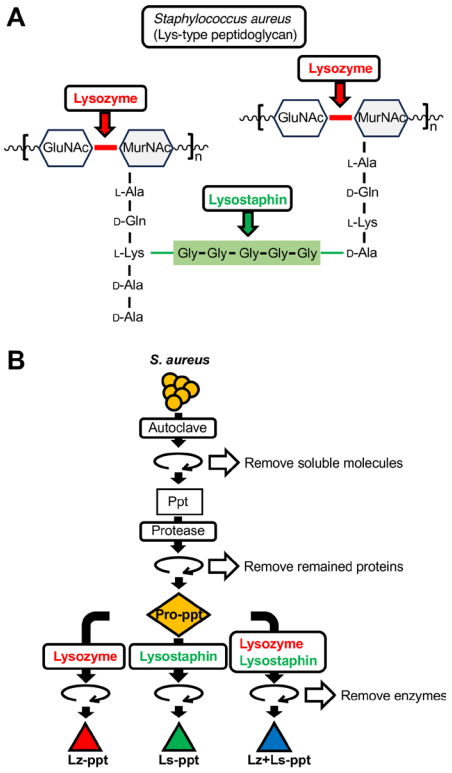

Figure 1. Preparation of PGN-degrading enzyme-treated *S. aureus* insoluble fractions. (A) Cleavage sites of lysozyme and lysostaphin on PGN of *S. aureus*. PGN structure of *S. aureus* was shown. Lysozyme cleaves the β-1,4 linkage between GlcNAc and MurNAc in *S. aureus* PGN (Red arrow). Lysostaphin cleaves the pentaglycine crosslinker in *S. aureus* PGN (Green arrow). GluNAc: *N*-acetylglucosamine, MurNAc: *N*-acetylmuramic acid, L-Ala: L-alanine, D-Gln: D-glutamine, L-Lys: L-lysine, D-Ala: D-alanine, Gly: glycine. **(B)** Autoclaved S. aureus cells were treated with protease K at 50°C for 1 h. The protease-treated *S. aureus* precipitate (Pro-ppt) was further treated with lysozyme and/or lysostaphin at 37°C for 2 h. Lysozyme-treated *S. aureus* precipitate: Lz-ppt, lysostaphin-treated *S. aureus* precipitate: Ls-ppt, lysozyme and lysostaphin-treated *S. aureus* precipitate: Lz+Ls-ppt.

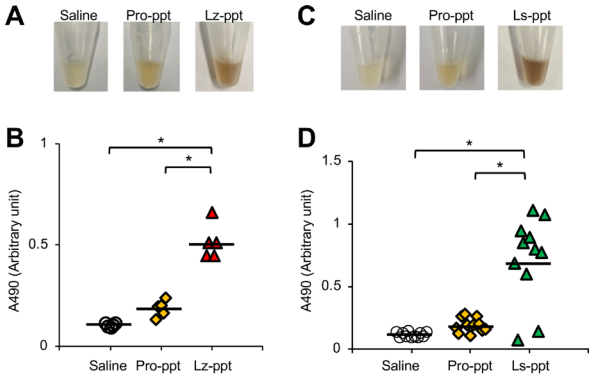

Figure 2. Induction of hemolymph melanization of silkworms by lysozyme or lysostaphin-treated *S. aureus*. (A, B) Sample solution (50 μL) of saline, Pro-ppt, or Lz-ppt, was injected to silkworms. Silkworm hemolymph was collected at 3 hours after injection. **(A)** Photograph. **(B)** Absorbance at 490 nm (A_{490}). n = 5/group. Statistically significant differences between groups were evaluated using the Tukey's test. *P < 0.05. **(C, D)** Sample solution (50 μL) of saline, Pro-ppt, or Ls-ppt, was injected to silkworms. Silkworm hemolymph was collected at 3 hours after injection. **(C)** Photograph. **(D)** Absorbance at 490 nm (A_{490}). n = 11/group. Statistically significant differences between groups were evaluated using the Tukey's test. *P < 0.05.

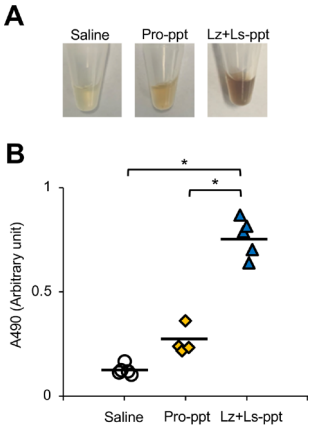

Figure 3. Induction of hemolymph melanization of silkworms by lysozyme- and lysostaphin-treated *S. aureus*. (A, B) Sample solution (50 μL) of saline, Pro-ppt, or Lz+Ls-ppt, was injected to silkworms. Silkworm hemolymph was collected at 3 hours after injection. **(A)** Photograph. **(B)** Absorbance at 490 nm (A_{490}). n = 4-5/group. Statistically significant differences between groups were evaluated using the Tukey-Kramer test. *P < 0.05.

hemolymph melanization is increased by cleaving the amino acid chain linker of *S. aureus* PGN with lysostaphin.

Experiments using PGN-degrading enzymes showed that the structure of the sugar linker or amino acid chain linker of *S. aureus* PGN affects its innate immune-inducing activity. Next, we examined the effects of simultaneous treatment with lysozyme and lysostaphin on *S. aureus* PGN. Administration of the Lz+Ls-ppt fraction enhanced silkworm hemolymph melanization compared to that of the Pro-ppt fraction (Figure 3). The result suggests that *S. aureus* PGN cleaved with lysozyme and lysostaphin exhibits the activity to induced silkworm hemolymph melanization.

PGN-degrading enzymes induce acute innate immunity in silkworms by affecting *S. aureus*. Lysozyme cleaved the β-1,4 linkage between GlcNAc and MurNAc in *S. aureus* PGN, and lysostaphin cleaved the pentaglycine cross-linker in *S. aureus* PGN. Therefore, these enzymes produce insoluble *S. aureus* PGN, in which the sugar chain and amino acid chain are partially cleaved. PGRP-S5 in the silkworm hemolymph binds to PGN and enhances melanization (*26*). On the other hand, Bmintegrin β3, an integrin on silkworm blood cells, binds to *S. aureus* and inhibits the melanization response (*27*). Therefore, the normal PGN structure of *S. aureus* may be involved in evading acute innate immune responses (Figure 4). Then, we hypothesized that melanization reaction was induced by partial degradation of *S. aureus* PGN, which facilitates binding to PGRP-S5 in the silkworm hemolymph or prevents binding to

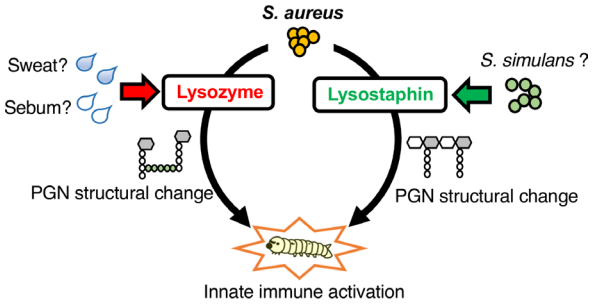

Figure 4. Model of acute innate immune activation of *S. aureus* by PGN-degrading enzymes. Lysozyme is present in sweat and sebum. Lysostaphin is produced by several coagulase-negative staphylococci including *Staphylococcus simulans*. Structural changes of *S. aureus* PGN by the PGN-degrading enzymes increases the acute innate immune responses of silkworms.

Bmintegrin β3. Binding experiments with PGRP-S5 and Bmintegrin β3 to *S. aureus* partially degraded by PGN-degrading enzymes will be the subject of future research.

Lysozyme and lysostaphin are enzymes present in the human skin and mucosa. Lysozyme is present in secretions such as sweat and sebum (*6,7*). *Staphylococcus simulans*, which is often present in human skin microbiomes, produces lysostaphin (*8–10*). Like lysostaphin, an endopeptidase ALE-1 produced by *Staphylococcus capitis* EPK1 also cleaves the pentaglycine linker (*28*). These coagulase-negative staphylococci may produce endopeptidases that affect *S. aureus* PGN. Therefore, the innate immune-inducing activity of *S. aureus* may increase under the influence of lysozyme and lysostaphin on the human skin surface and the follicular glands. In contrast, the silkworm melanization-inducing activity of *C. acnes*, a dominant bacterium on human skin, is reduced by lysozyme and lysostaphin (*24*). Therefore, the presence of lysozyme and lysostaphin on the human skin and nasal cavity may decrease the immune-inducing activity of *C. acnes*, while increasing that of *S. aureus*. Revealing the role of PGN-degrading enzymes in the skin microbiome to induce immunity is important.

In conclusion, partial degradation of *S. aureus* PGN by PGN-degrading enzymes promotes its innate immune-inducing activity.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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*Address correspondence to:
Yasuhiko Matsumoto, Department of Microbiology, Meiji Pharmaceutical University, 2-522-1, Noshio, Kiyose, Tokyo 204-8588, Japan.
E-mail: ymatsumoto@my-pharm.ac.jp

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