Brief Report

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Properties of induced antimicrobial activity in *Musca domestica* larvae

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Summary **Insects produce antimicrobial molecules that contribute to their innate immune responses to eliminate invading microorganisms. To explore the potential utility of these antimicrobial molecules, we focused on larvae of the house fly *Musca domestica*, which is an efficient processor of organic waste and a good resource of protein and oil for animal feeding. The induction of hemagglutinating activity, which is usually accompanied by activation of innate immune responses in fly larvae, was observed in the hemolymph following needle injury. Hemolymph collected from injured larvae demonstrated potent antimicrobial activities against both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Furthermore, the antimicrobial activity was significantly retained in hemolymph after heat-treatments, suggesting that pasteurization of animal feed prepared from fly larvae would be a useful sterilization method. These observations indicate that injured *Musca domestica* larvae are a source of antimicrobial agents, and highlight the utility of preparing animal feed from these larvae.**

Keywords: House fly, hemolymph, antimicrobial agents, innate immunity

1. Introduction

Insects respond to bacterial infection using innate immunity, consisting of germline-encoded sensing and effector molecules, among which antimicrobial peptides are prominent (*1*). Recognition of microorganisms induces synthesis of potent antimicrobial peptides in the fat body. Secretion of antimicrobial peptides into the hemolymph plays a role in inhibiting the growth of invading microorganisms. Over 150 insect antimicrobial peptides have been purified or identified, and they have potential applications in medicine and agriculture (*2*).

Antimicrobial peptides contain a region of positively-charged amino acids that specifically bind to negatively-charged surface molecules, such as bacterial lipopolysaccharide (*3-5*). This interaction disrupts the bacterial membrane and leads to cell lysis and/or cell death (*6-8*). Antimicrobial peptides were biochemically identified in the hemolymph of insects such as *Sarcophaga peregrina* (flesh fly) larvae and silkworm larvae (*9-13*). Furthermore, the excretions or secretions of medicinal maggots of the blowfly *Lucilia sericata* contain antimicrobial peptides in the absence of injury or invading bacteria (*14*). These findings indicate that insects express inducible and/or constitutive antimicrobial peptides.

Antimicrobial peptides are considered to be a novel class of antibiotics because they exhibit broad-spectrum antimicrobial activities, and they are not likely to induce resistance (*15*). Chemically synthesized or modified antimicrobial peptides were developed based on amino acid sequences of insect antimicrobial peptides. For example, the undecapeptide KLKLLLLLKLK-NH_2 was developed by modifying the primary structure of an antimicrobial peptide of *Sarcophaga peregrina*, Sapecin B (*16,17*). KLKLLLLLKLK-NH_2 exhibits broad-spectrum antimicrobial activities against Gram-positive bacteria, Gram-negative bacteria, and fungi (*17*). It also enhances mammalian immune responses, and its potential usefulness as an adjuvant has been previously demonstrated (*18-20*). Furthermore, KLKLLLLLKLK-NH_2 synthesized using D-amino acids displays higher antimicrobial activity than its L-form (*21,22*). These observations highlight the importance of native

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antimicrobial peptides as a resource for antimicrobial agents as well as immune modulators.

The house fly (*Musca domestica*) is a well-known carrier of pathogens that affect human and animal health. However, *Musca domestica* larvae are efficient processors of organic waste, and are a good source of protein and oil for animal feed (*23,24*). Animal feed prepared from *Musca domestica* larvae, in which antimicrobial peptide were induced, may be beneficial to overall animal health because it is well established that the addition of antimicrobial supplements to animal feed increases animal weight (*25*). Therefore, we sought to examine whether injury can induce antimicrobial activities in *Musca domestica* larvae. Induced antimicrobial molecules are useful sources of antimicrobial agents and valuable supplements in animal feeding.

2. Materials and Methods

2.1. *Fly larvae, bacteria, and reagents*

Musca domestica larvae were provided by E's Inc. (Tokyo, Japan). *Staphylococcus aureus* (NBRC100910), *Staphylococcus epidermidis* (NBRC100911), and *Pseudomonas aeruginosa* (NBRC12689) were purchased from National Institute of Technology and Evaluation (Kisarazu, Chiba, Japan). *Escherichia coli* XL1-blue was purchased from Stratagene (Agilent Technologies, Santa Clara, California, USA). Mannitol salt agar and cetrimide agar were purchased from Nissui Pharmaceutical (Tokyo, Japan). Mueller-Hinton II broth was purchased from Becton Dickinson (Franklin Lakes, New Jersey, USA). LB broth was purchased from Nacalai tesque (Kyoto, Japan). Agar for bacterial culture was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. *Injury of larvae and collection of hemolymph*

Musca domestica larvae were anesthetized by incubation on ice prior to being pricked once with a 0.30 × 12 mm needle (Dentronics, Tokyo, Japan). The injured larvae were incubated in an insect saline solution (130 mM NaCl, 5 mM KCl, 1mM $CaCl_2$) at 30°C. After 24 h, the anterior tip of the larva was cut off using fine scissors and the hemolymph was collected in a tube on ice. Approximately 500 µL of hemolymph was collected from 500 larvae. The hemolymph was centrifuged for 10 min at 100× g to remove hemocytes, and the supernatant was stored at –30°C.

2.3. *Assay for antimicrobial activity*

Bacteria were grown in LB broth and log-phase cells (OD_{600} = 0.15-0.3) were used for the analysis. The bacteria in growth medium (2 µL) was mixed with 18 µL of hemolymph sample or Mueller-Hinton II broth. To evaluate the effect of pH, 16 µL of the hemolymph sample or Mueller-Hinton II broth were mixed with 2 µL of 0.2 M phosphate buffer or 0.2 M phosphate/0.1 M citrate buffer for pH adjustment, and the pH adjusted samples were mixed with 2 µL of the bacterial suspension. The bacteria/hemolymph assay mixtures were incubated at 30°C for 1 h, and then serially diluted with Mueller-Hinton II broth. The diluted assay mixtures (100 µL) were plated onto agar plates and incubated at the appropriate temperature (30 or 37°C) for 1 or 2 days. The appropriate selective medium was used for each bacterial strain: mannitol salt agar (for *S. aureus* and *S. epidermidis*), LB agar containing 50 µg/mL tetracycline (for *E. coli* XL1-blue), and cetrimide agar (for *P. aeruginosa*). After cultivation, the colony forming units (CFU) in the bacteria/hemolymph assay mixtures were determined. Means of CFU were determined from triplicate or duplicate agar plates, and standard deviations (SD) were determined from triplicate plates.

2.4. *Assay for hemagglutinating activity*

Commercially available rabbit red blood cells were washed twice with 5 volumes of buffered insect saline (10 mM Tris/HCl (pH 7.9) containing 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$), and suspended in 10 volumes of phosphate buffered saline. Hemagglutinating activity was measured using serial two-fold dilutions of hemolymph in microtiter V-plates, and activity unit was determined as $titer^{-1}$. Each well contained a 50 µL suspension of red blood cells and 50 µL of hemolymph diluted with buffered insect saline. Agglutination was determined as previously described (*26*).

3. Results and Discussion

3.1. *Induction of antimicrobial activity in the hemolymph of Musca domestica larvae by injury*

We collected hemolymph from *Musca domestica* larvae injured with a needle and from larvae which were not injured as an experimental control. Initially, we examined the induction of hemagglutinating activity using rabbit red blood cells because previous reports indicated that hemagglutinating activity occurred concomitantly to the induction of innate immune responses in *Sarcophaga peregrina* larvae (*9,26*). Hemolymph collected from injured larvae usually exhibited 4- to 8-fold higher hemagglutinating activity than those from uninjured larvae, suggesting that needle injury induced innate immune responses in *Musca domestica* larvae.

Antimicrobial activities of the hemolymph were examined against several bacterial species including *S. aureus*, *S. epidermidis*, and *P. aeruginosa*. They were selected based on the availability of selective growth medium, which supports the growth of the desired bacteria while repressing the growth of environmental

bacteria. As shown in Figure 1, CFUs of *S. aureus*, *S. epidermidis*, and *P. aeruginosa* were reduced by treatment with hemolymph collected from injured larvae. However, CFUs were not reduced by treatment with hemolymph collected from larvae without injury. These observations indicate that antimicrobial activities against *S. aureus*, *S. epidermidis*, and *P. aeruginosa were* induced in the hemolymph of injured *Musca domestica* larvae.

3.2. *Characterization of the induced antimicrobial activity of hemolymph*

Charge-based interactions between bacterial membranes and antimicrobial peptides are essential for their antimicrobial activity, and it is likely that pH plays a critical role. Therefore, antimicrobial activity of hemolymph from injured larvae was examined across various pH conditions. As shown in Figure 2A, antimicrobial activity against *S. epidermidis* was increased as pH decreased, indicating that acidic environments are desirable for optimal antimicrobial effects. Moreover, antimicrobial activity against *E. coli* was observed at pH 5.8 but was not apparent at pH 7.8 (Figure 2B). These observations indicate that pH is an important factor for induced antimicrobial activity in *Musca domestica* larvae.

Stability of antimicrobial activity following heat-treatment was examined. Hemolymph collected from injured larvae was incubated at 65 or 75°C for 10 min. After heat-treatment, antimicrobial activity against *P. aeruginosa* was examined. Antimicrobial activity was retained in hemolymph samples subjected to heat-

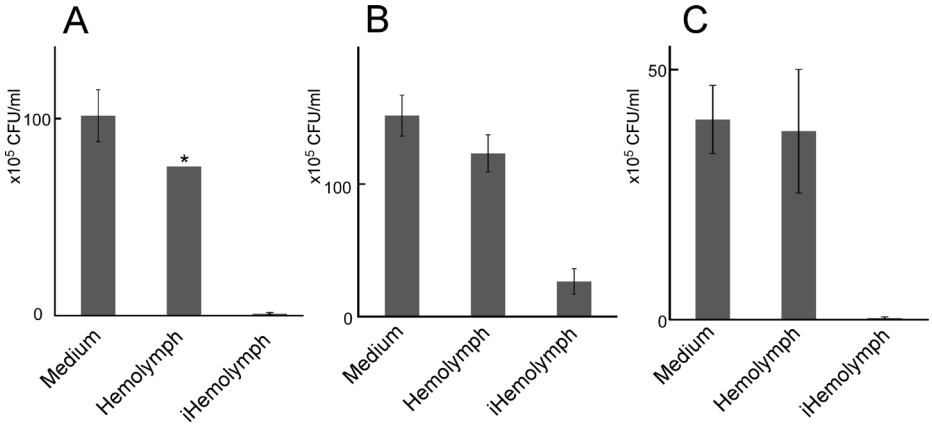

Figure 1. Antimicrobial activities were induced in hemolymph of Musca domestica larvae by needle injury. *S. aureus* **(A),** *S. epidermidis* **(B)**, and *P. aeruginosa* **(C)** were used to detect antimicrobial activities. The bacteria were combined with Mueller-Hinton II medium (Medium), hemolymph collected from larvae without injury (Hemolymph) or hemolymph collected from injured larvae (iHemolymph). The CFUs of the assay mixtures were determined, and the bars indicate the mean from duplicate plates (*) or the means ± SD from triplicate plates.

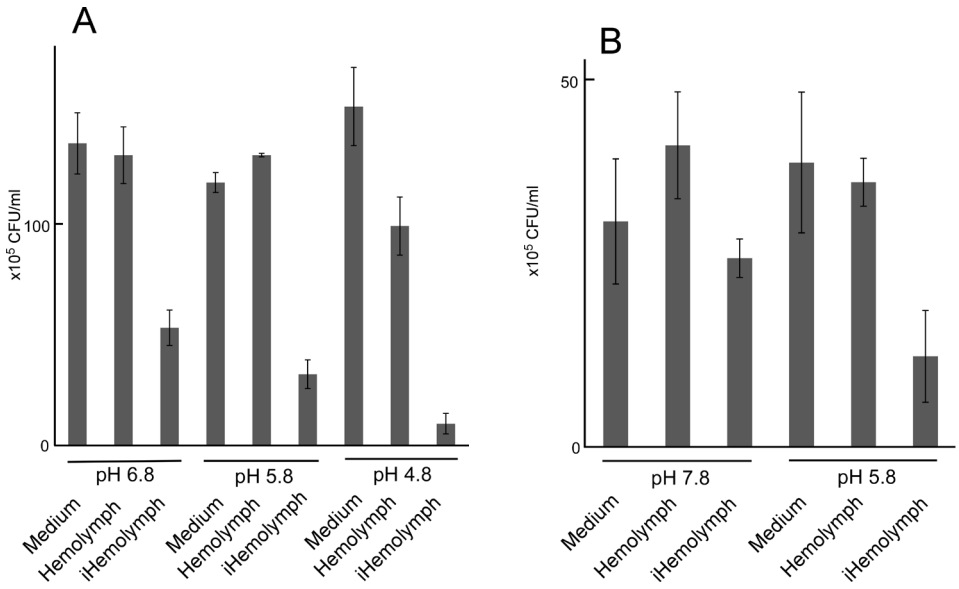

Figure 2. Effect of pH on the antimicrobial activity of hemolymph from injured larvae. *S. aureus* **(A)** and *E. coli* **(B)** were used for the analysis of antimicrobial activities. Values of pH in the assay mixtures were adjusted with phosphate/citrate buffer **(A)** and phosphate buffer **(B)**. CFUs in the assay mixtures (means ± SD) were presented.

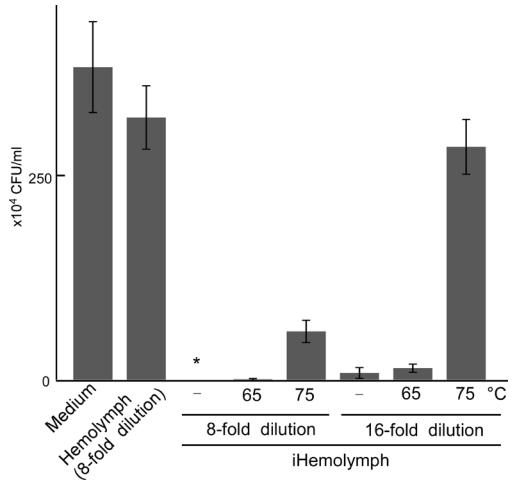

Figure 3. Heat-stability of antimicrobial activity of hemolymph from injured larvae. Diluted hemolymph in Mueller-Hinton II medium (18 μL of 8-fold and 16-fold) or Mueller-Hinton II medium alone were incubated at 65°C, 75°C, or on ice (–) for 10 min prior to combining with *P. aeruginosa*. CFUs in the assay mixtures (means ± SD) were presented. Asterisk (*) indicates that no colonies were detected (less than 8.3×10^3 CFU/mL).

treatment at 65°C (Figure 3). However, antimicrobial activity following heat-treatment at 75°C was only observed in 8-fold dilutions of hemolymph (Figure 3). Although these findings suggest that animal feed prepared from *Musca domestica* larvae should retain antimicrobial activity following pasteurization, future studies are needed to elucidate the optimal sterilization strategy.

In this study, we observed the induction of antimicrobial activity in *Musca domestica* larvae against several bacterial species following injury using a needle. Our current findings are consistent with previous reports on the upregulation of genes involved in innate immunity against invading bacteria in *Musca domestica* larvae (*27*). We speculate that the induction of antimicrobial activity was mediated by *imd* as well as *Toll* pathway, which were essential innate immune responses in *Drosophila* (*1*). The hemolymph collected from injured larvae contains various antimicrobial materials, including antimicrobial peptides, which may be ideal sources of antimicrobial agents. Furthermore, the antimicrobial properties of *Musca domestica* larvae make them a beneficial animal feed, because the addition of antimicrobial supplements to animal feed increases animal weight (*25*).

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Conflict of interest

K.K. is an advisor for E's Inc.

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