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Article

Antibacterial activity by hemolymph defensin from the hard tick *Haemaphysalis longicornis*

Yurika Yada[1], Melbourne Rio Talactac[1,2,5], Kodai Kusakisako[1,2], Emmanuel Pacia Hernandez[1,2], Remil Linggatong Galay[4], Masako Andoh[3], Kozo Fujisaki[6], and Tetsuya Tanaka[1,2,*]

[1]Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan; k915kn1010n@yahoo.co.jp (Y.Y.); talactacdvm@cvsu.edu.ph (M.R.T.); seigi26jp@yahoo.co.jp (K.K.); jhunemman@yahoo.com (E.P.H.)
[2]Department of Pathological and Preventive Veterinary Science, The United Graduate School of Veterinary Science, Yamaguchi University, Yoshida, Yamaguchi 753-8515, Japan
[3]Laboratory of Public Health, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan; masako@vet.kagoshima-u.ac.jp (M.A.)
[4]Department of Veterinary Paraclinical Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, Los Baños, Laguna 4031, Philippines; rlgalay.dvm@gmail.com (R.L.G.)
[5]Department of Clinical and Population Health, College of Veterinary Medicine and Biomedical Sciences, Cavite State University, Cavite 4122, Philippines
[6]National Agriculture and Food Research Organization, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan; acarikf@nifty.com (K.F.)
*Correspondence: k6199431@kadai.jp (T.T.)
Tel./Fax: +81-99-285-3570

Abstract: Ticks are key vectors of some important diseases of humans and animals. Although they are carriers of disease agents, the viability and development of ticks are not harmed by the infectious agents due to their innate immunity. Antimicrobial peptides directly protect hosts against pathogenic agents such as viruses, bacteria, and parasites. Among the identified and characterized antimicrobial peptides, defensins have been considerably well studied. Defensins, which contain intramolecular disulfide bridges between cysteine residues, are commonly found among fungi, plants, invertebrates, and vertebrates. The sequence of the tick hemolymph defensin (*HEdefensin*) gene from the hard tick *Haemaphysalis longicornis* was analyzed after identification and cloning from a cDNA library. HEdefensin has a predicted molecular mass of 8.15 kDa and a theoretical isoelectric point of 9.48. Six cysteine residues were also identified in the amino acids. The synthetic HEdefensin peptide only showed antibacterial activity against Gram-positive bacteria such as *Micrococcus luteus*. A fluorescence propidium iodide exclusion assay also showed that HEdefensin increased the membrane permeability of *M. luteus.* Additionally, an indirect fluorescent antibody test showed that HEdefensin binds to *M. luteus*. These results suggested that HEdefensin strongly affects the innate immunity of ticks against Gram-positive bacteria.

Keywords: HEdefensin; *Haemaphysalis longicornis*; bacteria

1. Introduction

Antimicrobial peptides (AMPs) are widely distributed in microorganisms, plants, and animals [1,2]. Most AMPs have certain common features, such as being made up of small molecules (10–50 amino acids), containing positive charges, and having an amphipathic structure. Based on their amino acid sequences, size, and structure, AMPs can be classified into several categories, such as peptides with α-helix and β-sheet structures, peptides stabilized by disulfide bridges, and peptides with a loop structure. The expression level of AMPs differs depending on the tissue and cell type; however, in most cases, AMPs are co-expressed as groups, then act together. One of the major AMP

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families that has been known to have biological effects is defensin [3]. Defensins have a characteristic cysteine motif duo to the intramolecular disulfide bridges [4]. These peptides can be found in different organisms, such as fungi, plants, insects, birds, and various mammals [5–7]. They are mainly effective against Gram-positive bacteria and also against some Gram-negative bacteria, fungi, yeasts, and parasites [4].

Ticks have been known as important vectors of a wide variety of disease-causing viruses, bacteria, parasites, and other pathogenic organisms [8]. Defensins have been characterized as molecules involved in the innate immune responses from several kinds of ticks. For instance, varisin, which is a cationic defensin, was isolated from hemocytes of the hard tick *Dermacentor variabilis* [9]. Varisin has a similarity to defensins of the members of insect families. Varisin has antimicrobial activity not only against Gram-positive and Gram-negative bacteria but also against *Borrelia burgdorferi*. A defensin-like molecule from *Ixodes ricinus* was observed to be induced following microbial challenge [10,11]. Two non-cationic defensin-like isoforms with antimicrobial activities against Gram-positive and Gram-negative bacteria have been identified in the hard tick *Amblyomma hebraeum* [12]. Defensins have also been characterized in the soft tick *Ornithodoros moubata*, where four different isoforms have been identified from different tissues [13].

The hard tick *Haemaphysalis longicornis*, which transmits various diseases, including severe fever with thrombocytopenia syndrome (SFTS) [14], human rickettsiosis, bovine theileriosis, and bovine and canine babesiosis, is distributed mainly in East Asia and Australia. Thus, knowledge of the antimicrobial peptides of *H. longicornis* is important for understanding the innate immunity of ticks and the role of this response in vector competence [15]. Previously, a sequence of antimicrobial peptide (longicin) of *H. longicornis* was cloned, and a functional characterization is being done at our laboratory [16]. Longicin (aa 1 to aa 73) and one of its synthetic partial analogs (P4) displayed antimicrobial activity, suggesting that they are potential chemotherapeutic agents against viruses and parasites [17–19]. Interestingly, three other synthetic partial analogues of longicin (P1, P2, and P3) were inactive against microorganisms [16]. This result indicated that the longicin P4 peptide (aa 53 to aa 73) might have a potential antimicrobial motif in longicin.

Depending on the specific antimicrobial mechanisms of the defensins studied, results showed direct disruption of the microbial cell membrane resulting in the death of the microbial cell. Disruption of lipid bilayers by defensins occurs through a variety of mechanisms [20]. In general, cationic peptides are attracted by electrostatic forces to the negative phospholipid head groups on the membrane surface, which include lipopolysaccharides (LPSs) in Gram-negative bacteria and teichoic acids (TAs), lipoteichoic acids (LTAs), and lysylphosphatidylglycerol in Gram-positive bacteria. After defensins gain access to the cytoplasmic membrane, they interact with the lipid bilayer [20]; this is followed by the displacement of lipids, alteration of the membrane structure, and creation of a physical hole that results in leakage of the cellular contents. Collectively, all of these are well-established models that are representative of the mechanisms for defensins. Each model provides a different view of peptide activity; however, none of them is capable of adequately explaining the effectiveness of these mechanisms *in vivo*. Salzman et al. [21] reported that this innate immune molecule was produced by Paneth cells in a human intestine model.

In the previous study, we identified and characterized a defensin-like encoding gene, *HEdefensin*, from the expressed sequence tag (EST) database of hemolymph from the hard tick *H. longicornis*. A synthetic HEdefensin peptide demonstrated significant virucidal activity against the Langat virus (LGTV) but not against the adenovirus in co-incubation virucidal assays [22]. However, it is unknown whether HEdefensin is also effective against bacteria. In this study, we have evaluated the potential biological activities of HEdefensin against bacteria *in vitro*.

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2. Results

2.1. HEdefensin shows antimicrobial activity

The antimicrobial activity of HEdefensin was tested against Gram-positive and Gram-negative bacteria. As shown in Table 1, HEdefensin showed significant antimicrobial activities against Gram-positive bacteria such as *M. luteus*, *B. cereus*, and *S. aureus* with a minimal inhibitory concentration (MIC) of less than 50 μM. For *M. luteus*, HEdefensin showed potent antimicrobial activities at 12.5 μM MIC and 3.125 μM half maximal (50%) effective concentration (EC50). However, HEdefensin was less effective against Gram-negative bacteria such as *E. coli* and *P. aeruginosa*.

Table 1. Antimicrobial activity of HEdefensin

Bacteria	MIC (μM)	EC50 (μM)
Gram-positive bacteria		
Micrococcus luteus (ATCC9341)	12.5	3.125
Bacillus cereus (ATCC11779)	>50	12.5
Staphylococcus aureus (ATCC29213)	>50	Not determined
Gram-negative bacteria		
Escherichia coli (ATCC25922)	No effect	—
Pseudomonas aeruginosa (ATCC27853)	No effect	—

M. luteus was exposed for 120 min at 37 °C to saline alone (Fig. 1A) or 50 μM HEdefensin (Fig. 1B). In Fig. 1, arrows indicate the bacterial destruction. In contrast, all of the bacterial cells incubated with saline appeared normal after Gram staining.

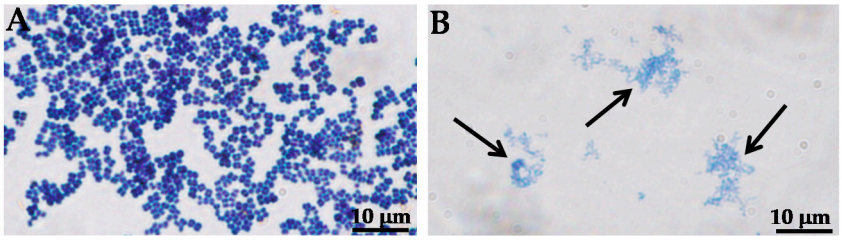

Figure 1. *Micrococcus luteus* exposed to HEdefensin. The bacterial cells were exposed for 120 min at 37 °C to saline alone (A) or 50 μM HEdefensin (B). Arrows indicate the destroyed bacteria.

The colony-forming unit (CFU) was notably reduced at 15 min, and the number of bacterial colonies significantly decreased in the presence of 12.5 μM HEdefensin compared with saline alone at 30 min (Fig. 2).

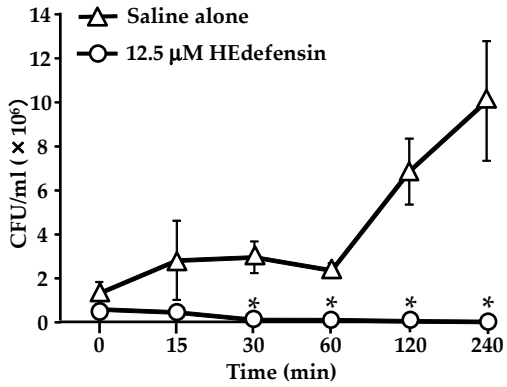

Figure 2. Time dependence of antimicrobial activity of saline alone (△) or HEdefensin (○) against *Micrococcus luteus*. The bacterial cells were exposed to 12.5 μM HEdefensin for 15 min, 30 min, 60 min, 120 min, or 240 min at 37 °C. $^{*}p<0.05$, saline alone vs. 12.5 μM HEdefensin.

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2.2. HEdefensin causes membrane disruption in M. luteus

To further investigate the result shown in Fig. 2, which suggested that HEdefensin exerts direct bactericidal activity by disrupting the bacterial membrane integrity, we performed a fluorescence propidium iodide exclusion assay (Fig. 3). The results of this assay showed that HEdefensin can disrupt the bacterial membrane.

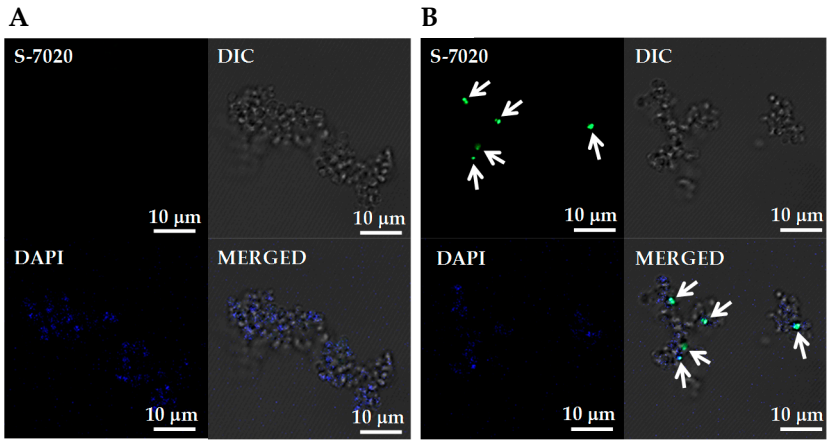

Figure 3. Fluorescence propidium iodide exclusion assay after co-incubation of *Micrococcus luteus* and saline only (A) or 12.5 µM HEdefensin (B) for 30 min at 37 °C. All bacterial cells' nuclei were stained positively with DAPI (blue), but only those with disrupted membranes were stained with S-7020 (green) as indicated by arrows. DIC, differential interference contrast panel; MERGED, three panels combined.

2.3. HEdefensin binds to M. luteus

To determine whether *M. luteus* interacts with HEdefensin, we used confocal microscopy to examine the binding activity of HEdefensin incubated with bacteria. Using a confocal microscope, HEdefensin was localized on bacterial cells, as indicated by arrows. The nuclei of bacteria were stained with DAPI (Fig. 4). These results indicate that HEdefensin has an affinity for the membrane of *M. luteus*.

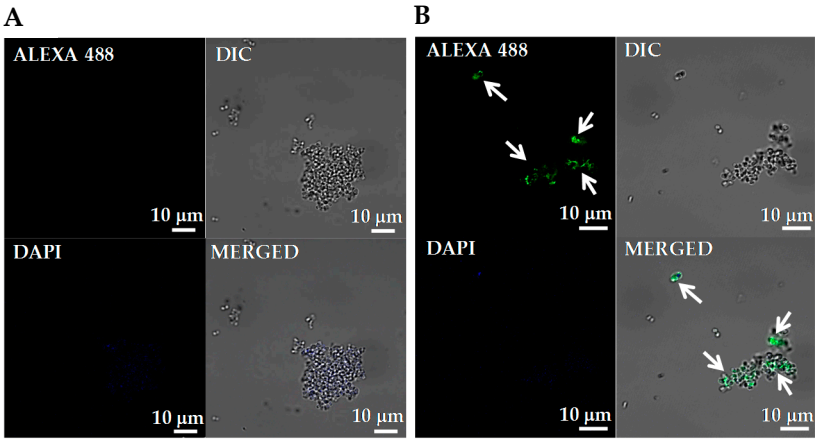

Figure 4. HEdefensin binds to *Micrococcus luteus*. Bacterial cells were exposed for 30 min at 37 °C to saline alone (A) or 12.5 µM HEdefensin (B). HEdefensin was localized as indicated by arrows, and the nuclei of bacterial cells were stained with DAPI. The confocal microscopy images suggested that the bactericidal effect of HEdefensin occurs via specific adherence to the bacterial membrane. DIC, differential interference contrast panel; MERGED, three panels combined.

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3. Discussion

Defensins are present in all types of organisms, from mammals and plants to arthropods. Defensins are AMPs that form the first line of defense against many pathogens. A common action of defensins from all organisms is the lysis of bacterial cells; however, the amino acid sequences of defensins show high diversity. Sequence analysis showed that HEdefensin had more than 65% homology at the amino acid level with defensin from hard ticks [19]. The differentiation could be influenced by a diverse strategy of blood intake and pathogens that each tick species has encountered during its evolutionary history and geographical isolation [23].

The incidence of tick-borne diseases has steadily increased over the past few years, and effective vaccines against most tick-borne pathogens are currently unavailable [24]. However, defensin is not affected by the antibiotic-resistant mechanism of pathogenic microorganisms [25]. For instance, two defensin isoforms, Def1 and Def2 from *I. ricinus*, showed significant bactericidal effects against Gram-positive bacteria; however, they did not show any effects against Gram-negative bacteria, yeasts, and viruses [26]. Another defensin-like peptide from *H. longicornis* has potent antimicrobial activities against bacteria and fungi and even shows strong antimicrobial ability against drug-resistant microorganisms [27]. The HEdefensin we identified from the hard tick *H. longicornis* also has a distinct antimicrobial profile against different microbes. HEdefensin showed strong antimicrobial potency on Gram-positive bacteria (*M. luteus*, *B. cereus*, and *S. aureus*) (Table 1) and LGTV [19] but had less of an effect on Gram-negative bacteria (*E. coli* and *P. aeruginosa*) (Table 1).

HEdefensin contains asymmetric clusters of basic amino acid residues, such as arginine, histidine, and lysin. Similar cationic peptides, such as defensins, polymyxins, and magainins, are known to show affinity for biological membranes and to disrupt the cytoplasmic membrane of various microorganisms [28]. The biochemical similarity and dissimilarity of the membranes of *M. luteus* and other microbial cell membranes are obscure. Nonetheless, it is conceivable that HEdefensin has an affinity for the membranes of *M. luteus*, as shown in Figs. 3 and 4, and disrupts their normal functional properties. However, HEdefensin did not show any affinity for *E. coli* membranes according to the binding assay using a confocal microscope (data not shown). We also speculated that HEdefensin might enter the cytoplasm of *M. luteus* and cause remarkable cellular damage through membrane pores. HEdefensin seems to gain entry into the cell through the pores and may disrupt internal membranes and organelles. Most defensins are amphipathic molecules that have clusters of positively charged residues as well as hydrophobic amino acid residues [29]. On the other hand, bacterial cell membranes consist of a lipid bilayer that is covered with negatively charged phospholipid head groups and hydrophobic fatty acids. Taken together, the mechanism of defensin molecules to kill bacteria is currently hypothesized to be direct electrostatic interaction with the bacterial cell membranes through their positively charged domains. As defensin molecules accumulate in microbial cell membranes, they change their location, resulting in the formation of pores in the microbial cell membrane [30,31]. As bacterial cell membranes are the primary target of defensin molecules, the occurrence of microbicide-resistance mutations is unlikely. In order to clarify the mechanisms of the bactericidal effect of HEdefensin, future studies are necessary to investigate the kinetics of interaction between HEdefensin and the cell membrane of the bacteria.

4. Materials and Methods

4.1. Synthetic peptide

HEdefensin, except for the signal peptides, was synthesized using a Perkin-Elmer Applied Biosystems 431 A Synthesizer with prederivatized polyethylene glycol polystyrene arginine resin, FastMoc chemistry, and double coupling for residues. The HEdefensin peptide characterization is as follows: (51 mer; Mw: 5865.69; pI: 9.43). The reduced peptides were purified using reverse-phase

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high-performance liquid chromatography (RP-HPLC), while peptide purity and integrity were assessed using MALDI-TOF mass spectrometry (MALDI-TOF MS) [19].

4.2. Preparation of mouse anti-HEdefensin sera

A mouse polyclonal antiserum was generated against a peptide (SEVAHLRVRRGFGC) consisting of the N-terminal 14 amino acids of HEdefensin, and then these peptides were conjugated by keyhole limpet hemocyanin (KLH). To prepare mouse anti-HEdefensin sera, each mouse was injected intraperitoneally with 50 µg of KLH-conjugated HEdefensin completely mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Immunization was repeated 14 and 28 days after the first immunization, but KLH-conjugated HEdefensin was mixed with an incomplete adjuvant (Sigma-Aldrich). All sera were collected at 14 days after the last immunization.

4.3. Bactericidal assay

Bactericidal activity was determined by a CFU assay [32]. Gram-positive bacteria, *Micrococcus luteus* (ATCC9341), *Bacillus cereus* (ATCC11779), and *Staphylococcus aureus* (ATCC29213), and Gram-negative bacteria, *Escherichia coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC27853), were used in this experiment. The bacterial culture was subcultured in 3% tryptic soy broth (TSB) with shaking at 37 °C overnight to obtain log-phase bacterial cells. The bacterial cells were washed and diluted to 1×10^6/ml in a 10 mM sodium phosphate buffer (pH 7.4) containing 1% TSB. The bacterial cell suspensions (90 µl) were mixed with 10 µl of HEdefensin (0, 3.125, 6.25, 12.5, 25, and 50 µM) solutions and incubated at 37 °C for 2 h. After incubation, bacterial samples were immediately stained with Gram-staining solution and assessed by light microscopy to observe bacteria conditions. Samples were then diluted 10,000-fold in 1% TSB and spread on TSB agar plates. Plates were incubated at 37 °C for 16–20 h, colonies were counted, and CFUs per ml were calculated.

For *M. luteus*, bacterial cells were washed and diluted to 1×10^6/ml in a 10 mM sodium phosphate buffer (pH 7.4) containing 1% TSB. The bacterial cell suspension (90 µl) was mixed with 10 µl of 12.5 µM HEdefensin solutions and incubated at 37 °C for 0, 15, 30, 60, 120, and 240 min.

The direct bactericidal activity of HEdefensin against *M. luteus* was assessed by fluorescence propidium iodide exclusion assay using a 5 µM florescent probe S-7020 (Invitrogen, Carlsbad, CA, USA), and a drop of VECTASHIELD® with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) mounting medium. Bacterial cells were observed under an LSM 700 confocal microscope (Carl Zeiss Microscopy, Jena, Germany).

4.4. Binding assay with M. luteus

A *Micrococcus luteus* bacterial culture was subcultured in 3% TSB with shaking at 37 °C overnight to obtain log-phase bacterial cells. Bacterial cells were then washed and diluted to 1×10^8/ml in a 10 mM sodium phosphate buffer (pH 7.4) containing 1% TSB. The bacterial cell suspensions (90 µl) were then mixed with 10 µl of 12.5 µM HEdefensin solutions and incubated at 37 °C for 30 min. The bacterial cells (1×10^6/ml) were washed three times by centrifugation at 350×*g* for 10 min and resuspended in saline, then incubated with anti-HEdefensin sera overnight at 4 °C in a final volume of 100 µl of saline. After centrifugation at 350×*g* for 10 min to remove the reaction buffer, the bacterial cells were incubated with Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen) for 60 min on ice in a final volume of 100 µl of saline. After centrifugation at 350×*g* for 10 min to remove the reaction buffer, the bacterial cells were resuspended in 100 µl of saline with DAPI for 60 min on ice. Finally, after centrifugation at 350×*g* for 10 min to remove the reaction buffer, the bacterial cells were added with Dako fluorescent mounting medium (Dako, Caminteria, CA, USA) and spotted on the glass slides. The bacterial cells were observed under an LSM 700 confocal microscope (Carl Zeiss Microscopy).

4.5. Statistical analysis

All samples were tested at least in triplicate. Data were statistically analyzed using Student's *t*-test. Results are presented as means ± S.D. Values of $p < 0.05$ were considered significant.

5. Conclusions

In summary, the HEdefensin of *H. longicornis* shows antimicrobial activity against Gram-positive bacteria. Therefore, the significant antimicrobial activity of the peptide against this group of bacteria *in vitro* offers a new potential antimicrobial agent against various Gram-positive bacteria in the medical field.

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Conflicts of Interest: The authors declare no conflict of interest.

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