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Hemolymph defensin from the hard tick *Haemaphysalis longicornis* attacks Gram-positive bacteria

Yurika Yada[a], Melbourne Rio Talactac[a,b,e], Kodai Kusakisako[a,b], Emmanuel Pacia Hernandez[a,b], Remil Linggatong Galay[d], Masako Andoh[c], Kozo Fujisaki[f], Tetsuya Tanaka[a,b,*]

[a] Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan
[b] Department of Pathological and Preventive Veterinary Science, The United Graduate School of Veterinary Science, Yamaguchi University, Yoshida, Yamaguchi 753-8515, Japan
[c] Laboratory of Public Health, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan
[d] Department of Veterinary Paraclinical Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, Los Baños, Laguna 4031, Philippines
[e] Department of Clinical and Population Health, College of Veterinary Medicine and Biomedical Sciences, Cavite State University, Cavite 4122, Philippines
[f] National Agriculture and Food Research Organization, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan

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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 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 including signal peptides 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.

1. Introduction

Antimicrobial peptides (AMPs) are widely distributed in microorganisms, plants, and animals (Brown and Hancock, 2006; Lai and Gallo, 2009; Li et al., 2017; Yang et al., 2017). Most AMPs have certain common features, such as consisting 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 families that has biological effects is defensin (Bulet et al., 2004; Falanga et al., 2017). Defensins have a characteristic cysteine motif duo to the intramolecular disulfide bridges (Bulet et al., 1999; Nigro et al., 2017). These peptides can be found in different organisms, such as fungi, plants, insects, birds, and various mammals (Boman, 1995; Hoffmann et al., 1999; Lehrer and Ganz, 1999). They are mainly effective against Gram-positive bacteria and also against some Gram-negative bacteria, fungi, yeasts, and parasites (Bulet et al., 1999).

Ticks are known as important vectors of a wide variety of disease-causing viruses, bacteria, parasites, and other pathogenic organisms (Sonenshine, 2014; Sonenshine and Macaluso, 2017), and defensins are involved in the innate immune response of several tick species. For example, varisin, a cationic defensin, was isolated from hemocytes of the hard tick *Dermacentor variabilis* (Johns et al., 2001). Varisin is similar to defensins of some insect families and has antimicrobial activity, not only against Gram-positive and Gram-negative bacteria but also against *Borrelia burgdorferi*. A defensin-like molecule from *Ixodes*

* Corresponding author at: Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan.
E-mail address: k6199431@kadai.jp (T. Tanaka).

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ricinus was observed to be induced following microbial challenge (Rudenko et al., 2005; Saito et al., 2009). 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* (Lai et al., 2004). Defensins have also been characterized in the soft tick *Ornithodoros moubata*, where four different isoforms have been identified from different tissues (Nakajima et al., 2001).

The hard tick *Haemaphysalis longicornis*, which transmits various diseases including severe fever with thrombocytopenia syndrome (SFTS) (Luo et al., 2015), human rickettsiosis, bovine theileriosis and bovine and canine babesiosis, occurs mainly in East Asia and Australia. Knowledge about the antimicrobial peptides of *H. longicornis* is important for understanding the innate immunity of ticks and the role of immune response in vector competence (Sun et al., 2017; Zhou et al., 2007). Previously, a sequence of antimicrobial peptide (longicin) of *H. longicornis* was cloned, and functional characterization is being done at our laboratory (Tsuji et al., 2007). Longicin (aa 1 to aa 73) and one of its synthetic partial analogs (*P*4) produced antimicrobial activity, suggesting that they are potential chemotherapeutic agents against viruses and parasites (Galay et al., 2012; Talactac et al., 2016; Tanaka et al., 2012). Interestingly, three other synthetic partial analogues of longicin (*P*1, *P*2, and *P*3) were inactive against microorganisms (Tsuji et al., 2007). This result indicated that the longicin *P*4 peptide (aa 53 to aa 73) might have a potential antimicrobial motif in longicin.

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 (Talactac et al., 2017). 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*.

2. Materials and methods

2.1. Synthetic peptide

HEdefensin, except for the signal peptides, was synthesized using a Perkin-Elmer Applied Biosystems 431 A Synthesizer with pre-derivatized polyethylene glycol polystyrene arginine resin, FastMoc chemistry, and double coupling for residues. The HEdefensin peptide characterization is as follows: EEESEVAHLRVRRGFGCPLNQGACHRH-CRSIRRRGGYCSGIIKQTCTCYRN (51 mer; Mw: 5.87 kDa; pI: 9.43). The reduced peptides were purified using reverse-phase high-performance liquid chromatography (RP-HPLC), while peptide purity and integrity were assessed using MALDI-TOF mass spectrometry (MALDI-TOF MS) in Supplementary Fig. 1 (Talactac et al., 2017).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jip.2018.07.005.

2.2. Preparation of mouse anti-HEdefensin sera

Twelve 4-week-old female ddY mice (Kyudo, Kumamoto, Japan) were used for anti-HEdefensin serum preparation. Experimental animals were kept at 25 °C and 40% relative humidity, with a constant supply of water and commercial feeds. The care and use of experimental animals in this study were approved by the Animal Care and Use Committee of Kagoshima University (approval number VM15056 for the mice).

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. All sera confirmed to react with synthetic HEdefensin by Western blotting (Supplementary Fig. 2).

2.3. Bactericidal assay

Bactericidal activity was determined by a CFU assay (Ghosh et al., 2002). 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 bacteria were 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).

2.4. 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 mouse normal sera or 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, bacterial cells with added Dako fluorescent mounting medium (Dako, Caminteria, CA, USA) were spotted onto glass slides. The bacterial cells were observed under an LSM 700 confocal microscope (Carl Zeiss Microscopy).

2.5. 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.