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Sequence characterization and expression patterns of two defensin-like antimicrobial peptides from the tick *Haemaphysalis longicornis*

Jinlin Zhou [a,b], *Min Liao* [a], *Mami Ueda* [c], *Haiyan Gong* [c], *Xuenan Xuan* [c], *Kozo Fujisaki* [a,c,*]

[a] *Department of Frontier Veterinary Medicine, Kagoshima University, Korimoto 1-21-24, Kagoshima 890-0065, Japan*
[b] *Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, 3 Lane 345, Shilong Road, Shanghai 200232, China*
[c] *National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan*

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ABSTRACT

Two cDNAs encoding defensin-like antimicrobial peptides were cloned and sequenced from the tick *Haemaphysalis longicornis*. The full-length cDNA of *Hlgut*-defensin (*H. longicornis* midgut defensin) is 333 bp, encoding an expected protein with 73 amino acids. The full-length cDNA of *Hlsal*-defensin (*H. longicornis* salivary gland defensin) is 382 bp, encoding an expected protein with 81 amino acids. The antibacterial activities of the synthetic peptides based on the Hlgut-defensin and Hlsal-defensin sequences were tested against a variety of Gram-positive and Gram-negative bacteria. Using real-time PCR, the tissue-specific expression of two defensin-like peptides were determined and it was also found that the gene transcripts of Hlgut-defensin and Hlsal-defensin were significantly induced by a lipopolysaccharide (LPS) injection.

1. Introduction

Antimicrobial peptides (AMPs) are widely distributed throughout the animal and plant kingdoms. Despite sharing some common features, such as a small size (often below 10 kDa) and a cationic character, most AMPs differ in their amino acid sequence and mode of action [7]. One of the major families of AMPs that has been characterized is the defensins [3]. Defensins conserve a characteristic motif of six cysteines constructing a homologous conformation by intramolecular disulfide bridges [2]. This peptide can be found in different organisms, such as plants, fungi, mollusks, scorpions, insects, and birds, and also in different cells of various mammals. Solution structure of the defensin-like peptide was determined and the primary structural similarity between members of the family suggests that the global fold is robust and that the nature of the side-chains determine the functional specificity [3,25]. They are mainly effective against Gram-positive bacteria and also have potent activity against some Gram-negative bacteria, fungi, yeasts, and protozoa [2].

Ticks are important vectors of a wide variety of disease-causing bacteria, viruses, protozoa, and other pathogenic organisms. Defensins have been isolated and characterized as part of the innate immune response from several species of ticks. A cationic defensin (varisin) was identified from hemocytes of the hard tick, *Dermacentor variabilis* [4,12]. Varisin has a similarity to members of the insect family of defensins. In addition to antimicrobial activity against Gram-positive

* *Corresponding author*. Tel.: +81 99 285 3569; fax: +81 99 285 3570.
E-mail address: tick@ms.kagoshima-u.ac.jp (K. Fujisaki).
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bacteria, varisin is also active against *Borrelia burgdorferi*. A defensin-like molecule from *Ixodes ricinus* was recently described and found to be induced following microbial challenge [23]. Two non-cationic defensin-like isoforms with antimicrobial activity against Gram-positive and Gram-negative bacteria have been identified in the hard tick *Amblyomma hebraeum* [14]. Two cysteine-rich antimicrobial peptides, a novel 10.2 kDa polypeptide and a 4.29 kDa peptide, were identified in the hard tick *Rhipicephalus* (*Boophilus*) *microplus*. Although both molecules contained six cysteine residues, only the smaller one was characteristic of the insect family of defensins [7]. More recently, a defensin-like gene was identified from tick *Ixodes scapularis* [11]. Defensins have also been identified in a soft tick, *Ornithodoros moubata*, where four different isoforms have been characterized from different tissues [17,18]. The hard tick, *Haemaphysalis longicornis*, is distributed mainly in East Asia and Australia, where it transmits a wide range of pathogens, including bovine theileriosis (*Theileria* spp.), bovine babesiosis (*Babesia ovata*), canine babesiosis (*Babesia gibsoni*), and human rickettsiosis (*Rickettsia japonica*) [29]. Thus, knowledge of the antimicrobial peptides of *H. longicornis* is important for understanding the innate immunity in this vector tick and the role of this response in vector competence. Previously, a sequence of antimicrobial peptides (longicin) of *H. longicornis* was cloned and submitted to the GenBank database under accession numbers AB105544 and a functional characterization is currently underway at our laboratory. In this study, we report the sequence characterization and expression patterns of two defensin-like antimicrobial peptides from the tick *H. longicornis*.

2. Materials and methods

2.1. Ticks and tissue collection

The parthenogenetic Okayama strain of the tick *H. longicornis* has been maintained by feeding on rabbits and mice for several generations in our laboratory [28]. For tissue collection, adult females of *H. longicornis* were infested on the ears of rabbits. Ticks were recovered from the rabbit ears after 4 days, and the tissues were immediately dissected under the microscope [28]. The sample materials were stored at −80 °C until used.

2.2. Construction of the tick midgut and salivary gland full-length cDNA library by vector capping and cDNA sequencing

A tick midgut full-length cDNA library was made using the vector-capping method described previously [29]. The tick salivary gland full-length cDNA library was constructed using the same method. Total RNA was prepared from the salivary glands of partially fed female adult ticks, which had remained attached to rabbit ears for 4 days. The cDNA was synthesized with 5 μg total RNA by the G-capping method [13] and ligated into plasmid vector pGCAP1; the resulting plasmid was transformed into electrocompetent cell DH12S (Invitrogen). A total of 10,000 recombinant transformants from the library were randomly selected for plasmid DNA purification and sequencing. Nucleotide sequences were determined using an automated sequencer (ABI PRISM 310 Genetic Analyzer, USA). The cDNA clones encoding defensin-like antimicrobial peptides were chosen from the two cDNA libraries for further analysis.

2.3. Synthetic peptides and antibacterial assay

Peptides were 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 reduced peptides were purified using RP-HPLC. The partial peptides were as follows: G1, LVRVRRGFGCPFDER; G2, ACHAHCQSVGRRGGYCGNFRMTCYCY; S1, GFRTAHVDLVCPDNPD; S2, NCIQQCVSKGAQGGYCTNEKCTCY (Fig. 1). According to the domain structures, synthetic peptides G1 and S1 include the loop domain of Hlgut-defensin and Hlsal-defensin, respectively; G2 and S2 include the α-helix and β-sheet domains of Hlgut-defensin and Hlsal-defensin, respectively. Peptide purity and integrity were assessed by MALDI-TOF MS.

A liquid growth inhibition assay was performed according to the method of Bulet et al. [1]. The Poor medium (0.1% Bactotryptone; 0.5% NaCl) was used for bacterial cultures. Four strains of Gram-positive bacteria (*Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus megaterium*, and *Bacillus sublitis*) and three strains of Gram-negative bacteria (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *E. coli* O157) were used in this experiment. Bacterial suspensions were prepared to give OD600 of 0.001, and 90-μL aliquots were incubated with 10 μL of serial dilutions of purified antibacterial peptides. Bacterial growth was assayed by measuring an increase of OD600 after incubation for 24 h at 37 °C. A dilution buffer was used as a negative control.

2.4. Real-time quantitative PCR

For the estimation of mRNA abundance, we used the one-step TaqMan real-time reverse-transcription polymerase chain reaction (R/T RT-PCR) method. Gene-specific primers and a fluorogenic probe were designed to target the defensin-like genes or control tick actin gene (accession number, AY254898) using the Primer Express software (Applied Biosystems, USA). The PCR primers and TaqMan probes used in this study are listed in Table 1. The one-step R/T RT-PCR reaction was conducted using the TaqMan one-step RT-PCR master mix reagent kit (Applied Biosystems, USA) according to the manufacturer's guidelines. Samples were amplified by using a program that included a reverse-transcription procedure consisting of one cycle of incubation at 48 °C for 30 min and 94 °C for 10 min followed by 45 cycles of a denaturation step at 94 °C for 15 s and an annealing/extension step at 60 °C for 1 min. We established the standard curves for cystatin and actin using serial dilutions (800–50 ng) of total RNA. Amplification and product detection were performed using the ABI PRISM 7900 HT sequence detection system (Applied Biosystems, USA). A positive result was determined by identifying the threshold cycle (C_T) value at which reporter dye emission could be observed above the background. The relative amount