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Purification and characterization of a novel antibacterial peptide from black soldier fly (*Hermetia illucens*) larvae

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ABSTRACT

In this study, we induced and purified a novel antimicrobial peptide exhibiting activity against Gram-positive bacteria from the immunized hemolymph of *Hermetia illucens* larvae. The immunized hemolymph was extracted, and the novel defensin-like peptide 4 (DLP4) was purified using solid-phase extraction and reverse-phase chromatography. The purified DLP4 demonstrated a molecular weight of 4267 Da, as determined using the matrix-assisted laser desorption/ionization–time-of-flight (MALDI–TOF) method. From analysis of DLP4 by N-terminal amino acid sequencing using Edman degradation, combined with MALDI–TOF and rapid amplification of cDNA ends–polymerase chain reaction (RACE–PCR), the amino acid sequence of the mature peptide was determined to be ATCDLLSPFKVGHAACAAHCIA RGKRGGWCDKRAVCNCRK. In NCBI BLAST, the amino acid sequence of DPL4 was found to be 75% identical to the *Phlebotomus duboscqi* defensin. Analysis of the minimal inhibitory concentration (MIC) revealed that DLP4 have antibacterial effects against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA). The expression of DLP4 transcripts in several tissues after bacterial challenge was measured by quantitative real-time PCR. Expression of the DLP4 gene hardly occurred throughout the body before immunization, but was mostly evident in the fat body after immunization.

1. Introduction

Antimicrobial peptides (AMPs) are evolutionarily ancient molecules and these peptides are found in all living organisms ranging from bacteria to humans. AMPs are an evolutionarily conserved component of the innate immune system. One of the major families of antimicrobial peptides that have been characterized is the defensins (Zasloff, 2002). Defensins were found in most species investigated to date. Insect defensins were first reported from cell cultures of the flesh fly *Sarcophaga peregrina* (Matsuyama and Natori, 1988) and from experimentally injured larvae of the black blowfly, *Phormia terranovae* (Lambert et al., 1989). In general, defensin-like peptides (DLPs) consist of 34–43 amino acids, and have a molecular weight of 3–4 kDa; they are characterized as cationic peptides and three pairs of disulfide bridges (Ganz, 2003; Hazlett and Wu, 2011). Since first discovered, the insect AMPs with these characteristics have come to be deemed as insect defensins (Hoffmann and Hetru, 1992). The defensin derived from *Allomyrina dichotoma* exhibited strong antimicrobial activity against Gram-positive bacteria including MRSA, while strong activity against Gram-negative bacteria was not observed (Miyanoshita et al., 1996). Insect defensins have long been known to be particularly resistant to Gram positive bacteria but some of defensins, such as *Sarcophaga* Sapecin, *Aedes* defensin, showed a small amount of activity against some Gram-negative bacteria (Lowenberger et al., 1995; Matsuyama and Natori, 1988; Tzou et al., 2002).

Recently, research has been actively underway to explore the biodefense mechanisms of insects in order to identify novel AMPs (Dang et al., 2010; Wei et al., 2015). However, research on AMP extracted from *H. illucens* has not yet been conducted. Against this background, we induced and purified a novel AMP from the immunized hemolymph of *H. illucens* in order to identify its structural characteristics, cDNA sequence, and antimicrobial activity against various Gram-positive and Gram-negative bacteria, including MRSA and *E. coli*. In addition, the gene expression of the larvae by tissue was examined.

2. Materials and methods

2.1. Insects, immunization, and hemolymph collection

Fifth instar larvae from the black soldier fly (*H. illucens*) were supplied by Nuree Inc., Baekgok, Korea. To induce the production of AMPs, the larvae of *H. illucens* were subjected to an immunization

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process. The larvae were first washed with water containing disinfectant, and then rinsed with deionized water. Excess water was removed using filter paper, after which each larva was pricked deeply with a fine needle dipped into *S. aureus* (KCCM 40881; $OD_{600} = 2.4$). The larvae were reared at 32 °C and 62% humidity for 24 h. Immunized hemolymph was collected in ice-cold tubes containing a few crystals of phenylthiourea. The hemolymph was centrifuged at 12,000 × *g* for 10 min to remove hemocytes and cell debris, and the supernatant was stored at −70 °C until use.

2.2. Purification of defensin-like peptide 4 (DLP4)

Immunized hemolymph was diluted with an equal volume of ice-cold aqueous trifluoroacetic acid (TFA) at 0.1%. The sample was then centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant was injected onto Sep-Pak C18 cartridges (Waters) and eluted in a stepwise fashion with 20 ml each of 10, 20, 30, 50, and 80% acetonitrile (ACN) in acidified water (0.05% TFA). The anti-MRSA fraction (30% ACN eluent) was lyophilized and kept at −70 °C until use.

After partial purification using Sep-Pak cartridges, the 30% ACN eluent powder was redissolved in 0.05% (v/v) TFA/water and injected onto Resource RPC (GE Healthcare), after which it was eluted with a gradient of 17.5–26.5% ACN in 0.05% aqueous TFA using fast protein liquid chromatography (FPLC). Fractions exhibiting antibiotic activity against MRSA, *E. coli*, or both, were separately collected and pooled. Pool number 1 (anti-MRSA fractions) was further purified by high-performance liquid chromatography (HPLC) on a 4.6 × 250 mm Shim-pack VP-ODS (Shimadzu, Japan) with a simple linear gradient of 20–30% ACN (0.05% (v/v) TFA) at a flow rate of 1 ml/min. The elution pattern was monitored at 214 nm, and the antibacterial activity was determined using aliquots of the fraction that had been vacuum-dried to remove the ACN.

Protein concentration of purified peptide was determined via enhanced BCA assay (Pierce) using bovine serum albumin as a standard and monitored at 262 nm. Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Schägger (2006) using mini (10 × 8 × 0.075 cm) polyacrylamide gels (16% T and 3% C).

2.3. N-terminal amino acid sequencing and matrix-assisted laser desorption/ionization–time-of-flight (MALDI–TOF) mass spectrometry (MS)

The amino acid sequence of purified DLP4 was determined by Edman degradation using an ABI492 protein sequencer (Applied Biosystems, USA). PTH-amino acids released at each cycle of Edman degradation were identified by manual comparison of each chromatogram to a standard mixture of 19 PTH–amino acid chromatogram run at the start of the analysis. The molecular mass of DLP4 was analyzed by matrix-assisted laser desorption/ionization–time-of-flight (MALDI–TOF) mass spectrometry (MS) on an AutoflexII (Burker Daltonics, Germany).

2.4. RNA isolation, cDNA production, and cloning of the full-length cDNA of DLP

To prepare a cDNA library, total RNA was isolated from induced fat bodies of *H. illucens* using Trizol (Invitrogen) according to the manufacturer's protocol and dissolved in water treated with DEPC. The full-length sequence of the DLP gene was revealed by rapid amplification of cDNA ends (RACE). First-strand cDNA was synthesized from 1 mg of total RNA using a SMARTer RACE cDNA Amplification Kit (Clontech, USA).

For 3′-RACE, a DNA fragment encoding a portion of DLP was amplified by polymerase chain reaction (PCR) using the Advantage 2 PCR Kit (Clontech, USA) with forward primers (Table 1) and universal primer mix (UPM) for the reverse primers. The DLP3-3RACE and DLP4-3RACE for forward primers were based on the N-terminal amino acid sequence of DLP (Pool1 peptide). The RACE-PCR product was cloned into the pGEM-T Easy vector (Promega) and the nucleotide sequence was determined in both directions using an ABI-3700 automatic DNA sequencer (Applied Biosystems, USA). For 5′-RACE, a gene-specific primer was designed from the internal sequence obtained from the previous 3′-RACE-PCR (Table 1). Nucleotide sequencing was conducted as described earlier. Alignment of the sequences was carried out using the Clustal W multiple sequence alignment program (Thompson et al., 1994).

2.5. Sequence analysis and statistical analysis

The cDNA of DLPs and the predicted protein sequences were analyzed using bioinformation software. Similarity searches were performed using BLAST and the NR database of the National Center for Biotechnology Information (NCBI). The theoretical isoelectric point (pI) and molecular weight (MW) were calculated using the Compute pI/MW Tool (http://www.expasy.org/tools/pi_tool.html). The pro-peptide cleavage site was predicted using ProP1.0 (http://www.cbs.dtu.dk/services/). A phylogenetic tree was compiled using the MEGA 5 program. The minimal inhibitory concentration (MIC) and expression pattern data were expressed as mean ± standard error. Significant differences between the groups were determined using Duncan's test ($P < 0.05$).

2.6. Antibacterial activity assays

In order to assess the antibacterial activities of DLP4, the following bacterial species were used: *Escherichia coli* (KCCM 11234), *Enterobacter aerogenes* (KCCM 12177), *Pseudomonas aeruginosa* (KCCM 11328), MRSA (methicillin-resistant *Staphylococcus aureus*, clinical isolated, multidrug resistant), *Staphylococcus aureus* (KCCM 40881, KCCM 12256), *Bacillus subtilis* (KCCM 11316), and *Staphylococcus epidermidis* (KCCM 35494). *E. coli* and *Staphylococcus* were grown in tryptic soy broth, while the other bacteria were grown in nutrient broth.

The antimicrobial activities of *H. illucens* hemolymph and chromatographic fractions against *E. coli* and MRSA were measured using the inhibition zone assay with slight modifications, as described previously (Park et al., 2013). Thin plates (1 mm) of 1% agarose containing 6×10^4 cells/ml were prepared and wells of 3 mm in diameter were punched out of the plates. Samples were loaded into the appropriate wells. After incubation overnight at 37 °C, the plates were stained with thiazolyl blue tetrazolium bromide in PBS (5 mg/ml) at 25 °C for 1 h, and then the diameters of the clear zones were measured.

Table 1
Primer sequences.

Primer name	Primer sequence[a]
DLP1-3RACE	5′-CTCGATCAGGCAGTGGAACT-3′
DLP2-3RACE	5′-CCTGGATACGCACTGGAACT-3′
DLP3-3RACE	5′-GCWACCTGTGACCTSTTG-3′
DLP4-3RACE	5′-TTYAARCCAGTAGARAARTTY-3′
DLP1-5RACE	5′-TGCGCAGGCGGCATGACCCACYTTGAA-3′
DLP2-5RACE	5′-TGCGCAGGCGGCATGACCCACYTTGAA-3′
DLP3-5RACE	5′-AACGGCTCGATCATCGCAC-3′
DLP4-5RACE	5′-GTCGACAACTAAAGGTTCAGACAAAC-3′
Actin-qRT-F	5′-AAGGACTCGTACGTGGGTG-3′
Actin-qRT-R	5′-GCCAACCGTGAGAAGATG-3′
DLP4-qRT-F	5′-GCAACCTGTGACCTSTTG-3′
DLP4-qRT-R	5′-GTGCGATGATCGAGCCGTT-3′

[a] The letters Y, R, W, and S in degenerate primers mean nucleotide mixtures of CT, AG, AT, and GC, respectively.