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Characterization and cDNA Cloning of a Cecropin-Like Antimicrobial Peptide, Papiliocin, from the Swallowtail Butterfly, *Papilio xuthus*

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Cecropin is a well-studied antimicrobial peptide that is synthesized in fat body cells and hemocytes of insects in response to hypodermic injury or bacterial infection. A 503 bp cDNA encoding for a cecropin-like peptide was isolated by employing annealing control primer (ACP)-based differential display PCR and 5′-RACE with immunized *Papilio xuthus* larvae. The open reading frame of the isolated cDNA encoded for a 62-amino acid prepropeptide with a putative 22-residue signal peptide, a 2-residue propeptide, and a 38-residue mature peptide with a theoretical mass of 4060.89 Da. The deduced amino acid sequence of the peptide evidenced a significant degree of identity with other lepidopteran cecropins. This peptide was named papiliocin. RT-PCR results revealed that the papiliocin transcript was detected at significant levels after injection with bacterial lipopolysaccharide (LPS). On the basis of the deduced amino acid sequence of papiliocin, a 38-mer mature peptide was chemically synthesized *via* the Fmoc method, and its antimicrobial activity was analyzed. The synthetic papiliocin peptide evidenced a broad spectrum of activity against fungi, Gram-positive and Gram-negative bacteria, and also evidenced no hemolytic activity against human red blood cells.

INTRODUCTION

Antimicrobial peptides perform pivotal functions in the systemic immune responses of insects against invading pathogens including bacteria, fungi, and viruses. They are produced rapidly in the fat bodies and other specific tissues of insects after septic body injury or immune challenge, and subsequently released into the hemolymph to act against microorganisms (Hoffman et al., 1999). In recent years, a large number of antimicrobial peptides, which exert broad-spectrum antimicrobial effects, have been identified and characterized from a variety of insects, including the Lepidoptera, Hymenoptera, Diptera, and Coleoptera. It has been generally accepted that insect antimicrobial peptides suppress bacterial infections, but have minimal toxic and allergic side effects in the host cells (Bulet et al., 1999; Lee et al., 2009). These peptides are classified on the basis of their amino acid sequences and secondary structures (Boman, 1995; Bulet et al., 1999). These are the cecropin-like peptides, insect defensins, proline-rich peptides, glycine-rich peptides, and lysozymes.

Cecropin was initially isolated from bacterially challenged *Hyalophora cecropia* pupa (Steiner et al., 1981). Since then, a number of cecropin-like peptides have also been identified in the Lepidopteran, Dipteran and Coleopteran insects, such as *Bombyx mori* (Morishima et al., 1990), *Drosophila melanogaster* (Kylsten et al., 1990), *Musca domestica* (Liang et al., 2006), *Acalolepta luxuriosa* (Saito et al., 2005) and *Helicoverpa armigera* (Li et al., 2007). Furthermore, these peptides have also been reported in tunicate (Zhao et al., 1977) and ascarid nematodes (Andersson et al., 2003), thereby suggesting the broad distribution of this peptide family. The majority of these peptides are synthesized as precursors consisting of 62 to 64 amino acid pre-propeptides, and the mature peptides were generated via post-translation processing (Boman et al., 1989). The active mature cecropins without cysteine residues are typically 35 to 39 amino acids in length, and form two amphipathic α-helices connected by a hinge region (Cociancich et al., 1994; Saito et al., 2005). They evidence broad-spectrum activity against both Gram-positive and Gram-negative bacteria, as well as certain fungal and metazoan parasites (Chalk et al., 1995; DeLucca et al., 1997).

In this study, we report the cDNA cloning and characterization of a novel gene encoding for the cecropin-like antimicrobial peptide, papiliocin, from the South Korean swallowtail butterfly, *Papilio xuthus*. We also synthesized the mature form of papiliocin peptide and assessed its antimicrobial activity.

MATERIALS AND METHODS

Immunization and collection of hemolymph

Final-instar *Papilo xuthus* larvae were used for immune challenge. A volume of 20 ul of lipopolysaccharide (LPS, sigma, 0.5

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mg/ml) dissolved in sterile insect Ringer was injected dorsolaterally into the hemocoel using 1 ml disposable syringes. For the antibacterial activity assay, hemolymph samples were directly collected 24 h after injection into sterile tubes.

Annealing control primer (ACP)-based differential display

Total RNA were extracted from whole larvae at 12 h post-injection or from untreated larvae using Trizol reagent (Invitrogen, USA) and then treated for 15 min with DNase I at 37°C to remove any residual genomic DNA. RNA integrity was verified by ethidium bromide gel staining and the quantities were determined via spectrophotometry. Total RNA was employed for the synthesis of first-strand cDNA by reverse transcriptase. Reverse transcription was conducted for 1.5 h at 42°C in a final reaction volume of 20 μl containing 3 μg of purified total RNA, 4 μl of 5x reaction buffer (Promega, USA), 5 μl of dNTPs (each 2 mM), 2 μl of 10 μM cDNA synthesis prime dT-ACP1, 0.5 μl of RNasin® RNase Inhibitor (40 U/μl; Promega, USA), and 1 μl of M-MLV reverse transcriptase (200 U/μl; Promega, USA). First-strand cDNA samples were diluted via the addition of 80 μl of ultra-purified water. For the screening of differentially expressed genes (DEG) in immune-challenged *p. xuthus* larvae, we utilized an ACP-based GeneFishing PCR kit (Seegene, Korea). In brief, ACP-based PCR was conducted using 120 pairs of arbitrary ACPs and dT-ACP2 to synthesize the second-strand cDNA. PCR analysis was conducted in a final 20 μl volume containing 4 μl of diluted first-strand cDNA, 1 μl of dT-ACP2 (10 μM), 1 μl of 10 μM arbitrary ACP, and 10 μl of 2× Master Mix (Seegene, Korea). The PCR protocol for second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After the completion of second-strand DNA synthesis, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 s, followed by 65°C for 40 s, 72°C for 40 s, followed by a 5 min final extension at 72°C. The amplified PCR products were separated in 2% agarose gel and stained with ethidium bromide. Differentially expressed bands were extracted and subcloned into pGEM-T easy vector (Promega, USA) and subjected to DNA sequencing. The sequence data of DNA fragments was analyzed via a BLAST search (http://www.ncbi.nlm.nih.gov).

5′-rapid amplification of cDNA ends (RACE)

To characterize the entire open reading frame (ORF) of the DEG 25 fragment, which was confirmed as the cecropin-like peptide, a 5′-RACE (5′-rapid amplification of cDNA ends) reaction was conducted using an available kit (Invitrogen, USA), resulting in the cloning of a full-length cDNA. Two antisense primers (5′-TATTCATCCTTTGACGACAGTTGC-3′ and 5′-AGAGTTGCCGCTTGTCCAACTACC-3′ as GSP1 and GSP2, respectively) were synthesized on the basis of the confirmed DEG 25 sequence. First-strand cDNA was synthesized with the gene specific primer (GSP1) from the total RNA of the immunized larvae. The addition of a polymeric C tail to the first-strand cDNA, PCR amplification using GSP1 and an abridged anchor primer, and subsequent nested PCR amplification using GSP2 and abridged universal amplification primer, were all conducted in accordance with the manufacturer's recommended protocols. The resultant PCR product (200 bp) was then subcloned into the pGEM-T easy vector (Promega, USA) and subjected to DNA sequencing.

Reverse transcription PCR (RT-PCR)

Total RNA was extracted from whole larvae at 0 h, 12 h, and 24 hours' post-injection using Trizol reagent (Invitrogen, USA), and then treated for 15 min with DNase I. The treated RNA samples (1 μg per sample) were employed for cDNA synthesis with oligo (dt) primer and SuperScript III reverse transcriptase (Invitrogen, USA). PCR amplifications were conducted using a pair of specific primers (5′-ATGGGATGGAAGATATTCAAGAAA-3′ and 5′-TCATCCTTTGACGACAGTTGCCGC-3′) under the following conditions: 94°C for 30 s, 62°C for 45 s, and 72°C for 1 min for 25 cycles with a final 10-min extension at 72°C. The amplified PCR products were electrophoresed on 1% agarose gel.

Peptide synthesis

The mature form of papiliocin (38-mer peptide) was synthesized with an automated solid-phase peptide synthesizer at the peptide synthesis facility, PepTron Inc. (Korea). The synthetic peptide was then re-purified via reverse-phase high-pressure liquid chromatography (RP-HPLC) using a Shiseido Capcell Pak C18 column. Elution was conducted with a water-acetonitrile linear gradient (0-80% of acetonitrile) containing 0.1% (v/v) trifluroacetic acid (TFA). The correct identity of the synthetic peptide was verified via ESI mass spectrometry (Platform II, Micromass, UK) and MALDI-TOF mass spectrometry (Voyager-DESTR, Applied Biosystem, USA).

Measurement of antimicrobial activity

The minimum inhibitory concentration (MIC) of the synthetic peptide against several types of bacteria were determined via a liquid growth inhibition assay with serial dilutions of peptide via the method recommended by the British Society for Antimicrobial Chemotherapy (BSAC), with some modifications. In brief, the bacteria were grown overnight at 37°C and shaken at 200 rpm in Tryptic Soy Broth (TSB, Difco). The cultures were then washed twice with autoclaved 10 mM sodium phosphate buffer (pH 7.4), and resuspended in fresh TSB to a final concentration of 2×10^4 CFU/ml. The stock peptide solution was prepared at 640 μg/ml in 0.01% acetic acid and then serially diluted twofold to 2 μg/ml. Aliquots (10 μl) from each of the dilution peptides were distributed to each well of 96-well polypropylene microtiter plates, then inoculated with 90 μl of bacterial suspension (2×10^4 CFU/ml) in TSB. After 18 h of incubation, the inhibition of growth was determined by measuring the absorbance at 600 nm with a microplate reader. The MIC values were expressed as intervals (A-B), in which A represents the highest concentration tested at which bacteria are still growing and B is the lowest concentration required for complete growth inhibition.

Hemolytic activity analysis

The hemolytic assay for human red blood cells was conducted for synthetic papiliocin, and melittin, a hemolytic peptide from bee venom, was used as a positive control peptide. The erythrocytes were washed three times in buffer (10 mM PBS, 150 mM NaCl, pH 7.4), and centrifuged at 1,000 × *g* and 4°C for 10 min. A 100 ul cell suspension diluted with 10 mM PBS (final concentration around 8%) was mixed with 100 ul of varying amounts of the peptide stock solution in 96-well microtiter plates, then incubated for 1 h at 37°C. After 5 min of centrifugation at 1,000 × *g* and 4°C, 100 ul of the supernatants were transferred to 96-well microtiter plates and the optical density was determined at 405 nm. The values for 0% and 100% hemolysis were determined using erythrocyte suspensions incubated in 10 mM PBS or Triton X-100, respectively.

RESULTS AND DISCUSSION

Isolation of immune inducible gene fragment from *P. xuthus*

We first compared the antibacterial activity of immune challenged

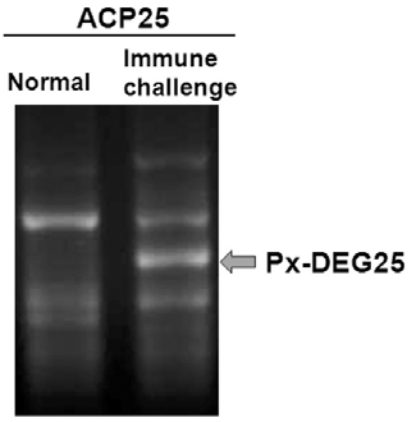

Fig. 1. ACP25 products of ACP system from normal and immune-challenged *P. xuthus* larvae were visualized via agarose gel electrophoresis and ethidium bromide staining. Candidate differential expressed gene (DEG25) is indicated with arrow.

and non-immune hemolymph from the larvae of the swallowtail butterfly, *P. xuthus,* against *E. coli* ML35 (data not shown). The results demonstrated that the immune-challenged larval hemolymph yielded higher activity than was seen with the non-immune hemolymph. In order to isolate the immune-related gene from the *P. xuthus* larva, we employed annealing control primer (ACP)-based differential display PCR. By comparing the band intensities of amplified cDNA fragments between the immune-challenged larvae and the non-immune larvae, we selected 26 fragments with different expression levels (data not shown). The 26 amplified bands were eluted from 2% agarose gel and subjected to DNA sequencing. According to the BLAST and FASTA homology search of sequence data for the identification of their gene annotations, one of these differently expressed genes (from DEG25, Fig. 1A) evidenced a high degree of similarity to the carboxyl terminal region of *Hyalophora cecropia* cecropin A antimicrobial peptide, and thus it was named papiliocin (derived from *Papilio xuthus*). The length of the papiliocin cDNA fragment was 271 bp including the poly (A) tail (gray color highlighted from 233 bp to 503 bp in Fig. 1B).

Cloning and sequencing of papiliocin cDNA

After the cloning and sequence verification of the papiliocin cDNA fragment, specific primers were synthesized on the basis of its nucleotide sequence for 5′-RACE (Rapid Amplification of cDNA Ends) extension, which eventually resulted in the amplification of its full-length cDNA, which consisted of 503 nucleotides. This cDNA contained an open reading frame of 192 nucleotides encoding for 62 amino acid residues (Fig. 1B). Its cDNA sequence also harbored a 93 bp 5′-untranslated region and a 221 bp 3′-untranslated region containing a putative polyadenylation consensus signal (AATAAA). The putative translational start site sequence (AAAATGAAT) conformed well to the Kozak consensus sequence (AAAATGAAG) already characterized in eukaryotic mRNA (Kozak et al., 1984). SignalP analysis showed that the cleavage site for the potential signal peptide was predicted between 22-Ala and 23-Ser. Further, a cleavage site between 24-Pro and 25-Arg was also predicted by the alignment of the amino acid sequence of this peptide with that of the cecropin A of several insects. These sequence analyses suggested that the putative mature peptide of papiliocin was composed of 38 amino acids, with a theoretical mass of 4060.89 Da (Fig. 1B). The deduced amino acid sequence comparison showed that the newly isolated papiliocin precursor from the swallowtail butterfly was highly similar to the cecropin A-type antimicrobial peptides from other lepidopteran insects (62-70% identity, Fig. 2). Like many insect cecropins, papiliocin also harbors a glycine residue for C-terminal amidation at the C-end, which suggests potential amidation. The majority of insect cecropin-like peptides, with the notable exception of *Bombyx mori*'s cecropin D (Hara et al., 1994), harbor amidated C-termini (Cociancich et al., 1994; Saito et al., 2005). As previously described in *H. cecropia* cecropin A (Callaway et al., 1993), the peptide activity was enhanced significantly by this C-terminal amidation. On the other hand, the amidation of *Anopheles gambiae*'s glycine-extended cecropin did not influence the antimicrobial activity (Vizioli et al., 2000). However, the amidation of glycine residue was previously theorized to protect the cecropin-like peptide against carboxypeptidase digestion (Liang et al., 2006). The papiliocin precursor harbors dipeptides (Ser-Pro) between the signal and mature peptide alignment, which is not conserved in terms of the tetrapeptides (Ala-Pro-Glu-Pro) in cecropin A and dipeptides (Ala-Pro) in cecropin D.

Expression of papiliocin gene

To confirm the expression of the *P. xuthus* papiliocin gene at the transcriptional level, RT-PCR analysis was conducted using total RNA prepared from whole larvae at different time-points after LPS injection (Fig. 3). Our results demonstrated that transcripts of the papiliocin gene were detected at extremely low levels in the larvae, but the transcript abundance increased significantly after immunization. The papiliocin gene transcript peaked at 12 h to 24 h after LPS injection. This indicated that the expression of the papiliocin gene was induced rapidly after challenge. In the *H. cecropia* larvae, the transcripts of cecropin

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  1 CGGATCCAGACGCTGCGCTTTGCTGGCTTTGATGAAAATACAGCAACAGTCTTTCAACGC
 61 TTTCATATTAAAATATTACATTCTTTTTTTAAAATGAATTTTGGTAAAATTTTATTTTTC
                                    M  N  F  G  K  I  L  F  F
121 GTAATGGCTTGTTTGGCTGCTCTTAGTTTAACAACGGCCAGTCCTAGATGGAAGATATTC
     V  M  A  C  L  A  A  L  S  L  T  T  A↑S  P  R  W  K  I  F
181 AAGAAAATTGAAAAAGTTGGTAGAAACGTTCGCGATGGTATCATCAAAGCGGGACCAGCA
     K  K  I  E  K  V  G  R  N  V  R  D  G  I  I  K  A  G  P  A
241 GTGGCGGTAGTTGGACAAGCGGCAACTGTCGTCAAAGGATGAATATTACCGTGTGTTTCC
     V  A  V  V  G  Q  A  A  T  V  V  K  G  *
301 GAGACTTAAATATCTGTGTAAAACATGTCATCCTTGTCTTTCATCTTTGACAAAACAGAG
361 ATAACAATATGTTTTAAACGGGGATATTATTCTCAGAAACCCATGGTTACTTCAAACTAG
421 TCTACATAATTACTCTAAGTTTTAATTAAGTTATTGTAAATACTGCCAATAAATTCTTAC
481 CTCATCAAAAAAAAAAAAAAAAA
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Fig. 2. Nucleotide and deduced amino acid sequence of the cDNA encoding for the cecropin-like peptide, papiliocin. The predicted signal peptide cleavage site is indicated with an arrow. The putative mature peptide is underlined and an asterisk indicates the stop codon. The polyadenylation signal (AATAAA) is boxed. The RT-PCR product (DEG25) is highlighted in grey.

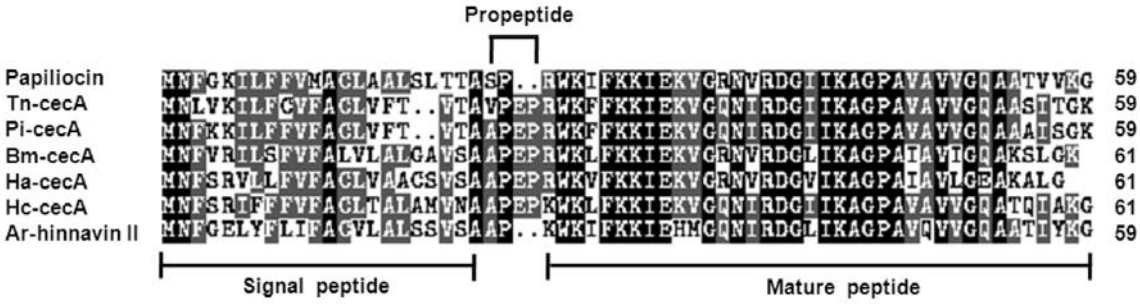

Fig. 3. Comparison of the papiliocin precursor amino acid sequence with those of other typical cecropin A precursors from lepidopteran insects. Multiple sequence alignment was conducted using the CLUSTAL W program. Tn-CecA, cecropin A from *Trichoplusia ni* (ABV68873); Bm-cecA, cecropin A from *Bombyx mori* (BAA31507); Ha-cecA, cecropin A from *Helicoverpa armigera* (EU041763); Hc-cecA, cecropin A from *Hyalophora cecropia* (AAA29186); Ar-Hinnavin II, *Artogeia rapae* hinnavin II (AAT94287).

Table 1. Antimicrobial activity of synthetic papiliocin

Microorganism	MIC (μM)	
	Papiliocin	Melittin
Gram-negative bacteria		
Escherichia coli	4	1
Klebsiella pneumoniae	0.5	2
Pseudomonas aeruginosa	1	2
Gram-positive bacteria		
Staphylococcus aureus	8	2
Enterococcus faecalis	8	2
Bacillus subtilis	8	2
Yeast fungus		
Candida albicans	10	5

The antimicrobial activity assay was replicated three times.

Table 2. Hemolytic activity of synthetic papiliocin against human red blood cells

Peptides	% Hemolysis (μM)				
	50	25	12.5	6.25	3.13
Papiliocin	0	0	0	0	0
Melittin	100	100	100	100	98

The hemolysis assay was replicated three times.

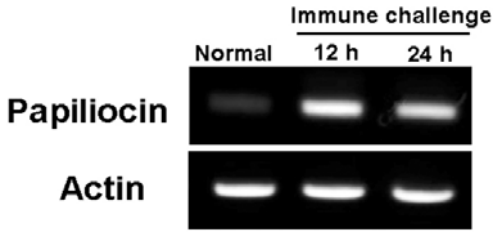

Fig. 4. RT-PCR analysis of papiliocin gene transcription in native larvae and the LPS-challenged larvae 12 h and 24 h post-injection. The gene for *Actin* was used as a control.

A and B were detected within 2 h after bacterial challenge (Gudmundsson et al., 1991).

Antimicrobial and hemolytic activity of synthetic papiliocin

To assess the antimicrobial effects of papiliocin, we synthesized papiliocin (38-mer peptide) using an automated solid-phase peptide synthesizer. The synthetic peptide was identified using an ESI mass spectrometer and a MALDI-TOF mass spectrometer (data not shown). The molecular mass of synthetic papiliocin was measured at 4060.5 Da, which is very consistent with the predicted molecular mass (4060.89 Da) of the mature peptide from the cDNA sequence. The antimicrobial activity of synthetic papiliocin was analyzed via a liquid growth inhibition assay by serial dilution against several Gram-positive and Gram-negative bacteria and *Candida albican*. The minimum inhibitory concentration (MIC) values are summarized in Table 1. As expected, papiliocin proved to be active against all microorganisms tested, with MIC values of 0.5-10 μM. It evidenced more profound growth-inhibitory effects on *E. coli* (MIC value of 0.5 μM), but these effects were less strong than those of melittin. The present antimicrobial spectrum data are consistent with the activity data reported for insect cecropin-like peptides (Li et al., 2007; Powers and Hancock, 2003). Papiliocin evidenced no hemolytic activity against human red blood cells, even at high concentrations (Table 2), thus suggesting that the peptide is not detrimental to eukaryotic cells.

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