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Identification of An Antibacterial Gene by Differential Display from Lipopolysaccharide-Stimulated Dung Beetle, *Copris tripartitus*

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A novel beetle antimicrobial protein from stimulated *Copris tripartitus* and the corresponding gene were isolated in parallel through differential display-PCR and expression in *Escherichia coli*. To find cDNA clones responsible for bacteria resistance, the suppression subtractive hybridization and GeneFishing differentially expressed genes system were employed in the dung beetle, *Copris tripartitus* immunized with lipopolysaccaride. One cDNA clone from eight subtracted clones was selected through dot blot analysis and confirmed by northern blot analysis. The 516-bp, selected cDNA clone was determined by 5' and 3' rapid amplication of cDNA ends and cloned into the GST fusion expression vector pGEX-4T-1 for expression of the protein. The expressed protein was predicted 14.7 kDa and inhibited the growth of gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*. These results implied that the expressed protein is related to immune defense mechanism against microorganism.

Key words: *Copris tripartitus*, Subtractive hybridization, GeneFishing, Antimicrobial protein

Introduction

Anti-microbial peptides (AMPs) are ubiquitous in nature, having been identified in and isolated from a diversity of sources within the living organisms (Bulet *et al*., 2004; Hancock and Lehrer, 1998). Over the past two decades, numerous antibiotic peptides have been studied from a wide variety of insects, amphibians and mammals including humans (Boman, H. G., 1995; Nicolasand Mor, 1995; Steiner *et al*., 1981). Even though the precise mechanism of action of these peptides is not fully understood, the activity of many AMPs depends mainly on membrane permeabilization or lysis, and several mechanisms of action have been proposed (Zelezetsky and Tossi, 2006). Living organisms are exposed daily to microbial infections and pathogens, and in order to defend themselves against the unclean environment, they have developed potent defensive mechanism that are part of innate and adaptive immunity. AMPs play important roles in the innate host defense mechanisms of most living organisms, including plants, insects, amphibians and mammals (Koczulla and Bals, 2003; Lehrer *et al*., 1993; zasloff, 2002). There has been particular interest in these peptides as a new class of antibiotics with potential clinical value. In addition, they are known to possess potent antibiotic activity against bacteria, fungi, and even certain viruses (De Lucca and Walsh, 1999).

Previous works in insect AMPs are classified into four major type by structure and size: (i) peptides with an a-helical conformation (insect cecropins, marginins, etc.), (ii) cyclic and open-ended cyclic peptides with pairs of cysteine residues (defensins, protegrin, etc.), (iii) peptides with an over-representation of some amino acids (proline rich, glycine rich, histidine rich, etc.), (iv) lysozymes. In recent years, a series of novel AMPs have been discovered as processed forms of large proteins. Despite the extreme diversity in their primary and secondary structure, all natural AMPs have the *in vitro* particularity to affect a large number of microorganisms (bacteria, fungi,

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yeast, virus, etc.) with identical or complimentary activity spectra.

In order to develop of peptides for clinical use, a cost effective and scalable method is required. To date, various expression systems, such as prokaryote (Skosyrev *et al.*, 2003; Xu *et al.*, 2007), yeast (Almida *et al.*, 2001; Jin *et al.*, 2006; Li *et al.*, 2005) and insect cells (Yamada *et al.*, 1990) have been used for production of recombinant antibacterial proteins. It is difficult that the expression of AMPs bring about cytotoxicity to host cells, sensitivity to proteolytic degradation and low expression level (Skosyrev *et al.*, 2003). In this study, we focused on how we have approached to seek a new AMPs candidate, recombinant novel protein from expressed in *E. coli*.

Materials and Methods

Experimental animal and immunization

Dung beetle, *Copris tripartitus* Waterhouse, was collected in the field of the Jeju Island, South Korea, and cultured in a room kept at 25°C (Bang *et al.*, 2003). Third-instar larvae were cooled on ice and injected with lipopolysaccaride (LPS, 2 μg) suspended in a saline buffer. The larvae were kept at 25°C for 24 hrs and then, were grinded in liquid nitrogen.

ACP-based GeneFishing™ PCR

Differentially expressed genes (DEGs) were screened by annealing control primers (ACP) based PCR methods using the GeneFishing DEGs kits (Seegene, South Korea). After total RNA isolation from third-inster larvae, RT-PCR is conducted using oligo dT-ACP to synthesize the first-strand cDNAs from normal and immunized *Copris tripartitus*. The second-strand cDNAs and subsequent PCR amplication were conducted with an arbitrary ACP and dT-ACP2.

RNA extraction and northern blot analysis

Total RNAs were extracted from third-instar larvae 24 hrs after immunization using SV total RNA isolation system (Promega, USA). Each aliquot of total RNA (10 μg) was separated on 1.2% (w/v) agarose, 3% (v/v) formaldehyde denaturing gel (McMaster and Carmichael, 1977), and transferred to nylon membrane (Amersham Biosciences). The cDNA probe was labeled with [α-^{32}P] dCTP using Prime-It II random primer labeling kit (Stratagene, USA) according to the manufacturer's instruction. Membrane was hybridized with a cDNA probe at 65°C for overnight, and analyzed by autoradiography. As an internal marker, 28S rRNA was visualized by ethidium bromide staining.

Dot blot hybridization assay and DNA sequencing

The cDNA clones, differentially expressed genes were arrayed on to Hybond-N membrane (Amersham Biosciences, Sweden) using 96-well format dot blotter (Bio-RAD, USA) after denaturation. For preparing probes, mRNAs were isolated from normal and immunized larvae. Then, ^{32}P-labeled cDNA probes were generated by reverse transcription of 0.5 ~ 1.0 μg of each poly(A)$^+$ RNA sample in the presence of [α-^{32}P]dCTP. Each cDNA probe was then hybridized with the membrane at 65°C. A hybridization solution containing 50% (w/v) formamide, 5 × SSC, 10 × Denhardt's solution (0.2% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone), 25 μg/ml sonicated salmon sperm DNA, and 50 mM sodium phosphate (pH 7.0) was used. After hybridization, membranes were washed for 30 min with increasing stringency, from 2 × SSC and 0.1% SDS to 0.1 × SSC and 0.1% SDS. After a high-stringency wash, membranes were then exposed to X-ray film (AGFA, Germany) for 1 or 3 days at −70°C. Sequencing of clone DNA confirmed by dot blot analysis was performed using an automatic sequencer (Applied Biosystems, model, Foster city USA). The sequences were compared with the NCBI protein database.

Construction of expression vectors

cDNA sequences were amplified for vector construction using the primers containing *BamHI* and *XhoI* sites: the forward primer FP-SC-23-3 (5'-AAAGGATCCTTACCATATCCAGAAATTGT) and the reverse primer RP-SC-23-3 (5'-AAACTCGAGTTAAAAGTCTTTCCTAACAT), respectively. PCR was executed as follows: preheating at 94°C for 5 min, and 25 cycles at 94°C for 30 sec, 60°C for 40 sec, 72°C for 60 sec, followed by an elongation at 72°C for 10 min. PCR products were visualized on a 1.2% ethidium bromide stained gel, purified from the gel by using QIAquick gel extration kit and then cloned into a pGEX-4T-1 vector using pGEM®-T Easy Vector System I (Promega, USA). The nucleotide sequence of pGEX-4T-1/SC-23-3 was confirmed by sequencing (ABI 310 PRISM™).

Expression and purification of fusion protein pGEX-4T-1/SC-23-3

Recombinant proteins were expressed in *E. coli* strain BL21. A single bacterial colony was inoculated in rich liquid LB medium containing ampicillin (final concentration, 100 μg/ml) and grown at 37°C (Hoffman *et al.*, 1995). Protein expression was induced by the addition of isopropyl-β-D-thiogalactoside (0.1 mM IPTG) with further growth at 37°C for 3 hrs. Cell were harvested by centrifugation (3000 g, 15 min). Cell pellet (1 g) was resuspended in 5 ml protein extraction reagent (Bug-

Buster® Protein Extraction Reagent, Novagen). Following Benzonase nuclease treatment, resuspended cells were incubated on a shaking at a mild condition for 20 min at room temperature. After incubation, the supernatants were recovered by centrifugation at 12,000 g for 20 min to remove the insoluble fraction. The supernatant was used for purification. Purification was performed essentially according to the instruction of GSTrap 4B column (GE Healthcare Bio-Sciences AB, Sweden).

Assay of antibacterial activity and hemolysis

Bacteria were grown to mid-log phase in 10 ml of full-strength (3%, w/v) trypticase soy broth (TSB) (BD Biosciences). The microorganisms were washed once with PBS, and then 2×10^4 bacterial colony-forming units (CFU) were added to 10 ml of the underlay-agarose gel (0.03% TSB, 1% low electroendosmosis-type agarose (Sigma), 1 mM sodium citrate, and 9 mM sodium phosphate (pH 7.4). The underlay was poured into a 100×15 mm dish. After agarose solidification, 3-mm-diameter wells were punched, and 5 μl of test peptide was added to each well. Plates were incubated at 37°C for 3 hrs to allow diffusion of the peptides. The underlay gel was then covered with 10 ml of molten overlay (6% TSB and 1% low electroendosmosis-type agarose in distilled H_2O). The antimicrobial activity of a peptide was visualized as a zone of clearing around each well after incubating 18~24 hrs at 37°C (Lehrer *et al*., 1991).

Erythrocytes freshly prepared from mouse blood were suspended in 10 mM Na-phosphate buffer (pH 7.4) containing 0.9% NaCl to a 10% suspension and then rinsed three times by centrifugation at 1000 g for 10 min at 4°C, and resuspended in PBS. Red blood cells (RBC) of the suspension (4% in PBS, v/v) were plated in 96-well microtiter plates, after which an equal volume of the peptide solution dissolved in PBS was added. Plates were incubated for 1 h at 37°C and then centrifuged at 1000 g for 5 min. Aliquots (100 μl) of the supernatant were then transferred to a new 96-well microtiter plates, where released hemoglobin was monitored using microplate ELISA reader (Molecular Devices) by measuring the absorbance at 405 nm. The degree of hemolysis was assessed by hemoglobin content in the supernatant of the incubated samples. Zero and total hemolysis were determined in PBS and 0.1% Triton X-100, respectively.

Results and Discussion

Differentially expressed genes in response to LPS treatment

To identify genes that are differentially expressed, we compared the mRNA expression profile of *C. tripartitus* larvae untreated and treated with LPS (2 μg). We extracted mRNAs from the larvae with or without LPS treatment, and subjected them to ACP RR-PCR analysis using a combination of 10 arbitrary primers (ACP_{16}-ACP_{25}) and oligo (dT) primer (dT-ACP2) (data not shown). After synthesizing cloned DNA from 8 DEGs, it practiced the sequencing homology analysis using BLAST search. It came to reveal 7 known genes and one unknown gene (Table 1). Differential expressions of eight genes were confirmed by dot blot hybridization (Fig. 1A). Unknown cDNA (SC-23-3) showed differentially expressed pattern through dot blot analysis and the clone was confirmed by northern blot (Fig. 1B).

Table 1. Sequence homology analysis of differentially expressed cDNA clones using GeneFishing PCR

Representative Clone (bp)	Homology found	Acc. No./score/E-value
SC-16-3 (617)	Apis mellifera similar to CG11638-PA	XM_392226/95/2e-15
SC-17-5 (210)	Sus scrofa mitochondrial solute carrier family 25 member 6 (SLC25A6)	NM_214418/54/1e-04
SC-19-5 (210)	Drosophila melanogaster CG7773-PA (fidipidine)	NM_079036/36/6.8
SC-19-6 (618)	Sus scrofa mitochondrial solute carrier family 25 member 6	NM_214418/54/4e-04
SC-20-1 (367)	Mus musculus BAC clone RP23-444O19 from 13	AC006979/40/3.2
	Homo sapiens BAC clone GS1-85B12 from 7	AC115299/40/3.2
	Human DNA sequence from clone RP11-470M1 on chromosome 13 Contains GSSs	AL158064/40/3.2
SC-20-4 (528)	Oryza sativa (japonica cultivar-group) chromosome 10	AE017104/42/1.2
SC-23-2 (398)	Homo sapiens chromosome 18, clone XXfos-83061E10	AC139070/40/3.4
	Homo sapiens chromosome 18, clone CTD-2382L18	AC137738/40/3.4
SC-23-3 (328)	Unknown	Unknown

The name, such as "SC-23-3" of differentially expressed cDNA was coined after stimulated *Copris tripartitus* (SC), a number used ACP primer and a number of electrophoresis band.

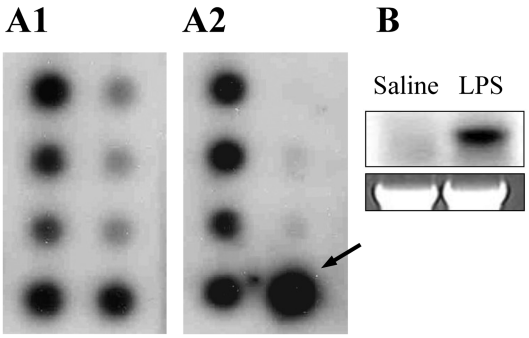

Fig. 1. Gene expression analysis using dot and northern blot. Two identical membranes were hybridized with radioactive labeled probes prepared from saline (A1) and LPS (A2) injected *Corpris tripartitus* larvae. One of the differentially expressed clones was marked with arrow (SC-23-3 in Table 1). Its expression was confirmed by northern blot (B).

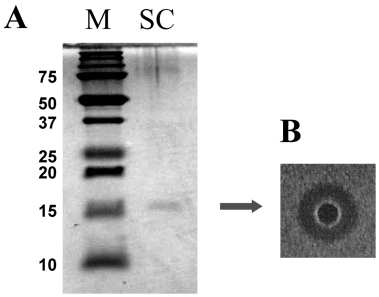

Fig. 3. SDS-PAGE analysis of purified the fusion protein and RDA. Purified fusion protein (0.1 μg) was analyzed on 13.5% SDS-PAGE and stained with Coomassie brilliant blue (A). M and SC represent protein molecular maker and purified fusion protein. B shows its capacity of growth inhibition against gram-negative bacteria (*E. coli*).

Nucleotide and deduced amino acid sequencing.
A full-length nucleotide sequence of the unknown cDNA clone (SC-23-3) was determined by 5' and 3' rapid amplication of cDNA ends (RACE) (Fig. 2).

The full cDNA coding sequence of this gene contains 649 bp, in which the initiation codon ATG is located at nucleotide number 58 and the stop codon TAA at 489, encoding a protein of 169 amino acids (Fig. 1). The calculated molecular mass of this gene based on the DNA sequence is 52,149 Da (pI=5.8). We also identified 85 extra nucleotides at the 5'-untranslated region, and the TATAAA consensus pre-adenylation signal from downstream of the stop codon. Poly (A) tail of this gene is located at 25 nucleotides downstream from the pre-adenylation signal. This is consistent with a typical pre-adenylation signal which is quite often present at 11~30 nucleotide upstream from the poly (A) tail of other genes (Fig. 1). The nucleotide sequence showed no significant homology to reported nucleotides.

Expression and purification of the fusion protein.
To assess the expression of the fusion protein, the recombinant expression vector was transformed into competent *E. coli* BL21 (DE3) cell. Upon IPTG induction, GST-4T-1/SC-23-3 was expressed as a fusion protein. *E. coli* cells were induced at an OD_{600}=0.5~1.0 with 0.1 mM IPTG and incubated for 3 hrs at 37°C. The fusion protein was extracted by BugBuster® system and purified using GSTrap 4B affinity column. It gave a single band about 15~16 kDa (Fig. 3A), which was close to the size of the fusion protein that has a calculated molecular weight of 14.7 kDa. In our works, the large proportion of the fusion protein was insoluble, thus was difficult to purify. There

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AGTTCACTTACAGTTAACAACCAGAACAGTTCTAATTAAACAAATTCATATTTGAAAATGGGTCCAATCCTAATC
                                  ▼                   M  G  P  I  L  I
GTTGCTGTTACGTGCATCGCCTCCATGGCTGCTGCTTTACCATATCCAGAAATTGTCGAAAATGAAGCTGGTGAT
 V  A  V  T  C  I  A  S  M  A  A  A  L  P  Y  P  E  I  V  E  N  E  A  G  D
GTCTTCCAACTGGTTCCGTTGGAAAGAGTAAGGAGGGGCAGCGTATATGGTAACGTTGATATAACAGATCCCGGT
 V  F  Q  L  V  P  L  E  R  V  R  R  G  S  V  Y  G  N  V  D  I  T  D  P  G
AGACTAATTGTTGGTGCTAAAGGTACTCCAATCGATAATGCTAATCACAGGTGGGATACACATGTTTTTGCCACC
 R  L  I  V  G  A  K  G  T  P  I  D  N  A  N  H  R  W  D  T  H  V  F  A  T
GACAACATCCGTCATCACTCTCCTTTGACAGTTGGCGGAGAAGCCAGTTACTTACATAAACCTACTGGAAGTACT
 D  N  I  R  H  H  S  P  L  T  V  G  G  E  A  S  Y  L  H  K  P  T  G  S  T
GCACACCTAGCTGGAACTCGTACCGAACAGTGGGGCACTGACCTGAAAGCATCTGGACGATACAATTTCTTCCAA
 A  H  L  A  G  T  R  T  E  Q  W  G  T  D  L  K  A  S  G  R  Y  N  F  F  Q
GATAAAACTACAAACGCAAATGTTGAGGCATTTGCTTCTAAACACATAGGAGGATTTCCCGGAAATCAGCCTACT
 D  K  T  T  N  A  N  V  E  A  F  A  S  K  H  I  G  G  F  P  G  N  Q  P  T
GATTACGGTGTTATGTTTAATGTTAGGAAAGACTTTTAAATTAATTTGAATTATTACTTTATTTATTTCTTGTAT
 D  Y  G  V  M  F  N  V  R  K  D  F  ***
TTTTTAAATAAAGCAATTTGTTATATAAAAAAAAAAAAAAAAA
```

Fig. 2. Nucleotide and deduced amino acid sequence of novel gene (SC-23-3). Complete sequence of cDNA was obtained by a combination of RT-PCR, based on 5'- and 3'-RACE. The predicted signal peptide cleavage site is marked with an arrowhead.

Table 2. Antibacterial activity of SC-23-3 fusion protein

	Bacteria	Activity	
		0.1 mg	0.2 mg
Gram –	*Escherichia coli*		
	Pseudomonas aeruginosa		
Gram +	*Micrococcus luteus*		
	Staphylococcus aureus		
Resistant	MRSA[1]		
	VRE[2]		

Protein was applied in 3 mm well. The inhibition zones: +, < 5 mm; ++, 5 - 7 mm; +++, > 7 mm; –, dose not observed.
[1]MRSA, methicillin-resistant staphylococcus aureus;
[2]VRE, vancomycin-resistant enterococcus.

was a small amount of fusion protein in the soluble fraction. About 2 mg of fusion protein was purified from 1L of culture medium.

Antibacterial activity and hemolytic assays

Antibacterial activity of SC-23-3 fusion protein was examined against various bacteria. SC-23-3 fusion protein was incubated with bacteria and then, the activity of growth inhibition was determined by radial diffusion assay (Fig. 3B). SC-23-3 fusion protein showed considerable antibacterial activity against gram-negative bacteria such as *E. coli* and *P. aeruginosa*. However, it didn't work against gram-positive bacteria and resistant bacteria (Table 2). On the other hand, cytotoxicity of fusion protein was not found in dose-dependent manner in the erythrocyte lysis assay although the fusion protein exhibited a slight increase in cytotoxicity at a concentration of 200 μg/ml (data not shown).

Most recombinant antimicrobial peptides have been expressed in *E. coli* as inclusion bodies to protect the host cells from the toxic effects of the recombinant peptide (Xu *et al*., 2007). The inclusion bodies should be refolded to become functional proteins, but its process does not always result in high ratio of native form and active form and, therefore, the recovery yield in this study was very low. So, its process of over expression must be rechecked (i.e., concentration of IPTG induction, incubation temperature, interchange with another host cell, etc.). In conclusion, a novel gene cDNA clone was isolated from stimulated *Copris tripartitus* using ACP-based GeneFishing PCR system and it produced a SC-23-3 fusion protein, which related to immune defense mechanism against microorganism. If the problem of protein expression in inclusion bodies could be solve. The aforementioned method, differentially display from LPS-stimulated insect, to find a novel AMPs is fairly valuable.

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