Int. J. Indust. Entomol. Vol. 3, No. 1, 2001, pp. 57~62

International Journal of
Industrial Entomology

Molecular Cloning of a Defensin Homologue Gene of a Novel Family Member from the Firefly, *Pyrocoelia rufa*

Kwang Sik Lee, Hye Jin Park, Seong Ryul Kim, Sang Mong Lee[1], Hung Dae Sohn and Byung Rae Jin*

College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Korea.
[1]Department of Sericultural and Entomological Biology, Miryang National University, Miryang 627-130, Korea.

(Received 18 May 2001; Accepted 31 July 2001)

A cDNA encoding the defensin homologue of a novel family member was isolated from the cDNA library of the firefly, *Pyrocoelia rufa*. Sequence analysis of the cDNA encoding the defensin homologue of *P. rufa* resulted that the 165 bp cDNA has an open reading frame of 55 amino acid residues. The deduced amino acid sequences of the defensin homologue gene from *P. rufa* showed identity to known mammalian defensins. Also 6 cystein residues in the *P. rufa* defensin homologue gene were conserved in the same position as those of known mammalian defensins. The result suggested that *P. rufa* defensin homologue is a novel member of the insect defensin family. Southern blot analysis suggests that there may be a single copy number of the *P. rufa* defensin homologue gene and their fat body-specific expression pattern at the transcriptional level was confirmed by Northern blot analysis.

Key words : Firefly, *Pyrocoelia rufa*, Defensin homologue gene

Introduction

The host defense of insects relies on cellular and humoral mechanisms. The humoral defense mechanism has been extensively studied from various insect species, and it is now clear that it is essentially based on the synthesis of antibacterial and antifungal peptides. In insects, these antimicrobial molecules are produced in the fat body and released into the hemolymph. Today, more than 150 antimicrobial peptides have been characterized from various insect species (Lamberty *et al.*, 1999). The antimicrobial peptides are often grouped into four families: cecropins, cysteine-rich peptides, proline-rich peptides, and glycine-rich peptides (Hoffman and Reichhart, 1997; Hetru *et al.*, 1998; Hoffman *et al.*, 1996).

Defensins are active against Gram-positive bacteria. These cystein-rich antibacterial peptides contain members with 1 to 4 disulfide bridges with molecular masses ranging from 2 to 6 kDa. Their presence has been reported from many insect orders (Miyanoshita *et al.*, 1996; Lamberty *et al.*, 1999) and also from scorpions (Ehret-Sabatier *et al.*, 1996) and mammals (Tarver *et al.*, 1998). Insect defensins are widely distributed in insects of the orders, Coleoptera (Moon *et al.*, 1994; Bulet *et al.*, 1991), Hymenoptera (Fujiwara *et al.*, 1990), Hemiptera (Cociancich *et al.*, 1994), Diptera (Lambert *et al.*, 1989; Matsuyama and Natori, 1988; Yamada and Natori, 1993; Dimarcq *et al.*, 1994; Hoffman and Hetru, 1992; Lowenberger *et al.*, 1995) and Odonata (Bulet *et al.*, 1992). Hitherto, they had not been isolated from Lepidoptera (Lamberty *et al.*, 1999).

Although insect defensins were originally thought to have structural similarity to mammalian defensins, three-dimensional structures and disulfide bond topologies of the peptides were found to be entirely different (Hoffman and Hetru, 1992; Hanazawa *et al.*, 1990). Interestingly, all 6 cystein residues and a tyrosine residue next to the N-terminus are completely conserved in 16 insect defensin species (Miyanoshita *et al.*, 1996).

Intriguingly, no defensin exhibiting structural similarity with mammalian defensins has been reported so far from insects, and no antimicrobial peptides have been recorded from the firefly. We present here the isolation of a cDNA encoding the defensin homologue of a novel family member from the firefly, *Pyrocoelia rufa*, the structure of which shares similarities with mammalian defensins.

*To whom correspondence should be addressed.
College of Natural resources and Life Science, Dong-A University, Busan 604-714, Korea. Tel.: 82-51-200-7594; Fax: 82-51-200-7594; E-mail: brjin@mail.donga.ac.kr

Materials and Methods

cDNA library screening

A cDNA library was constructed from poly (A^+) mRNA isolated from the whole body of *Pyrocoelia rufa* larvae by Uni-ZAP XR vector and Gigapack III Gold Packing Extract (Stratagene, La Jolla, USA) (Lee *et al.*, 2000, 2001). *E. coli* XL1-Blue MRF strain was infected by the Uni-ZAP XR library harboring *P. rufa* cDNA and cultured on the NZY agar medium. Each plaque was suspended in SM buffer [5.8 g/l NaCl, 2 g/l $MgSO_4 \cdot 7H_2O$, 0.05 M Tris-Cl (pH 7.5) and 0.01% gelatin solution] containing 0.02% (v/v) chloroform and stored at 4°C for 1 day. The pages were eluted into SM buffer. The pBluescript phagemids were *in vivo* excised from the Uni-ZAP XR vector using an ExAssist helper phage. *E. coli* strain, SOLR cell (Stratagene), was infected by the excised phagemids and plated on LB-Amp medium (50 μg/ml ampicillin). Plasmid DNA from the overnight culture was isolated by Wizard mini-preparation kit (Promega, Madison, WI). For the construction of the expressed sequence tags (ESTs) profile, cDNA clones randomly selected from the cDNA library were sequenced.

DNA sequencing and data analysis

The cDNA was sequenced using an automatic sequencer (model 310 Genetic Analyzer; Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences were compared using the DNASIS and BLAST programs provided by the NCBI. GenBank, EMBL and SwissProt databases were searched for sequence homology using a BLAST algorithm program. The hydropathic index for deduced amino acid sequence of the *P. rufa* defensin homologue was analyzed by the Kyte-Doolittle method (Kyte and Doolittle, 1982).

The sequence data of *P. rufa* defensin homologue gene obtained from this study have been deposited with the EMBL/GenBank/DDBJ libraries under the accession number AY048773.

Genomic DNA isolation and Southern blot analysis

Genomic DNAs were extracted from the larvae of *P. rufa* using a Wizard™ Genomic DNA Purification Kit, according to the manufacturers instructions (Promega). Genomic DNAs from *P. rufa* were digested with *Eco*RI or *Xho*I, and electrophoresed through 1.0% agarose gel. The DNA from the gel was transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany) and hybridized at 42°C with a probe in a hybridization buffer containing 5 × SSC, 50% formamide, 0.1% (W/V) *N*-lauroylsarcosine, 0.02% sodium dodecyl sulphate (SDS) and 2% blocking agent (Boehringer Mannheim, Mannheim, Germany). The probe used to detect the DNA fragment containing defensin homologue gene was a 168 bp *P. rufa* defensin homologue gene radiolabeled with [α-^{32}P] dCTP (Amersham, Arlington Heights, IL). After hybridization, the membrane filter was washed three times for 30 min each in 0.1% SDS and 0.2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C, and finally exposed to *X*-ray film.

RNA isolation and Northern blot analysis

Total RNAs were isolated from the whole body, fat body, light organ, gut and head part of the *P. rufa* larvae by using the Total RNA Extraction Kit (Promega). Total RNAs (10 μg/lane) from the larvae of *P. rufa* were denatured by glyoxalation (McMaster and Carmichael, 1977), transferred onto a nylon blotting membrane (Schleicher & Schuell) and hybridized at 42°C with a probe in a buffer containing 2 × PIPES, 50% formamide, 1% SDS and blocking agent (Boehringer Mannheim). The probe used to detect the defensin homologue gene transcripts was a 168 bp *P. rufa* defensin homologue gene radiolabeled with [α-^{32}P] dCTP (Amersham). The other procedures for washing the membrane filter and exposing *X*-ray film were performed as in the Southern blot analysis described above.

Results

The cDNA library was prepared from the whole body of *P. rufa* larvae (Lee *et al.*, 2000, 2001). The partial sequencing of randomly selected clones harboring cDNA inserts was performed to generate the *P. rufa* ESTs (expressed sequence tags). The *P. rufa* ESTs were aligned and putatively identified based on known genes. Of these ESTs, one clone was aligned and identified to mammalian defensins. The nucleotide sequence of cDNA from the clone was analyzed and its amino acid was deduced. As the result of the complete nucleotide and amino acid sequences in Fig. 1, the 165 bp defensin homologue gene

```
  1 ATGAAACTGTCAGTTTTTGTGCTAGTTGCAGTAATGCTTGTTTTGTTGTGTTGTGCGATG
  1 M  K  L  S  V  F  V  L  V  A  V  M  L  V  L  L  C  C  A  M

 61 CAGACGGAGGCACGTCGTCGTTGTCGTTCCTGTGTGCCATTTTGCGGCAGCAATGAACGT
 21 G  T  E  A  R  R  R  C  R  S  C  V  P  F  C  G  S  N  E  R

121 ATGATATCCACCTGTTTCTCTGGAGGAGTAGTTTGCTGCCCAAGATGATCAAAATTCCTA
 41 M  I  S  T  C  F  S  G  G  V  V  C  C  P  R  *

181 ATGTTGTTTTATATTTATTAATAAACTTATATTACATACCTATACCTTTTAAAAAAAAAA
```

Fig. 1. The nucleotide and deduced amino acid sequences of *P. rufa* defensin homologue gene. The start codon of ATG is boxed and the termination codon is single underlined with asterisk. The poly A signal is double underlined. The GenBank accession number is AY048773.

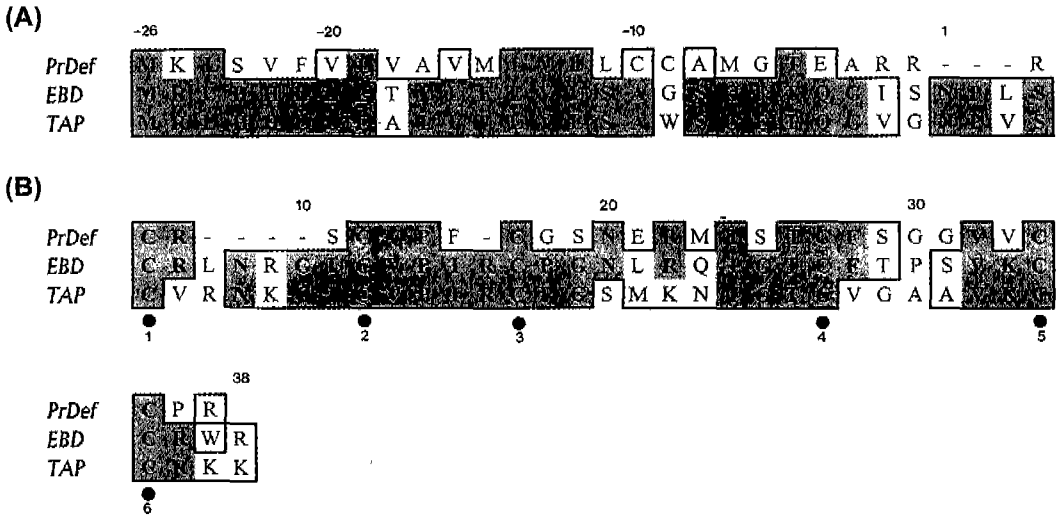

Fig. 2. Alignment of the signal peptide (A) and predicted mature peptide (B) of the *P. rufa* defensin homologue. Closed circles with number indicated the conserved 6-cystein residues. Shadow boxes indicated the conserved amino acid residues throughout three defensins. The *P. rufa* defensin homologue is aligned with protein sequences of EBD (Tarver *et al.*, 1998) and TAP (Diamond *et al.*, 1991, 1993).

has an open reading frame of 55 amino acid residues.

The amino acid sequences of *P. rufa* defensin homologue gene were aligned to those of known defensin genes. The *P. rufa* defensin homologue gene was not matched to the previously identified insect defensins. On the other hand, the deduced amino acid sequences of the defensin homologue gene from *P. rufa* showed identity to known mammalian defensins, EBD (enteric β-defensin) and TAP (tracheal antimicrobial peptide). When EBD and *P. rufa* defensin homologue are aligned, the *P. rufa* defensin homologue is suggested that the putative signal peptide is 26 amino acid residues and the predicted mature peptide is 29 amino acid residues (Fig. 2). The sequence of the predicted mature peptide of the *P. rufa* defensin homologue has 40% identity to that of EBD. Also, all 6 cystein residues in the *P. rufa* defensin homologue gene were conserved in the same position as those of known mammalian defensins, EBD and TAP (Fig. 2). The *P. rufa* defensin homologue is a cystein-rich protein and contains 6 cystein residues.

The hydropathic index for amino acid sequence of the *P. rufa* defensin homologue was analyzed by the Kyte-Doolittle method (1982). The signal peptide of the *P. rufa* defensin homologue, like EBD and TAP, is hydrophilic. Except for the signal peptide, three hydrophilic regions in the predicted mature peptide of the *P. rufa* defensin homologue were found (Fig. 3). The hydropathy of the *P. rufa* defensin homologue was similar to mammalian defensins, EBD and TAP.

To scrutinize the genomic organization of the defensin homologue gene in *P. rufa*, genomic DNA was digested

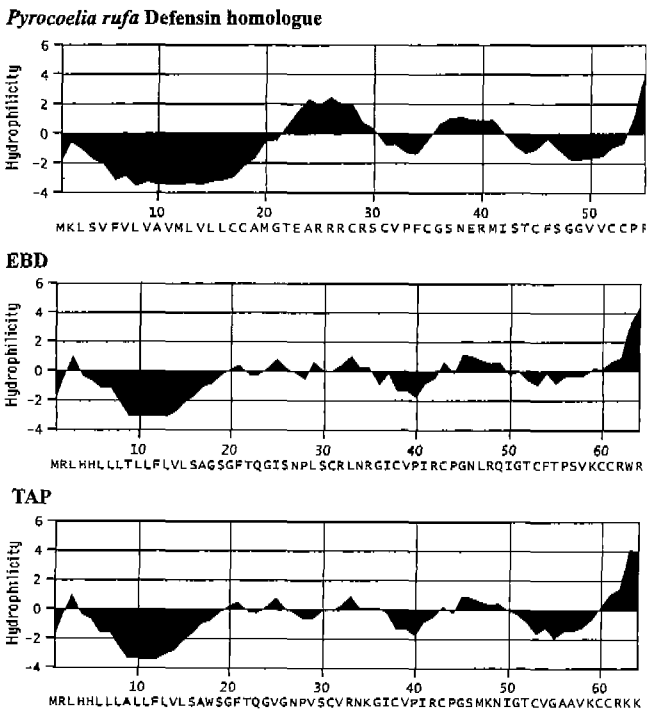

Fig. 3. Hydropathic analysis of the deduced protein of *P. rufa* defensin homologue. The hydropathic index for deduced amino acid sequences of *P. rufa* defensin homologue (A), EBD (B) and TAP (C) was analyzed by the Kyte-Doolittle method (1982).

with restriction enzymes without restriction site within defensin homologue gene and hybridized with the full-length defensin homologue gene amplified by PCR in this study as a probe. The Southern blot analysis revealed that

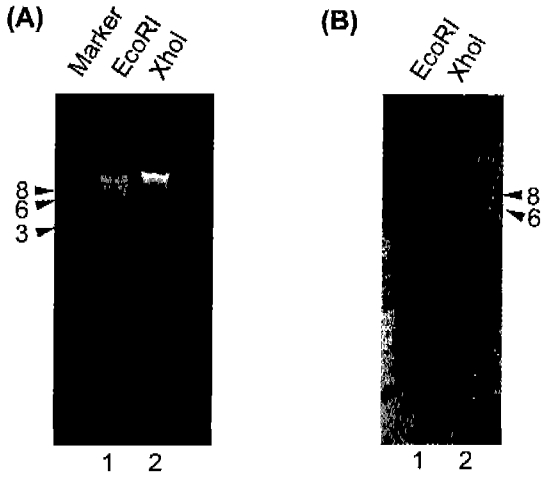

Fig. 4. Southern blot analysis of *P. rufa* genomic DNA for the defensin homologue gene. Genomic DNA was digested with restriction enzymes, *Eco*RI (lane 1) and *Xho*I (lane 2), separated by 1.0% agarose gel electrophoresis (A), and hybridized with the radiolabelled 168 bp defensin homologue gene in this study (B). Size markers are shown on the left of panel A. The hybridized bands indicated the arrow with molecular size.

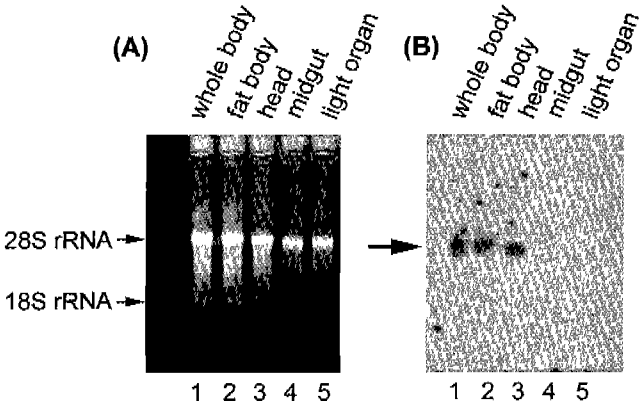

Fig. 5. Northern blot analysis of the *P. rufa* defensin homologue gene. Total RNAs were isolated from the whole body (lane 1), fat body (lane 2), head part (lane 3), midgut (lane 4), and light organ (lane 5). The RNAs were separated by 1.0% formaldehyde agarose gel electrophoresis (A), transferred on to a nylon membrane, and hybridized with the radiolabelled 168 bp defensin homologue gene (B). Transcripts of the defensin homologue gene are indicated on the left of panel B by a solid arrow.

the defensin homologue gene in *P. rufa* genome was detected as a single band pattern (Fig. 4).

In order to confirm the tissue-specific expression of the *P. rufa* defensin homologue gene at the transcriptional level, the Northern blot analysis was carried out using the full-length *P. rufa* defensin homologue gene amplified by PCR in this study as a probe (Fig. 5). The result of the Northern hybridization showed the expression of the *P. rufa* defensin homologue gene in the whole body, fat body and head part, but not detected in the midgut and light organ.

Discussion

We have cloned a cDNA encoding the defensin homologue in the firefly, *P. rufa*. The complete sequences of this cDNA comprised a 165 bp encoding the *P. rufa* defensin homologue of 55 amino acid residues. Above all, we compared the amino acid sequences of the various insect defensins isolated from 4 different insect orders (Miyanoshita *et al.*, 1996). However, the *P. rufa* defensin homologue gene was not matched to the previously identified insect defensins. On the other hand, the deduced amino acid sequences of the defensin homologue gene from *P. rufa* showed identity to known mammalian defensins, EBD and TAP (Diamond *et al.*, 1991, 1993; Tarver *et al.*, 1998). When mammalian defensins and *P. rufa* defensin homologue are aligned, the *P. rufa* defensin homologue is suggested that the putative signal peptide and the predicted mature peptide are 26 and 29 amino acid residues, respectively (Tarver *et al.*, 1998). Although the sequence of the predicted mature peptide of the *P. rufa* defensin homologue has 40% identity to that of EBD, all 6 cystein residues in the *P. rufa* defensin homologue gene were completely conserved in the same position as those of known mammalian defensins. The result suggested that the *P. rufa* defensin homologue has three disulfide bridges and showed structural similarity to mammalian defensins. The previous study indicated that, in the insect defensin family, defensins from Coleoptera (4 species), Hymenop- tera (1 species), Hemiptera (1 species) and Diptera (10 species) contained 6 cystein residues, and completely conserved in the same position (Miyanoshita *et al.*, 1996). Interestingly, all 6 cystein residues of the *P. rufa* defensin homologue are different for 16 insect defensin species. It is apparent that this *P. rufa* defensin homologue gene represents a new member of the insect defensin family.

The genomic organization of the defensin homologue gene in *P. rufa* was analyzed by Southern blot hybridization. By digestion of the restriction enzymes without cutting site within *P. rufa* defensin homologue gene, only one band was detected. This banding pattern suggests that the defensin homologue gene exists as a single copy number in *P. rufa*.

In general, the antimicrobial molecules in insects are produced in the fat body and released into the hemolymph (Lamberty *et al.*, 1999). The tissue-specific expression of

the *P. rufa* defensin homologue gene was confirmed by the Northern blot analysis. Interestingly, the result revealed that the *P. rufa* defensin homologue gene is expressed in the whole body, fat body and head part, and especially showed high-expression level in the head part. However, further investigation is required to fully understand its tissue-specific expression.

In conclusion, we report gene structure of the *P. rufa* defensin homologue gene for the first time. This gene suggested a novel member of the insect defensin family.

Acknowledgments

This paper was supported by the Dong-A University Research Fund in 2000.

References

Bulet, P., S. Cociancich, J. L. Dimarcq, J. Lambert, J. M. Reichhart, D. Hoffman, C. Hetru and J. A. Hoffman (1991) Isolation from a coleopteran insect of a novel inducible antibacterial peptide and of new members of the insect defensin family. *J. Biol. Chem.* **266**, 24520-24525.

Bulet, P., S. Cociancich, M. Reuland, F. Sauber, R. Bichoff, G. Hegy, A. Van Dorsselaer, C. Hetru and J. A. Hoffman (1992) A novel insect defensin mediates the inducible antibacterial activity in larvae of the dragonfly *Aeschna cyanea* (Paleoptera, Odonata). *Eur. J. Biochem.* **209**, 977-984.

Cociancich, S., A. Dupont, G. Hegy, R. Lanot, F. Holder, C. Hetru, J. A. Hoffman and P. Bulet (1994) *Biochem. J.* **300**, 567-575.

Diamond, G., D. E. Jones and C. L. Bevins (1993) Airway epithelial cells are the site of expression of a mammalian antimicrobial peptide gene. *Proc. Natl. Acad. Sci. USA* **90**, 4596-4600.

Diamond, G., M. Zasloff, H. Eck, M. Brasseur, W. L. Maloy and C. L. Bevins (1991) Tracheal antimicrobial peptide, a novel cystein-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. *Proc. Natl. Acad. Sci. USA* **88**, 3952-3956.

Dimarcq, J. L., D. Hoffman, M. Meister, P. Bulet, R. Lanot, J. M. Reichhart and J. A. Hoffman (1994) Characterization and transcriptional profiles of a *Drosophila* gene encoding an insect defensin. A study in insect immunity. *Eur. J. Biochem.* **221**, 201-209.

Ehret-Sabatier, L., D. Loew, M. Goyffon, P. Fehlbaum, J. A. Hoffmann, van Dorsselaer and P. Bulet (1996) characterization of novel cystein-rich antimicrobial peptide from scorpion blood. *J. Biol. Chem.* **271**, 29537-29544.

Fujiwara, S., J. Imai, M. Fujiwara, T. Yaeshima, T. Kawashima and K. Kobayashi (1990) A potent antibacterial protein in royal jelly: Purification and determination of the primary structure of royalisin. *J. Biol. Chem.* **265**, 11333-11337.

Hanazawa, H., I. Shimada, T. Kuzuhara, H. Komano, D. Kohda, F. Inagaki, S. Natori and Y. Arata (1990) *FEBS Lett.* **269**, 413-420.

Hetru, C., D. Hoffmann and P. Bulet (1988) Molecular mechanism of immune response in insects. Brey, P. T. and D. Hultmark (eds.), pp. 40-66, Chapman and Hall, London.

Hoffman, J. A. and C. Hetru (1992) Insect defensins: Inducible antibacterial peptides. Immunology Today **13**, 201-209.

Hoffmann, J. A. and J. M. Reichhart (1997) Drosophila immunity. *Trends Cell Biol.* **7**, 309-316.

Hoffmann, J. A., J. M. Reichhart and C. Hetru (1996) *Curr. Opin. Immunol.* **8**, 8-13.

Kyte, J. and R. F. Doolittle (1982) A simple method for displaying hydropathic character of a protein. *J. Mol. Biol.* **157**, 105-132.

Lambert, J., E. Keppi, J. L. Dimarcq, C. Wicker, J. M. Reichhart, B. Dunbar, P. Lepage, A. Van Dorsselaer, J. Hoffman, J. Fothergill and D. Hoffman (1989) Insect immunity. Isolation from immune blood of the dipteran *Phormia terranovae* of rabbit lung nacrophage bactericidal peptides. *Proc. Natl. Acad. Sci. USA* **86**, 262-266.

Lamberty, M., S. Ades, S. Uttenweiler-Joseph, G. Brookhart, D. Bushey, J. A. Hoffmann and P. Bulet (1999) Insect immunity: isolation from the lepidopteran *Heliothis virescens* of a novel insect defensin with potent antifungal activity. *J. Biol. Chem.* **274**, 9320-9326.

Lee, K. S., J. S. Bae, T. W. Goo, S. E. Kim, J. G. Kim, H. D. Sohn and B. R. Jin (2000) Analysis of expressed sequence tags of the firefly, *Pyrocoelia rufa. Int. J. Indust. Entomol.* **1**, 165-169.

Lee, K. S., H. J. Park, J. S. Bae, T. W. Goo, I. Kim, H. D. Sohn and B. R. Jin (2001) Molecular cloning and expression of a cDNA encoding the luciferase from the firefly, *Pyrocoelia rufa. J. Biotechnol.* In press.

Lowenberger, C., P. Bulet, M. Charlet, C. Hetru, B. Hodgeman, B. M. Christensen and J. A. Hoffman (1995) *Insect Biochem. Mol. Biol.* **25**, 867-873.

Matsuyama, K. and S. Natori (1998). *J. Biol. Chem.* **263**, 17112-17116.

McMaster, G. K. and G. G. Carmichael (1977) Analysis of single- and double-stranded nucleic acids on polyacryamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA* **74**, 4835-4838.

Miyanoshita, A., S. Hara, M. Sugiyama, A. Asaoka, K. Taniai, F. Yukuhiro and M. Yamakawa (1996) Isolation and characterization of a new member of the insect defensin family from a beetle, *Allomyrina dichotoma. Biochem. Biophys. Res. Commun.* **220**, 526-531.

Moon, H. J., S. Y. Lee, S. Kurata, S. Natori and B. K. Lee (1994) Purification and molecular cloning of cDNA for an inducible antibacterial protein from larvae of the coleopteran, *Tenebrio molitor. J. Biochem.* **116**, 53-58.

Traver, A. P., D. P. Clark, G. Diamond, J. P. Russell, H. Erdjument-Bromage, P. Tempst, K. S. Cohen, D. E. Jones, R. W.

Sweeney, M. Wines, S. Hwang and C. L. Bevins (1998) Enteric β-defensin: molecular cloning and characterization of a gene with inducible intestinal epithelial cell expression associated with *Cryptosporidium parvum* infection. *Infect. Immun.* **66**, 1045-1056.

Yamada, K. and S. Natori (1993) Purification, sequence and antibacterial activity of two novel sapecin homologues from Sarcophaga embryonic cells; Similarity of sapecin B to charybdotoxin. *Biochem. J.* **291**, 275-279.