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A 61kDa Antibacterial Protein Isolated and Purified from the Hemolymph of the American Cockroach *Periplaneta amreicana*

U.M. Seraj, M.I. Hoq, M.N. Anwar and S. Chowdhury
Department of Microbiology, University of Chittagong, Chittagong, Bangladesh

Abstract: Attempts were made to isolate, purify and characterize antibacterial peptides from the hemolymph of the American cockroach *Periplaneta americana*. Both non-induced and induced hemolymphs were tested for their antibacterial activity against several Gram-positive and Gram-negative bacteria. Induction was done by injecting *Escherichia coli* into the abdominal cavity of the cockroach. A time-induction study showed that antibacterial peptides were induced as early as ½ an hour with a peak at 9 h which started to decline around 24 h. The non-induced hemolymph showed activity only against *E. coli* whereas induced hemolymph showed activity against several Gram-positive and Gram-negative bacteria as well as against one antibiotic resistant *E. coli*. The induced hemolymph was subjected to SDS-PAGE to estimate the number and molecular weight of proteins present in the crude hemolymph. Seven distinct protein bands were detected by SDS-PAGE. The hemolymph was then subjected to gel filtration chromatography to purify the proteins responsible for the antibacterial activity. Twenty fractions, one ml each, were collected and all the fractions were tested against those bacteria which previously showed sensitivity to the crude hemolymph. Only six fractions were found to be effective against the tested bacteria. The protein concentrations in the active fractions were determined by spectrophotometry. The active fractions were finally subjected to SDS-PAGE to determine the molecular weight of the protein(s) which were responsible for the antibacterial activity. There was one protein in all these six fractions except fraction F_{13} where an additional protein of 67kDa was present. The approximate molecular weight of the isolated antibacterial protein present in all the fractions was 61kDa. It was found that only 2.87 μg of the protein could inhibit bacterial growth whereas approx.10 μg of conventional antibiotics was required to obtain similar result.

Key words: Antibacterial peptide, cockroach, *Periplaneta americana*, antibiotic resistance, hemolymph, gel filtration chromatography, SDS-PAGE, spectrophotometry

Introduction

Antibacterial peptides were initially isolated from the hemolymph of the cecropia pupae after challenging with live bacteria (Hultmark *et al*., 1980). Until now more than 150 antibacterial peptides have been purified from different insect species (Boman, 1998; Barra, 1998). These molecules are mainly produced by the fat body and their mRNAs are detected simultaneously as early as 1-3 h after injection of bacteria (Åsling, 1995; Morishima, 1995). Insects are fascinating in their ability to induce antimicrobial proteins, creating chemotherapeutic compounds in their hemolymph in response to outside stimuli (Natori, 1994). Antimicrobial peptides are important in the first line of host defence system of many animal species (Boman, 1995). They have mainly been studied in invertebrates. They are inducible peptides, although there are some antibacterial peptides such as lysozyme (Powning, 1973) and andropin (Samakovlis *et al*., 1991) which are not only inducible but also constitutive. Not only bacteria or LPS can induce this antibacterial production, but also fungi or trauma can cause induction (Per-Ove Thornqvist and Kenneth Soderhall, 1997). So far, five major groups of antibacterial peptides have been found (Hultmark, 1993): cecropins, insect defensins, attacin-like (glycine-rich) proteins, proline rich peptides and lysozymes. The mechanisms of antibacterial activity of cecropins and insect defensins have been studied extensively (Okada and Natori, 1984, 1985; Cociancich *et al*., 1993; Matsuyama and Natori, 1990; Yamada and Natori, 1994).Antibacterial peptides and proteins of invertebrate origin have drawn attention to a large number of scientists around the world. Since the first antibiotic agent, penicillin was isolated from green mold (*Penicillium* sp.). Antibiotics have saved millions of lives throughout the world. Although mankind has benefited from antibiotics for about half a century, many drug resistant bacteria have emerged. Besides, many of the available antibiotics are cost effective and have various side affects. As a result, scientists are constantly looking for new and effective antimicrobial agents from natural

sources such as plants, insects, etc. Scientists speculate that insects have acquired a unique survival strategy during their period of evolution. It is believed that if they can elucidate the mechanisms of this strategy, it is possible to have an insight of the problem of drug resistance. Hence they are looking for antimicrobial peptides from different insect species. Many antibacterial peptides show a remarkable specificity for prokaryotes with low toxicity for eukaryotic cells; a phenomenon which has favoured their investigation and exploitation as potential new antibiotics (Zasloff, 1992). Many insect species have been examined to discover antimicrobial agents and many insect-peptides have been discovered so far. In the present study we tried to isolate and characterize antibacterial peptides from the American cockroach *P. americana*. This is probably the first study of this kind in this country which may open up a new avenue of research in a search to combat against pathogenic microbes.

Materials and Methods

Bacterial strains: The bacterial strains that were used for the screening of antimicrobial peptides include *E. coli* ATCC 25922, *Bacillus subtilis* BTCC 17, *Bacillus cereus* BTCC19, *Bacillus megaterium* BTCC18, *Staphylococcus aureus* ATCC 6539, *Streptococcus* sp., *Pseudomonas aeruginosa, Salmonella typhi* AE 14612, *Salmonella paratyphi* AE 14613, *Shigella dysenteriae* AE 14396, *Vibrio cholerae* AE 14748 (Collected from Bangladesh type culture collection, Institute of Nutrition and Food Science, University of Dhaka, Bangladesh), *Shigella sonnei* (Cholera Research Laboratory, ICCDRB, Dhaka, Bangladesh), *Pasteurella maltocida,* six antibiotic resistant *E. coli* strains [S1-S6] (Chittagong Govt. Veterinary College, Chittagong, Bangladesh).

Animals and hemolymph collection: A colony of American cockroaches (*Periplaneta americana*) was maintained in a plastic container at room temperature with the supply of sterile water and biscuits. For collection of non-induced hemolymph, the cockroaches were anesthetized on ice, a pair of hind legs was cut off with fine scissors, and then 200 μl/animal of ice-cold modified Carlson's saline was injected into the abdominal cavity (Jomori and Natori, 1992). The hemolymph that exuded from the wound was collected and centrifuged at 1370 × g for 10 min. The supernatant was collected and stored at 4°C. For collection of induced hemolymph, each cockroach was injected with 100 μl of *E. coli* (10^6 cells /ml). Half an hour later, the cockroaches were anesthetized on ice. Then the hind legs were cut off with fine scissors and 200 il per animal of ice cold modified Carlson's saline was injected into the abdominal cavity. The hemolymph that exuded from the wound was collected by using sterile syringe. The hemolymph was centrifuged at 1370 × g for 10 minutes and the supernatant was collected and stored at 4°C.

Appearance of antibacterial activity: Six groups with five cockroaches in each group received an injection of *E. coli*, and their hemolymph was collected after various time intervals up to 24 h. The presence of antibacterial activity was monitored for each group of cockroach on an aliquot of cell-free hemolymph in the plate growth inhibition assay against *Streptococcus* sp.

Antibacterial assay: The antibacterial assays were done by paper disc method. Sterile Petri dishes (20 mm in diameter) received 20 ml of melted Luria Burtenii medium, pH 7.0. After solidification of the medium, the agar surface was inoculated with 0.1 ml (10^6cells/ml) of the test bacterial strain (24 h old) and spread with the help of a glass rod. Sterile paper disc soaked with 20 μl of the hemolymph was placed on the medium. The plate was incubated overnight at 37°C, and the diameters of the clear zones were recorded.

SDS-PAGE: The SDS-PAGE (Laemmli, 1970) was performed on 12% separating gel which was stained for proteins with Coomassie blue R-250. The electrophoresis was carried out at constant 100 volt for 3 h. Casein, bovine serum albumin and egg albumin were used as molecular weight markers.

Gel filtration chromatography: The hemolymph was applied to a Sephadex G-50 gel filtration column (1 × 50 cm) equilibrated with 0.1 M ammonium acetate buffer, pH 6.4. Gel column was eluted with the same buffer at 15 ml/h flow rate and 1 ml per tube was collected (Kyung and Donald, 2000).

Spectrophotometry: The protein concentration was measured in spectrophotometer (Spectronic-21). To determine the concentrations of proteins in the sample, standard curve of bovine serum albumin (BSA) was prepared at 660 nm by plotting the absorbance against concentrations. Then the absorbance of the sample was plotted in the standard curve.

Results

Antibacterial activity of the hemolymph: The non-induced hemolymph was found to be active only against *E. coli* (Fig. 1) but did not show any antibacterial activity against the antibiotic resistant *E. coli* strains. However,

Table 1: Antibacterial activity of the crude induced hemolymph of *Periplaneta americana* against the wild bacterial strains. '-' means no activity; '+' means active

Name of the bacteria	Activity	Zone of inhibition (diameter) in millimeter
Bacillus subtilis	+	14
Bacillus cereus	+	10
Bacillus megaterium	+	10
Staphylococcus aureus	+	27
Streptococcus sp.	+	15
Pseudomonas aeruginosa	-	-
Salmonella typhi	-	-
Salmonella paratyphi	-	-
Shigella dysenteriae	-	-
Shigella sonnei	-	-
Vibrio cholerae	+	11
Pasteurella maltocida	-	-
Escherichia coli	+	15

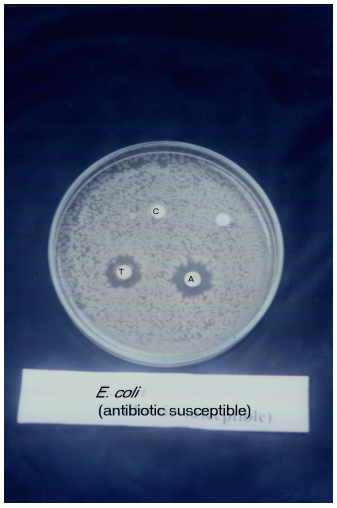

Fig. 1: Antibacterial activity of non-induced hemolymph against *E. coli* where 'T' indicates treatment, 'C' control and 'A' antibiotic. 10 μl hemolymph was absorbed into the disc. The clear zones around the discs indicate antibacterial activity

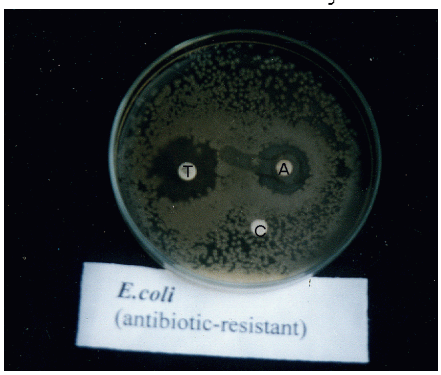

Fig. 2: Antibacterial activity of induced hemolymph against *E. coli* (antibiotic resistant) where 'T' indicates treatment 'C' control and 'A' antibiotic. 10 μl hemolymph was absorbed into the disc. The clear zones around the discs indicate antibacterial activity

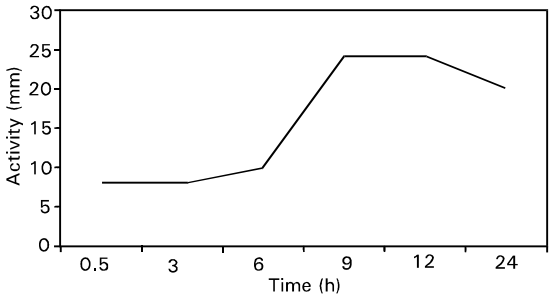

Fig. 3: Induction of antibacterial activity in hemolymph of *P. americana* after injection of *E. coli*. Each group of *P. americana* received an injection of *E. col.* Hemolymph was collected after various time intervals and freed from hemocytes by centrifugation. Antibacterial activity was assayed using 10 μl aliquots by the plate growth inhibition assay. Antibacterial activity is expressed in diameter (mm) of growth inhibition zone

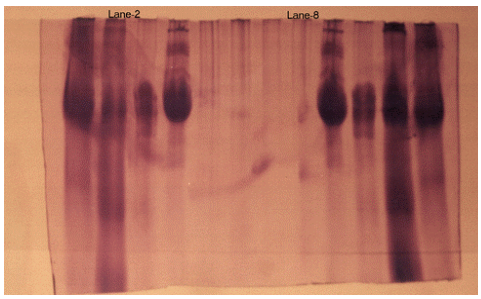

Fig. 4: SDS-PAGE of crude hemolymph.SDS-PAGE was performed as described by Laemmli (1970) using 12.5% acrylamide. Lane-2 shows different proteins in the crude hemolymph. The molecular weight markers are shown in lane-8

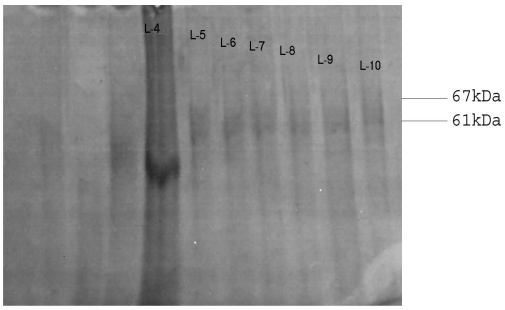

Fig. 5: SDS-PAGE of GFC fractions. SDS-PAGE was done as described by Laemmli (1970) using 12.5% acrylamide. Lane-4 shows different molecular mass markers and Lane 5-10 show various GFC fractions (L-5/F_{20}, L-6/F_{19}, L-7/F_{18}, L-8/F_{17}, L-9/F_{16}, L-10/F_{13})

the induced hemolymph was found to be active against all the Gram-positive bacteria tested but showed no antibacterial activity against the Gram-negative bacteria

except *E. coli* and *Vibrio cholerae* (Table 1). Moreover, the induced hemolymph was found effective against only one of the antibiotic resistant *E. coli* strains (Fig. 2). Appearance of antibacterial activity was monitored at various time intervals. The results show that strong antibacterial activity appeared between 9 to 12 h following injection of bacteria and the activity begins to decline at 24 h as tested (Fig. 3).

Purification of the antibacterial peptides: The hemolymph with antibacterial activity was subjected to Gel Filtration Chromatography (GFC) for separation and purification of the peptides. Twenty fractions, 1 ml each, were collected. All the fractions were subjected to antimicrobial susceptibility testing against those bacteria which previously showed sensitivity to the crude hemolymph. The fractions F_{13}, F_{16}, F_{17}, F_{18}, F_{19}, and F_{20} were found to show antibacterial activity against *Streptococcus* sp., *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis* and *Staphylococcus aureus*. However F_{13} also showed antibacterial activity against *E. coli*. The other fractions F_1 to F_{12}, F_{14}, and F_{15} did not show any activity against the tested bacteria. Again the GFC fractions were tested for their antibacterial activity against the 6 antibiotic resistant *E. coli* strains. Of the collected fractions, only F_{13} showed activity against one of the antibiotic resistant *E. coli* strains.

Determination of molecular weight: The crude hemolymph was subjected to SDS-PAGE. Seven proteins were detected with molecular weights 93, 89, 83, 67, 61, 38 and 16kDa.The molecular weight was measured against the standards used (Fig. 4). The 6 active GFC fractions were again subjected to SDS-PAGE to determine the molecular weight of the active peptide(s) responsible for the antibacterial activity. Two protein bands were detected in F_{13} and one band was detected in each of the other fractions (Fig. 5). The molecular weights of the 2 bands were determined as 61 and 67kDa.

Discussion

Our study establishes that orthopteran species respond to a bacterial challenge by the rapid appearance in their hemolymph of antibacterial peptides. The activity persists for a longer period, starting at ½ an hour, reaches a peak between 9 and 12 h and begins to decline after 24 h as tested.

In the present study, the non-induced hemolymph and the induced hemolymph of the American cockroach *P. americana* were screened for the antimicrobial activity against 13 human pathogenic bacteria and six antibiotic resistant *E. coli* strains. The non-induced hemolymph showed antibacterial activity against *E. coli* but it was not active against other bacterial strains tested. As its activity was observed only against the *E. coli*, the activity may be attributed to LPS-binding protein (LBP) in the hemolymph. Similar LPS binding protein (450 kDa) was also purified and characterized by Jomori and Natori, 1990 from *Periplaneta americana*. This activity may be due to the presence of constitutive peptides which might be active against *E. coli*. Some other antibacterial proteins with similar activity were also purified from different animal species. For example, L_6, a 27 kDa protein was purified from horse-shoe crab hemocytes (Saito *et al*., 1995). Another antibacterial protein of 11.5kDa was purified and characterized from *Carcinus maenas* which is cationic and hydrophobic in nature and active only against marine gram-positive bacteria (Juliet *et al*., 1999).

The non-induced hemolymph showed no activity against the antibiotic resistant *E. coli* strains. These may be due to the fact that the reasons for developing antibiotic resistance is strong enough to restrain the antimicrobial activity of the peptides or the resistant strains might have altered the genetic structure so that the peptides are not able to recognize them.

It has been observed in various insect species that bacteria injected into the hemocoel elicit the synthesis of a number of peptides and proteins which are active singly or in concert against the invaders and are secreted into the hemolymph (Gillespie *et al*., 1997). Induction is a common process in many insect species. In the present study, induction of such peptide(s) was done by injecting *E. coli* into the body cavity of the American cockroach *P. americana*. The induced hemolymph of *P. americana* showed antibacterial activity against all the gram-positive bacterial strains tested and it was not effective against all the Gram-negative bacteria tested except *E. coli* and *Vibrio cholerae*. These results suggest that cockroaches produce antibacterial peptides to combat bacterial infection. With an activity to Gram-positive bacteria, similar antibacterial peptides were reported by Hara and Yamakawa, 1995; Fehlbaum *et al*., 1996. Many other antibacterial proteins including sarcotoxin I (Okada and Natori, 1985), sapecin (Matsuyama and Natori, 1990), lebocin (Chowdhury *et al*., 1995), cecropin-B (Taniai *et al*., 1995) were isolated from different insect species with similar activity.

Some proteins present in the hemolymph of invertebrates may be both constitutive and inducible such as P^{47} of *Ceratitis capitata* (Charalambidis *et al*., 1996) and lysozyme (Gillespie *et al*., 1997). They are sometimes good antibacterial (lysozyme) and sometimes act as signal molecule (P^{47}). Some proteins tend to have higher activity against Gram-positive bacteria than the Gram-negative

ones. For example, moricin have higher activity against Gram-positive bacteria than Gram-negative bacteria (Hara and Yamakawa, 1995). It is possible that these peptide(s) induced in *P. americana* in this study might have similar feature like the proteins mentioned above, since they have higher activity against Gram-positive than Gram-negative bacteria.

Induced hemolymph showed antibacterial activity against several bacterial strains. It was subjected to SDS-PAGE to determine number and size of the proteins present in the hemolymph. Seven protein bands were detected in the gel but it was not possible to determine at this stage which protein(s) was responsible for the observed antibacterial activity. To determine the protein(s) responsible for the activity, the hemolymph was subjected to GFC.

Gel filtration chromatography was used to separate the proteins in the crude hemolymph. Twenty fractions, 1 ml each, were collected and tested against all the bacteria to which the crude hemolymph showed activity. Only six fractions i.e., F_{13}, F_{16}, F_{17}, F_{18}, F_{19} and F_{20} were found to be active. This might be due to the fact that these fractions contain peptide(s) which are active against the tested bacteria. Other proteins may be present in the non-active fractions of the hemolymph which might be involved in signal transduction or in recognition as a concert.

However, it was observed that the fraction F_{13} showed strong antibacterial activity against all the Gram-positive bacteria tested, the wild *E. coli* strain and one antibiotic resistant *E. coli* strain, S2. While the other fractions showed no activity against the wild *E. coli* and antibiotic resistant *E. coli*. These results suggest that the fraction F_{13} contains antibacterial protein(s) specific against the wild *E. coli* strain and the resistant *E. coli* which might be due to the presence of a specific peptide(s) or protein(s) in this fraction which is able to penetrate the Gram-negative bacteria. This result is well in agreement with hymenoptaecin, an antibacterial peptide isolated from the honeybee *Apis mellifera* (Casteels *et al*., 1993), moricin (Hara and Yamakawa, 1995).

The active fractions were then subjected to SDS-PAGE. Two proteins with approx. mol. wt. of 61kDa and 67kDa were detected in fraction F_{13} and one protein of 61kDa was detected in fractions F_{16}, F_{17}, F_{18}, F_{19} and F_{20}. Fraction F_{13} was effective against all the bacteria including wild type *E. coli* and antibiotic resistant *E. coli* but all other fractions were effective against all the bacteria tested except *E. coli*. This indicates that the 67kDa protein might be responsible for the activity against S_2, the antibiotic resistant *E. coli*. It could be of greater interest to isolate and characterize this protein which might be used commercially against those resistant strains because it has become a normal feature that bacteria are developing resistance to antibiotic after several uses.

The purified proteins showed activity predominantly against the Gram-positive bacteria. It suggests that the antibacterial activity of the peptides is related to the cell wall of bacteria. But the peptides of fraction F_{13} are also active against the Gram-negative bacteria such as *E. coli* and *Vibrio cholerae* which is in contrast to the assumption that the antibacterial activity is related to the structure of cell wall. Thus the antibacterial activity may be attributed to other characteristics of cell. Similar proteins with activity against both gram positive and gram negative bacteria were reported by Hara and Yamakawa, (1995). Moricin, an antibacterial peptide was reported to act against *E. coli*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Bacillus subtilis*, *B. megaterium*, *B. cereus*, *Staphylococcus aureus*, *S. xylosus*, *S. epidermidis*, *S. pyogenes* etc. Comparing to their study, it may be assumed that the proteins identified in this study might play an important role in the self-defense against bacterial infection in *P. americana* singly or in concert.

In conclusion, the 67kDa and 61kDa proteins appear to have an interesting potential for therapeutic application because of its activity against several bacterial strains at very low concentrations as compared to conventional antibiotics (2.9 μg of peptide : 10 μg of antibiotics). Special emphasis might be given on the 67kDa protein since it has activity against Gram-positive, Gram-negative and a resistant strain also.

References

Åsling, B., M. Dushay and D. Hultmark, 1995. Identification of early genes in *Drosophila* immune response by PCR-based differential display: the attacin A gene and the evolution of attacin-like proteins. Insect Biochem. Mol. Biol., 25: 511–518.

Barra, D., M. Simmaco and H. Boman, 1998. Gene-encoded peptide antibiotics and innate immunity. FEBS Lett., 430: 130–134.

Boman, H., 1998. Gene-encoded peptide antibiotics and the concept of innate immunity: an update review. Scand. J. Immunol., 48: 15–25.

Boman, H.G., 1995. Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol., 13: 61–92

Casteels, P., Ampe, C., Jacobs, F. and Tempst, P. 1993. Functional and chemical characterization of Hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (*Apis mellifera*). J. Biol. Chem., 268: 7044-7054.

Charalambidis, N.D, L.C. Foukas and V.J. Marmaras, 1996. Covalent association of lipopolysaccharide at the hemocyte surface of insects is an initial step for its internalization. Eur. J. Biochem., 236: 200-206.

Chowdhury, S., K. Taniai, S. Hara, Kadono-Okuda, Y. Kato, M. Yamamoto, J. Xu, S. Choi, C. Nitish C. Debnath and Minoru Yamakawa, 1995. cDNA cloning and gene expression of lebocin, a novel member of antibacterial peptides from the silkworm, *Bombyx mori*. Biochemical and Biophysical Research Communications, 214: 271–278.

Cociancich, S., A. Ghazi, C. Hetru, J.A. Hoffmann and L. Letellier, 1993. Insect defensin, an inducible antibacterial peptide, forms voltage dependent channels in *Micrococcus luteus*. J. Biol. Chem., 268: 19239-45.

Fehlbaum, P., P. Bulet, S. Chernysh, J.P. Briand and J.P. Roussel, 1996. Structure-activity analysis of thanatin, a 21-Residue inducible insects defense peptide with sequence homology to frog skin antimicrobial peptides. Proceedings of the National Academy of Sciences. USA, 93: 1221-25.

Gillespie, P.J., M. Kanost and T. Trenczek, 1997. Biological mediators of insect immunity. Annu. Rev. Entomol., 42: 611-613.

Hara, S. and M. Yamakawa, 1995. Moricin, a novel type of antibacterial peptide isolated from the silkworm, *Bombyx mori*. J. Biol. Chem., 270: 29923-27.

Hultmark, D., 1993. Immune reactions in *Drosophila* and other insects: a model for innate immunity. Trends in Genetics, 9: 178-183.

Hultmark, D., H. Steiner, T. Rasmuson and H.G. Boman, 1980. Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. Eur. J. Biochem., 106: 7–16.

Jomori, T. and S. Natori, 1992. Function of the lipopolysacharide-binding protein of *Periplaneta americana* as an opsonin. FEBS Lett., 296: 283-86.

Jomori, T., T. Kubo and S. Natori, 1990. Purification and characterization of lipopolysaccharide-binding protein from hemolymph of American cockroach *Periplaneta americana*. Eur. J. Biochem., 190: 201-206.

Juliet, M. Relf, June R.S. Chisholm, Graham D. Kemp and Valerie J. Smith, 1999. Purification and characterization of a cysteine-rich 11.5 kDa antibacterial protein from the granula hemocytes of the shore crab, Carcinus maenas. Eur. J. Biochem., 264: 350-357.

Kyung T. Chung and Donald D. Ourth, 2000. Viresin, a novel antibacterial protein from immune hemolymph of *Heliothis virescens* pupae. Eur. J. Biochem., 267: 677-683.

Laemmeli, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* (London), 227: 680-685.

Matsuyama, K., S. Natori, 1990. Mode of action of sapecin, a novel antibacterial protein of *Sarcophaga peregrina* (flesh fly). J. Biochem., 108: 201-206.

Morishima, I., T. Horiba, M. Iketani, E. Nishioka and Y. Yamano, 1995. Parallel induction of cecropin and lysozyme in larvae of the silkworm *Bombyx mori*. Dev. Comp. Immunol., 19: 357–363.

Natori, S., 1994. Antimicrobial peptides. Wiley, Chichester (Chiba Foundation Symposium, 186). pp: 123-134.

Okada, M., S. Natori, 1985. Ionophore activity of sarcotoxin I, a bactericidal protein of *Sarcophaga peregrina*. Biochem. J., 229: 453-458.

Okada, M.., S. Natori, 1984. Mode of action of a bactericidal protein induced in the hemolymph of *Sarcophaga peregrina* (flesh fly) larvae. Biochem. J., 222: 119-124.

Per-Ove Thornqvist and Kenneth Soderhall, 1997. Crustacean Immune Reactions, a Short Review: Diseases in Asian Acquaculture III, published by the Fish Health Section, Asian Fisheries Society, Philipines.

Powning, R.F. and W.J. Davidson, 1973. Studies on insect bacteriolytic enzymes - I. Lysozyme in haemolymph of *Galleria mellonella* and *Bombyx mori*. Comp. Biochem. Physiol., 45: 669-686.

Samakovlis, C.P., D.A. Kylsten, A. Kimbrell, Engstrom and D. Hultmark, 1991. The andropin gene and its product, a male specific antibacterial peptide in *Drosophila melanogaster*. EMBO J., 10: 163-169.

Saito, T., S. Kawabata, M. Hirata and S. Iwanaga, 1995. A novel type of limulus Lectin-6: purification, primary structure and antibacterial activity. The American Society for Biochemistry and Molecular Biology, 270: 14493-14499.

Taniai, K., K. Kadono-Okuda, Y. Kato, M. Yamamoto, M. Shimabukuro, S. Chowdhury and M. Yamakawa, 1995. Structure of two cecropin B-encoding genes and bacteria-inducible binding proteins which bind to the 5′upstream regulatory region in the silkworm, Bombyx mori. Gene., 163: 215-19.

Yamada, K. and S. Natori, 1994. Characterization of the antimicrobial peptide derived from sapecin B, an antibacterial protein of *Sarcophaga peregrina* (flesh fly). Biochem. J., 298: 623-628.

Zasloff, M., 1992. Antibiotic peptides as mediators of innate immunity. Curr. Opin. Immunol., 4: 3–7.