http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 6 (15): 1345-1353, 2003
ISSN 1028-8880
© 2003 Asian Network for Scientific Information

Isolation and Characterization of Antibacterial Peptides from the Mud-crab, *Scylla serrata*

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

Abstract: Both induced and non-induced hemolymphs were collected from the mud-crab, *Scylla serrata* and were subjected to antibacterial assay to investigate the presence of inducible and constitutive antibacterial protein(s). Induction was done by injecting *Escherichia coli* ATCC 25922. The induced hemolymph showed antibacterial activity against a range of different strains of marine or non-marine gram-positive and gram-negative bacteria including few antibiotic resistant strains, whereas the non-induced hemolymph was not appeared to be active against those tested. The induced hemolymph was subjected to SDS-PAGE and seven proteins were detected. The molecular weight of which were in the range from 36 to 84.5 kDa. The hemolymph proteins were fractionated by gel filtration chromatography. Repeated antibacterial assay of the chromatographic fractions against previously proved hemolymph sensitive bacteria showed that only a few fractions were responsible for the activity and that the fraction purified proteins showed better activity than the crude hemolymph. Total five different proteins were present in all the fractions detected by SDS-PAGE analysis. The molecular weights were 64 kDa, 61 kDa, 56.5 kDa, 49 kDa and 36 kDa.

Key words: Hemolymph, Antibacterial protein, mud-crab, *Scylla serrata,* Invertebrate immunity

Introduction

Invertebrates represent the most diverse taxon of animals on the planet, accounting for more species than all other animals combined. In addition to the experimental evidence, it makes sense that such successful animals would have evolved efficient means for combating infection. In many ways, invertebrates face the similar immune challenges to those experienced by vertebrates (Gillespie *et al.,* 1997). Invertebrates lack immune systems that involve antigen-antibody reactions and do not have an immune memory; therefore most invertebrate species show no evidence of acquired immunity (Hultmark, 1993). As a consequence, invertebrates have to rely on innate immune processes to combat pathogens which closely resembles the innate immune system of the vertebrates. The processes entail rapid, relatively nonspecific responses, both cellular and humoral, and may have appeared quite early in the evolution of animals.

Invertebrate immunity is comprised of both non-self recognition and effectors mechanisms. How an insect recognizes an invader as non-self (i.e. foreign) is still unclear, but it is the subject of much current research. Presently, much is known about effector mechanisms of insect immunity (i.e., how an insect rids itself of an invading pathogen or parasite (Vinson, 1974; Hultmark, 1993). The recognition of the pathogens and parasites by the invertebrate immune system may involve soluble proteins present in the hemolymph as well as proteins (receptors) localized at the surface of the hemocyte or other cells. The initial recognition may bring about communication to other population of cells through molecules that act as signals to stimulate a response. The recognition stimulus and the secondary signals trigger signal transduction pathways that produce action by a cell: cell adhesion followed by phagocytosis or encapsulation by hemocyte, release of signaling factors or induced transcription of genes for production of antimicrobial proteins (Gillespie *et al.,* 1997). The main site of synthesis of induced antibacterial peptides, however, is the fat body-an adipose tissue with somewhat analogous metabolic function to the mammalian liver. Smaller contributions are made by hemocyte and other tissues such as pericardial cells, malphigian tubules, and midgut (Dickinson *et al.,* 1988; Dunn *et al.,* 1994; Mulnix and Dunn, 1994; Russell *et al.,* 1990; Russell *et al.,* 1996). Antibacterial peptides can also be induced in epidermal cells in response to wounding or infection in the cuticles (Brey *et al.,* 1993; Lee and Brey, 1995). The whole process of synthesizing antibacterial proteins may take few minutes or hours after the challenge and these are secreted into hemolymph when invertebrates are in acute phase. Most of these proteins are small cationic molecules exhibiting a broad spectrum of activity against gram-positive and/or gram-negative bacteria. There are some antibacterial proteins that are not inducible such as lysozyme (Powning and Davidson, 1973) and andropin (Samakovlis *et al.,* 1991).

Antibacterial peptides have been isolated from a wide variety of invertebrate phyla, including insects (Boman, 1995), ascidians (Azumi *et al.,* 1990; Lee *et al.,* 1997),

chelicerates (Nakamura *et al.,* 1988), annelids (Milochau *et al.,* 1997) and mollusks (Hubert *et al.,* 1996; Charlet *et al.,* 1996). A very few invertebrate species from which antibacterial peptides and proteins have been studied intensively includes *Hyalophora cecropia, Manduca sexta, Bombyx mori, Drosophila melanogaster* and *Sarcophaga peregrina* (Boman, 1995; Chadwick and Aston, 1991; Faye and Hultmark, 1993; Hoffmann *et al.,* 1996; Hultmark, 1993; Meister *et al.,* 1994). However, cecropin is the first discovered inducible antibacterial peptide in invertebrates (Hultmark *et al.,* 1980). The first antibacterial protein to be isolated and partially characterized from a marine decapod was a 6.5-kDa proline-rich peptide with sequence similarity to bactenecin 7 from mammals (Schnapp *et al.,* 1996). These antibacterial proteins provide a useful way of assessing and studying them for their commercial or chemotherapeutic value.

When first discovered, antibiotics were touted as a miracle cure and they literally were. Pathogens that were virtually eliminated with the introduction of antibiotics are mutating, gaining strength, and resisting treatment. The spread of antibiotic resistances and the appearance of multiple-antibiotic-resistant pathogenic bacteria complicate medical treatment of bacterial infections. Researchers have engaged themselves in continuing quest for new and effective antibiotics from natural sources such as plants, invertebrates, etc. to fill the place of the old ones. In the present study, we surveyed antibacterial activity of the crude induced and non-induced hemolymphs of the mud-crab, *Scylla serrata* and so far, we have purified and characterized five inducible antibacterial peptides which showed strong antibacterial activity against both gram-positive and gram-negative bacteria with a predominant activity against gram-positive bacteria.

Materials and Methods

Experimental Animal : *Scylla serrata*, the common mud-crab were collected in baited baskets from Bay of Bengal, Bangladesh. *S. serrata* is a marine decapod of the *Portunidae* family. Healthy male and female animals at different stages of development were used throughout for experimental purposes and each animal was subjected to a single bleed. Collections were being done at the time of use.

Collection of Hemolymph: Induced and non-induced hemolymphs (approx 3 mlcrab^{-1}) were collected by cutting each walking leg of the animal with a fine sterile scissor. To avoid hemocyte degranulation and coagulation, the hemolymph was collected in the presence of sodium citrate buffer, pH 4.6 (2:1, v/v). Equal volume of Physiological saline (0.85% NaCl, w/v) was added to it. To remove hemocytes from plasma the hemolymph was centrifuged at 800 g for 10 min at 4°C, supernatant collected by aspirating and stored at -4°C. For induction of peptides, the animal was injected with 100 μl of the gram-negative bacteria *Escherichia coli* ATCC 25922 (10^5-10^6) through the genital pore of the crab. The induced hemolymph was collected 60-90 min after injection.

Antibacterial Assay**:** The spectrum of activity was studied using as test agents a range of 24 different strains of marine or non-marine gram-negative and gram-positive bacteria of which there were six antibiotic resistant strains. The following bacterial strains were generous gifts from different institutions: *Bacillus subtilis* BTCC 17, Bacillus cereus BTCC 19, *Bacillus megaterium* BTCC 18, *Staphylococcus aureus* ATCC 6539, *Escherichia coli* ATCC 25922, *Salmonella typhi* AE 14612, *Salmonella paratyphi* AE 14613, *Shigella dysenteriae* AE 14396, *Vibrio cholerae* AE 14748 (Bangladesh Type Culture Collection, Institute of Nutrition and Food Science, University of Dhaka, Bangladesh) *Shigella sonnei*, *Pseudomonas aeruginosa* (Cholera Research Laboratory, ICDDR,B), *Streptococcus pyogenes*, *Pasteurella multocida* and antibiotic resistant *Escherichia coli* strains (Chittagong Govt. Veterinary College, Bangladesh). Five marine bacterial strains were isolated from water samples of St. Martin island, Bangladesh by serial dilution technique (Greenberg *et al*., 1980) on 3% NaCl containing nutrient agar plates; they were selected on the basis of their colonial morphology viz. color, form, elevation, margin surface, optical characters etc. (Ekluned and Lankford, 1967; Bryan, 1950). Gram reaction of each isolate was established and one strain was identified up to species on the basis of their microscopic, cultural and biochemical properties following standard procedures. Non-marine strains were grown on Luria Burtenii agar or broth at 37°C for 18 h and maintained on slopes of LB agar at 4°C and subcultured into fresh broth before final suspension in fresh sterile saline. Marine strains were maintained on slopes of 3% NaCl containing nutrient agar at 4°C and grown to log phase in high salt conditioned broth for 18 h at 25°C.

The disc diffusion technique (Bauer *et al*., 1966) with little modification was applied for the *in vitro* investigation of antibacterial activity. All bacterial suspensions were calibrated and routinely standardized to a concentration of approximately 10^6 cfu•mL^{-1}. Test material was applied as 10μldisc^{-1}. Positive controls contained 10-30 μgdisc^{-1} of a standard antibiotic. Negative controls comprised buffer only. Antibacterial activity was expressed in terms of diameter of zone of inhibition in mm.

SDS-PAGE: SDS-PAGE was performed in 12% separating gels, according to the method described by Laemmli (1970). The reference proteins for molecular weight estimation were casein (23,6 kDa), egg albumin (45 kDa) and bovine serum albumin (66 kDa). The electrophoresis was carried out at constant 100 volt for 3 h. Following electrophoresis, the protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

Protein Concentration: The amount of protein was measured by spectrometry according to the method of Lowry *et al.* (1951) using a calibration curve prepared with different concentrations (0.1-0.5 $mgml^{-1}$) of bovine serum albumin as standard. Folin-Ciocalteu reagent was used as color reactant and concentration was calculated in response to absorbance at 660 nm in a spectrophotometer (Spectro UK-VIS RS).

Gel filtration Chromatography (GFC): Hemolymph preparation containing a mixture of proteins was applied to a 40 X 1.5 cm Sephadex G-50 column at room temperature, equilibrated with 0.5 M ammonium acetate buffer, pH 6.8. Bovine serum albumin of gel filtration standard (0.3 mg ml^{-1}) was used to determine the column void volume. Elution was carried out at a flow rate of 0.3 $mL \cdot min^{-1}$ at a constant hydrostatic pressure using buffer reservoir. Fractions (1 ml each) were collected and concentrations determined using spectrophotometry.

Results

Antibacterial Activity of S. serrata Hemolymph: The antibacterial activity of induced and non-induced hemolymph of *S. serrata* was investigated against a range of 24 different strains of gram-positive and gram-negative bacteria of which there were five marine isolates, six antibiotic resistant strains and the rest were wild strains. The results demonstrated that the induced hemolymph showed strong activity against all the tested wild strains except two gram-negative strains, *Vibrio cholerae* and *Shigella sonnei* (Table 1). The immune hemolymph also appeared to be active against four antibiotic resistant *E. coli* strains among the six tested (Table 2). Antibacterial activity of the crude induced hemolymph of *Scylla serrata* against *Bacillus megaterium* and an antibiotic resistant *Escherichia coli* strain (S_5) were shown in fig. 1 and 2. In this study, five marine bacterial strains were isolated and Gram reaction of those revealed that one was gram-positive and the rest were gram-negative. Antibacterial assay against the marine isolates showed that the induced hemolymph have activity only against the gram-positive one (Table 3). The cultural, microscopic and physiological characteristics of the marine isolate indicated that the organism is closely related to the genus *Erysipelothrix* and to the species *E. rhusiopathiae* (Migula) Buchanan 1918 but differ in gelatin liquefaction and lactose fermentation. Unlike induced hemolymph, there was no antibacterial activity recorded for the non-induced hemolymph against all the tested bacteria.

Table 1: Antibacterial activity of the crude induced hemolymph of *Scylla serrata* against wild bacterial strains determined by disc diffusion technique (Bauer *et al.*, 1966) where numbers indicate zone of inhibition; minus sign (-) indicates no antibacterial activity; bold numbers indicate marked inhibition zone; +ve indicates positive and –ve means negative

Name of the bacteria	Gram +ve/-ve	Activity	Zone of inhibition (mm in diameter) procured by induced hemolymph
Bacillus subtilis	+ve	+ve	14
Bacillus cereus	+ve	+ve	12
Bacillus megaterium	+ve	+ve	21
Staphylococcus aureus	+ve	+ve	11
Streptococcus pyogenes	+ve	+ve	11
Pseudomonas aeruginosa	-ve	+ve	11
Salmonella typhi	-ve	+ve	18
Salmonella paratyphi	-ve	+ve	13
Shigella dysenteriae	-ve	+ve	14
Shigella sonnei	-ve	-ve	-
Vibrio choleriae	-ve	-ve	-
Pasteurella multocida	-ve	+ve	15
Escherichia coli	-ve	+ve	9

Table 2: Antibacterial activity of the crude induced hemolymph of *Scylla serrata* against antibiotic resistant bacterial strains determined by disc diffusion technique (Bauer *et al.*, 1966) where numbers indicate zone of inhibition; minus sign (-) indicates no antibacterial activity; bold numbers indicate marked inhibition zone; +ve indicates positive and –ve means negative

Name of the bacteria	Gram +ve/-ve	Activity	Zone of inhibition (mm in diameter) procured by induced hemolymph
Escherichia coli (S_1)	-ve	+ve	9
Escherichia coli (S_2)	-ve	+ve	10
Escherichia coli (S_3)	-ve	-ve	-
Escherichia coli (S_4)	-ve	+ve	14
Escherichia coli (S_5)	-ve	+ve	15
Escherichia coli (S_6)	-ve	-ve	-

Table 3: Antibacterial activity of the crude induced hemolymph of *Scylla serrata* against marine isolates determined by disc diffusion technique (Bauer *et al.*, 1966) where numbers indicate zone of inhibition; minus sign (-) indicates no antibacterial activity; bold numbers indicate marked inhibition zone; +ve indicates positive and –ve means negative

Name of the bacteria	Gram +ve/-ve	Activity	Zone of inhibition (mm in diameter) procured by induced hemolymph
Strain no M_1	-ve	-ve	-
Strain no M_2	-ve	-ve	-
Strain no M_3	+ve	+ve	22
Strain no M_4	-ve	-ve	-
Strain no M_5	-ve	-ve	-

SDS-PAGE: The crude induced hemolymph that showed antibacterial activity was subjected to SDS-PAGE to estimate the number and molecular weight of proteins present in it. Casein, egg albumin and bovine serum

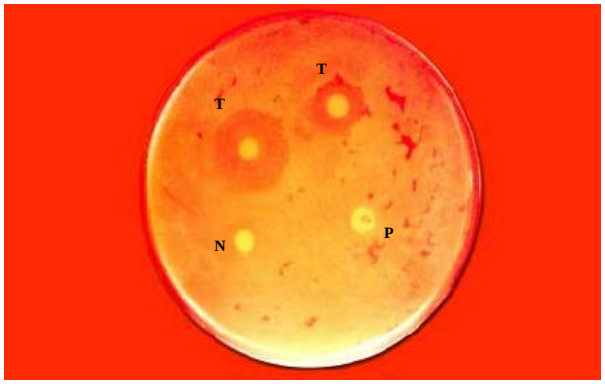

Fig. 1: Antibacterial activity of the crude induced hemolymph of *Scylla serrata* against *Bacillus megaterium* where positive control (P) contained 20 μg of ampicillin and negative control (N) comprised buffer only. Test material (T) was applied as 10μldisc^{-1}

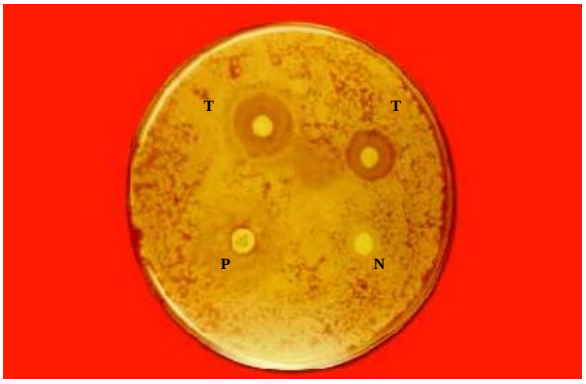

Fig. 2: Antibacterial activity of the crude induced hemolymph of *Scylla serrata* against an antibiotic resistant *Escherichia coli* strain (S_5) where positive control (P) contained 20 μg of cephalexin and negative control (N) comprised buffer only. Test material (T) was applied as 10μldisc^{-1}

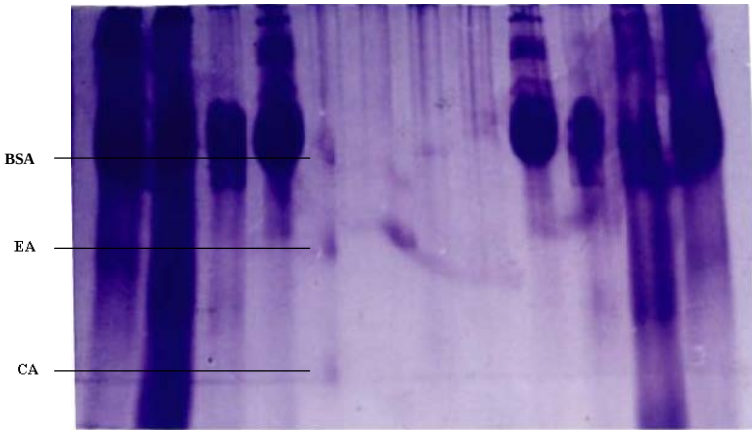

Fig. 3: SDS-PAGE of crude induced hemolymph of *Scylla serrata* where M indicates marker; H hemolymph proteins; BSA bovine serum albumin; EA egg albumin and CA casein.

albumin were used as standard to determine molecular weight of hemolymph proteins. The stained gel revealed that the hemolymph contained a complex population of proteins (Fig. 3). Seven clear bands were detected in the gel that represented proteins of 84.5, 71.5, 64, 61, 56.5, 49 and 36 kDa.

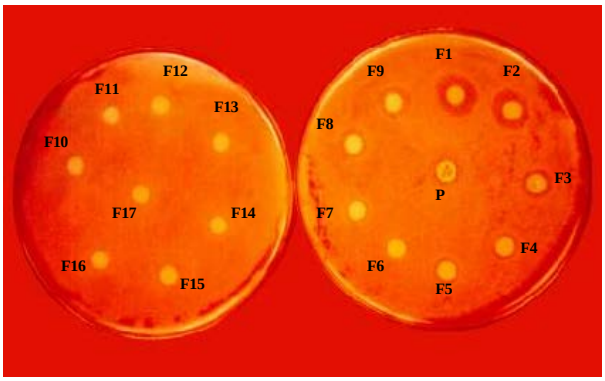

Fig. 4: Antibacterial activity of the chromatographic fractions of induced hemolymph of *Scylla serrata* against *Pasteurella multocida* where F1-F17 indicates the fractions. Test material was applied as 10μldisc^{-1} and positive control (P) contained 20 μg of ampicillin

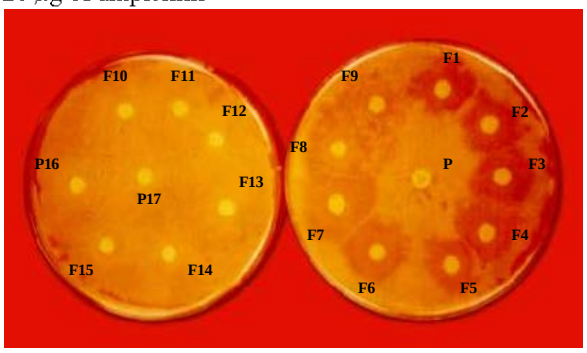

Fig. 5: Antibacterial activity of the chromatographic fractions of induced hemolymph of *Scylla serrata* against *Streptococcus pyogenes* where F1-F17 indicates the fractions. Test material was applied as 10μl disc^{-1} and positive control (P) contained 20 μg of tetracycline

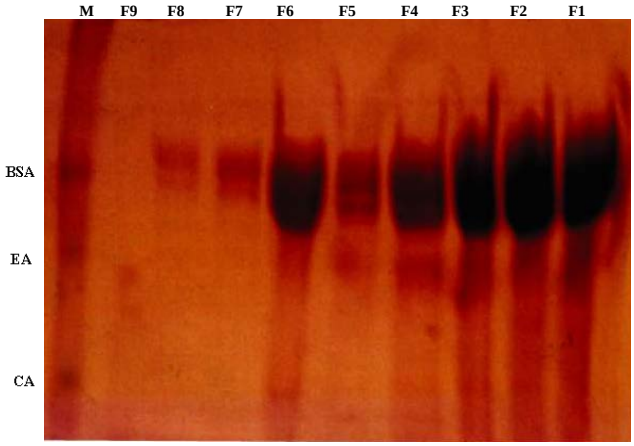

Fig. 6: SDS-PAGE of the chromatographic fractions of induced hemolymph of *Scylla serrata* where M indicates marker; F1-F9 indicate the fractions; BSA bovine serum albumin; EA egg albumin and CA casein

Estimation of protein concentration and fraction purification of the Antibacterial Proteins: Protein concentration of the induced hemolymph with antibacterial activity was measured using a spectrophotometer. The hemolymph with a protein content of 7550 μg•mL^{-1} was subjected to gel filtration chromatography (GFC) to fractionate the proteins into pure fractions. Seventeen fractions (F1-F17), 1 ml each were collected in Eppen-dorf tubes.

All the fractions were subjected to antimicrobial

sensitivity testing against those bacteria that should activity to the crude induced hemolymph previously. The fractions F1 to F9 were found to show activity against those tested organisms that have already shown sensitivity to the crude induced hemolymph, whereas other fractions F10 to F17 did not show any activity against the tested bacteria. Antibacterial activity of the chromatographic fractions of induced hemolymph of *Scylla serrata* against *Pasteurella multocida* and *Streptococcus pyogenes* were shown in Fig 4 and 5. Protein concentration of the active fractions (F1 to F9) was recorded in the range from 290-8650 μg mL^{-1}.

Determination of molecular weight of the fractionated peptides: All the purified fractions having activity were loaded on SDS-PAGE gel to determine the molecular weight of the protein(s) responsible for the antibacterial activity. Three bands were observed in fractions F1-F6, two bands in F7 and F8 and only one band in F9 after staining of the gel (Fig 6). The molecular weight of the proteins was determined with the calibration curve of casein, egg albumin, bovine serum albumin as standard and the results showed that total five different proteins were present in different active fractions. F1-F6 contained three proteins of 56.5 kDa, 49 kDa, and 36 kDa; F7 and F8 contained two proteins of 64 kDa and 61 kDa; F9 contained only one protein of 49 kDa.

Discussion

In the present investigation, both induced and non-induced hemolymphs were collected from the mud-crab, *Scylla serrata* and were subjected to antibacterial assay to investigate the presence of inducible and constitutive antibacterial protein(s). A gram-negative bacterium *Escherichia coli* ATCC 25922 was used for the induction of peptides in the crab hemolymph. Morishima *et al.* (1992) tested various bacteria as elicitor of antibacterial protein synthesis in *B. mori* larvae. Some species of gram-positive and gram-negative bacteria were effective elicitor. Peptidoglycan purified from *E. coli* and *Bacillus megaterium* and lipopolysaccharide from *E. coli* were also effective elicitors. The result of our present work is in agreement with the above report (Table-1) where *E. coli* was used to elicit antibacterial proteins/peptides. Although most of the invertebrate antibacterial proteins are inducible, there are some proteins which are not inducible but constitutive such as lysozyme (Powning and Davidson, 1973) and andropin (Samakovlis *et al.*, 1991). Lysozyme occurs constitutively in invertebrates but can also be induced by prior immunization. The result of present investigation showed that the non-induced hemolymph of *Scylla serrata* have no activity against all the tested organisms. This possibly indicates either that the mud-crabs contain constitutive antibacterial proteins in its hemolymph which appeared to be active against other organisms which were not included in the test. It has been observed in various invertebrate species that bacteria injected into the hemocoel elicit the synthesis of a number of antibacterial peptides and proteins, which are secreted into the hemolymph and are active singly or in combined (Gillepsie *et al*., 1997). In the present study, the induced hemolymph showed antibacterial activity against a range of different strains of marine or non-marine gram-positive and gram-negative bacteria including few antibiotic resistant strains as shown in Fig. 1 and 2. The results suggest that the mud-crab can produce antibacterial substances instantly to combat bacterial infection. Induction of antibacterial peptides were also observed in case of sarcotoxin I (Okada and Natori, 1985) and sapecin (Matsuyama and Natori, 1988) in *Sarcophaga peregrine*, moricin (Hara and Yamakawa, 1995), lebocin (Chowdhury *et al*., 1995) and cecropin-B (Taniai *et al*., 1995) in *Bombyx mori*. As the hemolymph showed antibacterial activity against both gram-positive and gram-negative bacteria, it offers to comment that broad-spectrum antibacterial peptides were secreted in response to immunization. Similar observations were also found by Nakamura *et al.* (1988) in *Tachypleus tridentatus*, Morishima *et al*. 1992 in *Bombyx mori*, Gudmundsson *et al*. 1991 in *Hyalophora cecropia*.

It is an interesting finding that crabs, being marine animal has the ability to dispose the bacteria upon infection. As the bacterium is a human pathogen, it is important that sea water should be free from this type of bacteria. Usually it should not be in the water and the peptides can kill more efficiently than the conventional antibiotics. The induced hemolymph of *S. serrata* showed activity against all the gram-positive and six gram-negative wild bacterial strains tested except *Shigella sonnei* and *Vibrio cholerie* (Table 1). The induced hemolymph also inhibited growth of four antibiotic resistant strains among the six tested (Table 2). It also showed activity against only one gram-positive marine bacterium among five strains tested (Table 3). The cultural, microscopic and biochemical characteristics of the hemolymph sensitive marine isolate (Strain no. M_3) indicates that the organism is closely related to the genus *Erysipelothrix* and to the species *E. rhusiopathiae* (Migula) *E. rhusiopathiae* (or *E. insidiosa*) is the single member of the genus *Erysipelothrix*. It causes swine erysipelas and septicemia in mice. In humans, it usually causes a self-limited infection of the fingers and hand (erysipeloid), but may cause bacteremia with arthritis and endocarditis. An erythematous, elevated, spreading lesion develops at the point of

entrance of the organism; the disease generally can be traced on contact with animals or animal products (Sydney *et al*., 1978). It is surprising to find this bacteria in this area of the sea and also the response of the mudcrab against these bacteria.

Not all peptides induced are antibacterial, some acts as signal molecule and some as antibacterial peptides but all work as a concert. Some proteins present in the hemolymph of invertebrates may be both constitutive and inducible for example, P47 of *Ceratitis capitata* (Marmaras and Charalambidis, 1992). They are sometimes good antibacterial and sometimes act as signal transducer, also sometimes show activity more in gram-positive than gram-negative bacteria for example, moricin (Hara and Yamakawa, 1995). The peptide/protein(s) induced in *Scylla serrata* in the present study might have similar feature like those mentioned above and it is possible to draw an inference that upon bacterial challenge *Scylla serrata* can produce better antibacterial response against gram-positive bacteria than those of gram-negative ones. Similar observations were recorded by Matsuyama and Natori (1988) and Destoumieux *et al.* (1997). Destoumieux *et al.* (1997) isolated three members of a new family of antibacterial peptides from the hemolymph of shrimps *Penaeus vannamei.* The three molecules display antimicrobial activity against fungi and bacteria with a predominant activity against gram-positive bacteria. This may be due to the recognition molecules present in the peptides which have an affinity towards gram-positive bacterial cell wall.

As induced hemolymph showed antibacterial activity against several bacterial strains, it was subjected to SDS-PAGE to estimate the number and molecular weight of proteins. After electrophoresis, seven clear bands were detected in the gel which represented proteins of molecular weight 84.5, 71.5, 64, 61, 56.5, 49 and 36 kDa (Fig. 3). It, however, could not be determined at this level which of the proteins was responsible for the antibacterial activity. Schagger and Jagow (1987) explained tricine-sodium dodecyl-sulphate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa which fits well with the range of antibacterial peptides found in this work.

To fractionate the proteins into pure fractions, the induced hemolymph was subjected to gel filtration chromatography and 17 fractions (F1-F17) were collected. All the fractions were tested against the wild strains, antibiotic resistant *E. coli* strains and a marine bacterium against which the crude hemolymph has already shown activity. Fractions F1-F9 showed activity against the tested bacteria but the other fractions (F10-F17) appeared to be inactive (Fig. 4 and 5) which explains that fractions F1-F9 contain protein(s) which are active against the tested bacteria and the remaining fractions did not have activity against those bacteria tested. However, that does not rule out the role of those peptides in the killing process. Those non-sensitive proteins might be involved in signal transduction or in recognition as a concert of antibacterial activity. A comparison of the antibacterial activity of the purified fractions and the crude hemolymph showed that the purified peptides have better activity than the crude hemolymph. It means that the crude hemolymph has several peptides together which gives the concentration of the active one less than the purified one. Due to the purification, the concentration of the active peptide (if one or more) in the purified fractions was high and showed more activity than in combination. The concentration of proteins in the active purified fractions was determined using spectrophotometry and recorded in the range from 290-8650 μg ml^{-1}. A concentration of 2.9 μg disc^{-1} has shown the activity reported here which is much less than the conventional antibiotic (10 μg disc^{-1}) used in the test.

The active purified fractions were subjected to SDS-PAGE to determine molecular weight of the protein(s) comparing with molecular markers (bovine serum albumin, egg albumin, casein). Total five different proteins were present in different active fractions (Fig. 6). F1-F6 contained three proteins of mol. wt. 56.5 kDa, 49 kDa, and 36 kDa; F7 and F8 contained two proteins of 64 kDa and 61 kDa; F9 contained only one protein of 49 kDa. When the results were compared with the proteins in the crude hemolymph, it showed that all the proteins were present in the crude induced hemolymph. Similar molecular weight proteins were also isolated by Okino *et al.* (1995) from horseshoe crab hemocytes. The analysis of protein bands in the SDS-PAGE gel indicated that F1-F6 showed similar protein band patterns and F7 and F8 also showed similar pattern but F1-F5 are different from F6 and F7 both in turns of migration and size of the proteins. F9 showed a band of 49 kDa protein which was also found in F1-F6 fractions. Antibacterial activity of the chromatographic fractions revealed that this 49 kDa protein is responsible for a moderate activity to the tested organisms. In *Ceratitis capitata* larvae, a 47 kDa protein on the surface of some hemocytes is involved in the internalization of LPS. A hemolymph protein of 47 kDa named hemolin, composed of repeated immunoglobulin domains, is thought to have a role in immune recognition and in modulation of defensive responses in *H. cecropia* and *M. sexta* (Faye and Kanost, 1996; Kanost and Zhao, 1996 and Lanz and Faye, 1996). Hemolin is present in the hemolymph at a low constitutive level and induced to concentrations of 1-7 mgml^{-1} after injection of bacteria.

In the present study, the antibacterial peptides are only partially purified. Research is also needed for extreme purification of the peptides in order to determine the structure and sequence of the peptides. Thus the appropriate molecular biology could be examined for possible exploitation for human welfare. However, this study indicates that the inducible antibacterial peptides of *Scylla serrata* would be a good source of antibacterial agents and would replace the existing inadequate and cost effective antibiotics.

References

Azumi, K., H. Yokosawa and S. Ishii, 1990. Halocyamines: novel anti-microbial tetrapeptide-like substances from the hemocytes of the solitary ascidian, *Halocynthia roretzi*. Biochem., 29: 159–165.

Bauer, A.W., W.M.M. Kirby, J.C. Sherris and M. Turck, 1966. Antibiotic susceptibility testing by a standardized single disc method. Am. J. Clin. Pathol., 45: 493-496.

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

Brey, P.T., W.J. Lee, M. Yamakawa, Y. Koizumi and S. Perrot, *et al.,* 1993. Role of the integument in insect immunity: epicuticular abrasion and induction of cecropin synthesis in cuticular epithelial cells. Proc. Natl. Acid. Sci. USA. 90: 6275-79.

Bryan, A.H., 1950. Manual of methods of pure culture study of bacteria. 12: Leaflet. I-X. McCraw Hill Book Co. Inc. New York, London.

Chadwick, J.S. and W.P. Aston, 1991. Antibacterial immunity in Lepidoptera. In: Immunity in Insects and other Arthropods, Ed. AP Gupta, Boca Raton: CRC Press. pp: 347-70.

Charlet, M., S. Chernysh, H. Philippe, C. Hetru, J.A. Hoffmann and P. Bulet, 1996. Innate immunity. Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. J. Biol. Chem., 271: 21808-21813.

Chowdhury, S., K. Taniai, S. Hara, Kadono-Okuda, Y. Kato, M. Yamamoto, J. Xu,, S. Choi, C. Nitish Debnath, Hong Kyu Choi, Akihiro Miyanoshita, Masao Sugiyama, Ai Asaoka 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

Destoumieux, D., P. Bulet, D. Loew, A.V. Dorsselaer, J. Rodriguez, E. Bachère, 1997. Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). J. Biol. Chem., 272: 28398-28406.

Dickinson, L., V. Russell, P.E. Dunn, 1988. A family of bacteria-regulated, cecropin D-like peptides from Manduca sexta. J. Biol. Chem., 263: 19424-19429.

Dunn, P.E., T.J. Bohnert and V. Russell, 1994. Midgut Antibacterial Defenses of Manduca sexta Following Infection and During Metamorphosis. In: Phylogenetic Perspectives in Immunity: The Insect Host Defense, Hoffmann, J., C.A. Janeway and S. Natori (Eds.)., R.G. Landes Co., Austin, TX., pp: 105-111.

Ekluned, C. and C.F. Lankford, 1967. Laboratory Manual for General Microbiology. Prentice' Hall, Inc., Englewood. Cliffs, New Jersey.

Faye, I. and D. Hultmark, 1993. The insect immune proteins and the regulation of their genes. In: Parasites and Pathogens of insects, Vol. 2, Pathogens, ed. NE Beckage, SN Thompson, BA Federici, San Diego: Academic, pp: 25-53.

Faye, I. and M. Kanost, 1996. Function and regulation of hemolin. In: Molecular Mechanisms of Immune Responses in Insects, Ed, P Brey, D Hultmark. New York: Chapman and Hall. In press.

Gillespie, J.P., M. Kanost and T. Trenczek, 1997. Biological mediators of insect immunity. Annu. Rev. Entomol., 42: 611-43.

Greenberg, A.E., J.J. Connors, D. Jenkins and M.A. Frason, 1980. Standard Methods for the Examination of Water and Waste-Water. 15th Edn., American Public Health Association, Washington, DC., USA.

Gudmundsson, C.G., D.A. Lidholn, B. Asling, R. Gan and H.G. Boman, 1991. The cecropin locus: cloning and expression of a gene cluster encoding three antibacterial peptides in *Hyalophora cecropia.* J. Biol. Chem., 266: 11510-17.

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.

Hoffmann, J.A., J.M. Richeart and C. Hetru, 1996. Innate immunity in higher insects, Current Opinion in Immunology, 8:8-13.

Hubert, F, T. Noel and P.H. Roch, 1996. A member of the arthropod defensin family from edible Mediterranean mussels (*Mytilus galloprovincalis*). Eur. J. Biochem., 240: 302–306.

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.* European Journal of Biochemistry, 106: 7-16.

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

Kanost, M.R. and L. Zhao, 1996. Insect hemolymph proteins from the Ig super-family. Adv. Comp. Environ. Physiol., 23: 185-97.

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

Lanz, H. and I. Faye, 1996. Immunoglobulin super-family proteins in invertebrates. New Directions in Invertebrate Immunology. Fair Haven, NJ: SOS Publ. In press.

Lee, H,, C. Zhao, Y. Cho, S.S.L. Harwig, E.L. Cooper and R.I. Lehrer, 1997. Clavanins, alpha-helical anti-microbial peptides from tunicate hemocytes. FEBS Lett., 400: 158–162.

Lee, W.J. and P.T. Brey, 1995. Isolation and characterization of the lysozyme-encoding gene from the silkworm *Bombyx mori*. Gene, 161: 199-203.

Lowry, O. H, N.J. Rosebrough, A.F. Farr and R.J. Randall, 1951. Protein measurement with the Folin-Phenol reagent. J. Biol. Chem., 193: 265-275.

Marmaras, V.J. and N.D. Charalambidis, 1992. Certain hemocyte proteins of the medfly, *Ceratitis capitata*, are responsible for nonself recognition and immobilization of *Escherichia coli* in *in vitro*. Arch. Insect Biochem. Physiol., 21: 281-88.

Matsuyama, K. and S. Natori, 1988. Purification of three antibacterial proteins from the culture medium of NIH-Sape-4, an embryonic cell line of *Sarcophaga peregrine*. J. Biol. Chem., 263: 17112-17116.

Meister, M., P. Georgel, B. Lamaitre, C. Kappler, M. Lagueux, *et al.,* 1994. Immune gene expression in *Drosophila.* Phylogenetic Perspectives in Immunity: The Insect Host Defense. Boca Raton: CRC Press.

Milochau, A., M. Lassegues and P. Valembois, 1997. Purification, characterization and activities of two hemolytic and anti-bacterial proteins from coelomic fluid of the annelid *Eisenia fetida andrei*. Biochim. Biophys. Acta., 1337: 123–132.

Morishima, I., K. Yamada and T. Ueno, 1992. Bacterial peptidoglycan as an elector of antibacterial protein synthesis in larvae of the silkworm *B. mori*. Insect biochem. Mol. Bio., 22: 363-367.

Mulnix, A.B. and P.E. Dunn, 1994. Structure and induction of a lysozyme gene from the tobacco hornworm *Manduca sexta.* Insect Biochem. Mol. Biol., 24: 271-81.

Nakamura, T., H. Furanaka, T. Miyata, F. Tokunaga, T. Muta, S. Iwanaga, M. Niwa, T. Takao and Y. Shimonishi, 1988. Tachyplesin, a class of anti-microbial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*). J. Biol. Chem., 263: 16709–16713.

Okada, M. and S. Natori, 1983. Purification and characterization of an antibacterial peptide from hemolymph of *Sarcophaga peregrine* (fresh fly) larvae. Biochem. J., 211: 727-734.

Okino, N., S. Kawabata, T. Saito, M. Hirata, T. Takagi and S. Iwanaga, 1995. Purification, characterization, and cDNA cloning of a 27-kDa lectin (L10) from horseshoe crab hemocytes. J. Biol. Chem., 270: 31008-31015.

Powning, R.F. and W.J. Davidson, 1973. Studies on insect bacteriolytic enzymes-I. Lysozyme in hemolymph of *Galleria mellonella* and *Bombyx mori.* Comp. Biochem. Physiol., 45: 620-29.

Russell, V.W. and P.E. Dunn, 1990. Lysozyme in the pericardial complex of *Manduca sexta.* Insect Biochem., 20: 501-9.

Russell, V.W. and P.E. Dunn, 1996. Antibacterial proteins in the midgut of *Manduca sexta* during metamorphosis. J. Insect Physiol., 42: 65-72.

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

Schägger, H. and G. von Jagow, 1987. Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem., 166: 368–379.

Schnapp, D., G.D. Kemp and V.J. Smith, 1996. Purification and characterization of a proline-rich anti-bacterial peptide, with sequence similarity to bactenecin 7, from the haemocytes of the shore crab, *Carcinus maenas*. Eur. J. Biochem., 240: 532–539.

Sydney, M.F., J.M. William and G.S. Elvyn, 1978. Bailey and Scott's Diagnostic Microbiology: A textbook for the isolation and identification of pathogenic microorganisms, Ed. 5. The C. V. Mosby Company, Saint Louis, USA, pp: 217.

Taniai, K., K. Kadono-Okuda, Y. Kato, M. Yamamoto, M. Shimabukuro, S. Chowdhury, J. Xu, E. Kotani, S. Tomino and M. Yamakawa, 1995. Structure of two cecropm B-encoding genes and bacteria-inducible DNA-binding proteins which bind to the 5 prime-upstream regulatory region in the silkworm, *Bombyx mori*. Gene, 163:215-219.

Vinson, S.B., 1974. The role of the foreign surface and female parasitoid secretions on the immune response of an insect. Parasitol., 68:27-33.