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Isolation and Purification of antibacterial proteins from hemolymph of Marine gastropods dwelling at rocky shores of Kanyakumari, Southeast coast of India

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ABSTRACT

Proteins from body fluid (hemolymph) of two marine gastropods *Nerita albicilla* and *Purpura bufo* collected from the coastal regions of Kanyakumari were precipitated with ammonium sulphate and purified using ion-exchange chromatography. The fractions obtained were tested for their antibacterial potential against human pathogens. Fractions F2 and F4 from *N.albicilla* showed higher inhibitory activity against *Salmonella typhi*, *Streptococcus mutans* and *Staphylococcus aureus*. The molecular weight of this antibacterial protein present in the fractions was determined with the help of SDS PAGE and it was found to be 30KDa. Further, characterization of this active protein paves the way for the development of new antibiotic against harmful human pathogens in future.

Keywords: Gastropods, hemolymph, peptides, pathogens, antibiotics

INTRODUCTION

Ocean serves as a reservoir for newly discovering potential therapeutic agents. Numerous compounds with interesting pharmaceutical activities have been reported from marine organisms during the past decades. Hence, marine organisms are considered as important source of bioactive molecules to treat various diseases. Emergence of antibiotic resistant microbes has increased the demand for the production of novel and effective antimicrobial compounds (Villa *et al*., 2010 and Blunt *et al*., 2011). Marine invertebrates lack acquired memory type immunity which differentiates it from vertebrate immune system (Ratcliff, 1989). Innate immune mechanisms including humoral and cellular responses are observed in these organisms. Circulating hemolymph in themselves make this innate immune system more effective because it contains biologically active substances such as complement, lectin, clotting factors and antimicrobial peptides that fight against invading microorganisms (Menzel *et al*., 2002; Miyata *et al*., 1989). The hemolymph

also contains water, sugar, salts, amino acids and white cells as found in blood. It helps in the transport of digestive and excretory product and also facilitates all chemical exchange within the tissues (Dimitriadis, 2005). The most interesting phyla that contribute to pharmacologically active marine compounds include bacteria, fungi, algae, sponges, soft corals, molluscs and bryozoans (Faulker, 2000).

Molluscs contribute mainly to the biomedical area among them antimicrobial peptides play a major role in host defense system of many animal species (Boman, 1995). Antimicrobial peptides have recently drawn attention as the host defense compound due to their properties and diversity (Smith and Chisholm, 2001). Gastropods have a well-developed internal defense system and it is principally carried out by both the cellular and humoral components (Ottaviani, 1992). The hemolymph in them contain proteins which are unique in composition, as they do not contain immunoglobulin or albumin like proteins and its composition varies in relation to physiological and functional state of the animal (Terwilliger *et al*., 1998). Bioactive compounds from *Phyllidae* sp (Ilangedone *et al*., 1999), bivalves (Chellaram *et al*., 2004) and gastropods (Kagoo *et al*., 1992) exhibiting antitumour, antileukaemic, anti-bacterial and antiviral activities have been reported. Studies on antimicrobial proteins of mollusc group and also on whole body homogenates of some marine molluscs for their antimicrobial compounds may provide valuable information for newer antibiotic discoveries (Amutha and Ulagesan, 2016). Bioactive potentials of hemolymph have attracted pharmacologist to focus their search for potential metabolites from marine gastropods (Amruthalakshmi and Yogamoorthi, 2015). Therefore, the aim of the present study is to evaluate the antibacterial property of hemolymph proteins of the two marine gastropod species against different human pathogens and to determine the molecular weight of the active protein present in the hemolymph using SDS-PAGE which is responsible for antibacterial property.

MATERIAL AND METHODS

Collection of samples

Marine gastropod species such as *Nerita albicilla* and *Purpura bufo* were collected by hand picking from the rocky shores of Chinnamuttom (8.094345^{o}N and 77.561445^{o}E) and Colachel (8.17^{o}N and 77.24^{o}E) coastal areas in Kanyakumari district, Tamilnadu, India. They were brought to the laboratory and rinsed with tap water. Shells were broken and kept in a tray to collect the hemolymph.

Purification of hemolymph proteins

Hemolymph was collected from each animal by puncturing the pedal blood sinus with a syringe needle. Haemocytes and cellular debris were removed by centrifugation at 10,000 rpm for 20 min at 4°C. The supernatant was stored at −20°C until further use. The hemolymph proteins of two marine gastropod species namely *N.albicilla* and *P.bufo* were precipitated using 80% ammonium sulphate solution and dialysed against Tris HCl buffer. The dialysed protein samples were subjected to Ion Exchange Chromatography for further purification. The column was packed with DEAE cellulose and previously equilibrated by 20mM Tris buffer, P^{H} 7.4 and then the column was washed by 0-1 M NaCl. The flow rate was fixed as 2ml/min. The column fractions were measured for absorbance at 280nm. The active fractions were pooled and checked for antibacterial activity.

Antibacterial analysis

The purified fractions obtained for each sample were subjected to antibacterial assay. The assay was performed by well diffusion method against seven human pathogenic MTCC strains namely *Bacillus subtilis* (strain no.1134), *Escherichia coli* (strain no.1671), *Klebsiella pneumoniae* (strain no. 7407) *Pseudomonas aeruginosa* (strain no.6538), *Salmonella typhi* (strain no.733), *Staphylococcus aureus* (strain no.916) and *Streptococcus mutans* (strain no.1936). Petri plates containing 20ml Muller Hinton medium were seeded with 24hr culture of bacterial strains. Wells of approximately 9 mm was bored using a well cutter and, 50µl of sample was added to the well. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well (NCCLS, 1993). Streptomycin was used as a positive control. The analyses were carried out in triplicate and results are reported as the mean±standard deviation (SD).

Molecular weight determination was done by Sodium dodecyl Sulphate polyacrylamide gel electrophoresis. Purified protein fractions present in the sample were separated by SDS-PAGE analysis in 10% polyacrylamide gel (Laemmli, 1970). 10 µl sample with different molecular weight markers (14.4 – 97.4 KDa) were

loaded in the well and run the gel. Gels were then stained with Coomassie brilliant blue and destained for clear visualization of bands.

RESULTS

Partial purification of hemolymph proteins

Altogether 30 fractions were obtained from each dialysed hemolymph protein samples. Among them, 8 fractions were selected based on protein absorbance at 280nm and tested for antibacterial activities. The selected fractions were named as F1, F2, F3, F4, F5, F6 F7 and F8. The chromatograms of dialysed hemolymph protein samples are represented in Figure 1 and 2.

Antibacterial assay of purified protein fractions

In this assay, the fractions F2 and F4 obtained from *N. albicilla* showed maximum zone of inhibition against the seven pathogenic bacterial strains. The zone of inhibition for fraction F2 was measured as 18.33±0.57, 14.33±0.57, 13±1, 13±1, 12±1, 10.33±0.57 and 10.33± 0.57 mm and for fraction F4 it was observed as 14±1, 13.33±0.57, 19.33±0.52, 15.33±0.57, 11.6±0.57 and 11.6±0.57mm in *S. mutans, E. coli, S. typhi, S. aureus, P. aeruginosa, B. subtilis and K. pneumoniae* respectively. The other fractions such as F1, F3, F5, F6, F7 and F8 also showed zone of inhibition against the pathogenic bacterial strains when compared to control which are represented in Table 1.

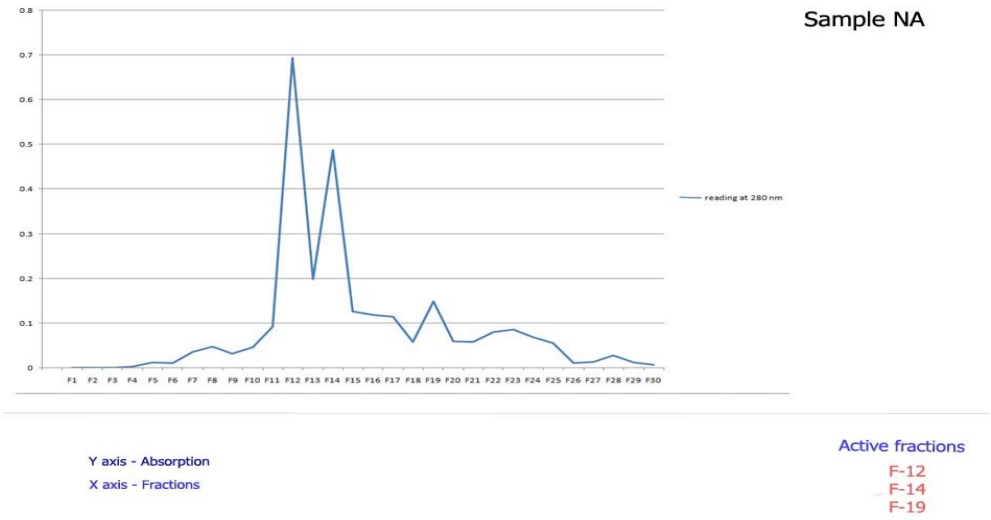

Fig 1. Ion-exchange chromatogram of dialysed hemolymph protein sample from *N. albicilla*

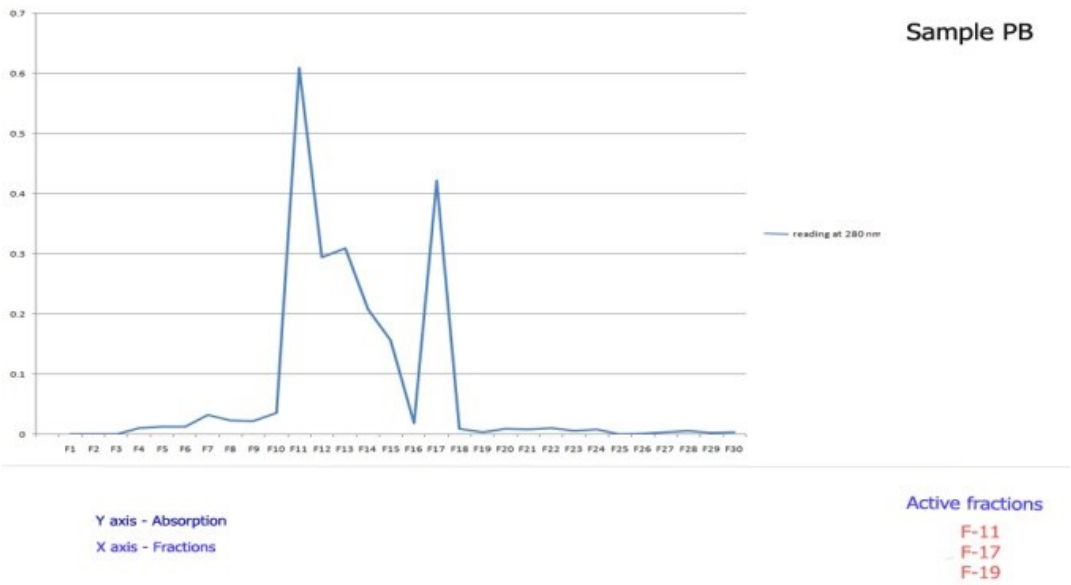

Fig 2. Ion-exchange chromatogram of dialysed hemolymph protein sample from *P. bufo*

Table1. Antibacterial assay of purified fractions from *N. albicilla.* The data was expressed as mean of triplicates ±SD measurements

Pathogenic Bacteria	Zone of inhibition (mm)								
	Control	F1	F2	F3	F4	F5	F6	F7	F8
S. mutans	37 ±0.57	11.33 ±0.57	18.33 ±0.57	11.6 ±0.57	14 ±1	-	-	12 ±1	17 ±0.57
E.coli	38.3 ±0.57	10.33 ±0.57	14.33 ±0.57	8.6 ±0.57	13.33 ±0.57	-	-	10.33 ±0.57	10.66 ±0.57
S. typhi	36.3 ±0.57	10.33 ±0.57	13 ±1	18.33 ±0.57	19.33 ±0.52	10.66 ±0.57	17.33 ±0.57	12.33 ±0.57	15.33 ±0.57
S. aureus	15.3 ±0.57	11.33 ±0.57	13 ±1	10.33 ±0.57	15.33 ±0.57	-	10.33 ±0.57	10.33 ±0.57	10.66 ±0.57
P. aeruginosa	39.3 ±1.5	10.33 ±0.57	12 ±1	-	-	-	-	-	-
B. subtilis	29 ±1	-	10.33 ±0.57	10.33 ±0.57	11.6 ±0.57	10.66 ±0.57	17 ±1	17 ±1	17.66 ±0.57
K. pneumoniae	27.3 ±0.57	-	10.33 ±0.57	10.33 ±0.57	11.6 ±0.57	10.33 ±0.57	12 ±1	-	12.33 ±0.57

Table 2: Antibacterial assay of purified fractions from *P. bufo*. The data was expressed as mean of triplicates ±SD measurements.

Pathogenic Bacteria	Zone of inhibition (mm)								
	Control	F1	F2	F3	F4	F5	F6	F7	F8
S. mutans	37.66±0.57	-	-	-	-	-	-	-	-
E.coli	34.66±0.57	8.6±0.57	10.66±0.57	10.66±0.57	10.66±0.57	-	-	-	-
S. typhi	35.33±0.57	-	-	-	-	-	-	-	-
S. aureus	37.66±0.57	8.6±0.57	10.66±0.57	8.6±0.57	10.33±0.57	-	-	-	-
P. aeruginosa	39.3±1.5	-	-	-	-	-	-	-	-
B.subtilis	29±1	-	-	-	-	-	-	-	-
K. pneumoniae	27.3±0.57	-	-	-	-	-	-	-	-

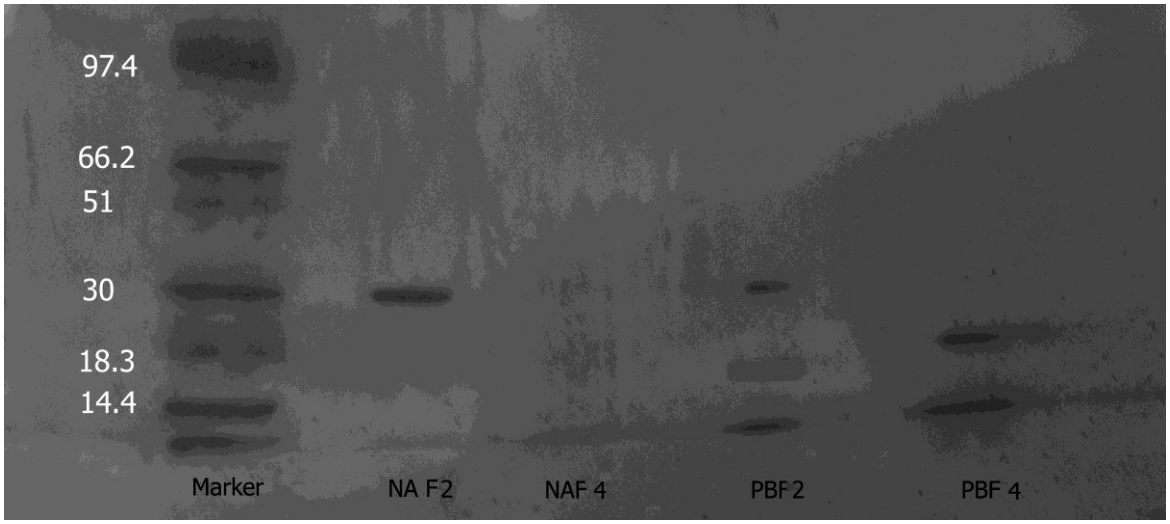

Plate 1: SDS Gel Electrophoresis of purified fractions F2 and F4 from *N. albicilla* and *P. bufo*

Likewise, the purified protein fractions obtained from *P.bufo* were also subjected to antibacterial assay and tabulated which is shown below. Among 8 fractions selected, fractions such as F1, F2, F3 and F4 showed zone of inhibition 8.6±0.57, 10.66±0.57, 10.66±0.57 and 10.66±0.57mm against *E.coli* and the same fractions also showed zone of inhibition 8.6±0.57, 10.66±0.57, 8.6±0.57 and 10.33±0.57mm respectively against *S. aureus*. All the 8 fractions (F1–F8) did not show any positive result against the remaining bacterial strains namely *S. mutans, S. typhi, P. aeruginosa, B. subtilis* and *K. pneumoniae* compared to control.

SDS gel electrophoresis of purified fractions

Since, two fractions F2 and F4 from both *N. albicilla* and *P. bufo* hemolymph protein samples showed maximum zone of inhibition, these two fractions were selected for further studies. These fractions were loaded in SDS gel electrophoresis to identify the molecular mass of the respective protein fractions. The F2 fraction of *N. albicilla* showed a single band indicating the presence of a single protein with the molecular weight 30KDa. No bands were observed for the fraction F4. Also, the fraction F2 from *P. bufo* showed a single band with molecular weight 30 kDa and the other fraction F4 showed two bands with molecular weight less than 20 KDa.

DISCUSSIONS

Marine metabolites or marine natural products receive the attention of researchers from various fields because 25% of all drugs contain active principles that are obtained from these natural products. Among all the classes in the phylum Mollusca, class Gastropoda includes single shelled animal found in shallow, intertidal areas and deep sea. The opercula of gastropods are used traditionally in Indian medicine as an ingredient to combat different diseases (Periyasamy *et al*., 2012).

The respiratory proteins, hemocyanins occur freely dissolved in the hemolymph of molluscs. The salting out procedure has been usually employed for protein separation. The high solubility property of salt ammonium sulphate makes it widely used for the salting out process and is relatively inexpensive (Idakieva *et al*., 2009). In our present work, ammonium sulphate salt was used to precipitate hemolymph proteins and the precipitated proteins were dialyzed against Tris-HCl buffer. The dialyzed protein samples were further purified using ion-exchange chromatography technique. In a study made by Idakieva *et al*. (2009), hemocyanin isolated from marine gastropod *Rapana thomasiana* using ion-exchange chromatography were found to be eluted as single and symmetrical peaks, indicating that the proteins were homogenous in size. The fractions obtained in our study were tested for their antibacterial potentials against seven human pathogens. Among eight fractions selected for antibacterial studies two fractions from each gastropod species showed maximum zone of inhibition. It correlates with the study conducted by Wirawan *et al*. (2007) in which the purified protein fractions isolated from hemolymph of *C. belcheri* (oyster) exhibited antibacterial activity against Gram negative bacteria *vibrio* sp.

The antibacterial proteins act as an important one in the first step of defense against pathogenic microorganisms. The hemolymph, egg mass or the whole body extracts have been tested for antibacterial activity in most of the molluscan species namely oyster (*C. virginica*), mussel (*Mytilus edulis*), muricid mollusc (*Dicathais orbita*) and sea hare (*Dolabella auricularia*) (Anderson and Beaven, 2001; Ijiima *et al*., 2003). It has been reported that small antimicrobial peptides and large protein hemocyanin are also isolated from the hemolymph of molluscs (Coates *et al*., 2014; Zhuang *et al*., 2015). Broad spectrum of antimicrobial activity against Gram positive and Gram negative bacteria were reported by peptides/proteins from the hemolymph of molluscs and arthropods (Rong *et al*., 2013; Gabriel *et al*., 2011). In a study conducted by Dolashka *et al*. (2011) pharmacologically active peptides has been isolated from the hemolymph of marine snail *Rapana venosa* which showed high antimicrobial activity against *S. aureus* and low activity against *K. pneumonia*. Destomieux *et al*. (1997) isolated antibacterial peptides from the hemolymph of shrimp *Penaeus vannamei*. They were found to possess antimicrobial activity against fungi and bacteria mainly Gram positive bacteria. It suggested that these peptides had greater affinity towards Gram positive bacterial cell wall. The present study also demonstrates the presence of antibacterial proteins in hemolymph of collected marine gastropod species such as *N. albicilla* and *P. bufo*.

Hoq *et al*. (2003) reported that hemolymph from bacterial induced mud crab *Scylla serrata* showed antibacterial activity against both Gram positive and Gram negative bacteria namely *B. subtilis, B. cereus, B.*

megaterium, S. aureus, S. pyrogenes, P. aeruginosa, S. typhi, S. paratyphi, S. dysenteriae, P. multocida and E. coli, whereas the hemolymph of native one had none and he found that the components responsible for antibacterial activity in the induced hemolymph were five different proteins with molecular weight of 64, 61, 56.5, 49 and 36 KDa respectively. It shows that antibacterial peptides were secreted in response to immunization in hemolymph of *S. serrata*. Similar observations were made by Nakamura *et al*. (1988) in *Tachypleus tridentatus*, Morishima *et al*. (1992) in *Bombyx mori*, Gudmundsson *et al*. (1991) in *Hyalophora cecropia*. Antimicrobial peptides reported from insect hemolymph lack adaptive immune system which play a major role in fighting against invading pathogens. These antimicrobial peptides are released into hemolymph due to microbial infections or body injury in insect fat body where they act against microorganisms (Tzou *et al*., 2002; Dunphy *et al*., 2003, Irving *et al*., 2004).

The present study reveals that the active fractions contained active peptides with molecular mass 30 KDa for both species. Similar observation were found by Wirawan *et al*. (2007) in oyster *Crassostrea belcheri* and two antibacterial proteins were isolated from hemolymph which had molecular weight 30.5 and 25 KDa respectively. Andreas *et al*. (1997) reported that gloverin, an antibacterial protein isolated from hemolymph of *Hyalophora pupae* inhibited the growth of *E. coli* by affecting the integrity of the permeability barrier and the molecular weight of these proteins were found to be 13.8KDa. Bactericidal lectin obtained from hemolymph using partial characterization by affinity chromatography inhibited both Gram positive and Gram negative microbial growth with molecular weight 4.8 – 5KDa (Chattopadhyay *et al*., 1996). Active peptide with molecular weight 35KDa has been isolated from hemolymph of marine gastropods *Rapana rapiformis* by Amruthalakshmi and Yogamoorthi, (2015). Likewise, Pitchiah *et al.* (2013) isolated proteins from hemolymph of shoe crab *Ocypoda macrocera* with molecular weight ranging between 15.43 – 60.34 KDa with antimicrobial potency. Hemolin, a hemolymph protein with molecular weight 47 KDa containing repeated immunoglobulin domains have an important role in immune recognition and in defensive responses in *H. cecropia* and *H. sexta* (Faye and Kanost, 1996; Kanost and Zhao, 1996).

CONCLUSION

The proteins isolated from the hemolymph of two marine gastropods *Nerita albicilla* and *Purpura bufo* showed inhibitory activity against human pathogenic strains and their molecular weight was found to be 30 KDa. Research is needed for further purification of the peptides in order to determine the structure and sequence of the peptides. However, this study indicates that the antibacterial peptides of *N. albicilla* and *P. bufo* would be a good source of antibacterial agents.

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