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Bioactive Potential of Proteins from Deep Sea Organisms

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Abstract

Marine environment rich in prokaryotes and eukaryotes is a reservoir of novel bioactive molecules with wide chemical diversity. For this study, deep sea organisms were collected by dredging and trawling during FORV Sagar Sampada Cruise # 291 Leg I and Cruise # 305. Around 60 different marine organisms were sampled and screened for antimicrobial compounds and proteins against an array of human pathogens such as *Klebsiella pneumoniae, Bacillus cereus, Salmonella typhimurium, Escherichia coli*, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Vibrio cholerae* by agar well diffusion method and disc diffusion method. Solvents such as ethanol, methanol, chloroform, butanol and 5% acetic acid were used. Thin layer chromatography and bioautography assays of bioactive extracts confirmed the presence of antimicrobial compounds in them. The separation of the compound was done using cyclograph. Ray fish, star fish, *Loligo* sp., spider crab, *Maxmulleria* sp. and mussel showed presence of an antimicrobial protein that was purified by TLC, cation exchange chromatography and tricine-SDS PAGE.

Keywords: Antimicrobial activity, pathogens, disc diffusion, bioautography, TLC, SDS – PAGE

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Introduction

The marine environment is tremendously rich in species diversity, which is estimated to be approximately half of the total global biodiversity, while estimates of the marine macro fauna alone range between 0.5 and 10×10^6 different species (Bouchet, 2006; de Vries & Hall, 1994). The marine environment varies substantially from terrestrial and sweet water habitats and remains relatively uncharted. The unique habitats in the ocean harbour animals that through evolution have developed molecules for defense against pathogens and predators and for paralyzing/killing of prey (Krug, 2006). Furthermore, marine animals are in close proximity with microbes. The estimated density of bacteria in sea water and sediment is in the order 10^5–10^7 ml^{-1} and 10^8–10^{10} g^{-1}, respectively (Austin 1988). This constant pressure from potentially harmful microbes, combined with the evolutionary success among marine invertebrates, suggests that the immune effectors found in these animals are highly effective in bacterial killing/inhibition.

One major difference between the marine and terrestrial environments is the relative stability of the physical factors especially in the deep pelagic waters. Life there is usually confronted with low temperature, elevated pressure, absolute darkness and high salinity (Sigmund et al., 2011).

Unusual compounds have been obtained from marine organisms because they live in a very exigent, competitive and aggressive surrounding very different in many aspects from the terrestrial environment, a situation that demands the production of quite specific and potent active molecules. Knowledge of the bioregulatory role of different endogenous peptides in organisms as well as the understanding of the molecular mechanisms of action of some new bioactive peptides obtained from natural sources on specific cellular targets, contributed to consider peptides as promising lead drug candidates. Recently marine peptides have opened a new perspective for pharmaceutical development (Tincu & Taylor, 2004).

Cyclic and linear peptides discovered from marine animals have increased our knowledge about new potent cytotoxic, antimicrobial, ion channels specific blockers, and many other properties with novel

chemical structures associated with original mechanisms of pharmacological activity. These facts introduce marine peptides as a new choice of lead compounds for biomedical research (Aneiros & Garateix, 2004).

Antimicrobial peptides (AMPs) are classically described as small cationic, amphipathic, gene-encoded molecules (<10 kDa, 15-100 amino acids) that differ considerably in amino acid sequence and structural conformation. They are commonly found in the blood or epithelial (mucosal) surfaces that are most exposed to microorganisms. The AMP mode of action is basically determined by structural conformation, cationic charge and amphipathicity. It is generally agreed that AMPs predominantly act by disrupting the membrane integrity of the cell target. The cationic portion of the peptide is first attracted to the negatively-charged bacterial and fungal cell walls and/or membranes and following this first electrostatic interaction, the peptide inserts into and permeabilizes the microbial cell membranes through its hydrophobic portion (Rosa & Barracco, 2010).

Fishes possess a strong innate immune system, which act as the first line of defense against pathogen infections. Fish AMPs and their possible applications are reported by researchers (Rajanbabu, 2011). These fish origin AMPs have positive charge and amphipathic characteristics like other AMPs from terrestrial animals. Researchers have focused on preparation of fish AMPs from fish secretes, organ extractions, or transgenic expression (Song et al., 2012).

In the present study, the focus was to screen almost 60 different deep sea organisms obtained from the dredge and trawl for the presence of antimicrobial peptides.

Materials and Methods

Fishes were collected from deep sea (200 m) by dredging and trawling during Sagar Sampada Cruise #291 Leg I and Cruise # 305 and were stored in -80^{0}C deep freezer for further studies. Different parts like, skin tissue, flesh, gut region and mouth parts were used for antibacterial compound isolation.

Five grams of tissue was homogenized using tissue homogenizer (PRO, UK), 5 ml of hand bearable hot tissue and cell lysis buffer (1% Triton X 100 for lysis, 50 mMTris (pH 8.0), 150 mM sodium chloride containing aproteinin as protease inhibitor) was added, vortexed for 10 minutes and allowed to stand at 45^{o}C for 20 min and the total protein was determined by Lowry's method (Lowry et al., 1951).

The protein extract was acetone precipitated by adding 4 volumes of cold acetone to 1 volume of crude protein extract in a centrifuge tube followed by incubation at -20^{o}C deep freezer for four hours (Wessel & Flugge, 1984). The total protein was precipitated out whereas other chemical compounds in the buffer added were removed along with the supernatant. The supernatant was discarded after centrifugation at 12000 rpm for 15 min at 4^{o}C and the air dried pellet was suspended in phosphate buffer of pH 7.2. The antibacterial activity of the suspension was checked by agar well diffusion method.

To study the presence of antibacterial compounds 10 g of homogenized sample was mixed with 5 ml of organic solvent/5% acetic acid and mixed continuously overnight in a shaker (Orbitek, Germany) at 120 rpm at room temperature. The tubes were centrifuged at 10000 rpm for 10 min (Hermle, Germany). The supernatant was collected and subjected to agar well diffusion method to check for antimicrobial activity, with the solvent alone as control.

Bacterial strains for antibacterial studies were obtained from NCIM, Pune, India. The strains used were *Klebsiella pneumonia* (NCIM No. 5082*), Bacillus cereus* (NCIM No. 2217), *Salmonella typhimurium* (NCIM No. 2501), *Escherichia coli* (NCIM No. 2574*)* and *Staphylococcus aureus (*NCIM No. 2079*).*

Antibacterial assay was conducted by Agar well diffusion method (Bauer et al., 1966). The 18 h old bacterial cultures grown in nutrient broth were spread on Mueller Hinton agar plates (Himedia, India) and wells of 0.2 cm diameter were punctured. 30 µl of the extract was added to each well (Perez et al., 1990). The zone of inhibition was measured. The buffer itself was used as negative control and ampicillin (HiMedia, Mumbai, India) as positive control.

Thin layer chromatography (TLC) was done on silica gel G25 plates (Merck, Germany). About 40 µl (0.5 mg mL^{-1}) of sample was loaded on the plate. This was then developed with the solvent n-butanol: acetic acid:water (5:1:5) (Pillay & Mehdi, 1970). The plates were dried and stained with 0.2% ninhydrin.

The band formed by the active peptide was located by bioautographic assay (Sule et al. 2011). A thin layer of Mueller – Hinton Agar (HiMedia, Mumbai, India) with 2% agar was plated in a sterile petri dish and allowed to solidify. The unstained TLC plate, developed as detailed above, was placed over the agar medium. This was overlaid with molten MH agar (0.8%) containing 1 ml (0.1OD 600 nm) of the test organism, *Staphylococcus aureus*. The plates were incubated at 37°C for 24 h. Zones of inhibition of 6 mm diameter were visualized by adding dehydrogenase- activity- detecting reagent, *viz*., a 2, 3, 5-triphenyltetrazolium chloride (TTC 1 mg 6 ml^{-1}) into the plates (Hamburger & Cordell, 1987). Metabolically active bacteria convert the colorless tetrazolium salt into the corresponding intensely colored formazan. The area where metabolically active bacteria was not present appeared as a clear zone against red background. TLC plate submerged in the mobile phase was used as control.

Further purification and characterization was carried out with two samples, mollusc-1 and star fish by Cation exchange chromatography and analyzed by SDS- PAGE.

Almost 2 ml (8 mg ml^{-1}) of the acetone precipitated sample, dissolved in 0.01 M phosphate buffer was loaded into the cation exchange column (Hunter & Carta, 2000), Unosphere S (Biorad, USA) was filled up to 25 cm in a 30 x 1 cm^2 Econocolumn to get a final volume of 25 ml. Prior to loading, the column was equilibrated with 0.01 M phosphate buffer of pH 7.4 and eluted in FPLC (fast protein liquid chromatography, Biorad, USA). The unbound proteins were eluted by 0.01 M phosphate buffer. Then the column was gradient eluted with 0.1, 0.2, 0.3, 0.5 and 1M NaCl in 0.01M phosphate buffer. The fractions were collected, quantified spectrophotometrically and used for further study.

The active fractions were separated on tricine-SDS PAGE (Schägger & von Jagow, 1987) (Biorad, USA). Active fraction was mixed with loading dye containing sample buffer (prepared with glycerol, tris-HCl and bromophenol blue). The samples were loaded on to 15% tricine Polyacrylamide gel along with molecular weight markers (Biorad, USA).Silver staining was performed for visualizing the bands. The molecular weight and the purity of the loaded samples was determined.

Results and Discussion

Among the sixty organisms screened only ten organisms gave positive results (Table 1).

In certain cases, particular portions like mouth parts, tail, gut region etc were only used for homogenizing, whereas in some cases, the whole organisms were used as such. In the case of mussel, ray tail, *Benthobatis* sp., *Loligo* sp., *Octopus* sp., maxmulleria and spider crab the extract by extraction buffer gave activity while in mollusc 1, star fish and jelly fish gave activity in 5% acetic acid extract.

Table 1. Organisms showing positive results in screening of antibacterial peptides

Sample	*Salmonella typhi*	*Salmonella typhimurium*	*Escherichia coli*	*Klebsiella pneumoniae*	*Staphylococcus aureus*
Mytilus sp. (Mussel)	-	-	+	-	+
Manta sp. (tail)	-	-	-	-	+
Bursa sp. (Mollusc 1)	-	+	+	+	-
Maja sp. (Spider Crab)	-	-	-	+	-
Asteroidea (Star fish)	+	++	++	++	++
Aurelia sp. (Jelly fish 1)	+	-	-	-	-
Benthobatis sp.	+	+	-	-	+
Loligo sp.	-	-	-	-	+
Octopus sp.	+	-	-	-	+
Maxmulleria sp.	-	-	-	-	+
Positive control (Ampicillin)	+	+	+	-	+

*All the values are deducted from control values, + zone size below 10mm. ++ zone size 20mm or greater

After acetone precipitation, the activity was obtained in the pellet dissolved in phosphate buffer, showing that the activity was due to some peptides in the extracts. TLC and bioautographic assay also confirmed the presence of bioactive proteins in the extract (Fig. 1)

In ion exchange chromatography, the active fraction with peptide content of 0.67 mg mL^{-1} was obtained in elution with 1M NaCl in phosphate buffer for mollusc -1 (Fig. 2). While for star fish, the active fraction with peptide content of 0.43 mg mL^{-1} was found in the single fraction in 0.5 M NaCl (Fig. 3).

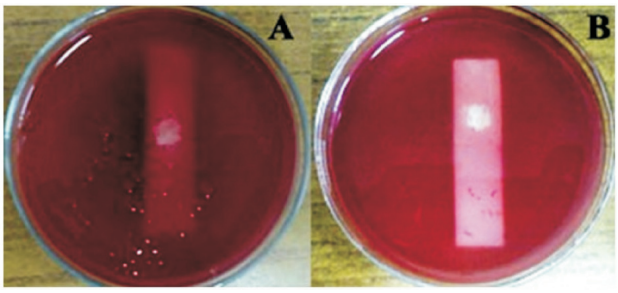

Fig. 1. Bioautographic assay. A: *Bursa* sp., B: Asteroidea (Star fish).

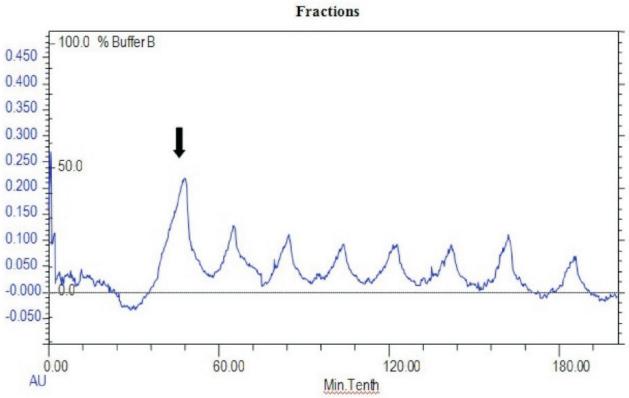

Fig. 2. Ion exchange chromatography of Mollusc-1. The first peak showed the activity. The peaks correspond to UV absorption.

This shows their high cationic nature. SDS - PAGE shows the presence of low molecular weight proteins of approximately 20 kDa and 18 kDa for mollusc-1 and star fish respectively (Fig. 4).

Though there are variations in structure and sequence, the AMPs are widely regarded as cationic and hydrophobic molecules which attack the bacterial or fungal cells by a pore forming action or

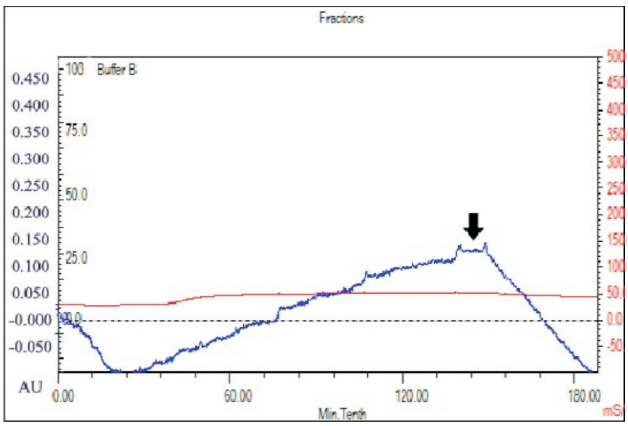

Fig. 3. Ion exchange chromatography of bioactive compound from Star fish

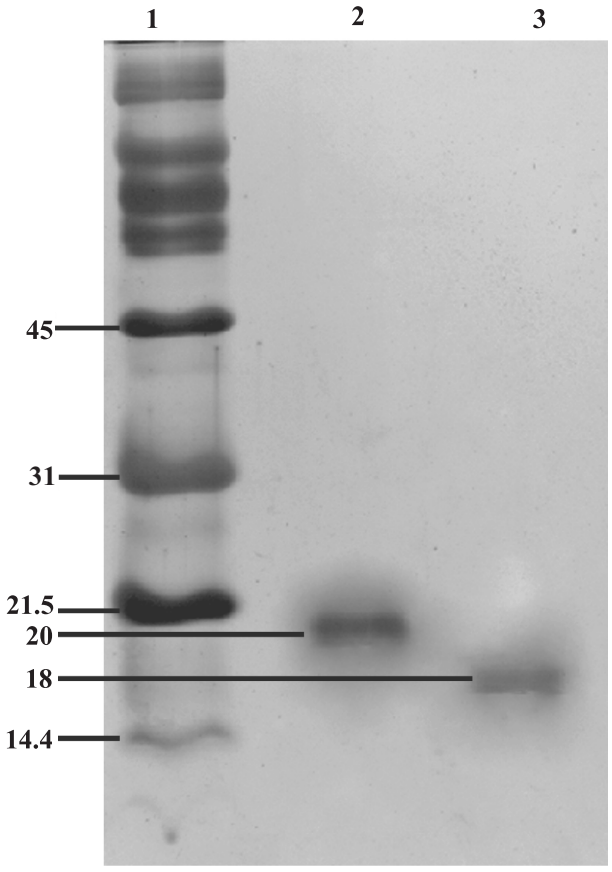

Fig. 4. Tricine – SDS-PAGE of the purified extracts.Lane 1: Low molecular weight protein marker; Lane 2: Purified extract of Mollusc-1 and Lane 3: Purified extract of star fish.

detergent effect. AMPs have been shown to exert little or no lethal effect on normal eukaryotic cells, due to its specificity in action. Their real target is either prokaryotic cells or abnormal eukaryotic cells.

Thus AMPs are considered as promising candidates for screening of new antibiotics (Li et al., 2009).

The origin and role of bioactive peptides in these organisms, in many cases are unclear. Many of these compounds have potent activities but not always clearly related to their *in situ* role. Examples are antitumor, antiviral, anti inflammatory and immunosuppressive properties. This indicates the need for further research into the bioactive potential of these antimicrobial peptides. A multidisciplinary and cooperative effort with the use of more sensitive and fast methods in the analysis of the structure of peptides and an evaluation of the pharmacokinetic and pharmacodynamic parameters will drastically speed up the entry of novel active peptides from marine sources to the pharmaceutical sector. To conclude, the results of this study provide useful information for investigation of antibacterial peptides derived from deep sea organisms.

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