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Characterization of a Bioactive Protein with Antimicrobial Property from *Loligo* sp.

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Abstract

Loligo is a genus of squids. Due to their abundance and economic importance in the export market, squids have been studied for their biology and fishery characteristics. The aim of the current study was to investigate anti-microbial activity associated with proteins extracted from this organism. Whole body tissue was homogenized with protein extraction buffer and its antimicrobial activity was screened against *Klebsiella pneumoniae, Bacillus cereus, Salmonella typhimurium, Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Vibrio cholera*e by agar well diffusion method. The protein was concentrated by acetone precipitation. Purification was done in different steps employing cut off membrane filtration, thin layer chromatography (TLC) and Sephadex G-50 gel filtration column. The molecular weight of the protein was determined by SDS-PAGE. A 45 kDa protein was isolated and purified from *Loligo* with a total yield of 0.06% which showed bioactivity against *V. cholerae.* MALDI and MASCOT analysis showed that the 45 kDa protein had a similarity score of 40 with parvalbumin protein of *Fundulus similis.*

Keywords: Antimicrobial activity, *Vibrio cholerae*, Sepahdex G-50, SDS-PAGE, MALDI, MASCOT

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Introduction

Emergence of multidrug resistance in human pathogens has become a major threat in treatment of microbial diseases caused by them. Most of the antimicrobials to date have been isolated from terrestrial organisms and these are well mined. With the costs of developing new drugs sky-rocketing, the search for antimicrobials has dipped in recent years. Marine environment is even today underutilised as a resource of new drugs. The reason for the same are manifold; unavailability of samples and difficulty of sampling from great depths to name a couple. However, in recent years, several bioactive compounds have been unearthed from diverse groups of marine animals like tunicates, sponges, sea hares, nudibranchs, bryozoans, sea slugs and marine microorganisms (Donia & Hamann, 2003; Haefner, 2003). The search for new metabolites from marine organisms has resulted in the isolation of about 10,000 metabolites (Fueseatni, 2002), many of which are endowed with pharmacodynamic properties.

Cephalopods include squids, cuttlefishes, octopuses and nautilus. Several bioactive molecules have been obtained from cephalopods; molecules which are linked to their survival or are part of their defence mechanisms (Nair et al., 2011). *Loligo* is a genus of squids and a representative of the widely distributed groups of myopsid squids. Three species of *Loligo* such as *Loligo forbesii, Loligo reynaudii* and *Loligo vulgaris* are extensively exploited by commercial fisheries.

V. cholerae is a Gram-negative bacterium. Two serogroups of *V. cholerae*, O1 and O139, cause outbreaks of cholera (Siddique et al., 1991; Yu et al., 2012) which is due to their colonization in the small intestine and secretes cholera toxin, a protein that causes profuse, watery diarrhoea. Commercially available antibiotics commonly administered as part of the treatment include tetracyclines, fluoroquinolones, macrolides and trimethoprim/sulfamethoxazole (WHO, 2004; Kitaoka et al., 2011). Several reports have documented tetracycline and fluoroquinolone resistant *V. cholerae*, and multidrug

resistance is increasing rapidly (Kitaoka et al., 2011). Antimicrobial drug resistance in *Vibrio* spp. can develop through mutation or through acquisition of resistance genes on mobile genetic elements, such as plasmids, transposons, integrons and integrating conjugative elements (ICEs) (Karlsson et al., 2011; Kitaoka et al., 2011).

This study looked at the potential of *Loligo duvaceli* as a source of antimicrobial molecules against some test organisms including human pathogens.

Materials and Methods

Sample collection

Loligo duvaceli samples were collected from the Quilon fishing harbour, Kerala, India. The samples were packed in sterile polythene bags and transported in ice to Department of Biotechnology, Cochin University of Science and Technology, India where they were stored at -80°C until further analysis.

Analytical grade standards and chemicals like Mueller-Hinton Agar (HiMedia), RIPA (Radio-Immunoprecipitation Assay) buffer, Sephadex G-50 (Sigma), Slilica gel (Merck), Acetone (SRL), Butanol (SRL), Acetic acid (SRL), Tris- HCL (pH 8), Glycerol (MercK), SDS (SRL), β-mercaptoethanol (SRL), Bromophenol blue (SRL), Tricine (Merck), Coomassie brilliant blue (SRL), were used in the study.

Protein extraction

Tissue was washed properly and five grams was excised from the whole organism and homogenized using tissue homogenizer (Pro-Scientific, USA) by adding 5mL of buffer. Protein extraction was done using hot (bearable to touch) protein extracting RIPA (Radio-Immuno-precipitation Assay) buffer (containing 1% Triton X100, 150 mM sodium chloride, 12 mM Sodium deoxycholate, 0.1% SDS, 1 M sodium dihydrogen phosphate, 10 mM disodium hydrogen phosphate, 0.2 % Sodium azide, 0.95 mM fluoride, 2 mM phenyl methyl sulfonyl fluoride, 50 μg mL^{-1} Aproteinin, 50 mM leupeptin), vortexed for 10 min and allowed to stand at 85°C for 45 min. Protein was estimated by Lowry's method (Lowry et al., 1951).

Detection of Antimicrobial activity

Agar well diffusion method was used to detect antimicrobial activity. (Cleidson et al., 2007). Mueller-Hinton (MH) agar plates were swabbed with the test organisms wells of 0.2 cm diameter were punctured using sterile borer and 25 μL of the protein sample (0.01 $mgmL^{-1}$) was loaded. The plates were observed for zone of inhibition after incubation at 37°C overnight.

The pathogenic bacterial strains used to study the antimicrobial activity of *Loligo* spp. were obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India and included *K. pneumoniae* NCIM no. 5082, *B. cereus* NCIM no. 2217, *S. typhimurium* NCIM no. 2501, *E. coli* NCIM no. 2574, *S. aureus* NCIM no. 2079, *P. aeruginosa* NCIM no. 2036 and *V. cholerae* (DBT, CUSAT)

Protein concentration and purification

Protein was concentrated by acetone precipitation. One volume of the tissue extract was mixed with four volumes of ice cold acetone and incubated at -80°C in the deep freezer for four hours. After incubation, the precipitated proteins were separated by centrifugation (Hermle, Model Z 36H, Germany) at 15 000 rpm for 12 min. The obtained pellet was air dried and mixed with 100 μL of phosphate buffer pH 7.2 and protein concentration was estimated (Lowry et al., 1951). Crude protein samples were also subjected to initial 10 kDa and later 30 kDa cut off membrane filtration (Amicon Ultra, USA).

Thin layer Chromatography

Thin layer chromatography was performed on ready to use Silica gel 60 F254 (Merck KGaA, Darmstadt, Germany) plates. 20 μL of acetone precipitated sample was loaded and air dried. This was then separated using the solvent n-butanol: acetic acid: water in the ratio 5:1:5 and was visualized under UV light (Boudjelal et al., 2011; Pyka, 2014).

Gel filtration Chromatography

Gel filtration chromatography was performed using Sephadex G50 column (BioRad, USA) of 55 cm height and 1 cm diameter. The column was washed initially with 150 mL of 0.05 M Tris HCl buffer (pH 8.0), the sample was applied and eluted with the same buffer. Bioactivity of all the fractions was checked and the bioactive fractions were pooled together. Later the pooled fractions were lyophilized to obtain a concentrated sample.

SDS-PAGE

Polyacrylamide gel electrophoresis of the concentrated protein sample was performed at reducing conditions using a 15% resolving and 4% stacking gel system according to Laemmli UK, 1970 using a gel electrophoretic apparatus (Biorad, USA). Briefly 15 μL of lyophilized protein sample (diluted in appropriate volume of phosphate buffer, pH 7.2),was mixed 5 μL of sample buffer (5x) (containing 20% Glycerol(v/v), 10% SDS(w/v), β-mercaptoethanol, 10 mM, Tris-HCl 0.2 M, (pH 6.8) and Bromophenol blue 0.05%), heated at 100°C for 5 min and loaded onto precast gel. The current was given at 15 mA for 3 h. The molecular weight of the active protein was determined after Coomassie brilliant blue staining. PAGE analysis was done employing samples available at each step of purification.

MALDI

The purified protein band with activity was excised from SDS-PAGE and subjected to MALDI-MS on an Ultraflex TOF/TOF model (at Vimta laboratory Pvt Ltd, Hyderabad), (Bruker Daltonics, Germany) with Flex Analysis 2.0, Bruker software in linear mode with N2 Laser, 337 nm, 50 Hz and average of 800 shots was used. Equal amount of trypsin digested protein gel and matrix were mixed and spotted on a MALDI plate (MTP 384 ground steel, Target plate-Bruker). MASCOT search was performed for the obtained results.

Results and Discussions

Squids, a major group of Cephalopods are a potent source of many classes of antimicrobial compounds. Ghiretti (1959) reported a proteinaceous bioactive compound 'cephalotoxin' from posterior salivary glands of *Sepia officianalis*. Apart from salivary glands which act as a good source for a large number of bioactive molecules, accessory nidamental glands (ANG) provide some antimicrobial property as reported by Barbieri et al. (1997). The antimicrobial property of ANGs is generally attributed to the higher levels of unsaturated fatty acids like DHA, oleic acid, arachidonic acid EPA etc within the mature ANGs. Ink in some Cephalopods particularly *Sepia* find its place in homeopathic medicines. The ink extracts from *Sepia* are known to be used to treat urinary tract infections, gynaecological, pregnancy related complaints etc in women. The molecules extracted from the ink of squid *L. duvauceli* showed decreasing antibacterial activity against *E. coli* followed by *Salmonella* spp., *V. cholerae* and *Staphylococcus* spp. (Patterson & Murugan, 2000). Vino et al. (2013) studied the antibacterial activities of tissue extracts and polysaccharide fractions from two squid species *L. duvauceli* and *D. sibogae* against pathogenic strains including *V. cholerae*. In general, the various parts of these cephalopods such as squid body tissue, ink, ANG, polysaccharide fractions etc can be studied for their bioactivity. The present study also focused on these regions for bioactive molecules. Initial experiments were done using four extracts, tissue extract, ink, accessory nidamental gland extract and polysaccharide fraction. Only the tissue protein extract provided positive results against *V. cholerae* (Fig. 1). Hence tissue extract was selected for the purification of antimicrobial fraction.

The tissue extract of *Loligo* sample after acetone precipitation and 30 kDa ultrafiltration was screened for activity against 7 different human pathogens *viz., K. pneumoniae, B. cereus, S. typhimurium, E. coli, S. aureus, P. aeruginosa* and *V. cholerae* by agar well diffusion method. Antimicrobial activity was observed as zone of inhibition (16 mm) against *V. cholerae* (Fig. 1), which is a human pathogen generally present in potable water bodies, causing fatal infections. The protein concentration was calculated as 0.01 mg mL^{-1}.

The acetone precipitated protein sample was subjected to an initial 10 kDa cut off and later 30 kDa molecular weight cut off membrane filtration-centrifugations. Both the upper and lower phases of 30 kDa filtrate was checked for bioactivity. The

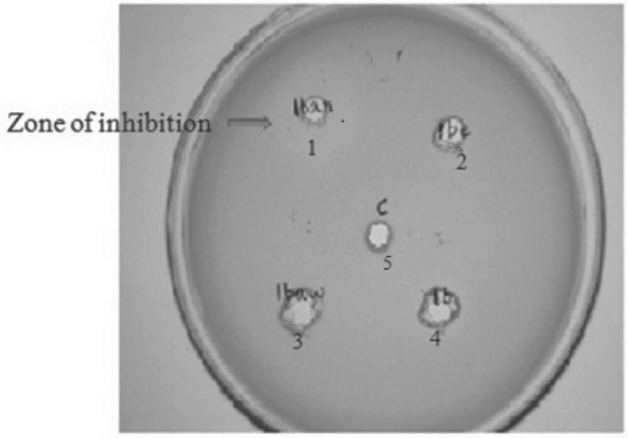
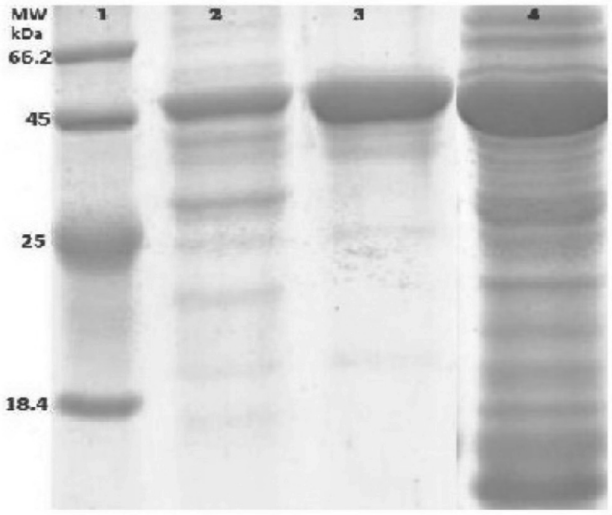


Fig. 1. Purified 45 kDa protein showing antibacterial activity against *Vibrio*

fraction above 30 kDa showed antimicrobial activity, indicating that the antimicrobial protein size was >30 kDa. PAGE analysis of the upper fraction with anti-bacterial activity showed prominent band with a molecular weight of 45 kDa when compared with a protein marker. Ultra-filtered fraction was then subjected to next step of purification employing Sephadex G-50 column.

After gel filtration on G-50 the protein yield was estimated as 0.06%. The eluted fraction (column fraction) was analysed using SDS-PAGE and it was confirmed that the activity was represented with a prominent 45 kDa single band as shown in Fig. 2.



Lane 1-Protein molecular weight marker (Fermentas, India)
Lane 2- Upper fraction of 30 kDa cut off
Lane 3- Sephadex G50 column purified
Lane 4- Crude protein extract

Fig. 2. SDS-PAGE of protein samples at each stage of purification

Thin layer chromatograph of the purified protein showed the presence of a single band with R_f 0.17 (Fig. 3), which showed associated antimicrobial activity.

Marine sources play a major role in yielding new antimicrobial products (Tincu & Taylor, 2004). Antimicrobial proteins of high molecular weight have been reported earlier (Rajaganapathi et al., 2002; James et al., 1996). MALDI and MASCOT analysis of the purified protein revealed that this protein has sequence similarity with parvalbumin

Fig. 3. TLC of protein sample after acetone precipitation and 30 kDa membrane filtration showing single band under UV illumination

protein of *Fundulus similis.* The aminoacid sequence obtained by peptide mass fingerprinting is represented below

1 MADEEIAALV VDNGSGMCKA GFAGDDAPRA VFPSIVGRPR HQGVMVGMGQ
51 KDSYVGDEAQ SKRGILTLKY PIEHGIVTNW DDMEKIWHHT FYNELRVAPE
101 EHPVLLTEAP LNPKANREKM TQIMFETFNT PAMYVAIQAV LSLYASGRTT
151 GIVMDSGDGV THTVPIYEGY ALPHAILRLD LAGRDLTDYL MKILTERGYS
201 FTTTAEREIV RDIKEKLCYV ALDFEQEMAT AASSSSLEKS YELPDGQVIT
251 IGNERFRCPE ALFQPSFLGM ESCGIHETTF NSIMKCDVDI RKDLYANTVL
301 SGGTTMYPGI ADRMQKEITA LAPSTMKIKI IAPPERKYSV WIGGSILASL
351 STFQQMWISK QEYDESGPSI VHRKCF

Further studies are needed to characterize the protein and identify the gene. Thus in this study, a 45 kDa protein with antimicrobial activity against *V. cholerae* was isolated from *Loligo* and partially characterized, thereby confirming that the marine environment and the organisms therein are a bountiful resource of bioactive molecules needing more study.

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