

Longfin snake eel, *Pisodonophis cancrivorus* (Richardson, 1848), as a potential source of antimicrobial peptides

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Original Article

Abstract

Antimicrobial peptides are small, cationic, amphipathic and evolutionarily conserved molecules of the innate immune system of eukaryotes, which they use to wade off a wide range of microbes. The present study was carried out to evaluate longfin snake eel, pisodonophis cancrivorus as a potential source of antimicrobial peptides. The crude peptide extract was obtained by modified acetic acid-acetone precipitation method and its activity was detected by disc diffusion assay. The crude peptide extract was subjected to solid phase extraction using Sep-pak®C-18 cartridges (Waters, USA). The 40% Sep-pak® fractions eluted was further purified by cation exchange chromatography which yielded 5 fractions. Among the five fractions, Pc40-4 and Pc40-5 exhibited the highest antimicrobial activity and showed inhibition against all tested bacterial strains namely Edwardsiella tarda, Bacillus cereus, Vibrio fluvialis, Vibrio cholera, Aeromonas hydrophila and Staphylococcus aureus. Pc40-3 exhibited remarkable antibacterial activity against the Gram-negative bacteria Aeromonas hydrophila (98%). This is the first report on AMPs from Pisodonophis cancrivorus.

Keywords: Antimicrobial peptides, *Pisodonophis cancrivorus*, antimicrobial activity, peptide extract, teleost fish.

Introduction

The aquatic environment offers a large biodiversity of flora and fauna. These organisms thrive in a very competitive and aggressive surrounding which demands the production of several bioactive compounds. Often during pathogenic invasion, endogenous peptides, which are constitutively expressed or induced, provide a fast and effective means of defence against the pathogen. This group of molecules termed as 'antimicrobial peptides' (AMPs) constitute the innate immune defence mechanism (Reddy et al., 2004; Gordon et al., 2005). Antimicrobial peptides are relatively small (molecular weight <15kDa) cationic and amphipathic peptides of variable length, sequence and structure. AMPs are found among all classes of life and are potent, broad spectrum antibiotics which exhibit bactericidal, fungicidal, virucidal and tumouricidal properties (Powers and Hancock, 2003; Lehrer, 2004). Most of these gene-encoded peptides are mobilized shortly after microbial infection and act rapidly to neutralize a broad range of microbes and share several common properties.

AMPs have attracted extensive research attention worldwide as candidates for clinical development because of their selectivity, their speed of action and inability of bacteria to easily develop resistance against them. The main advantage of AMPs for innate defence is that they are small molecules and can be synthesized easily, within few hours unlike components of adaptive immune response which take days to eliminate the pathogen. Moreover they can eliminate two or more intruders at the same time without requiring specific recognition for each foreign invader and has the ability to diffuse rapidly to the site of infection.

Fishes represent organisms that have managed to survive in a milieu of pathogenic microorganisms (Pickering, 1974). They use an array of antimicrobial peptides to defend themselves against pathogens. A 33- amino acid pore forming peptide paradaxin was one of the discovered antimicrobial peptides isolated from the secretions of the Red Sea Moses solefish, *Pardachirus marmoratus* (Lazarovici *et al.*, 1986). A number of other AMPs have also been isolated from different fish species including pleurocidin from winter flounder, *Pleuronectes americanus* (Cole *et al.*, 1997) dicentracin from sea bass, *Dicentrarchus labrax* (Salerno *et al.*, 2007) hepcidin from hybrid striped bass (Shike *et al.*, 2002) misgurin from loach, *Misgurnus anguillicaudatus* (Park *et al.*, 1997) cathelicidin from Atlantic cod (*Gadusmorhua*) and Antarctic charr, *Salvilinus alpinus* (Maier *et al.*, 2008).

The present study is focused on bioassay guided partial purification of antimicrobial peptides from the longfin snake eel, *Pisodonophis cancrivorus* and determining its antimicrobial activity *in vitro*.



Fig.1. Animal used for the study- Long fin snake eel, Pisodonophis cancrivorus TL :45 cm

Material and methods

Animal used for the study

The organism used for the study was *Pisodonophis cancrivorus* (Richardson, 1848) commonly known as burrowing snake-eel or longfin snake-eel. It is a burrowing form and mostly inhabits coastal lagoons and estuaries. The eel which had a length of 45 cm and mean weight of 200 g was collected from Vypin, Cochin backwaters (90 40[°] & 100 12[°]N and 760 10[°] & 760 30[°]E). The sample was then stored at -80°C.

Peptide Extraction

The crude peptide extract was obtained by using modified

acetic acid-acetone precipitation method (Rivillas and Soriano, 2007). The whole animal sample was cut into small pieces and homogenized in five volumes (w/v) of 10% acetic acid. The homogenate was incubated overnight at 4°C and thereafter centrifuged at 13500 rpm at 4°C for 30 minutes. To the supernatant were added two volumes (v/v) of ice cold acetone and stored overnight at 4°C. The crude peptide precipitate was collected by centrifugation at 13500 rpm at 4°C for 30 minutes. The supernatant was discarded and the crude peptide pellet was collected and stored at -80°C until use.

Antimicrobial Assay of crude peptide

The antimicrobial activity was tested using disc diffusion assay (Bauer *et al.*, 1966). The crude protein sample was reconstituted in sterile MilliQ water at a concentration of 200 mg/ml. 20 μ l of crude sample was loaded on to 6mm diameter discs (Whatman No: 1) using a micropipette with sterile tip. These impregnated discs were placed on plates seeded with microbial strains (Table 1). The crude peptide sample was also tested against fungal strains to detect its antifungal activity. The fungal strains *Aspergillus flavus, Fusarium solani* and the yeast strain *Candida haemulonii* were used for the test. The plates were kept at 4°C for 30 minutes and then incubated overnight at 37°C. The plates were then observed for zone of inhibition.

Table 1. Microbial strains used for antimicrobial assay

S.No	Gram-positive strains	Gram-negative strains	Fungal and Yeast strains	
1	Bacillus cereus	Edwardsiella tarda	Aspergillus flavus	
2	Staphylococcus aureus	Aeromonas hydrophila	Fusarium solani	
3	Streptomyces peuceticus	Vibrio proteolyticus	Candida haemulonii	
4		Vibrio fluvialis		

Solid phase extraction

Partial purification of peptides was carried out in Sep-Pak® C-18 cartridges (Waters). Crude protein extract was reconstituted in 0.1% tri fluoro acetic acid (TFA). The cartridges were equilibrated with 0.1% Tri Fluoro Acetic acid (TFA). The sample was loaded, followed by a wash with 0.1% TFA. The analyte trapped in the cartridges were eluted with 6 ml each of 5%, 40% and 80% acetonitrile in 0.1% TFA. Fractions were labelled as 5%, 40% and 80% fractions, lyophilized and stored at -80°C.

Cation Exchange Chromatography

The lyophilized 40% Sep-Pak® fraction was reconstituted in HPLC grade water and subjected to further purification by cation exchange chromatography using Duo Flow- Fast protein liquid chromatography (FPLC) system (Biorad). The column used was UNOTM QI (Q1 BioRad). Solvents used in mobile phase were solution A (25 mMTris-HCl) and solution B (1 M NaCl in 25 mMTris-HCl). The column and baseline were equilibrated with solution A, followed by sample loading. Linear gradient elution was achieved using 0-50% of solution B. Flow rate was maintained at 2 ml/min. The chromatography was monitored at 215, 225, 260 and 280 nm wavelength using Quad Tec detector (BioRad). Each peak was collected as separate fractions, which was lyophilized and stored at -80°C.

Preparation of Microbial Suspension

Pathogenic strains of both Gram positive (*Bacillus cereus* and *Staphylococcus aureus*) and Gram negative (*Edwardsiella tarda, Aeromonas hydrophila, Vibrio cholera*e, *Vibrio fluvialis*) strains were selected for the liquid growth inhibition assay on the basis of the initial disc diffusion assay. The strains were obtained from the Microbiology Laboratory repository of the Department of Marine Biology, Microbiology and Biochemistry, CUSAT. The bacterial cells were scrapped off from nutrient agar slants using sterile inoculation loop and were added into sufficient quantity of Hydroxy ethyl piprazine ethane sulfonic acid (HEPES) buffer and was mixed well. The Optical Density (OD) of the bacterial suspension was adjusted to 1 OD to determine the number of Colony Forming Units (CFU) of each bacterium. The bacterial suspension was then diluted so that 10µl of bacterial suspension contained approximately 100 CFU.

Antimicrobial Assay of FPLC fractions

The antimicrobial activity of the fraction was determined by the cell viability assay, estimated calorimetrically with MTT. Lyophilized column chromatography fractions were reconstituted in MilliQ water. 10 μ l of the 40% Sep-pak FPLC fractions and 10 μ l of the OD adjusted bacterial suspension were loaded onto the microtitre wells. To the blank only the HEPES buffer and the solution B (1 M NaCl in 25 mm TrisHCl) of the cation exchange chromatography was added. A set of negative and positive controls were maintained. To the negative controls the bacterial suspension and the solution B of the cation exchange chromatography was added. The positive controls composed of the bacterial suspension and the antibiotic tetracycline (5 μ M). After 2 hours incubation, 80 μ l of nutrient broth was added to each well, followed by incubation for another 5 hours at 37°C.25 μ l of MTT [3- (4, 5- Di Methylthiazol- 2- yl)- 2, 5- diphenyltetrazolium- bromide)] was added and incubated at room temperature for 30 minutes. Then 125 μ l of acidified isopropanol was added and kept at room temperature on a shaker. The OD was measured using a Microplate Reader (Tecan, USA) at a wavelength of 570 nm.

Results

Disc Diffusion Assay

Antimicrobial activity of the crude protein from the eel, *Pisodonophis cancrivorus* was tested against different microbial strains by the disc diffusion assay Antimicrobial activity could be observed against both Gram negative and Gram positive forms (Table 2). The highest activity was recorded against *Bacillus*

cereus (15 mm) followed by *Vibrio proteolyticus* (10 mm), *Vibrio fluvialis* (10 mm) and *Edwardsiella tarda* (9 mm). There was no zone of inhibition against the bacterial strains *Aeromonas hydrophila, Staphylococcus aureus* and *Streptomyces peuceticus*. No significant activity was observed against fungal strains.

Table 2. Anumicionial activity of the clude peptide against the microorganisms							
S.No.	Microbial strains	Zone of inhibition (mm)					
1	Staphylococcus aureus	0					
2	Bacillus cereus	15					
3	Streptomyces peuceticus	0					
4	Aeromonas hydrophila	0					
5	Edwardsiella tarda	9					
6	Vibrio proteolyticus	10					
7	Vibrio fluvialis	10					
8	Aspergillus flavus	0					
9	Fusarium solani	0					
10	Candida haemulonii	0					

Table 2. Antimicrobial activity of the crude peptide against the microorganisms

Cation Exchange Chromatography (FPLC)

Cation exchange chromatography of 40% Sep- pak fraction yielded five fractions which were labelled as Pc40-1, Pc40-2, Pc40-3, Pc40-4 and Pc40-5 on the basis of retention time (Fig. 2)



Fig. 2. Cation exchange chromatography analysis of 40% Sep- pak fractions of crude peptide from $\it Pisodonophis \ cancrivorus$

Antimicrobial Assay: Liquid Growth Inhibition Assay

The FPLC fractions Pc40-1 to Pc40- 5 (10μ l each) demonstrated antibacterial activity against one or more of the tested strains. Fractions Pc40-4 and Pc40-5 inhibited all the six bacterial strains tested namely *Edwardsiella tarda*, *Bacillus cereus*, *Vibrio fluvialis*, *Vibrio cholera*, *Aeromonas hydrophila* and *Staphylococcus aureus*. Fractions Pc40-1 and Pc40-2 were found to posses only a mild activity against the bacterial stains, but Pc40-3 exhibited a very high antibacterial activity against the Gram-negative bacteria *Aeromonas hydrophila* (Table 3).

					% Inhibition		
S.No	Fractions	Edwardsiella tarda	Bacillus cereus	Vibrio fluvialis	<i>Vibrio cholera</i> e	Aeromonas hydrophila	Staphylococcus aureus
1	Pc40-1	0	0	21	0	57	48
2	Pc40-2	0	0	51	26	42	0
3	Pc40-3	0	0	0	34	98	43
4	Pc40-4	94	82	94	92	94	74
5	Pc40-5	96	91	99	97	98	84
6	Tetracycline (5 μ M)	82	89	99	97	97	94

Table 3. Antibacterial activity of FPLC fractions from Pisodonophis cancrivorus

Discussion

The study on "The antimicrobial peptides from the longfin snake eel, *Pisodonophis cancrivorus*" was undertaken with the presumption that they produce antimicrobial peptides as an innate defence mechanism to combat the attack of pathogenic microorganism. It was reported that aquatic sources contain thousands of fish species and each secretes AMPs with structural differences which can be used by the pharmaceutical industry to develop novel drugs to treat drug-resistant pathogens (Rajanbabu and Chen, 2011).

In the present study the peptide fractions extracted from the longfin snake eel was initially screened to detect the antimicrobial activity by the disc diffusion assay. The crude peptide sample from eel displayed the highest activity against *Bacillus cereus* which produced a zone of inhibition of 15 mm. The liquid growth inhibition assay of all the 40% FPLC fractions (Pc40-1 to Pc40-5) exhibited antibacterial activity against one or the other bacterial strains selected.

Edwardsiella tarda was inhibited only by fractions Pc40-4 and Pc40-5 which showed a very high percentage of inhibition of 94% and 96% respectively. Pc40-4 and Pc40-5 showed much higher inhibition than positive control which indicate that these fractions are more potent than the antibiotic tetracycline. Bacillus cereus exhibited a zone of inhibition of 15 mm in the initial disc diffusion assay. This may be due to the combined effect of the active compounds of the fractions Pc40-4 and Pc40-5 which showed an inhibition of 82% and 91% respectively against this bacterial strain in liquid growth inhibition assay. Vibrio fluvialis was inhibited by almost all the FPLC fractions except Pc40-3. The fractions Pc40-4 and Pc40-5 were found to be highly potent against this bacterial strain which recorded a growth inhibition of 94% and 99% respectively. The prominent effect in the initial disc diffusion assay may have been contributed by the active compounds in the fraction Pc40-5. Fraction Pc40-4 showed a growth inhibition of 94% against Vibrio cholerae and about 97% of inhibition was exhibited by fraction Pc40-5. Though V. cholerae was not tested during the initial disc diffusion assay other pathogens of the same genus namely Vibrio proteolyticus and Vibrio fluvialis were tested.

Aeromonas hydrophila was inhibited by all the five 40% FPLC fractions. The lowest activity was recorded by fraction Pc40-1 and Pc40-2 which produced only 57% and 41% of growth inhibition. Fractions Pc40-3, Pc40-4 and Pc40-5 were found to possess a very high activity. None of the 40% FPLC fractions displayed growth promotion of *Aeromonas hydrophila*. It is presumed that some compounds responsible for the growth promotion of the microorganism may have been present in the 5% and 80% FPLC fractions in the crude peptide sample have resulted in the absence of inhibition in the initial disc diffusion assay.

Compared to the other bacterial strains only a mild activity was detected against *Staphylococcus aureus* in the liquid growth inhibition assay with Pc40-5 contributing the highest inhibition of 84% followed by Pc40-4 (75%), Pc40-1 (48%) and Pc40-3 (43%). The initial crude peptide sample contained all the active compounds of different fractions and in the liquid growth inhibition assay the purified 40% FPLC fraction (Pc40-2) were found to exhibit growth promoting activities against *Staphylococcus aureus*, and it is assumed that the synergistic effect of growth inhibiting properties and growth promoting properties of the different fractions in the crude peptide sample against *Staphylococcus aureus* resulted in the absence of inhibition in the initial disc diffusion assay.

The results suggest that the Longfin snake eel is a source of potent antimicrobial peptides. The array of AMPs produced by this eel to fight the bacterial infections in a highly fluctuating estuarine environment can be utilized by pharmaceutics to develop novel therapeutic agents.

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