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PURIFICATION AND PARTIAL CHARACTERIZATION OF ANTIMICROBIAL PEPTIDES IN THE GILL EPIDERMAL MUCOPROTEIN OF THE RED BANDED GROUPER, EPINEPHELUS FASCIATUS, (FORSSKAL, 1775)

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ABSTRACT

The study of the identification and purification of a novel antimicrobial peptide in the gills of the red banded grouper, Epinephelus fasciatus, a teleost fish, was investigated for the purpose of using compounds of the innate immunity in aquaculture and for anti-infective agents in animals. The purification procedure involved cation exchange chromatography and reverse phase HPLC followed by solid phase extraction on C18 Sep-Pak cartridges. The 50% of acetonitrile elutes (30 µg) caused a zone inhibition with a diameter of 0-16.4 mm depending on the salt concentration and the microbe tested. The Fourier transform infrared (FTIR) spectrum of epinisin in 50 mM sodium phosphate buffer was alpha-helix and ß-turn with tyrosine as amino acid side chains. The molecular mass of the antimicrobial factor was estimated to be approximately 72 kDa by SDS-PAGE. The antibacterial activity of the purified peptide was thermos table, remaining present even after incubation at 800C for 10 min. It can be concluded that epinisin showed a strong antimicrobial activity in vitro against a broad spectrum of microorganisms without significant hemolytic activity and was about 3 times more potent than magainin 2.

INTRODUCTION

Antimicrobial peptides (**AMPs**) are widespread in nature, being produced by bacteria, plants, and a wide variety of vertebrates and invertebrates[1, 2]. These peptides are thought to be important in the natural defense against invading microorganisms. In animals, AMPs are found in tissues that are likely to be in contact with microorganisms, such as at mucosal surfaces and within immunogenic cells and tissue. Most **AMPs** are cationic, containing 20 to 50 amino acid residues, and they are often amphipathic and/or hydrophobic, which reflects the fact that many of these peptides interact with and permeabilize target cell membranes[3]. Membrane permeabilization is thought to be mechanism by which most AMPs kill their target cells, although other mechanisms may be involved for some AMPs[4]. Antimicrobial peptides (AMPs) play a crucial part in the innate immunity and can be regarded as host defensive peptides. They are usually amphiphilic, have high cysteine content and are positively charged in their active forms. The AMPs can protect the hosts against a broad range of pathogenic infections which makes them attractive as therapeutic agents[5]. Marine AMPs are structurally diverse, display a wide spectrum of anti-infective activities, a low bio-deposition rate in body tissues, and are highly specific to targets. They provide different and unique sources of potential anti-infective drug candidates[6]. The antimicrobial peptide epinecidin-1 from grouper (*Epinephelus coioides*) demonstrated antibacterial activity against *Pseudomonas aeruginosa*, *Staphylococcus coagulase*, *Streptococcus pyogenes*, and *Vibrio vulnificus*. Plasmid DNA coding for a green fluorescent protein (EGFP)-epinecidin-1 fusion protein under the control of the cytomegalovirus (CMV) promoter was introduced with electroporation into decapsulated Artemia cysts. The resulting EGFP-epinecidin-1 protein suppressed *V. vulnificus* growth. Zebrafish fed on transgenic Artemia expressing CMV-gfp-epi combined with commercial fodder exhibited enhanced resistance to *V. vulnificus* and an increased survival rate. In addition, feeding of transgenic Artemia to zebrafish affected the immunomodulatory response to *V. vulnificus* infection; expression of immune-responsive genes, including hepcidin and defbl2, was altered, as shown by qPCR. These findings suggest that feeding transgenic Artemia expressing CMV-gfp-epi to larval fish has antimicrobial effects, without the drawbacks of introducing drug residues or inducing bacterial drug resistance[7]. The 6621-Da chitotriosidase-like antimicrobial peptide mytichitin-CB with

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three disulfide bonds from *Mytilus coruscus* hemolymph exhibited antifungal and antibacterial activities. The mRNA expression level of its precursor mytichitin-1 in the gonad was heightened following bacterial infection signifying a role in the host immune response against intruding bacteria[8]. The structurally unique tongue sole (*Cynoglossus semilaevis*) NK-lysin NKLP27 displayed bactericidal activity dependent on the 5 *C*-terminal residues NKLP27. NKLP27 disrupted bacterial cell membrane integrity, entered into the cytoplasm, and fragmented genomic DNA. NKLP27 curtailed bacterial and viral pathogen dissemination and replication in the fish tissues and elevated expression of immune genes[9]. Some of the marine antimicrobial peptidescould be used to combat drug-resistant microbes like tilapia piscidin-4[10] and epinecidin-1[11] (Naravan *et al*., 2015) against *Helicobacter pylori*. The molecular mechanism with which some of these peptides such as peptide H-P-6 (Pro-Gln-Pro-Lys-Val-Leu-Asp-Ser) from microbial hydrolysates of *Chlamydomonas* sp. inhibited *H. pylori* has been elucidated[12] .

The polar fraction of the organic extract of the Red Sea sponge *Theonella swinhoei*, contained a bicyclic glycopeptide, theonellamide G. Theonellamide G exhibited strong antifungal activity toward wild and amphotericin B-resistant strains of *Candida albicans* with IC50 of 4.49 and 2.0 μM, respectively. It exerted cytotoxicity against the human colon adenocarcinoma cell line (HCT-16) with IC50 of 6.0 μM. These data furnish further information pertaining to the different chemical structures and bioactivities of this class of compounds[13]**.**

Although fish live in a microbe-rich environment, they have primitive immune systems compared to those of higher vertebrates[14]. The innate or unspecific immune system, of which AMPs are a part, seems to an important component in their defense against infectious agents. Several AMPs have recently been purified from fish, especially from the skin and gill mucus. These include paradoxin[15]**,** pleurocidin[16], moronecidin[17], misgurin[18], bass hebcidin[19], parasin[20], hipposin[21], cathelicidins[22], epinecidin-1[23], dicentracin, hepcidins[24], and NK-lycin[25]. These peptides kill a wide range of Gram-negative and Gram–positive bacteria, and some of the peptides are also active against fungi.

Fish gills are constantly being flushed with water that may contain fish pathogens, but are covered with only a thin layer of protective mucus and are constructed of only a single layer of cells that separate the vascular system from the external environment. Thus, they are a very important site of pathogen penetration. Therefore, potent antimicrobial peptides can be expected to be found in fish gills to prevent such penetration. However, there is a paucity of information on nonspecific defense systems in the gills. An antimicrobial peptide has been identified in the gills of only two species, hybrid striped bass (*Morone saxatilis* x M. chrysops) and red sea bream (*Chrysophrys major*) chrysophsin[26]. In this study, we therefore tried to purify and charecterize antimicrobial peptides from the gills of the grouper *Epinephelus fasciatus* and found that a novel antimicrobial peptides. They exhibit potent bactericidal activity against Gram-negative and Gram-positive pathogens of human.

Experimental Section

Tissue Collection and Purification of Antimicrobial Peptides

Adult grouper were collected from the Marine Aquarium, CAS in Marine Biology, Parangipettai, and Tamil Nadu in India. Tissue collection and purification of Antimicrobial peptides were followed as previously described by Lauth (2002) and Iijima (2003). After starvation for 1 day, the fish were killed by stabbing the brain with a knife, and gills filaments were immediately collected, weighing 200-300 g and stored at -20 ^{0} C until use. Frozen samples were ground in to powder with a mortar and pestle under liquid nitrogen. Proteins were extracted in 10 % acetic acid supplemented with the protease inhibitor, aprotinin (1.5 μm, final concentration) by shaking on an ice-cold water path for 3 h. After centrifugation (3500 X g for 20 min), the supernatants were filtered (0.45 μm, Millex ^{TM}; Millipore Corp), prepurified, and loaded on to 12-ml Sep-Pak Vac C_{18} cartridges (Waters, Hertfordshire, UK) equilibrated with 10 % acetic acid. The cartridges were washed with acidified water (0.05 % trifluoroacetic acid), and two successive stepwise elution were performed with 50 % and 100 % acetified actonitrile (ACN), 0.05 % trifluoroacetic acid. Both effluents were lyophilized and resuspended in water.

Microbial Strains

Bacillus subtilis strain (ATCC 1774), *Planococcus citreus* strain (NCIMB 1493), *Escherichia coli* strain (ATCC 11229), *Listonella anguillarum* strain (NCIMB 2129), and *Candida albicans* strain (ATCC 90029) were used to analyze the antimicrobial activity in the gill mucus extracts. All strains were grown to logarithmic phase in LB agar and YM medium at the appropriate temperature (please see section 4.2.2).

Antimicrobial Assays

The antimicrobial activity was examined during each purification step by the radial diffusion assay on B.substilis lawn as described by Lehrer *et al*. (1991). A 20 ml culture of B.substilis cells in mid-logarithmic phase was washed with cold 10 mM sodium phosphate buffer (NAPB), pH 7.4, and resuspended in 10 ml of cold NAPB. A volume containing 1 X 10^6 bacterial CFU was added to 6 ml of underlayer agar (10 mM sodium phosphate, 1 % (v/v) trypticase soy broth, 1 % agarose, pH 6.5) and with salt medium (medium E: 0.8 mm $MgSO_4$, 9.5 mm citric acid, 57.5 mm K_2HPO_4, 16.7 mm $NaNH_4HPO_4$ and with 480 mM NaCl); for fungi Sabouraud was used and the mixture was poured in to a Petri dish. Extracts of (30 μg) diluted in 5 % DMSO (Dimethyl sulphoxide) and filtered (Millex-GV unit 0.22 μm Millipore pore size) were placed in in the glass wells and allowed to diffuse for 2 h at 4^0C. Samples were added directly to the 3-mm wells made on the solidified underlayer agar. After incubation for 3 h at 37^0 C, the underlayer agar was covered with a nutrient-rich top agar overlay and incubated overnight at 37^0 C for the bacteria, and at 30^0 C for the fungi. Antimicrobial activity was determined by observing the zone of inhibition of bacterial growth around the 3-mm wells. All inhibition assays and controls were carried out in duplicate.

Minimum Inhibitory Concentration MICs

The minimal inhibitory concentrations (MICs) of the isolated peptide against the microorganisms were determined as

described by Moore *et al*., (1991) and for the fungi Shadomy *et al*., (1985). 100 µl microorganism suspensions (10^5 CFU/ml) in 3 % w/v TSB was mixed with serial two-fold dilutions of the isolated peptide in a sterilized 96-well microtitre plate (Nunc F96 micrtitre plates, Denmark) and with NaCl to a total concentration of 10 g/liter. The final concentrations of the peptide ranged over 0.5-200 µg/ml. The 96-well plate was incubated overnight at 37^0 C and the inhibition growth was determined by measuring the absorbance at 620 nm on a model 550 Microplate Reader (Bio-Rad, Hercules, CA, USA). MIC was defined as the lowest concentration of peptide that inhibited growth. Bacteria used were *Bacillus substilis*, *Listonella anguillarum*, *Planococcus citreus*, *Escherichia coli* and fungi was *Candida albicans.*

Protein Purification

The reconstituted lyophilized extract was applied to CM Macro-Prep column for cation exchange chromatography at pH 7.0 as detailed in Fernandes *et al*., 2002. The active fractions with retention times between 30 and 40 min were acidified to a final concentration of 0.1% (v/v) TFA and subjected to solid phase extraction on Sep-Pak Vac 5g C_{18} cartridges (Waters Hertfordshire, UK) previously equilibrated with 0.1% (v/v) TFA. The proteins of interest were eluted with 0.1% (v/v) TFA in 50% (v/v) acetonitrile (ACN), lyophilized, reconstituted in deionized water containing 0.1% (v/v) TFA. The 50 % ACN effluents from the gill extracts were subjected to reverse phase (RP)-HPLC) purification through a C_{18} preparative column (10 X 220 mm; Phenomenex) on a 0-50 % ACN linear gradient over 50 min (gill extract) at a flow rate of 2 ml/min. The 100 % ACN effluents from the gill were purified as above, using a linear biphasic gradient of acidified ACN (0-50 % over 25 min/ 50-80% over 40 min). Fractions were monitored for absorbance at 220 nm. Each peak was collected, lyophilized, resuspended in water, and screened for antimicrobial activity by the liquid growth inhibition assay.

Fourier-transform Infrared Spectra

The peptide samples were dissolved in a solution of 50 mM sodium phosphate buffer (NaPB) and trifluoroethanol (TFE, 50 % in 50 mM NaPB, (V/V) at a concentration of 2 mg/ml. Fourier-transform infrared spectra were recorded on a Perkin-Elmer spectrometer equipped with a TGS detector. A Perkin-Elmer model 4000 data station was used for acquisition, storage and analysis. Samples (volume up to 50 µl) were placed in a thermostatically controlled Beckmann FH-01CFT micro cell fitted with CaF2 windows. The sample compartment was continuously purged with dry air to eliminate absorption by water vapor in the spectral region of interest. Spectral conditions (defined more fully in Haris *et al*., (1986) and Perkins *et al*., (1988) were as follows: number of scans, 200; spectral resolution, 4 cm^{-1}; sample temperature, 20^0C. After subtraction of the background measured with ^2H_2O buffer, amide I region (1700-1600 cm^{-1}) was used for peptide structure determination.

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis (SDS-PAGE) was conducted using the discontinuous Tris-glycine buffer (pH 8.3) system, with 5% resolving gel and 3% stacking gel. Protein samples were dissolved in 0.02 M sodium phosphate buffer (pH 7.2) to a final concentration of 0.1% (m/v). Then the dissolved protein samples were mixed with sample buffer at the ratio of 1:1 (v/v). After electrophoresis, the gel was stained using 0.5% Coomassie Brilliant Blue R-250, and the band intensities were analyzed using a digital gel image analysis system. Nine reference proteins, Myosin (440), ß-galactosidase (120), Bovine serum albumin (91), glutamate dehydrogenas (52), ovalbumin (52), carbonic anhydrase (38), myoglobin (26), lysozyme (19), and aproline (9) were used as to calibrate the column[33].

Protein Quantification

Total protein concentration was determined by the method of Bradford, (1976) using bovine serum albumin (Himedia) as standard.

Hemolytic Assay

Hemolytic activity was assayed as described by Aboudy *et al*. (1994) with a slight modification. Freshly packed goat erythrocytes (3 ml) (Gibson, 1991) was washed with isotonic phosphate-buffered saline (PBS), pH 7.4, until the color of the supernatant turned clear. The washed erythrocytes were then diluted to a final volume of 20 ml with the same buffer. To 190 µl of the cell suspension in microfuge tubes, peptides samples (10 µl), serially diluted in PBS, and were added. Following gentle mixing, the tubes were incubated for 30 min at 37^0 C and then centrifuged at 4000 rpm for 5 min. One hundred microliters of supernatant was taken, diluted to 1 ml with PBS, and OD_{567} was determined. The relative optical density, as compared with that of the cell suspension treated with 0.2% Triton X-100, was defined as percent hemolysis.

Proteolysis Digestion

Antibacterial activity of the purified peptide was assessed by radial diffusion assay as above and digestion with 60 µg/ml (final concentration) proteinase K for 60 min at 37 ^{0}C. The assay was done in triplicate.

Thermal Stability Assay

Heat stability of the antibacterial activity against *B.subtilis* was tested by comparing clear zone areas produced by samples incubated for 10 min at 80 ^{0}C. Samples were also tested for activity against *B. subtilis*.

RESULTS AND DISCUSSION

Purification of Antimicrobial Peptide/Protein

After fractionation of the acid extracted gill powder on Sep-Pak C_{18}, fraction showed the most bactericidal activity was further separated by cation exchange-HPLC (fig.1A). The fraction obtained after elution with 50% and 100% acetonitrile were analyzed by RP-HPLC and the HPLC fractions were tested for antimicrobial activity against *E.coli* with NaCl 480 mM medium included in the assay. While no activity was found in the HPLC fractions obtained from the 100% cation exchange-HPLC (data not shown), a fraction from the 50% acetonitrile cation exchange HPLC elutes had antimicrobial activity (**Fig.1A**). A component active against *E.coli*, eluted at 50% acetonitrile in the RP-HPLC (retention time 20 and 30 min in

Fig.1A), and purified to apparent homogeneity at 50% acetonitrile in the last step (F**ig.1B**). The molecular mass of the antimicrobial factor was estimated to be approximately 73 kDa by SDS-PAGE and these antimicrobial peptides is provisionally named epinisin, after the genus of red banded grouper, *Epinephelus* (Fig.1C).

Determination of secondary structure of protein/peptide

Secondary structure of epinisin was estimated using a FTIR spectrometer in the presence of 50 mM sodium phosphate buffer (NaPB). The contents of α-helix, ß-turn and random coils were calculated according to Barth, 2007 (Table 1). The FTIR spectrum of epinephenisin in 50 mM NaPB was alpha-helix and ß-turn with tyrosine as amino acid side chains (Table 1). In the presence of 50% trifluoroethanol, the peptide became structured, exhibiting a high level i.e. of alpha-helical folding structure. Then, the alpha-helical conformation of epinephenisin was confirmed in the presence of structure promoting solvent.

Antimicrobial Activity of the gill Epidermal mucus extract

The gill extract comprising approximately 45% protein, was assayed for antimicrobial activity against gram-positive *Bacillus subtilis Planococcus citreus; Gram-negative E.coli, Listonella anguillarum* and fungi *Candida albicans*. The 50% acetonitrile elutes (30 µg) caused a zone inhibition with a diameter of 0-16.4 mm, depending on the salt concentration and the microbe tested (Fig. 3), while the 100% acetonitrile elutes exhibited no activity. Without addition of medium E with 480 mM NaCl to the agarose, the gram-positive bacteria *B.subtilis* and *P.citreus* was the most sensitive to gill extract (Fig. 3). With addition of salt medium, the gram-negative bacteria and gram-positive bacteria were found equally sensitive the extract, while the activity against fungi *C.albicans* was fully eliminated (Fig. 3). In contrast to *C.albicans*, the extract showed significantly greater ($p<0.01$) activity against gram-negative bacteria when medium E with 480 mM NaCl was added to the assay (Fig.3). No difference was noted in activity against the gram-positive bacteria, with or without salt medium.

The grouper fish epinisin showed different lytic activity against gram-positive and gram-negative bacteria (Table 2). The MIC for most bacterial species and /or strains tested was 3 times higher than MIC of magainin 2 (Table 2). The grouper epinphenisin could inhibit the growth of most bacteria below 32 µg. It was interesting to note that the lytic activity of the peptide to different genus of gram-positive and gram-negative bacteria varied. The MIC to *Bacillus subtilis* was 16 µg and to *Planococcus citreus* was 28 µg; gram-negative bacteria *Escherichia coli* was 16µg and *Listonella anguillarum* was 32; and the peptide could inhibit fungi *Candida albicans* at 32µg. In addition, as compared to magainin 2 purified from *Xenopus laevis*; epinepenisin was approximately 3 times more potent against a broad range of microorganisms. However, no appreciable hemolytic activity was observed for epinephenisin (up to 100 µg /ml) when tested against goat red blood cells (Table 3).

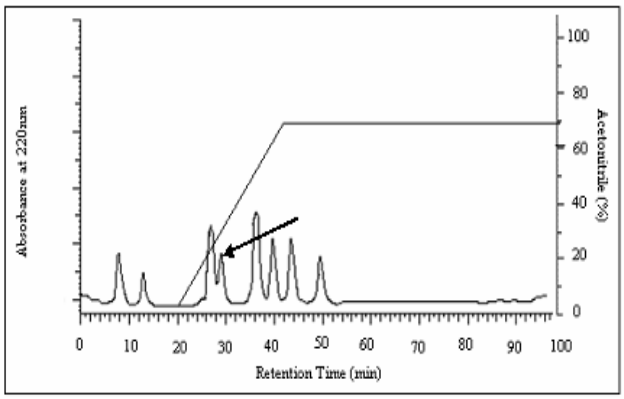


Fig.1 (A)

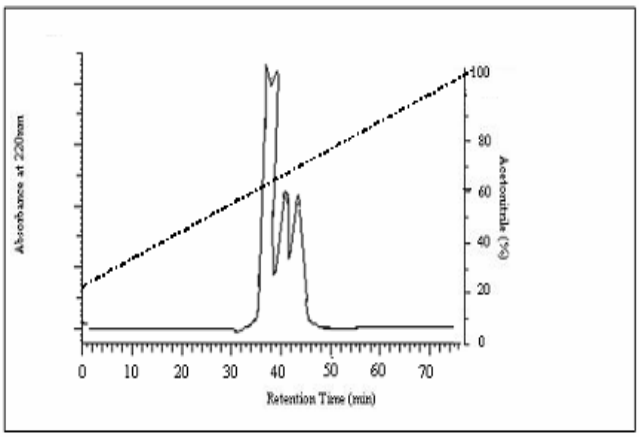


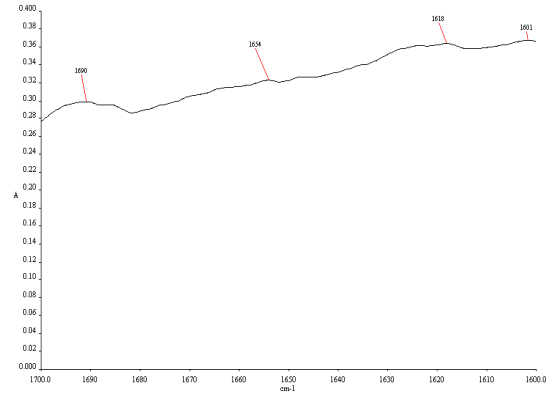
Fig 1 (B)

Fig.1(C)

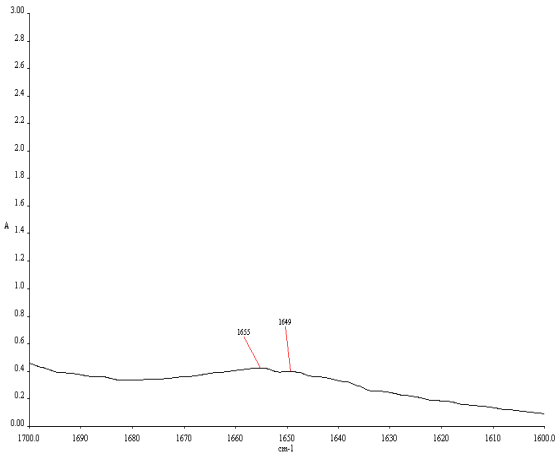
Fig 1 Purification of antimicrobial components from 100mg protein/peptide extract, prepared from the gill mucus of *E.fasciatus*, by use of HPLC and monitoring the antimicrobial activity. C_{18} reverse phase HPLC of the 50% acetonitrile elutes from $^{1}C_{18}$ solid phase extraction. Acid-soluble skin mucus extracts were subjected to cation exchange chromatography and the active fractions eluting between 32 and 48 min were pooled and concentrated by solid phase extraction on $^{1}C_{18}$ Sep-Pak cartridges (A). The 50% acetonitrile elutes was lyophilized, reconstituted in 0.1% TFA in deionized water and fractionated by C_{18} HPLC. Absorbance was monitored at 220 mm (B). The bar indicates the peak of interest. The bibasic water/ acetonitrile gradient is represented by the dotted line. (C): Tris/Tricine SDS-PAGE analysis of the active fractions of Gill epidermal mucus of *E.fasciatus*, GEM-EF. Sample: Lane 1; protein standards lane M-Markers. Purified antimicrobial protein after RP-HPLC. Each lane contains 7.5 µl of sample. The numbers on the left correspond to the molecular mass marker (kDa). Protein of interest is indicated by an arrow.

Table 1 FTIR spectra peak location and assignments for gill epidermal mucus protein of *E. fasciatus* (Barth, 2007).

Band position (cm^{-1})	Assignment	Reference
1690	ß-turn	Susi & Byler, 1986
1654	α-helix	Susi & Byler, 1986;
1618	Tyr, side chain	Chirgadze *et al*., 1975
1601	Tyr, side chain	Venyaminov and Kalnin, 1990



(A)



(B)

Fig 2 FTIR spectra (A) and (B) of gill epidermal mucus extract of *E.fasciatus*. The FTIR spectrum of active fraction was measured in 50 mM NaPB (A) and 50% TFE in 50 mM NaPB (B).

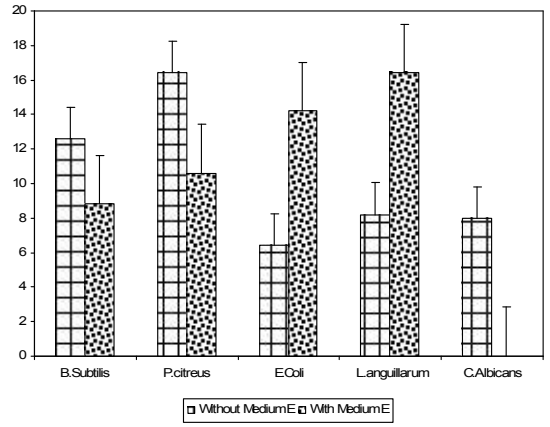


Fig 3 Antimicrobial activity of 30 µg protein/ peptide extract from the gills epidermal mucus of red banded grouper, *E.fasciatus* against the Gram-positive bacteria *B.subtilis* and *P.citreus*; the Gram-negative bacteria *E.coli* and *L.anguillarum*; and the fungi *C.albicans*, as measured by an inhibition zone assay. The activity was tested with and without medium E in the agarose. Each experiment is the average of at least 3 experiments. No activity was recorded against *C.albicans* when medium E was added to the agarose.

Table 2 Antimicrobial activities of epinephenisin and magainin 2. Means of results of two independent experiments are shown.

Peptide	Minimal inhibitory concentration (µg/ml)				
	B.subtilis	***P.citreus***	***E.coli***	***L.anguillarum***	***C.albicans***
epinephenisin	16	28	16	32	32
Magainin 2 (Park *et al.*, 1998)	50	50	100	NA*	25

NA*, Not applicable.

Table 3 Hemolytic activities of epinephenisin and magainin 2.

Concentration (µg/ml)	% of hemolysis of goat red blood cells	
	epinephenisin	Magainin 2[20]
5	0.00	0.19
10	0.00	0.19
25	0.23	0.57
50	0.43	0.95
100	0.46	1.14

DISCUSSION

The present investigation shows that 73 kDa antimicrobial proteins are expressed by grouper gill epithelium. The protein has a broad spectrum of activity, is highly potent and no significant hemolytic activity. FTIR spectra analysis revealed that it is likely to be highly cationic peptides. The antimicrobial protein was successfully purified by a combination Sep-Pak, cationic exchange HPLC and reverse phase HPLC. Active fractions were fractionated further by C_{18} reversed-phase HPLC, yielding one main fraction with antibacterial activity against *E.coli* that correspond to two peaks with retention time of 20 and 35 min (Fig. 1A& 1B). The antibacterial activity of the purified peptide was thermostable, remaining present even after incubation at 80^0Cfor 5 min. Activity was abolished after digestion with proteinase K, confirming that this factor has a proteinaceous nature. The protein thus purified featured the molecular mass of approximately 73 kDa under both reducing and non reducing condition.

It has been proposed that amphipathic α-helical peptides show antimicrobial activity by interacting electrostatically with anionic bacterial membrane, adapting an amphipathic alpha-helical conformation that allows them to insert the hydrophobic face into the lipid bilayers and form a pore[40,41,42]. The amphipathic ß–sheet and alpha-helical structure of epinisin was predicted by FTIR spectra analysis (Fig.2A). The epinephenisin form alpha-helical structure in the structure-forming solvent, trifluoroethanol (Fig.2B). This suggests that ß–sheet and alpha-helical structure in the water environment will form alpha-helical conformation after contacting with bacterial membrane. Thus, epinephenisin will show antimicrobial activity in a similar way to other previously studied amphipathic alpha-helical antimicrobial peptides[43].

The epinephenisin showed antibacterial activity against gram-positive and gram-negative bacteria in the presence of 480 mM NaCl, except for fungi *C.albicans*. The result indicates that epinephenisin show broad-spectrum bactericidal activity against pathogenic bacteria up to 480 mM NaCl. This suggests that the antimicrobial components are salt dependent, might be affected by the levels of salt in water[44]. Medium E with NaCl is known to enhance the antibacterial activity α-helical peptides[45]. These salt-dependent alpha-helical peptides, participating in the activity against gram-negative bacteria are active at the salt levels present in seawater. However, the activity against *C.albicans* is salt sensitive as the antifungal activity was abolished when medium E with 480 mM NaCl added. The increased concentration needed to inhibit growth of gram-positive bacteria at 480 mM NaCl concentration (Table 1) can be explains by the cations interfering with the electrostatic

interaction of the positively charged microbial surface[46]. In addition, as compared to magainin 2 purified from *Xenopus laevis*, epinephenisin was approximately 3 times more potent against a broad range of pathogenic microorganisms.

The peptide of grouper fish epinephenisin contains ß–sheet and alpha-helical structure with tyrosine as amino acids chain i.e. cationic residues, contributes additional positive charge to the mature peptide and enhances its antibacterial activity. Although this peptide shows equivalent lytic activity with *B.subtilis* and *E.coli* but it has limited effect on *P.citreus* and *L.anguillarum* as well as fungi C.albicans. One possible explanation is that there may exists some differences in cellular membranes in different species of pathogen and also in different strain of the same genus and species (Sun *et al*., 2007). To a certain extent, the difference in resistant to the peptide in different species may account for this differences in pathogenicity[47]. The fact that the gill extract components are found to be active against both gram-positive and gram-negative bacteria in a condition that is likely to mimic the natural environment of grouper further supports the role of the gill epidermal mucus as a defense barrier. Our results in this study indicate that grouper produce a strong antimicrobial peptide ß–sheet and alpha-helical structure in the gill epithelial mucosal layer to protect against the invasion of pathogenic microorganisms.

CONCLUSION

The fact that mucus components are found to be active against both gram-negative and Gram-positive bacteria in a condition that is likely to mimic the natural environment of tongue sole further supports the role of the mucus as a defense barrier. Because we observed an abolition of the extract activity by pepsin treatment we concluded that the activities are of protein/peptide origin. Prominent antimicrobial activity suggests that the mucus layer of the grouper is an important tissue in gill surface defense of *E. fasciatus*, and most likely protects the fish from infections caused by pathogenic microbes. We have demonstrated that the acidic extract of grouper gill epidermal mucus contains the amphipathic ßeta–sheet and alpha-helical structure of epinisin with tyrosine as amino acid side chains.

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