Purification and Characterization of Antimicrobial Protein from the Skin epidermal mucoprotein of Parrot fish, Scarus ghobban, (Forsskal, 1775)

1Elaperumal Natarajan, 1Subramanian Sankarlal, and 2Seetharaman Rathakrishnan

1Centre of Advanced Study in Marine Biology, Faculty of Marine Science, Annamalai University-608502
2Department of Botany, Faculty of Science, Annamalai University-608002

Received: 10 May Revised: 18 May Accepted: 26 May

Abstract

The study of the Isolation and identification of antimicrobial proteins/peptides in skin epidermal mucoprotein of Parrot fish, Scarus ghobban, a teleost fish, was investigated for the purpose of using compounds of the innate immunity in aquaculture and for human pathogens. The purification procedure involved by a combination of Sep-Pak, cationic exchange-HPLC and RP-HPLC. The Fourier transform infrared (FTIR) spectra in sodium phosphate buffer (NaPB) were characteristic of α-Helix, β-sheet and random coils structure. The 50% acetonitrile elutes (30 μg) caused a zone inhibition with a diameter of 0-13.4 mm, depending on the salt concentration and the microbe tested. HPLC chromatograms revealed that maximum absorbance was at 236 nm, 279 nm, 384 nm and 414 nm. A fraction from the 50% acetonitrile in the cationic exchange-HPLC eluted had antimicrobial activity. The protein thus purified featured the molecular mass of approximately 40 kDa by SDS-PAGE and the action against on both Gram-positive and Gram-negative bacteria as well as fungi. Prominent antimicrobial activity suggests that the mucus layer of parrot fish is an important tissue in surface defense, and most likely protects the fish from infections caused by pathogenic microbes.

Keywords: Antimicrobial protein, Scarus ghobban, Parrot fish, skin epidermal Mucoprotein, FTIR.

Introduction

Fish epithelial cells act as a seal between the animal and its surroundings. They possess microridges, which are raised, actin-rich structures that serve to maintain and aid in the controlled release of mucus on the outer surface of the fish (Webb et al., 2008). They serve as microchannels and greatly increase the surface area (Figure 3). Beneath and in between this surface layer there are, among other differentiated cells (Figure 1), the mucous goblet cells of the
stratum spinosum that supply the mucus (Figure 4). Goblet cells are structurally similar to its mammalian counterpart (Harris and Hunt, 1975), and their produced mucus contains neutral and acid (carboxylated and sulfated) glycoconjugates, disulfides, and other components, which are continuously accumulated in the direction of the external epidermal regions. They make the mucus a highly effective protection (Meyer et al., 2005). This protection shield includes most of the known piscine AMPs and is also made up of additional, AMP-independent anti-infection substances, including alkaline phosphatase, cathepsin B, complement, transferrin, lysozyme, and C-reactive protein (Zhao et al., 2008). Figure 3b shows the detection of glycoconjugates in some scale-derived epithelial cells. The substances lead to a specific glycocalyx composition, and their carbohydrate composition may change with stress, infection, or environmental conditions (Neuhaus et al., 2007). When goblet cells of the fish skin release the mucus, they are not able to synthesize and discharge mucus a second time. Hence, there is a continuous turnover of these cells in the outer layers of the epidermis. The physicochemical characteristics of fish skin mucus layer, as determined by the presence of bioactive substances and the epidermal migration of inflammatory cells and their secretions, may impair the establishment and proliferation of ectoparasitic bacteria, copepods, ciliates, or monogenean helminths (van der Marel et al., 2010). Yet, continuous mucus production and replenishment is costly, and mucus production itself may promote the growth of some bacteria and fungi that exploit it as a nutrient (Morgan and Adams, 2009).

Fish mucus as a rich source of potent AMPs Owing to their antibacterial and immunomodulatory activity, fish AMPs are of interest in veterinary medicine, namely in the context of fish aquaculture (one of the fastest growing industries in the past decades). During the past few years, a number of studies on piscine skin AMPs (Wei et al., 2010; Table 2) have raised the possibility that fish mucus extract may be an excellent antimicrobial agent for treating human pathogens as well. Piscine skin generates a large variety of AMPs such as hepcidin (Cuesta et al., 2008), defensin-like peptides (Zou et al., 2007; van der Marel et al., 2012), cathelicidins (Scocchi et al., 2009), certain apolipoproteins (Villarroel et al., 2007), piscidin (Silphaduang et al., 2006), and pleurocidin (Birkemo et al., 2003), often with selective properties against pathogenic bacteria, fungi, algae, viruses, or parasites (Urquhart et al., 2009). Fish AMP research could also increase its impact on research of innate immunity in human skin (Jensen et al., 2011). On one hand, understanding the mechanisms of AMP regulation in fish could lead to a better understanding of human AMP regulation.

Antimicrobial peptides (AMPs) are molecules known to be essential components of the innate immune response that can also participate in certain organisms as immune modulators (Zanetti, 2004). The living organisms use the AMPs as an important vital tool for survival. They combat the invading pathogens to the host via a broad spectrum of antimicrobial activity that can vary according to the nature of the pathogens (Lehrer et al., 1983; Ganz et al., 1985; Zasloff, 1987). The wide spectrum is in part attributed to the diversity in the structures of AMPs that vary from alpha helix to beta strands (Hancock, 2001). AMPs play defensive role for the producing
organisms. When it comes to their selective toxicity, the AMPs explore the differences in the lipid composition of the membrane between eukaryotic and prokaryotic organism that serves as their target of action sparing the host from harmful effects. Nevertheless, the selectivity of these molecules also arises from the effect of the uniform net charge and hydrophobicity which are characteristic traits for AMPs. The majority of these compounds have been described to be carrying a uniform positive charge ranging from +2 to +9, with only few reported to be anionic in nature (Zasloff, 2002).

The cationic nature accounts for the AMPs mechanism of action, as it enables their binding to the negatively charged lipopolysaccharides invading pathogen membrane via electrostatic attractive forces. During this interaction, the AMPs acquire an amphipathic confirmation that optimizes the binding to the pathogen’s membrane. This kind of AMPs are classified to be membrane permeabilizing peptides due to their capability of forming pores in the membrane after their electrostatic interaction, thus resulting in expulsion of cellular content and subsequent cell death (Sengupta et al., 2008). In addition to the previously mentioned mechanism, AMPs can act by interfering with some vital cellular processes inhibiting them after their translocation across the cellular membrane. These peptides are classified as non membrane permeabilizing peptides (Kondejewski et al., 1999; Brogden, 2005).

Despite the promising activity of the AMPs, they suffer from the following drawbacks. (i) limited stability at certain pHs; (ii) disrupt the cellular membrane of eukaryotic organisms causing hemolytic side effects; (iii) elevated production cost and technical issues are limitations of their manufacturing (Mygind et al., 2005); (iv) lack of data on their toxicity, pharmacodynamics, and pharmacokinetics properties (Michalopoulos et al., 2005); (v) reduced activity in the presence of some cations such as calcium, iron and certain serum conditions (Smith et al., 1996; Thackray and Moir, 2003).

The aim of this study was to isolate, identify and characterize antimicrobial proteins and peptides in epidermal mucus from healthy Parrot fish, Scarus ghobban (Forsskal, 1775). Increased knowledge of compounds taking part in innate defenses can be of great importance, both as a means for using compounds of the innate immunity in aquaculture and for anti-infective agents in animals and human. The species is a member of the fish family Scaridae, commonly known as Parrot fishes. It occurs in tropical marine waters of the Indo-West and Central Pacific, from South Africa and the Red Sea, north to Japan, south to Australia and east to French Polynesia. The Parrotfish is usually found in shallow lagoons, seagrass beds and reefs habitats. It is often seen in murky, turbid waters. It feeds by scraping algae from rocks and corals. Juveniles often form school, but adults are usually seen as solitary individuals.

2. Materials and Methods
2.1. Sample Collection:
Live specimens of the fish Scarus ghobban were collected from Nagapattinam as by-catch. Mucus scrapping: Epithelial mucus was sampled by scraping a dull scalpel blade along
the dorsal flank of live fishes, anterior to posterior. Mucus of the fish was collected from the dorsal region of the skin using blunt edged scalpel. Mucus was not collected from Ventral side of the fish to avoid urine and intestinal excreta (Chong et al., 2005). The fish was placed on a flat non slippery surface with its head and eyes covered by palms to reduce the photophobic response (fear of light). Using a dull blade, mucus was gently scraped off the entire dorsal flank of fish as described by Zamzow (2004). Mucus sample was taken from the anterior section by moving from the head towards the anus using a spatula and stored in the sterile Amber bottle and stored in ice, to avoid bacterial contamination and proteins degradation during the transportation.

**Sample Preparation:** 0.1002 g of sample was weighed and dissolved in 10 ml of methanol and diluted to 25 ml with methanol (Stored at -4º C). **Preparation of Mobile Phase:** Preparation of 0.2 % Acetic acid: 0.2 ml of Acetic acid mixed with 100 ml of distilled water, 75 ml of 0.2 % Acetic acid mixed with 25 ml of methanol and Filtered through and 0.45 μm nylon vacuum filter and sonicated. **Sonication:** Each sample was then mixed using a sonicator in an ice bath (Unisonics) for 20 min and left to leach for 24 h at room temperature. **Centrifugation:** The extracts were then centrifuged for 5 min at 18 000 × g and the supernatant was used for laboratory spectral UV analysis. **Sample extraction:** Samples were extracted in 1.5 ml of 100% methanol and homogenized. **Partial purification of Parrot fish Scarus ghobban fish mucus:** Partial purification of fish mucus was carried out by Silica gel chromatography.

### 2.2. Protein Estimation
Total protein concentration was determined following the method of Bradford, (1976) using Bovine serum albumin (Hi media) as standard.

### 2.3. Sodium Dodecyl sulphate -Polyacrylamide Gel Electrophoresis (SDS-PAGE)
SDS-PAGE (Laemmli, 1970) was used to separate mucus protein from the Parrot fish Scarus ghobban.

### 2.4. Fourier Transform Infrared Spectroscopy (FTIR):
FTIR characterization of FME was performed with a Perkin Elmer-Spectrum RX1 instrument. FME were prepared in the forms of pellet. Then the sample was mixed with KBr (Potassium Bromide) to make a 13 mm diameter pellet. The spectra of the FME sample were obtained with a frequency range of 400-400 cm-1 resolution. 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 were placed in a thermostatically controlled Beckmann FH-01CFT microcell fitted with CaF2 windows. The sample compartment was continuously purged with dry air to eliminate absorption by water vapour in thespectral region. Spectral conditions were analysed following procedure explained by Haris et al., (1975) and Perkins et al., (1988) and the conditions were as follows: number of scans, 200; spectral resolution, 4 cm-1; sample thickness of 50 μm using a Teflon spacer; sample temperature, 20ºC.

### 2.5. High Performance Liquid Chromatography: Qualitative Analysis by HPLC (Dunlap and Chalker, 1986)
**Chromatographic Conditions:** Column : Supelco C18 Silica, ODS (250×4.6 mm× 5 μm), Mobile phase : 0.2 % Acetic acid in water: Methanol (75:25) (v/v), Flow Rate :
1.0 ml/min, Detector : UV, Injection Volume : 20 μl, Diluent : Methanol, Column temperature : Ambient i.e. 25°C (± 1°C) and Run Time : 15.0 minutes.

2.6. Microbial Strains

Bacillus subtilis strain (ATCC 1774), S. aureus strain (NCTC 6571), E.coli strain (ATCC 11229), P. aeruginosa strain (ATCC 10145), and Candida albicans strain (ATCC 90029) were used to analyze the antimicrobial activity in the mucus. For each antimicrobial experiment, bacterial colonies were seeded from frozen stocks and grown on Luria-Bertani (LB) agar (Himedia) plates containing streptomycin (100 μg/ml). Yeast cultures were prepared from frozen stocks and grown on agar plates containing YM medium (Himedia). The plates were incubated at 37°C for 24 h. Colonies were picked from the agar plates and the bacteria suspended in 20 mL LB broth, or YM broth for yeast cells, and incubated at 37°C with shaking until the desired cell density was reached (D590 = 0.6).

2.7. Minimum inhibitory concentration

The Minimum Inhibitory Concentration Assay is a technique used to determine the lowest concentration of a particular antibiotic needed to kill bacteria. This assay is typically performed on planktonic (free floating) bacterial cells. A pure culture of a single microorganism is grown in Mueller-Hinton broth or other broth as appropriate. The culture is standardized using standard microbiological techniques to have a concentration of very near 1 million cells per milliliter. The more standard the microbial culture, the more reproducible the test results. The antimicrobial agent is diluted a number of times, usually 1:1, through a sterile diluent (typically Mueller-Hinton broth). After the antimicrobial agent has been diluted, a volume of the standardized inoculum equal to the volume of the diluted antimicrobial agent is added to each dilution vessel, bringing the microbial concentration to approximately 500,000 cells per milliliter. The inoculated, serially diluted antimicrobial agent is incubated at an appropriate temperature for the test organism for a pre-set period, usually 18 hours. The more consistent the incubation period, the more reproducible the test results. After incubation, the series of dilution vessels is observed for microbial growth, usually indicated by turbidity and/or a pellet of microorganisms in the bottom of the vessel. The last tube in the dilution series that does not demonstrate growth corresponds with the minimum inhibitory concentration (MIC) of the antimicrobial agent.

2.8. Haemolytic assay

The micro haemolytic test was performed in 96 well micro titre plates. Different rows were selected for chick, goat, cow and human blood. The crude, aqueous, acidic and organic extracts were checked for their haemolytic efficacy at 10 μL/mL diluted using sterile phosphate buffer saline. Its efficacy was tested using 100 μL of 1% RBC. Appropriate controls were included in the test such as 0.1% SDS as the positive control and 0.5% dimethylsulfoxide as the negative control as per Hoffman standard. The plate was gently shaken and then allowed to stand for 2 h at room temperature and the results were recorded. Uniform red colour suspension in the wells was considered as positive haemolysis and a button like formation in the bottom of these wells
was considered as lack of haemolysis. The absorbance was then read at 542 nm using an ELISA plate reader.

3. Results

3.1 Protein Quantification: Bradford Method (1976):
Standard Used: Bovine Serum Albumin, BSA Stock Concentration: 10 mg/ml, Concentrations of standard: 1, 2, 4, 6, 8, 10 μg/ml, Sample Volume: 1 μl and Absorbance: 595 nm.

Table 1. Total protein concentration of Parrot fish, Scarus ghobban SEM

<table>
<thead>
<tr>
<th>Std/conc µ/ml</th>
<th>OD_{595nm} Replicate-1</th>
<th>OD_{595nm} Replicate-2</th>
<th>OD_{595nm} Replicate-3</th>
<th>OD_{595nm} Replicate-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;0.56</td>
<td>&lt;0.56</td>
<td>&lt;0.56</td>
<td>&lt;0.56</td>
</tr>
<tr>
<td>1</td>
<td>1.2</td>
<td>0.8</td>
<td>10.2</td>
<td>&gt;10.5</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>1.5</td>
<td>&lt;0.56</td>
<td>&lt;0.56</td>
</tr>
<tr>
<td>4</td>
<td>4.3</td>
<td>4.0</td>
<td>2.0</td>
<td>0.8</td>
</tr>
<tr>
<td>6</td>
<td>6.9</td>
<td>6.5</td>
<td>8.7</td>
<td>8.4</td>
</tr>
<tr>
<td>8</td>
<td>8.5</td>
<td>8.0</td>
<td>0.6</td>
<td>&lt;0.56</td>
</tr>
<tr>
<td>10</td>
<td>10.0</td>
<td>8.8</td>
<td>1.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 2: Conc. < 0.500 indicates the total protein concentration is too low in the samples and doesn’t fit in the range of the standard curve.

<table>
<thead>
<tr>
<th>Curve Name</th>
<th>Curve Formula</th>
<th>A</th>
<th>B</th>
<th>R2</th>
<th>Fit F prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curve</td>
<td>Y=A*X+B</td>
<td>0.014</td>
<td>0.194</td>
<td>0.98</td>
<td>??</td>
</tr>
</tbody>
</table>
3.2. SDS-PAGE of *Scarus ghobban* SEM
The methanolic extracts of *Scarus ghobban* parrot fish epidermal mucus showed the protein bands lower than 40 KDa.

**Sodium Dodecyl sulphate -Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

![Molecular weight pattern of the Parrot fish *Scarus ghobban* SEM](image)

Fig. 1. Molecular weight pattern of the Parrot fish *Scarus ghobban* SEM. Lane 1-Marker, Lane 2-Fish Mucus Extract: Lane 2 shows that the Skin epidermal mucoprotein (SEM) Containing proteins lower than 40 KDa. It revealed that the presence of low molecular weight proteins.

3.3. FTIR analysis of *Scarus ghobban* SEM
FTIR spectra of methanolic extracts of *Scarus ghobban* parrot fish epidermal mucus showed functional groups such as carboxylic acids, aliphatic amines, aromatics, phenol and alkynes. Mostly aliphatic amines presence showed in the spectra showed in the Figure. **Fourier transforms infrared spectroscopy (FTIR):** Lyophilized Fish mucus sample was mixed with KBr (IR grade) and made into a pellet. The pellet was immediately put into the sample holder and FTIR spectra were recorded in the range of 450-4000 cm\(^{-1}\) for sample. Characterization of the FME was carried with a Spectrum one FT-IR Spectrometer. **Determination of secondary structure of protein/peptide:** Secondary structure of skin epidermal mucoprotein was estimated using a FTIR spectrometer in the presence of 50 mM sodium phosphate buffer (NaPB) and IR peak observed in the range of 1600 to 1700 cm\(^{-1}\) (fig.2). The contents of α-helix, β-turn and random coils were calculated according to Barth, 2007 (Table 3). The FTIR spectrum of mucoprotein for E.tauvina confirmed the presence of α-helix, and β-turn with tyrosine as amino acid side chains. In the presence of 50% trifluoroethanol, the peptide became structured, exhibiting a high level i.e. of α-helical folding structure. Then, the α-helical conformation of mucoprotein was confirmed in the presence of structure promoting solvent.
Table 3: FTIR spectra peak location and assignments for *Scarus ghobban* SEM

(Barth, 2007)

<table>
<thead>
<tr>
<th>Sample Lyophilized powder</th>
<th>Band position (cm⁻¹)</th>
<th>Assignment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyophilized powder</td>
<td>1690</td>
<td>β-turn</td>
<td>1689 ± 2 Susi &amp; Byler, 1986; Byler &amp; Susi, 1986; Dong <em>et al.</em>, 1994</td>
</tr>
<tr>
<td></td>
<td>1654</td>
<td>α-helix</td>
<td>1653 ± 4 Susi &amp; Byler, 1986; Byler &amp; Susi, 1986; Dong <em>et al.</em>, 1994</td>
</tr>
<tr>
<td></td>
<td>1618</td>
<td>Tyr, side chain</td>
<td>1612-1618 Chirgadze <em>et al.</em>, 1975</td>
</tr>
<tr>
<td></td>
<td>1601</td>
<td>Tyr, side chain</td>
<td>1602 ± 2 Venyaminov and Kalnin, 1990</td>
</tr>
</tbody>
</table>
Fig. 2. FTIR spectra of *S. ghobban* SEM

3.3. High Performance Liquid Chromatography (HPLC) of *Scarus ghobban* SEM

HPLC chromatograms revealed that maximum absorbance was at 236 nm, 279 nm, 384 nm and 414 nm. Figure 3A-D illustrate the absorbance and peaks and their retention time.

Qualitative Analysis by High Performance Liquid Chromatography (HPLC) of SEM

Sample: 

Fig 3A: UV detection at 236 nm
Fig 3B: UV detection at 279 nm

Fig 3C: UV detection at 384 nm
3.4. Antimicrobial activity of the gill epidermal mucus extract

The skin epidermal mucus extract comprising approximately 38% protein, was assayed for antimicrobial activity against gram-positive *Bacillus subtilis, Staphylococcus aureus; Gram-negative E.coli, Pseudomonas aeruginosa* and fungi *Candida albicans*. The magnitude of MICs was *P.aeruginosa* and *C.albicans*< *S.aureus*< *B.subtilis* and *E.coli*. The magnitude of MICs was *P.aeruginosa* and *C.albicans*< *S.aureus*< *B.subtilis* and *E.coli*. The greasy grouper skin epidermal mucus extract showed different lytic activity against gram-positive and gram-negative bacteria (table 1). The grouper mucus extract 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 *Staphylococcus aureus* was 28 µg; gram-positive bacteria *Escherichia coli* was 16µg and *Pseudomonas aeroginosa* was 32; and the peptide could inhibit fungi *Candida albicans* at 32µg.

Table 1. Antimicrobial activity of the Epidermal mucoprotein of the Parrot fish *scarus ghobban*

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Microbial pathogens</th>
<th>Minimum Inhibitory Concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Test Sample</td>
</tr>
<tr>
<td>1.</td>
<td><em>Bacillus subtilis</em></td>
<td>16</td>
</tr>
<tr>
<td>2.</td>
<td><em>Staphylococcus aureus</em></td>
<td>28</td>
</tr>
<tr>
<td>3.</td>
<td><em>Escherichia coli</em></td>
<td>16</td>
</tr>
<tr>
<td>4.</td>
<td><em>Pseudomonas aeroginosa</em></td>
<td>32</td>
</tr>
<tr>
<td>5.</td>
<td><em>Candida albicans</em></td>
<td>32</td>
</tr>
</tbody>
</table>
3.2. Haemolytic activities of epidermal mucus of the Parrot fish *Scarus ghobban*na

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Concentrations(mg/mL)</th>
<th>% of haemolysis of goat red blood cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>0.23</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>0.43</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Discussion

Synthetic drugs and food preservatives are notably affected the ecosystem to a considerable extent, due to their higher persistency and constant accumulation in the biological system. To overcome this, considerable investigations are being carried out to develop safer source. The skin mucous layer and epidermis are important in fish defense because they are the first sites of interaction between the host and potential pathogens. Within these layers are many enzymes and antimicrobial proteins, which are thought to be involved in innate immunity of the fish (Dalmo *et al.*, 1997). Fish live in intimate contact with their aqueous environment, which is densely populated with microorganisms. The protective role of the epidermal mucus of fish has been known for many years (Ellis, 2001; Hellio *et al.*, 2002; Magnadottir, 2006), indicating a source for isolation of antimicrobial components.

Over the past years, it has also been shown that mucus plays a role in the prevention of colonization by parasites, bacteria and fungi and the antibacterial role of mucus has been known for many years (Austin and McIntosh, 1988). Fish mucus was found as a source of antimicrobial products (Hellio *et al.*, 2002). The mucus contains many antibacterial substances including antibacterial peptides, lysozyme, lectins and proteases (Baranes *et al.*, 2003). According to Boman(1995) and Andreu and Rivas(1999) most of the antimicrobial peptides kill bacteria by a common mechanism, which involves direct electrostatic interactions with negatively charged phospholipids on microbial cell membranes followed by physical disruption and solubilization. Fish mucus is believed to play an important role in the prevention of colonization by parasites, bacteria and fungi and thus acts as a chemical defense barrier.

As for other natural drugs, the structure elucidation of MAAs was achieved by chemical degradation, spectroscopic techniques (UV-spectra, IR-spectra, 1H NMR, etc.). Here, we report the UV-B absorbing properties of mucus from the body surface of marine fishes. Furthermore, we found that the absorbing compounds were not contained within the skin cells, but were secreted into the epithelial mucus, and thus provided a protective coating to the animal. All MAAs found here had their peak absorbance within the UV-A range (320 to 400 nm) but also extended into the UV-B range (280 to 320 nm) of the light spectrum. Possession of several different types of MAAs with different absorption maxima provides organisms with a means of
UV absorption over a broad range (Tartarotti et al., 2004), thus, these fishes appear to have an effective strategy for UV protection. Our result showed that maximum absorbance peaked between 279-290 nm and SDS-PAGE result showed that the protein bands were below the molecular weight of >40 KDa. Similarly the results of (Dunlap et al., 1989) also correlate, in algal-invertebrate symbioses. MAAs are present in soluble form and not associated with any protein species whereas asymbiotic metazoans are protein-associated and occur especially in the epidermis (Dunlap et al., 2000), but also in the ocular tissues of many shallow water fishes (Dunlap et al., 2000; Dunlap et al., 1989) and some cephalopod molluscs such as Sepia officinalis (Shashar et al., 1998). Asterina-330 and gadusol exist in association with soluble proteins in fish lenses. The highly unstable major complex with absorption λmax = 330 nm, when dissociated, yields asterina-330 (λmax = 330 nm) and a protein of MW 80–100 kDa (λmax = 280 nm). The second, relatively stable minor complex of λmax = 323 nm, under similar conditions, yields gadusol (λmax = 269 nm) and a protein of MW 20–30 kDa (λmax = 280 nm) (Dunlap et al., 2000). FTIR has been used extensively to study the structure of proteins and lipids (Akkas et al., 2007) in fishes. The Fourier Transform Infra-Red Spectroscopy (FTIR) analysis spectrum of SEM (Figure.2) revealed the following groups. Peaks at 1690 cm\(^{-1}\), 1654 cm\(^{-1}\), 1618 cm\(^{-1}\)and 1601 cm\(^{-1}\) characterized a protein secondary structure of Beta turn, alpha helix with tyrosine amino acid as side chains (Susi & Byler, 1986, Dong et al., 1994, Chigadze et al., 1975, Venyaminov and Kalnin, 1990) Which is indicated the presence of certain groups like aliphatic, amino, amine groups, there are 2 major peaks it showing the presence of amine group and alcohol group. Stochaj et al. (1994) have showed that the chromatographic separation of the acidic compounds can be improved if the methanol content of the mobile phase is increased up to 75%. In this condition, the highly polar compounds interact with the weak anion exchange properties of the silanol groups to give an improved chromatographic separation of these compounds.

In algal-invertebrate symbioses MAAs were present in soluble form and not associated with any protein species whereas in asymbiotic, metazoans are protein-associated and they occur especially in the epidermis (Shick and Dunlap, 2002), but also in the ocular tissues of many shallow water fishes (Dunlap et al., 1989; Shick and Dunlap, 2002) and some cephalopod molluscs such as Sepia officinalis (Shashar et al., 1998). Asterina-330 and gadusol exist in association with soluble proteins in fish lenses. The highly unstable major complex with absorption λmax = 330 nm, when dissociated, yields asterina-330 (λmax = 330 nm) and a protein of MW 80–100 kDa (λmax = 280 nm). The second, relatively stable minor complex of λmax = 323 nm, under similar conditions, yields gadusol (λmax = 269 nm) and a protein of MW 20–30 kDa (λmax = 280 nm) (Shick and Dunlap, 2002). The HPLC chromatogram, shown in Figure.3A-D. Illustrates the result of SEM, and the presence of amines compound at 279 nm absorbance.

The classical HPLC method is based on reverse-phase low silanol-free group octadecylsilica (C18) columns and isocratic elution with 0.02% acetic acid as mobile phase at 15 °C (Shick et al., 1999). Recently Volkmann and Gorbushina, (2006) using isocratic C18
(endcapped) reversed phase liquid chromatography/mass spectrometry has successfully separated and identified eight different mycosporines of terrestrial fungi and five cyanobacterial MAASs. The protein with <40 KDa was detected in SDS-PAGE analysis as shown in figure 1 and it was confirmed by UV max at 279 nm and the same was observed in HPLC performance, the protein expressed protein as major functional groups. The result of FTIR revealed a protein of Beta turn, alpha helix with tyrosine as amino acid side chains. The instrumentation spectrum details supported the compound could be a peptide with tyrosine aminoacid was present in the SEM.

Fish have evolved to thrive in an aqueous environment rich in microbial flora and are presumed to use their innate immune system as the first line of defense against microbial invasion. In the last decade, many species-specific antimicrobial peptides have been isolated from fish (misgurin, pleurocidin, paradaxins, hagfish intestinal antimicrobial peptides, and parasin I), some of them showed a broad spectrum of activity against Gram-positive and Gram-negative bacteria (Zasloff, 2002; Silphaduang & Noga, 2001). Compared with largely found antimicrobial peptides from aquatic animals, less antimicrobial proteins from aquatic species are reported. Carp antimicrobial proteins, 27 kD and 31 kD proteins, had potent microbicidal activities against different strains of Gram-negative and Gram-positive bacteria (Lemaitre et al, 1996). Furthermore, it has been shown that larger antimicrobial proteins, such as aplysianin A from the sea hare (Takamatsu et al, 1995) and achacin from the giant African snail (Obara et al, 1992), have strong antimicrobial activities.

The data of present study indicate that the antibacterial activity of the fish mucus may be due to the presence of the above said substances. The mode of action of mucus is yet to be determined but studies have proposed various killing mechanisms for fish derived AMPs such as cytoplasmic membrane disruption, pore or channel formation (Syvitski et al., 2005) and inhibition of cell wall and nucleic acid synthesis (Partzykat et al., 2002; Brogden, 2005).

Although graesy groupers are susceptible to many infectious agents including bacteria such as vibrios (Herrera et al, 2006), antimicrobial proteins and peptides have not been previously reported. In this study, we report an antimicrobial protein from Scarus.ghobban skin epidermal mucus. The purified protein, is a 40 kD protein. The protein thus purified featured the molecular mass of approximately 40 kDa and the action against on both Gram-positive and gram-negative bacteria. The following antibacterial protein have so far been found in skin secretions or mucus, although most of them have been characterized; hydrophobic proteins with 27 and 37 kDa from carp C.carpio, 45 kDa protein from eel Anguilla anguilla, 65 kDa protein from rainbow trout O.mykiss and a 49 kDa protein from tench Tinca tinca. These antibacterial proteins are assumed to form ion channels in bacterial membrane and kill both Gram-positive and Gram-negative bacteria (Ebran et al., 1999; 2000). Nagashima et al. (2001; Lee et al., 2008; Chongsiriwatana et al., 2008), reported the antibacterial glycoprotein with a high molecular mass of approximately 400 kDa from rabbitfish Siganus fuscescens. Besides them, antibacterial protein, achacin, was isolated from the body surface mucus of giant African snail Achatina fulica and characterized
(Kubota et al., 1985; Obara et al., 1992). Achacin has a molecular mass of 160 kDa, exerts antibacterial activity against a wide range of both Gram-positive and Gram-negative bacteria. The antibacterial protein obtained from the tongue sole is different from the antibacterial agents described above in view of molecular mass and antimicrobial action.

A 40 kDa was the second antimicrobial peptide to be purified from S. ghobban and it was therefore named Scarin. This peptide is very potent, displaying broad-spectrum antibacterial activity at microgram level concentrations. In particular, Scarin is active against B. subtilis, the causative agent of vibriosis in fish. Vibriosis, also known as “red plague” or “saltwater furunculosis”, is a systemic bacterial infection that affects primarily marine and estuarine fishes (Bullock, 1987). The potential role of histone H1 in fish immunity is further supported by two recent reports. Richards et al. (2001) have isolated a 20.7 kDa antibacterial protein from the liver of Atlantic salmon, Salmo salar. This protein, which is active against E. coli D31 at submicromolar concentrations, was identified by tandem nanoelectrospray mass spectrometry as being histone H1 (Richards et al., 2001). A 26-residue antimicrobial peptide has also been purified from the mucus and serum of coho salmon, O. kisutch (Patrzykat, 2001). This 40 kDa protein displays antibacterial activity against B. subtilis, V. anguillarum, E. coli and P. citreus (Patrzykat, 2001). Moreover, this study also showed that the expression of the antimicrobial peptide was up-regulated following immunological stimulation and that it coincided with an increase in antibacterial activity of mucus.

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 (Zasloff, 2002; Tossi et al., 2000; Matsuzaki, 1998). The amphipathic β–turn and alpha-helical structure of Scarin was predicted by FTIR spectra analysis (Fig.5.2A). The Scarin form alpha-helical structure in the structure-forming solvent, trifluoroethanol (Fig.5.2B). This suggests that β–turn and alpha-helical structure in the water environment will form alpha-helical conformation after contacting with bacterial membrane. Thus, Scarin will show antimicrobial activity in a similar way to other previously studied amphipathic alpha-helical antimicrobial peptides (Iijima et al., 2003).

The peptide of grouper fish scarin contains β–turn 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 S. aureus and P. aeruginosa as well as fungi C. albicans. One possible explanation is that there may exist 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 (Sun et al., 2007). 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 β-sheet and alpha-helical structure in the gill epithelial mucosal layer to protect against the invasion of pathogenic microorganisms.

Previous studies have reported MICs in the range of 15 to 115 μg protein/mL for the mucus extracts of eel (A. Anguilla), tench (Tinca tinca), trout (O. mykiss) turbot (S. maximus) and carp (C. carpio) (Ebran et al., 1999) and 180 μg protein/mL for rockfish (Sebastes schlegeli) mucus (Nagashima et al., 2003). The acidic mucus extract of parrot fish showed antimicrobial activity in the range of 16to 32 μg protein/mL in the current study, which was lower than carp and eel acidic mucus extracts (Table 2). Parrot fishes are scavengers and inhabit corals and seagrass bed. Our results suggest that to survive in such conditions they secrete a larger volumes of mucus containing potent antimicrobial components such as lysozyme and proteases (Subramanian et al., 2007) and possibly bioactive peptides/proteins.

The mucus secretion of S.ghobban has proteinaceous substances which show potent bioactivity when mixed with blood cells of goat. Mucus extracts exhibits high level of hemolytic activity when the concentration increases. Some antimicrobial agents present in the mucus of bony fishes which bind with microbes and destroy the blood cells Hellio et al.,2000. Hellio et al.,2000 reported that lysozyme in the mucus has bacteriostatic properties and was upiquitous in its distribution among living organisms.

Conclusion

The detection of antimicrobial and haemolytic properties in the biological secretions and tissues of the examined fish species stresses the need for their further characterization. Further efforts are necessary for the chemical purification and isolation of active antimicrobial compounds. Extraction of antimicrobial peptides could provide not only a good and sustainable way for the utilization of fish by-products like mucus and skin, but it could also contribute in advancing current understanding of the function of these substances in the mucosal defense mechanisms of fish, in order to establish their possible applications as natural therapeutic compounds against infectious diseases. Further studies on the characterization and sequencing of the antimicrobial substances in these acidic mucus extracts will further our understanding of the composition and function of the antimicrobial components in the mucosal defense mechanism of these fish species.

Acknowledgement

The authors are thankful to the Dean and Director and university authority for providing facilities for carrying out this work. The second author also thanks the University Grand Commission-New Delhi-RGNF for providing financial support during his tenure of research work.
References


Noga EJ, Silphaduang U, Park NG et al. Piscidin 4, a novel member of the piscidin
Kristensen, H.H., Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. Nature. 2005, 437 (7061), 975–980.


Lee, M.T., Huang, W.C., Chen, F.Y., Huang, W.H., PNAS, Mechanism and kinetics of


**Corresponding Author:** Elayaperumal Natarajan, CAS in Marine Biology, Annamalai University, Parangipettai-608502. Email Id: menrajancas@gmail.com.