Assessment of Antimicrobial peptides from mucus of fish

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A B S T R A C T
Fish are a diverse group of animals, highly specialized for their aquatic existence and comprising almost half the number of vertebrate species in existence today. Fish are in intimate contact with their environment, which can contain very high concentrations of bacteria and viruses. The immune system is composed of numerous organs and cells that act together in a dynamic network in the defense against infection, disease and foreign substances. The bacterial pathogens was tested against several organisms and maximum inhibition was recorded against Staphylococcus aureus (12 mm), Lactobacillus vulgarius (10mm) and Klebsiella pneumonia (7 mm). The protein estimation of fish mucus was observed as (0.865 mg/ml). SDS -PAGE of fish mucus showed many protein bands with molecular size ranging from 22 to 65 K da.

Introduction
Aquaculture is the cultivation of aquatic animals such as fish (or) Shellfish (or) of plants such as seaweed in a controlled and sometimes enclosed body of water. In Aquaculture, according to the intensive system of production to reduce the cost, fish are kept in high densities and the possibility for exposure to pathogens which can be bacteria, parasites (or) virus, throughout production cycle was becoming high (Laidler et al., 1999). Under such conditions, the problems of the Infectious fish diseases become serious and have considerable effects on Aquaculture. In fact, bacterial and viral diseases of farmed fish have lead to high mortalities and reduced economical income for the fish farming industry (Munro 1933; Pilcher 1980). Though aquaculture is very profitable, it also creates serious economic threat when diseases outbreaks. Avoiding disease epidermis in aquaculture is very important to get economic benefit and it is possible only when fish species are reared in a good environmental condition and given priority in fish welfare. Fish welfare or animal right law is related to farmed fish. It means that fish have right to live a life as good as possible and express its natural behaviour as much as possible and free from negative experiences. Fish are in intimate contact with their environment through the large surface of their gills, skin, and of necessity they defecated into the medium in which they live, so water quality (in terms of dissolved oxygen, CO₂, ammonia and pH) and the presence of contaminants (organic and inorganic pollutants) are probably the most critical aspects of the environment for fish welfare and also the best defined (Mellor and Stafford 2001).

Optimal conditions vary markedly between species for example, catfish do poorly in clear water, whereas salmon do poorly in cloudy water and cyprinid fish are very tolerant of low dissolved oxygen levels where as salmonid fish are not (Kramer 1987). The mechanism barrier of the skin impedes entry of the majority of microorganisms into the body (Bressler and Bressler et al., 1989) Mucous membranes lining the alimentary, respiratory, and urogenital tracts are equipped with a layer of mucus which functions to entrap foreign microorganisms out of the body. The major components of the mucus layer are produced by goblet cells and these cells start to differentiate in the basal part of the epidermis, and then grow in size and move towards the surface where they release their content (Pickering, 1977). The mucus is a dynamic coat, which passively flows over and covers the fish (Powell et al., 1992).

Mucus slime is the material that makes fish slippery. Its 'slipperiness' is the result of its high water content and the presence of high-molecular weight, gel-forming, macromolecules. The skin of fish is a dynamic tissue whose cellular makeup known to be influenced by factors such as season, stress, diseases,
development stage and environmental conditions (Blackstock and Pickering, 1982). A great deal of research supports the notion that layers of mucus accumulate on the skin and gills of fish that are stressed by disease, adverse environmental conditions and handling (Handy and Eddy, 1991). The skin surfaces support considerable concentration of gradients, particularly for sodium and chloride in fresh water or seawater. Fish biologists suspected that mucus might be involved in ion regulation.

The present study demonstrated the Antibacterial activity and protein content in mucus of several fish species (Austin and McIntosh, 1988) yet this activity seems to vary from fish species to fish species and can be specific towards certain bacteria (Noya et al., 1995).

Materials and Methods

Fish Collection and Maintenance

Mucus sample was collected from *Oncorhynchus mykiss*, *Channa striatus* were obtained from a local fish market in Kanchipuram. Then the fish were stocked into the 500 L capacity circular plastic tanks. The fish acclimatized to laboratory conditions in a tap water and they were maintained for one week. During this period the fish were fed with commercial feed once a day at *ad libitum*. Every day 50 % of the water was changed. After one week of acclimatization the fish were used for mucus collection. Only healthy fish were chosen for mucus collection. Dead fish or fish with skin lesions were removed from the tanks.

Mucus Collection

Mucus was collected by a modified method of (Subramanian et al 2008). Fish was starved for one day prior to mucus collection. On the day of mucus collection fish was washed and transferred into a sterile polyethylene bag for 10 to 20 minutes and moved front and back to slough off the fish mucus. Then, the fish was returned to recovery tanks. Fish mucus samples obtained from five fish was then pooled and stored in refrigerator at 4°C until further use.

Mucus Extraction

The aqueous extract of fish mucus was prepared using a method as described by (Hellio et al 2002). Fifty ml of fish mucus was mixed with 50 ml of distilled water and homogenized using a polytron homogenizer. The mixture was then centrifuged at 30,000 g for 30 minutes at 4°C. Supernatant was then collected and filtered with Whatman no.1 filter paper. The filtrate was then collected and stored in refrigerator at 4°C.

Protein Estimation

The protein concentration of the samples was determined by the method of (Lowry et al., 1951) with bovine serum albumin as standard. To 5ml of Lowry reagent, add 1ml of suitably diluted sample and the mixture was kept at room temperature for 10min. to this add 0.5ml of Folin’s reagents and kept at dark condition for 30mins. The absorbance was taken at 640nm.

Protein separation and Quantification

Most commonly the strong anionic detergent SDS is used combination with a reducing agent and heated to disassocate the proteins before they are loaded on the gel. The amount of SDS bound is always proportional to molecular weight of the poly peptides and is independent of their sequence. The protein were separated by SDS-PAGE electrophoresis and size of polypeptide chains of given protein can be determined by comparing its electrophoretic mobility in SDS-PAGE gel with mobility marker proteins of known molecular weight (Laemmli, 1973).

Antimicrobial assays

*Staphylococcus aureus*, *Salmonella typhi*, *Salmonella paratyphi*, *Klebsiella oyxata*, *Pseudomonas aeruginosa*, *E.coli*, *Proteus mirabilis*, *Lactobacillus vulgaris*, *Vibrio cholera* and *Klebsiella pneumonia*. All the bacteria mentioned above were incubated at 35±0.1°C for 24h by inoculation into Nutrient Broth while the fungal cultures were incubated in Potato Dextrose Broth at 25±0.1°C for 48h. These cultures were spread-plate on Mueller Hinton Agar using a sterile cotton swab and wells were made in the plates with the help of a cork borer (0.85cm). The test compound (0.1ml) was introduced into the well and the plates were incubated (Russell et al., 1977).

Results

Protein content

The fish mucus was estimated for its protein level and the results shows that the fish mucus contains 0.865mg/ml of protein content.

Protein separation and Quantification

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is an excellent tool to identify and monitor proteins during purification and to access the homogeneity. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) of fish mucus showed many
protein bands with molecular size ranging from 22KDa as shown in Fig 1.

Antibacterial Assays
Antibacterial activity was determined and the bacterial pathogens (Staphylococcus aureus, Salmonella typhi, Salmonella paratyphi, Klebsiella oxytoca, Pseudomonas aeruginosa, E.coli, Proteus mirabilis, Lactobacillus vulgaris, Vibrio cholera and Klebsiella pneumonia) were susceptible to fish mucus. Maximum inhibition was recorded against Staphylococcus aureus (12mm), Lactobacillus vulgaris (10mm), Salmonella and Vibrio cholera (8mm) whereas Klebsiella pneumoniae were shown slightly lower inhibition (8mm) as shown in the Table 1 and Fig 2.

Discussion
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 skin mucus 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). Differences in activities of ntimicrobial enzymes, such as lysozyme and proline, may relate to the structure and composition of mucus and epidermal layers, may also relate to the differences observed in disease resistance. Lysozyme, an antimicrobial, hydrophobic protein found in fish blood and tissues has been studied most extensively in association with disease resistance (Lie et al., 1989) and cortisol stress response (Fevolden and Roed, 1993). The antibacterial activity in mucus samples of five salmon and five cod was tested in different protein concentrations, against four different bacterial strains (E.coli, Lactobacillus anguillarum, Cladstridium glutamicum and Staphylococcus aureus). Growth curves of the bacteria were investigated in different protein concentrations, against four different bacterial strains (E.coli, Lactobacillus anguillarum, Cladstridium glutamicum and Staphylococcus aureus). Antibacterial activity was conducted in the serum and mucus of rainbow trout (Onkorhychus mykiss) and found a number of antibacterial factors increase in concentration following immunization and that these probably played a role in protection against microbial disease (Rainger and Rowley, 1993).

Elutes from the solid phase extraction were tested for antibacterial activity against E.coli, Lactobacillus anguillarum, Cladstridium glutamicum and Staphylococcus aureus was detected in extracts from several tissues in all species tested, but mainly in the haemolymph and haemocyte extracts. L.anguillarum and C.glutamicum were found to be the most sensitive micro-organisms. However, Hellio et al., 2002 studied antibacterial, antifungal and cytotoxic activities of extracts from fish epidermis and epidermal mucus and found antifungal and antibacterial activities in the fish mucus. This peptide may play a role in protection against intracellular or extracellular pathogens (Fernandes and Smith, 2002). It was demonstrated that there were significant histological and biochemical differences between the skin and mucus of rainbow trout, Coho and Atlantic salmon, which may change as a result of different environments. Variation in these innate immune factors is likely to have differing influence on each species response to disease processes (Fast et al, 2002)

Table 1: Antibacterial activity of fish mucus

<table>
<thead>
<tr>
<th>Strains</th>
<th>Zone of Clearance (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>12</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>8</td>
</tr>
<tr>
<td>Salmonella paratyphi</td>
<td>8</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>7</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>10</td>
</tr>
<tr>
<td>E. coli</td>
<td>10</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>9</td>
</tr>
<tr>
<td>Lactobacillus vulgaris</td>
<td>10</td>
</tr>
<tr>
<td>Vibrio cholera</td>
<td>8</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>7</td>
</tr>
</tbody>
</table>

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