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ORIGINAL ARTICLE

# EXTRACTION, BIOCHEMICAL CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF FISH MUCUS.

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**Abstract:**

*Antimicrobial proteins and peptides play key roles in innate immunity and they had been observed from a wide variety of organisms in the last few years. The present study was undertaken to characterize antimicrobial peptides from the epidermal mucus of fishes collected from the Latur fish market. Antimicrobial properties of the fishes (Channa gachua, Channa punctatus, Cyprinus carpio and Arius dussumieri ) were tested against pathogenic bacteria and pathogenic fungi. The results of the present investigation reported that the mucus of the fishes having remarkable antimicrobial activity. In the present study efforts have been made to find the antimicrobial effect of the mucus of fresh water and river fishes namely Channa punctatus , Channa gachua, Cyprinus carpio and Arius dussumieri. Fish mucus was tested against pathogenic bacteria such as Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus aureus, A.niger and C. albicans. The molecular masses of the peptides were 15 kDa and 200kDa as determined by SDS-polyacrylamide gel electrophoresis. This peptide had shown very effective activity against various human pathogens. The present findings suggest that fish mucus has very good antimicrobial activity against pathogenic microbes.*

**KEYWORDS -**

SDS PAGE, Antimicrobial activity, fish skin mucus, fishes.

## INTRODUCTION

Fish mucus is dynamic biological interface between fish and their aqueous environment composing of biochemically diverse secretion from epidermal goblet and epithelial clavate cells (Pickering, 1974; Powell et al., 1992; Ellis, 1999, Rathinam Vennila et al, 2011.). It is composed mainly of water and gel forming macromolecules including mucins and other glycoproteins (Negus, 1963; Shephard, 1994, Rathinam Vennila et al, 2011.)

Fish mucus contain biologically active substances such as lysozyme, lectins, flavoenzymes, immunoglobulins, C-reactive protein, apolipoprotein A-I and antimicrobial peptides which gives protection to fish from potential pathogens (Ellis A.E. 2001, Kitani Y et al., 2007). The role of fish mucus is very large like respiration, ionic and osmotic regulation, reproduction, excretion, disease resistance, communication, nest building and protection (Fletcher, 1976; Ingram,1980; Trust 1986; Austin and MacIntosh, 1988; Fouz et al., 1990; Lemaitre et al., 1996; Ebran et al., 2000; Kosuga et al., 2000). The proteins and peptides present in the mucus of fresh water fishes play key roles in innate immunity (Ramasamy Anbuchezhian et al., 2011). In the present study fish species like Channa gachua, Channa punctatus, Cyprinus carpio and Arius dussumieri was selected because of its high commercial value. Lysozyme a bioactive compound which attack peptidoglycans and hydrolyze the glycosidic bond present in bacterial cell wall shows antibacterial activity exhibited by mucus (Fleming, 1922). There are different

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molecular weight proteins present in the fish mucus which act as bioactive compound. Hence the present study on biochemical characterization of fish mucus will provide useful information for prospecting of novel bioactive compounds from the aquaculture industry.

## MATERIALS AND METHODS

### Fish collection and extraction of mucus

The fishes were collected from Latur fish market and extraction of mucus sampling was performed on-site by 'skin-scraping' from the bodies of test subjects. Minimum amount of mucus volume was taken from each fish and the ventral area of all test subjects was avoided to reduce possible contamination by urine (Chong et al., 2005). With suitable force, mucus sample were taken from the anterior section by moving from the head towards the anus using a blunt sterile spatula. Mucus sampling was performed immediately after the fish was caught and smeared onto sterile vials with a drop of sterile water. The mucus samples were kept frozen in ice at 0°C to avoid bacterial growth and proteins degradation.

### Purification of fish mucus

Prior to analysis, mucus samples were thawed and kept in ice. By pouring into a centrifuge tube, 0.5 ml of sample was extracted once with 2 ml of sodium chloride by centrifugation at 8000 rpm and 4°C for 30 minutes. The supernatant was transferred into a new tube. The process followed by 3 times extraction of the pellet each with 1 ml of 1M of sodium chloride centrifuged at 15000 rpm and 4°C for 30 minutes. Each time the supernatant was transferred to the new tube as the first supernatant and stored at –20°C until next analysis (Mozumder, 2005). The extract was dialyzed against 0.1 M sodium chloride and then used for further analysis.Antimicrobial activity test

Bacterial and fungal isolates used in this study were collected from Indrayani Lab, Latur. The isolates consisted of E.coli, Klebsiella pneumoniae,Pseudomonas aeruginosa, Proteus vulgaris, Staphylococcus aureus, Aspergillus niger, and Candida albicans culture on Nutrient agar and Potato Dextrose Agar. Antimicrobial Activity was performed by using disc diffusion method. The paper discs soaked in fish mucus samples as well as standard drug solution (Streptomycin and flucanazole 1 µg/ml) were placed aseptically on sterile Nutrient Agar and Potato Dextrose Agar plates which were then incubated at 37 °C for 24 h for bacteria and 28°C for 48 h for fungi and the zones of inhibition were subsequently measured in mm (Mukherjee et al., 1995).

### Thin Layer Chromatography

Thin-Layer chromatography profiling was done for the fish mucus samples extract in solvent system of Butanol, Acetic acid and Water (B: A: W) in proportions of 5:1:4 (Ramasamy Anbuchezhian et al., 2011).

### Biuret test

The test was performed by adding an equal volume of 1 % sodium hydroxide and then few drops of 1 % of copper(II) sulfate to the sample solution.

### Lowry assay

Protein content was measured by using Lowery assay. Absorbance of mucus samples was recorded at 750 nm. Bovine serum albumin was used as a standard (Lowry et al, 1951).

### SDS-PAGE

The protein separation were analysed by SDS- PAGE 12% Separating gel , topped by 4% stacking gel (Laemmli, 1970). Electrophoresis was performed under constant voltage at 120V for 45 min.Anthrone test

The qualitative test of carbohydrate in all fish mucus sample was successfully determined by Anthrone reaction. The color change was observed (Morris, 1978).

**Phenol-sulfuric acid reaction:**

1ml of 5% phenol solution was added into each test tubes containing diluted glucose solution as standard and mixed thoroughly. 5.0 mL of concentrated sulphuric acid was added rapidly and directly on sample. The samples were left at room temperature for 20 minutes. The samples were incubated at 25-30°C. The Optical density was determined at 490 nm (Dubois et al., 1956).

**Free fatty acid test**

The qualitative estimation of of Lipid in all fish mucus samples was successfully determined by free fatty acid test reaction. The amounts of sodium hydroxide drops were counted for qualitative estimation of Lipid

**Acid value of lipid test**

The samples were suspended in 25 ml of the organic solvent (1:1-95% (v/v) alcohol: ether neutralized in phenolphthalein). 3 to 4 drops of phenolphthalein solution was added and mixed thoroughly. 0.05 M potassium hydroxide was titrated until the faint pink color persists for 10 to 20 seconds. (Zaharah et. al., 2005). (Note: 1 ml of 0.05 M KOH contains 2.8 mg KOH).

**RESULTS**

**Antimicrobial activity test**

Mucus sample from Channa gachua, Channa punctatus, C. carpio and Arius dussumieri was tested for their antimicrobial property against Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus vulgaris, Candida albicans and Aspergillus niger. The antibacterial activity was evaluated by quantifying the clear zone of inhibition in the producer lawn around the filter paper disk (Messi et al., 2003). The zone of inhibition in the discs was measured by millimeter scale (Tortora, 2007). The results showed antibacterial activity against E. coli, S. aureus, P.aeruginosa and C.albicans and absence of activity against K. pneumonia, P.vulgaris and A. niger. But only C. carpio did not showed activity against P.aeruginosa and C.albicans as well as Arius dussumieri showed activity against A. niger . (Table no. 1)

**Table No. 1 : Antimicrobial susceptibility test**

| Type of microbial | Zone of inhibition (mm) *C. gachua* | | | Zone of inhibition (mm) *C. punctatus* | | | Zone of inhibition (mm) *C. carpio* | | | Zone of inhibition (mm) *A. dussumieri* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *E. coli* | - | 15 | 16 | 12 | 12 | 14 | 16 | 15 | 15 | 14 | 14 | 14 |
| *P. aeruginosa* | 12 | 12 | 12 | 9 | 6 | 6 | - | - | - | 10 | 11 | 10 |
| *S. aureus* | 5 | 6 | 6 | 7 | 6 | 10 | 8 | 6 | 6 | 4 | - | 3 |
| *P. vulgaricus* | - | - | - | - | - | - | - | - | - | - | - | - |
| *K. pneumoniae* | - | - | - | - | - | - | - | - | - | - | - | - |
| *Candida albicans* | - | 3 | 5 | - | 9 | 12 | - | - | - | 4 | 6 | 12 |
| *Aspergillus niger* | - | - | - | - | - | - | - | - | - | 10 | 10 | 16 |

**Thin Layer Chromatography**

The plates when developed in the solvent system showed pink spots, when the TLC plates where spread with ninhydrin indicating the presence of amino acids and peptides.

**Biuret test**

The presence of protein in four fish mucus samples was qualitatively determined by Biuret reaction. Change in color occurred in the fish mucus samples from blue to violet or purple which indicates that protein is present in the fish mucus sample. In a positive test, a copper (II) ion is reduced to copper (I), which forms a complex with the nitrogens and carbons of the peptide bonds in an alkaline solution (Smith, et al., 1985).

**Lowry assay**

From the standard curve, R2=0.959 and y = 2.550x, where y equals to absorbance while x equals to concentration of BSA solution.Protein concentration in mg/ml was calculated by substituting the y value (absorbance of mucus sample), the average x value (protein concentration of mucus samples) Channa gachua (Sample 1):3.4039, Channa punctatus (Sample 2):1.7882, Cyprinus carpio (Sample 3):2.3372 and Arius dussumieri(Sample 4):2.5882 mg/ml respectively as shown in Table No.2 .It is the method of choice for accurate protein determination for cell fractions, chromatography fractions and enzyme preparations (Lowry et al., 1951).

**Table No. 2: Protein OD (mg/ml) of fish mucus protein in Lowry assay**

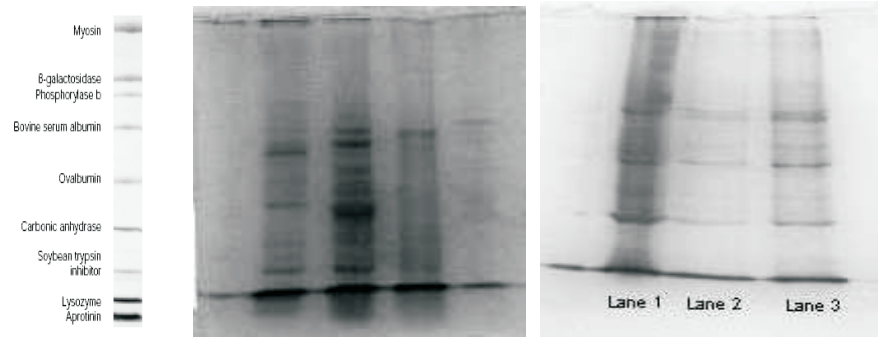
| Absorbance, OD750 | | | | Protein concentration, mg/ml | | | |
|---|---|---|---|---|---|---|---|
| *Channa gachua* | *Channa punctatus* | *Cyprinus carpio* | *Arius dussumieri* | *Channa gachua* | *Channa punctatus* | *Cyprinus carpio* | *Arius dussumieri* |
| 0.434 | 0.228 | 0.298 | 0.330 | 3.4039 | 1.7882 | 2.3372 | 2.5882 |

**SDS-PAGE**

Protein molecular weight standard curve was constructed by plotting Log 10 MW value against Rf value of each protein band in protein molecular weight standards. From protein molecular weight standard curve y = -1.542x + 5.412. Rf value (x value) for each band in the mucus was calculated. Then, Log 10 MW (y value) of each band was calculated based on the equation above. From Log 10 MW (y value); the molecular weight of each band in the mucus sample was determined in Table No.3 .

**Table 3:** Similarity in MW of marker and unknown mucus protein

| **Band of Protein** | **Marker MW (kDa)** | **Unknown protein MW (kDa)** |
|---|---|---|
| **Myosin** | 200.00 | (A)199.365 |
| **Ovalbumin** | 45.00 | (K)43.752 |
| **Carbonic anhydrase** | 31.00 | (N)33.950 |
| **Trypsin inhibitor** | 21.50 | (O)23.804 |
| **Lysozyme** | **14.40** | (R)15.079 |




Marker (0-50 KD), Lane (1) Crude fish mucus of Channa punctatus, Lane (2) Crude fish mucus of Cyprinus carpio, Lane (3) Crude fish mucus Arius dussumieri, Lane(4) Crude fish mucus of Channa gachua

The result showed that there are 17 bands (protein) in the mucus of Channa gachua, Channa punctatus, Cyprinus carpio, Arius dussumieri. The molecular weight for proteins was ranging from 15 kDa to 200 kDa. The gel showed five bands that probably matches protein molecular weight standard standard protein marker: (A) with myosin, (K) with ovalbumin, (N) with carsonic anhydrase, (O) with trypsin inhibitor and (R) with Lysozyme. The other 12 proteins detected were classified as unknown proteins.Myosin is a group of actin-binding proteins that produce movement typically toward the barbed end with respect to the actin filament. Myosins play an enormously diversity of motile functions in cells from muscle contraction to phagocytosis (Berg et al., 2001). The carbonic anhydrases form a family of enzymes that catalyze the rapid conversion of carbon dioxide to bicarbonate and protons .(Badger and Price, 1994). Trypsin inhibitors act as a defense mechanism to protect legume seeds against insect predation (Howard et al., 2006).

Lysozyme is having antibacterial property against pathogens.The enzyme functions by attacking peptidoglycans (found in the cells walls of bacteria, especially Gram-positive bacteria) and hydrolyzing the glycosidic bond that connects N-acetylmuramic acid with the fourth carbon atom of N acetylglucosamine. It does this by binding to the peptidoglycan molecule in the binding site within the prominent cleft between its two domains. This causes the substrate molecule to adopt a strained conformation similar to that of the transition state. The lysozyme then distorts the 4th sugar in hexasaccharide (the D ring) into a half-chair conformation. In this stressed state the glycosidic bond is easily broken. Therefore, Channa gachua, Channa punctatus, Cyprinus carpio, Arius dussumieri mucus protein(R) that highly resembles lysozyme in MW may be the compound that is responsible for the antibacterial activity exhibited by mucus.

**Anthrone test**

The presence of carbohydrate in all fish mucus sample was successfully determined by Anthrone reaction. Colour change occurred in the mucus samples from light yellow to blue-green complex. This indicates that carbohydrate is present in the fish mucus sample. It bases on the principle of which the concentrated sulphuric acid hydrolyses glycosidic bonds to give the monosaccharides which are then dehydrated to furfural and its derivatives. The furfural reacts with anthrone (10-keto-9, 10-dihydroanthracene) to give a blue green complex (Morse, 1947; Morris, 1948).

**Phenol-sulfuric acid reaction :**

Based on the standard glucose curve (Figure 4.10), $y = 3.98x + 0.981$. Carbohydrate concentration (x value) of four fish mucus sample was determined by substituting the absorbance (y value) of four fish mucus samples. The average carbohydrate concentration of fish mucus sample in Table No.5 Total neutral carbohydrate content is measured by the phenol–sulfuric acid method of using D-glucose as a standard (Dubois et al., 1956).

**Table No. 4 : Absorbance for phenol-sulfuric acid reaction**

| Absorbance, OD490 | | | | Carbohydrate concentration, mg/ml | | | |
|---|---|---|---|---|---|---|---|
| *Channa gachua* | *Channa punctatus* | *Cyprinus carpio* | *Arius dussumieri* | *Channa gachua* | *Channa punctatus* | *Cyprinus carpio* | *Arius dussumieri* |
| 0.253 | 0.164 | 0.350 | 0.212 | 1.271 | 0.8241 | 1.758 | 1.065 |

**Free fatty acid test**

The mucus from fish species (Channa gachua, Channa punctatus, Cyprinus carpio, Arius dussumieri) showed positive results by giving pink colour solution in respond to the addition of dilute alkaline (0.1% NaOH). This indicated that free fatty acids were present in the fish mucus samples. (McKee and McKee, 2003).

**Acid value of lipid test**

The KOH needed to fully react with the mixture of solution; (fish mucus samples, organic solvent and phenolphthalein) and faint pink color is developed. Acid value of mucus samples (Channa gachua, Channa punctatus, Cyprinus carpio, Arius dussumieri) are shown in Table No. 6. Note: 1 ml of 0.05 M KOH contains 2.8 mg KOH.

**Table No. 5: Result of acid value of lipid**

| Fish mucus samples | Amount of KOH (ml) | Acid value (mg/ml) |
|---|---|---|
| *Channa gachua* | 0.6 | 1.68 |
| *Channa punctatus* | 0.6 | 1.68 |
| *Cyprinus carpio* | 0.6 | 1.68 |
| *Arius dussumieri* | 0.5 | 1.40 |

**CONCLUSION:**

Analysis of protein, carbohydrate and lipid showed that proteins, lipids, free fatty acid and carbohydrates are present in Channa gachua, Channa punctatus Cyprinus carpio and Arius dussumieri fish mucus. There are 17 proteins found in the epidermal mucus of this fishes. Five of them are similarly sized to myosin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme. The other 12 proteins were classified as unidentified protein compounds. There is strong presence of lysozyme like protein, making it the primary candidate responsible for the antibacterial activity of the fish mucus. The epidermal mucus of Channa gachua, Channa punctatus, Cyprinus carpio and Arius dussumieri exhibited antimicrobial activities.

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