Original Article

Lysozyme, Protease, Alkaline phosphatase and Esterase activity of epidermal skin mucus of freshwater snake head fish *Channa striatus*

Loganathan K, Arulprakash A, Prakash M and Senthilraja P
Department of zoology, Annamalai University, Annamalai Nagar, Chidambaram- 608002, Tamilnadu, India.
E-mail: dnaprakash@yahoo.com

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Abstract
The potential of living organisms as a source of biologically active products is largely unexplored. Fish epidermal mucus provides the first line of defense against pathogenic microorganisms. The indiscriminate use of antibiotics highlight the urgent need for development of novel strategies to treat microbial infections. The aim of the present study was to estimate the activity of lysozyme, protease, alkaline phosphatase and esterase activity in the skin mucus of snakehead fish *Channa striatus*. All the activities were estimated by using the spectrophotometer set kits and turbidometric assay. The *Micrococcus lyzodeikticus* bacterium was used as a substrate for estimating the lysozyme activity. The results reveals that the protease activity is more in the fish mucus when compared to all other activity.

Key words: Skin mucus, lysozyme, protease, alkaline phosphatase and esterase activity.

Introduction
Virtually all fishes are covered with an integument mucus that is involved in many aspects of their biology, ranging from disease resistance to rearing of young ones to shelter and locomotion. The epithelial tissues produce antimicrobial molecules which serve as the first line of a host’s defense against microbial invasion in a variety of vertebrates including humans (26). Fish are more susceptible to bacterial infection than terrestrial animals, because the aquatic environment is a rich source of pathogenic bacteria (33). Bacterial infection often produces various fish diseases, and it is a global problem affecting both freshwater and marine fish. This problem is of major importance when fish live under stressful condition, such as poor water quality and intensive culture practices (14). Hag fish are, therefore, thought to display strong innate defense mechanisms for protection against pathogens. Various innate immune parameters including lysozyme, cathepsin B and other proteases have been observed in the epidermal mucus of hagfish (29).

Fish serum and skin mucus are known to contain a number of anti-pathogenic substances such as lysozyme, complement, alkaline phosphatase, C-reactive protein, lectins and other substances (35). Most mucus in animals is secreted by goblet cells, though other cells including those in the submucosal glands produce mucus (26,32). The main structural proteins of mucus are high molecular mass (~106 kDa) glycoproteins called mucins (30). Lysozyme is a representative endogenous antibacterial agent, which also called as muramidase and catalytically hydrolyzes the bond between N-acetylmuramic acid and N-acetylglucosamine in the cell wall of bacteria. Lysozyme was detected from the skin mucus of numerous fish such as channel cat fish *Ictalurus punctatus* (22), *Cyprinus carpio* (31), *Oncorhynchus mykiss* (18,28) and the ayu *Plecoglossus altivelis* (13). Alkaline phosphatase has been demonstrated as a potential stress indicator in the epidermal mucus of Atlantic salmon (25). The present study was to investigate the specific activities of lysozyme, protease, alkaline phosphatase and esterase in the skin mucus of snake head fish *Channa striatus*.

Materials and methods
Experimental fish
The healthy *Channa striatus* was collected from Sirkali fish market, Nagai, Tamilnadu, India of an average weight 300 ± 5.67g. The fish were kept in large aerate concrete tank containing potable tap water (pH 7.5 ± 0.5) facilitated with water and air pumps. The tank were treated with disinfectant sodium hypochloride, with the concentration of 200 ppm for 1 hrs and washed three times with fresh tap water prior to the introduction of the fish in the water (3). Mucus collection
The mucus was collected from the acclimatized healthy fish. The mucus was carefully scraped from the dorsal surface of the fish. Before collection of mucus any
anesthetic chemicals are not given. Mucus was not collected in ventral side to avoid anal and sperm contamination.

**Crude skin mucus extract**
The collected mucus was centrifuged at 10,000 rpm at 4°C for 15 minutes in a refrigerated centrifuge and supernatant was lyophilized and stored at 4°C until use.

**Peptide purification by precipitation**
The protein from the crude fish skin mucus sample was precipitated by ammonium sulphate (75%) and stored at 4°C overnight. The precipitate was collected by centrifuging at 15,000 rpm for 20 minutes at 4°C (REML) and the pellet was resuspended in an acetate buffer (50mM; pH 5.0), stored at 4°C.

**Total protein estimation**
The total protein estimation was determined by using the method of Lowry et al. (20) modified by Patterson, (23). Bovine serum albumin (BSA) solution was used as a standard.

**Lysozyme activity**
Lysozyme activity was used a turbidometric method of Shugar, (27). 50µL of the crude mucus sample and ammonium precipitate peptide separately mixed sample were diluted with 40mM sodium phosphate buffer (pH 6.5). Each sample were transferred into separate 96 well plate and incubated at 30°C for 15 minutes. Lyophilized Micrococcus lysodeiktus cells (50µL in 40mM sodium phosphate buffer, pH-6.5) were then added and the absorbance was measured continuously for 50 minutes at 30°C. The initial rate was used to calculate the activity. One unit of activity was defined as the amount enzyme that catalyzed a decrease in absorbance at 450 nm.

**Protease activity**
Protease activity in the skin mucus was quantified by azocasein hydrolysis assay as described by (25). Briefly, an equal volume of each sample (100µl) was incubated with 100mM ammonium bicarbonate (pH 7.8) buffer containing 0.7% azocasein for 19 hrs at 30°C on shaker, the reaction was stopped by adding trichloroacetic acid (4.6% final concentration) and cooled in ice. The reaction mixture was centrifuged at 13,000 rpm for 5 minutes and 100µl of supernatant was added to the microtiter plate well containing an equal volume of 0.5M NaOH (Sigma). Trypsin and assay buffer were used instead of samples as positive and negative controls, respectively. The protease activity was measured as the increase in the optical density values at 450nm on a microplate reader (Biorad, USA).

**Alkaline phosphatase activity**
Alkaline phosphatase assay was followed by method of (25). Alkaline phosphatase activity was measured by incubating an equal volume of skin mucus and ammonium precipitate peptide separately mixed with 4mM p-nitrophenyl phosphate (sigma, St, Louis, MO) in 100mM ammonium bicarbonate buffer containing 1mM magnesium chloride, pH-7.8 at 30°C as described by Ross et al., 2000. The increase in OD was measured continuously at 5 minutes regular intervals over 2-3 hrs at 405 nm on a microplate reader (Benchmark, Biorad, USA). The initial rate of the reaction was used to calculate the activity. One unit (U) of activity was defined as the amount of enzyme require to release 1 µmol of p-nitrophenol product in 1 minute. The extinction coefficient of p-nitrophenol in the microplate wells was experimentally determined.

**Esterase activity**
Esterase activity was determined continuously over 2-3 hrs at 405 nm using p-nitrophenyl myristate (MP, Biomedical, Inc, France.) as described in Ross et al., (25). An equal volume of skin mucus and ammonium precipitate peptide was separately incubated with 0.4 mM p-nitrophenyl myristate buffer containing 0.5% triton X-100, pH-7.8 at 30°C. This activity was determined as for alkaline phosphatase.

**Results:**

**Protein estimation**
The amount of protein present in the freshwater fish Channa striatus crude mucus was 246 µg/mg and Ammonium precipitate mucus extract was 190 µg/mg.

**Enzyme activity**
In the present study the Lysozyme activity of snake head fish crude skin mucus showed 65±1.40 Umg⁻¹ and its ammonium precipitate mucus extract was 49.50±1.58 Umg⁻¹. The protease activity in the crude mucus showed 96.28±1.59 Umg⁻¹ and ammonium precipitate extract showed 76.50±1.08 Umg⁻¹. Alkaline phosphatase activity in the crude mucus sample was 1.36±0.08 Umg⁻¹ and ammonium precipitate mucus extract showed 0.98±0.06 Umg⁻¹. The analyses of Esterase activity in crude mucus showed 0.94±0.04 Umg⁻¹ and ammonium precipitate mucus extract was 0.34±0.02 Umg⁻¹ (Table A).

**Table A:** Showed lysozyme activity of fish mucus

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Parameter</th>
<th>Enzyme activity fish mucus Umg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Crude skin mucus</td>
</tr>
<tr>
<td>1.</td>
<td>Lysozyme</td>
<td>65±1.40</td>
</tr>
<tr>
<td>2.</td>
<td>Protease</td>
<td>96.28±1.59</td>
</tr>
<tr>
<td>3.</td>
<td>Alkaline phosphatase</td>
<td>1.36±0.08</td>
</tr>
<tr>
<td>4.</td>
<td>Esterase</td>
<td>0.94±0.04</td>
</tr>
</tbody>
</table>

* Data are represented as mean±SD.

**Discussion**
The skin of fish plays a passive role in protective immunity, serving as an anatomical and physiological barrier against the external environment. Skin mucus, secreted by mucous cells localized in the epidermis is considered the first line of defense, as observed in other fish species (37). The mucus such as that produced by the skin of the stingrays, may include amino acids, peptides, complex carbohydrates, glycopeptides, glycolipids and other chemicals (17). Trypsin like protease individually or in cooperation with other mucosal immune substances may play a important role protecting the invading agent. Proteases could act directly on the pathogen or prevent invasion indirectly by modifying mucus consistency that...
results in increased sloughing of mucus and pathogen removal from the body surfaces. The ability of bacteria to adhere to host surfaces considered necessary for colonization, and there by eliciting of the disease. As bacterial colonization is required for pathogenicity, genes involved in bacteria colonization have been regarded as virulence genes. External and internal epithelial surfaces of fish are covered with a mucus layer providing protection against environmental factors like microorganisms, toxins, pollutants, acidic pH and hydrolytic enzymes. Secretory mucins are the major constituents of the mucus layer in which several biochemical compounds have been identified; like lysozyme (11), antimicrobial peptides (5) and antibodies (11).

The secretion and immunological role of several enzymes other than proteases, lysozyme, alkaline phosphatase and to some extent acid phosphatase in the skin mucus of fish (36). The mucus is known to comprise a number of immune components such as lysozyme, immunoglobulin, complement, carboxic anhydrase, lectins, crinotozins, calmodulin, c-reactive protein, proteolytic enzymes and antimicrobial peptides (1,4). Alkaline phosphatase, esterase and several enzymes secreted by *Olive flounder* skin mucus may act individually or in cooperation with other immune substances in the mucus in defending against pathogens or in healing wounds on the body surface (25).

Lysozyme, an enzyme that cleaves the glycosidic bonds of the peptidoglycan layer of bacteria, was present in the mucus of fish, lysozyme has been primarily studied from the serum, lymph, kidney, spleen, stomach, gills, gastrointestinal tract and other organs or tissues in various fish species (11,9). Lysozyme and anti fungal peptides are more important components of the innate immunity in animals (6,7,16). In sea lamprey (*Petromyzon marinus*) plasma; the lysozyme and antifungal peptide as very important in resistance to bacterial and fungal infections. Fungal infections must be important in lamprey health and innate immunity (24).

The blood or mucus of fish has bacteriostatic or bactericidal properties *in vitro* but this has generally been assumed to be due to the presence of lysozyme, agglutinins, thermolabile complement factors or immunoglobulin (34). Lysozyme has been found in fish mucus, serum, and ova (8). Fish lysozyme occurs in the two forms and one of this appears to be much more bactericidal than lysozyme of higher vertebrates. Alkaline phosphatase are widely distributed in nature and are characterised by a high pH optima and a broad substrate specificity (10). Alkaline phosphatase is a lysosomal enzyme suggested to have a protective role in fish during the first stages of wound healing (12).

Atlantic salmon (*Salmo salar*) during smoltification, changes in the levels of tissue and blood alkaline phosphatase isoenzymes were associated with smolting and gonadal development (15). In comparative study on the innate immune parameters in the epidermal mucus of various fish species (sea water and freshwater species), observed that *Cyprinus carpio* mucus had the highest alkaline phosphatase specificity activity among the freshwater species. The protective role of the skin mucus of *Clarias batrachus* was previously investigated by Loganathan et al. (19), study the mucus has an strong an anti-bacterial and anti-fungal activity against different pathogenic microorganisms. In present study the presence of lysozyme, protease, alkaline phosphatase and esterase of hydrolytic enzymes in the skin mucus of fish may have a direct effect on the innate immune response against pathogenic microorganisms.

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**References**


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