The Composition of Mucus in Freshwater Fishes: A Comparative Study

H.D. Kaher

Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar.

ABSTRACT:

Fish epidermal mucus provides the first line of defense against pathogens. The present work reports on the comparative study of variation in mucous composition and defense markers among some of the fresh water fishes mrigal (*Cirrhinus mrigala*), grass carp (*Ctenopharyngodon idella*), bighead carp (*Aristicthys nobilis*) snake head (*Channa punctatus*), walking catfish (*Clarius batrachus*) and tilapia (*Oreochromis niloticus*) with respect to their role in innate immune system. The defense markers analyzed include activities of protease, lysozyme, alkaline phosphatase and acid phosphatase in epidermal mucous. Among the samples, protease (1.228±0.01 U/mg) and acid phosphatase (1.06±0.06 U/mg) activities were quite high in grass carp; the total protein (0.313±0.0543 mg/gm), carbohydrate (0.127±0.0016 mg/gm), triglyceride (1.3075±0.0185), alkaline phosphatase activity (0.486±0.003 U/mg) and shear viscosity (100.84 mPa.s) were found to be highest in snake head; and lysozyme activity (5.68±0.09 U/mg) was found to be higher in tilapia in comparison to other fishes. The mucus samples have not demonstrated antimicrobial activity against selected human pathogenic bacteria and further analysis is required. The protein analysis of mucous samples by SDS-PAGE have shown expression of low molecular weight proteins mainly 93 kDa, 65 kDa, 41 kDa, 27 kDa and 21 kDa in snake head; 64 kDa, 36 kDa and 12 kDa in walking cat fish; and 69 kDa, 63 kDa, 33 kDa, 22 kDa and 14 kDa in tilapia.

Keywords: Mucus, Defense, Enzymes, Skin, Snake Head Fish.

1. INTRODUCTION

Body surfaces of multicellular organisms are defended by epithelia, which provide a physical barrier between the internal milieu and the external world. Skin is the structure that covers the body and protects it not only from the entry of pathogens or allergens, but also from the leakage of water, solutes, or nutrients. These outside-in and inside-out barrier functions are dependent on the epidermis. While mucus covers the epidermis. Skin is unique and histologically diverse. It is very different from that of mammals, because it secretes mucus which is involved in immune functions. The aquatic environment is rich in pathogenic organisms, hence, the skin of aquatic vertebrates is extremely important as the first line of defence against the invasion of environmental pathogens (Estaban 2012). The innate defense mechanisms present at mucosal surfaces, the mucus is one of the most important ones (Gomezet al., 2013). Mucus is composed of water and glycoproteins,which have wide range of function including disease resistance and protection, respiration, ionic, osmotic regulation, reproduction, excreation, communication, feeding, and nest building (Subramanian et al., 2007). The fish skin mucus acts as a natural, physical, biochemical, dynamic, and semipermeable barrier that enables the exchange of nutrients, water, gases, odorants, hormones, and gametes. Mucus is continuously secreted and replaced, which prevents the stable colonization of potential infectious microorganisms as well as invasion of metazoan parasites. Mucins are strongly adhesive, play a major role in the defence of the mucosae, form a matrix in which a diverse range of antimicrobial molecules can be found and impart viscoelastic and rheological properties to mucosal layers (Estaban 2012). A complex mixture of other proteins, ions and lipids are also found in mucus, creating an ideal niche for microbial adherence and growth. Mucus composition determines its adhesiveness, viscoelasticity, transport and protective capacity (Gomezet al., 2013). Fish mucosal secretions carry a wide variety of innate immune molecules including complement proteins, lysozyme, proteases, esterases, AMPs, immunoglobulins, lectins, agglutinin, calmodulin, interferon, C-reactive protein, proteolytic enzymes and vitellogenin (Gomezet et al., 2013) and (Guardiola et al., 2014). Lysozyme kills bacteria by catalyzing the hydrolysis of β-(1→4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine in the bacterial cell wall (Nagashima et al., 2009). Acid and alkaline phosphatases, which are important lysosomal enzymes and are associated with the innate immune system in fishes, have also been identified in fish skin mucus (Estaban 2012). Alkaline phosphatase is thought to act as an antimicrobial agent through its hydrolytic activity (Nagashima et al., 2009). The release of proteases into skin may act directly on a pathogen (they can kill bacteria by cleaving their proteins) or may prevent pathogen invasion indirectly by modifying mucus consistency to increase the sloughing of mucus and thereby the removal of pathogens from the body surfaces..

2. MATERIAL AND METHODS

Fish collection:

Hypophthalmicthys nobilis, cirrhina mrigala, ctenopharyngodon idella and tilapia were obtaind from juhu-krupa farm, pij village, taluka nadiad (dist. Kheda). From there we are transport the fish in polythene bag with airated water and immediately collected the mucus.

Mucus collection:

Mucus collection was carried out by using the method of Ross et al., (2000).the fishes were transfered in to polythene bags conaining 50mM Nacl solution at the rate 10ml for 100g fish. The bags were shaken by hand for 5 minutes to collect mucus and following this treatment fish were retuurned to recover in the beaker. The mucus were immediately transffered into 15ml sterile centrifuge tubes and placed on dry ice.the sample was centrifuged at 1500 rpm for 10 min at 4C and the supernatant obtained was alliqouted into 2ml centrifuge tubes, freeze dried and stored at -20c for further analysis.

Composition of mucus

The total protein concentration from the mucus samples was estimated by Lowery et al., (1951) method. The carbohydrate content of the mucus sample was analysed by phenol-sulphuric acid method. The presence of triglyceride was estimated by GPO method by Triglyceride Kit. From the standard curve of the the activity tyrosine of protease samples can be determined in terms of units, which is the amount in micromoles of tyrosine equvivalents released from casein per minute. Lysozyme activity was measured by turbidometric assay method. Alkaline phosphatase is estimated by king's method by ALP kit. Acid phosphatase is determined by king's method by ACP kit. The mucus protein expression determine by SDS-PAGE analysis.

3. RESULTS AND DISCUSSION

In the present experiment, the total protien content in epidermal mucus has been analyzed in all six selected species. The highest protien content of epidermal mucus has been detected in *C. Punctatus mucus sample; and in C.batrachus, Tilapia, C.mrigala, A.nobilis and C.idella*, the protien content is found comparatively lower showed in table no.1. The result indicate that a clear variation in protein content of selected fishes have been observed. This suggest that diffrent fishes express diffrent concentration of proteins in diffrent environment and it is also closely associated with their function. The highest protein concentration in the epidermal mucus sample of C. Punctata indicate its role in protection as mucus in reported to be rich in protein like Ig, C-reactive Protein, Lectins, Lysozyme, Complenment Proteins, Proteolytic enzymes and Various other antibacterial Protein and Peptides.

The highest concentration of Carbohydrate content has been detected in the mucus of *Channa Punctatus; and in C.batrachus, Tilapia, C.idella, C.mrigala and A..nobilis* the carbohydrate content is found comparatively lower showed in table no.1. The carbohydrate content of mucus appears to be associated with maintaining the fluidity of mucus. The mucopolysaccarides are negetively charged molecules and are also associated with protection. The high carbohydrate content of mucus collected from *Channa Punctatsus* uggested its strong protective role in this fishes.

The highest concentration of lipid content has been detected in the mucus of *Channa Punctatus; and in A.nobilis, C.batrachus, Tilapia, C.idella and C.mrigala* the carbohydrate content is found comparatively lower showed in table no.1. Lipids are found in mucus, creating an ideal niche for microbial adherence and growth.The presence of lipid (Triglyceride) determines its adhesiveness, viscoelasticity, transport and protective capacity (Gomez ei al., 2013). Lipids in mucus secretions, including covalently attached fatty acids, contribute to fiber-fiber interactions that increase the viscoelasticity of the gel (Estaban 2012).

The mucus content of fishes is reported to be rich in innate immune factors like Ig, Creactive Protein, Lectins, Lysozyme, Complenment Proteins, Proteolytic enzymes and Various other antibacterial Protein and Peptides (Shepherd, 1994; cole et al., 1997). Protease are known to be involved in the innate immune mechnism. This protease are also to be associated with modulation of Ig and antimicrobial peptide. In the present study the level of protease activity has been compared in the fish skin mucus of selected freshwater fishes. The highest Protease activity found in *Ctenopharyngodon idella*; and in A.*nobilis*, *C.batrachus*, *Tilapia*, *C.Punctata and C.mrigala* the Protease activity is found comparatively lower showed in table no.2. The highest protease activity indicates strong innate immune mechanism in *Ctenopharyngodon idella*. 36 Another important enzyme is lysozyme, a glycanohydrolase is bacteriolytic enzyme; and has been detected in the various organism inciuding fishes.

The lysozyme activity has been reported in mucus of many fish species like Arctic char, brook trout, koi carp, haddock, cod, hagfish (subramanian et al., 2007).In the present study the level of lysozyme activity has been compared in the fish skin mucus of selected freshwater fishes. The highest lysozyme activity found in *Tilapia; and in C.Punctatus, C.batrachus, A.nobilis, C.idella and C.mrigala* the lysozyme activity is found comparatively lower showed in table no.2. Lysozyme activity is known to change according to state of health, stess, sex, season, temperature, and degree of sexual maturity (Fletcher and White).

The present experiment, Alkaline phosphatase activity in epidermal mucus has been analyzed in all six selected species.the highest Alkaline phosphatase activity of epidermal mucus has been detected in *C. Punctatus* mucus sample; and in *C.batrachus,Tilapia,C.mrigala, A.nobilis and C.idella* showed in table no.2. Alkaline phosphatase activity is found comparatively lower. Alkaline phosphatase is considered as a potential stress indicator. Very high ALP activity in the mucus sample in diffrent species including koi carp has been reported by subramanian et al., 2007.

In the present experiment, Acid phosphatase activity in epidermal mucus has been analyzed in all six selected species. The highest Acid phosphatase activity of epidermal mucus has been detected in *C.idella* mucus sample; and in *C.mrigala, A.nobilis ,C.Punctatus,Tilapia* and *C.batrachus,* Acid phosphatase activity is found comparatively lower showed in table no.2.. Acid phosphatase are known to be involved in the innate immune mechnism.

The high level of sher viscosity has been detected in the mucus of *Channa Punctatus*; and in *C.batrachus, A.nobilis, Tilapia, C.mrigala* and *C.idella* the sher viscosity levelis found comparatively lower showed in table no.3.. Many other factors contribute to regulation of mucus viscoelasticity, including secreted lipids, trefoil factor, pH, calcium, and nonmucin glycoproteins. Toxic and irritating substances can greatly stimulate mucus secretion, increasing the thickness of the mucus blanket (Estaban 2012). the high level of the sher viscosity in *C. Punctatus* indicate highly protection again pathogen.

The SDS PAGE gel electrophoresis of mucus sample collected from diffrent fishes indicates change in expression pattern of Proteins. In *Channa Punctatus*, mucus sample 5 bands with approx. Mol. Wt 93.0 kDa, 65.0 kDa, 41.0 kDa, 27.0 kDa, 21 kDa were observed. New expression of proteins of Mol. Wt 21 kDa were identified. In *Clarius batrachus*, mucus sample 3 bands with approx. Mol. Wt 64.0 kDa, 36.0 kDa, 12.5 kDa were observed. New expression of proteins of Mol. Wt 65.0 kDa, 36.0 kDa, were identified. In *Tilapia mossambica*, mucus sample 5 bands with approx. Mol.Wt 69.0 kDa, 66.0 kDa, 33kDa, 22kDa, 14.2 kDa were observed. New expression of proteins of Mol. Wt 66.0 kDa was identified. In *Cirrhina mrigala, Ctenopharyngodon idella and Aristhichthys nobilis* mucus sample no band were observed. Over expression as well as expression of new protiens in mucus sample of *C. Punctatus, C.batrachus and tilapia mossembica* appear to be associated with the strong protective mechanisms in fishes showed in diagram no.1.

Table and graph No.1 Total Protein, Total carbohydrate, Triglyceride concentration in mucus samples of Six different freshwater fish

Parameter / Fish	Total Protein	Total carbohydrate	Triglyceride
Snake Head	0.313	0.127	1.3075
Cat fish	0.206	0.1	0.99
Mrigal	0.146	0.043	0.677
Grass Carp	0.0793	0.0448	0.71
Bighead carp	0.093	0.037	1.2805
Tilapia	0.194	0.0937	0.963

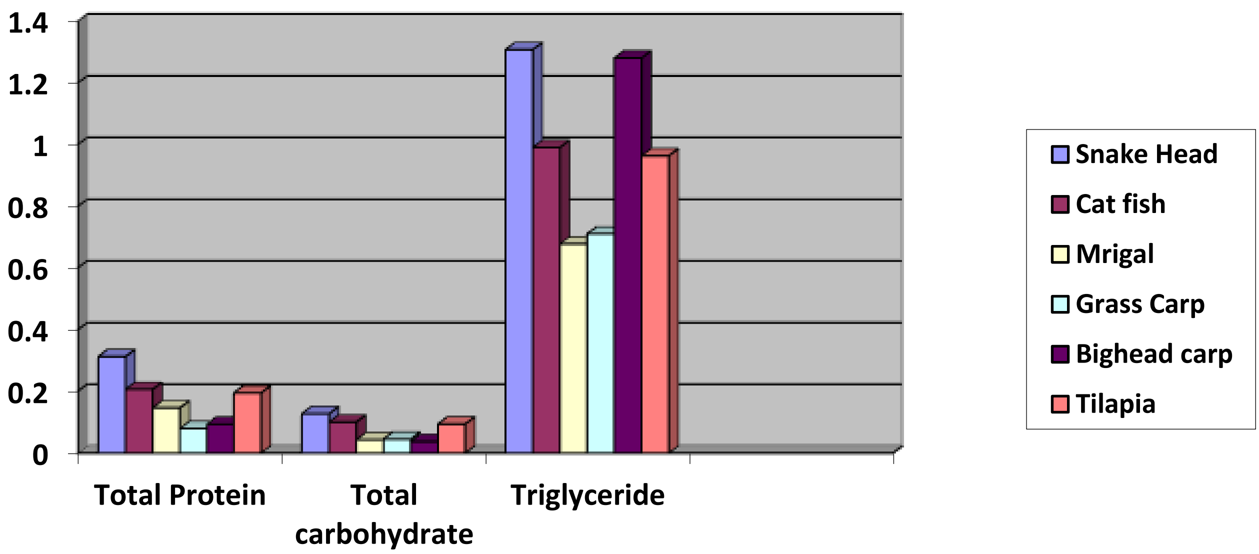


Table and graph no.2. Protease, Lysozyme, Alkaline phosphatase, Acid phosphatase concentration in mucus samples of Six different freshwater fish.

Parameter / Fish	Protease	Lysozyme	Alkaline Phosphatase	Acid Phosphatase
Snake head	**0.653**	**4.84**	**0.486**	**0.411**
Cat Fish	**0.954**	**3.65**	**0.352**	**0.302**
Mrigal	**0.45**	**2.27**	**0.268**	**0.791**
Grass Carp	**1.228**	**2.685**	**0.198**	**1.06**
Bighead Carp	**1.029**	**3.25**	**0.221**	**0.54**
Tilapia	**0.945**	**5.68**	**0.318**	**0.35**

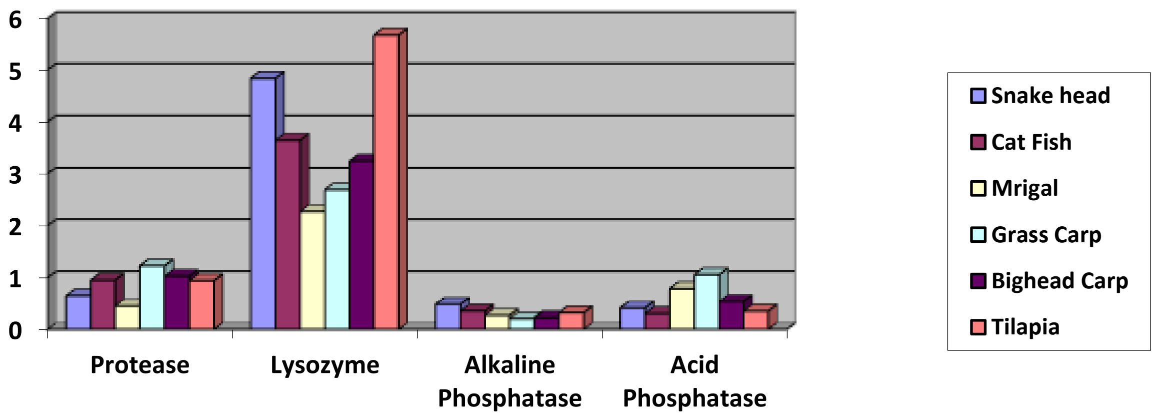
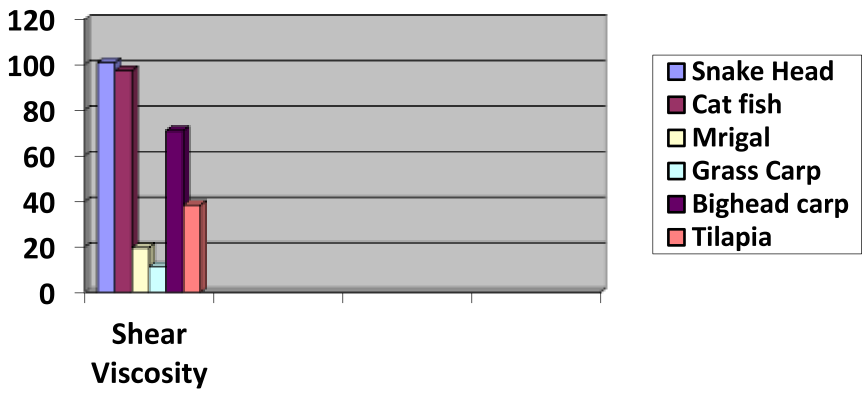
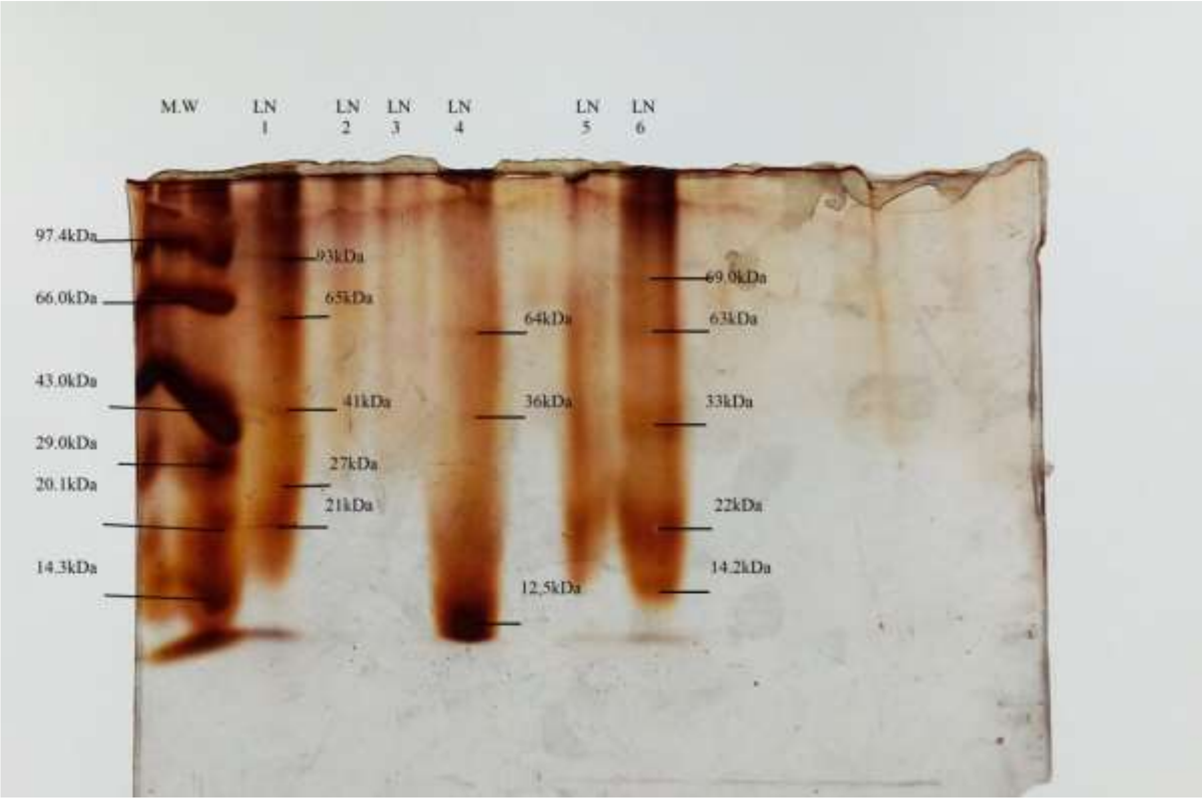


Table and graph no.3 Shear Viscosity in mucus samples of Six different freshwater fish



Parameter Fish	Shear Viscosity
Snake Head	100.84
Cat fish	97.42
Mrigal	19.7
Grass Carp	11.36
Bighead carp	71.09
Tilapia	38.53

Plate No.1 SDS PAGE gel showing the protiens bands in the mucus samples of fishes
M.W- Molecular weight Marker
LN 1- *Channa Punctatus* LN 2- *Cirrhina mrigala* LN 3- *Ctenopharyngodon idella*
LN 4- *Clarius batrachus* LN 5- *Aristhichthys nobilis* LN 6- *Oreochromis niloticus*



4. CONCLUSION

The major findings are as follows: in the present experiment protein, carbohydrate and triglyceride were determined in epidermal mucus and variation observed in diffrent species and highest level of all of these parameter obtained in *channa punctatus.* In the present study lysozyme and protease activites were determined in the epidermal mucus of the fish species and the variation observed in the enzyme activites can be correlated to their hbitat, so defense mechanism played by this can be observed in the fishes. The ALP and ACP activities observed in the mucus of the fishes shows the role in innate immunity of the fishes and their function in the defence mechanism. The viscosity was determined in the mucus sample of the fishes shows role in defence mechanism and prevent pathogen adhesion to the skinand highest level of viscosity obtained in *channa punctatus* . The mucous protein analysis by SDS-PAGE indicated several newly expressed proteins have been observed in the mucus samples of *Channa Punctata*, Clarius batrachus and Tilapia mossambica these proteins play a significant role in the defense mechanism of these fishes. Thus, present work suggest that the primary defense system in terms of chemical and physical properties of mucus is rhobust in *channa punctatus* quite weaker in *Ctenopharyngodon idella.*

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REFERENCES

1. Esteban, M. Á., & Cerezuela, R. (2015). *Fish mucosal immunity: skin*. *Mucosal Health in Aquaculture*.ElsevierInc.
2. Gobinath, C., & Ravichandran, S. (2011). Antimicrobial Peptide from the Epidermal Mucus of Some Estuarine Cat Fishes Faculty of Marine Sciences, CAS in Marine Biology,*12*(3),256–260.
3. Gomez, D., Sunyer, J. O., & Salinas, I. (2013). Fish & Shell fish Immunology The mucosal immune

system of fish :The evolution of tolerating commensals while fi ghting pathogens. Guardiola, F. A., Haro, J. P. De, Díaz-baños, F. G., Cuesta, A., & Esteban, M. Á. (2015). Terminal carbohydrate composition, IgM level and enzymatic and bacteriostatic activity of European sea bass (Dicentrarchus labrax) skin epidermis extracts. *Fish and Shellfish Immunology*.

4. Haniffa, M. A., Viswanathan, S., Jancy, D., Poomari, K., & Manikandan, S. (2014). Antibacterial studies of fish mucus from two marketed air-breathing fishes – Channa striatus and Heteropneustes fossilis, *5*(2), 22–27.
5. Nagashima, Y., Tsukamoto, C., Kitani, Y., Ishizaki, S., Nagai, H., & Yanagimoto, T. (2009). Comparative Biochemistry and Physiology , Part B Isolation and cDNA cloning of an antibacterial L -amino acid oxidase from the skin mucus of the great sculpin Myoxocephalus 53 polyacanthocephalus. *Comparative Biochemistry and Physiology*, *Part B*, *154*(1), 55–61.
6. Subramanian, S., Mackinnon, S. L., & Ross, N. W. (2007). A comparative study on innate immune parameters in the epidermal mucus of various fish species, *148*, 256–263.