Comparative biochemical analysis of skin mucous secretions from certain freshwater teleosts

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Abstract: The present study revealed significant differences in the biochemical composition of the skin mucus of Cirrhinus mrigala, Labeo rohita, Catla catla, Rita rita and Channa punctata, inhabiting different ecological niches. Protein contents in aqueous phases of the mucus were higher in L. rohita (54.77%) and R. rita (50.21%), than in C. catla (39.33%), C. punctata (20.64%) and C. mrigala (19.99%). Proteins in detergent soluble phases of skin mucus of these fish species, in general, were less than 3%. Mucus also contained small amounts of carbohydrates (1.28 - 4.53%) and lipids (2.51 - 8.60%). Electrophoretic profiling of aqueous phase proteins revealed a series of high molecular weight protein bands ranging from 50 kDa to 205 kDa in C. mrigala, L. rohita and C. catla. Such bands were relatively less in C. punctata. Protein bands in R. rita were mainly between 17 kDa and 50 kDa. Nature of mucus proteins on the basis of their molecular weight have been correlated with their biological significance in relation to ecological niches inhabited by the fishes.

Key words: Skin mucus, Protein

Introduction

Fish are the group of the animals that have evolved to thrive in an aqueous environment often rich with pathogenic agents. In general, fish body remains covered by mucus layer, which is secreted by different types of cellular constituents in the epidermis – the mucus cells, the club cells, the sacciform cells and the epithelial cells. Mucus is gel like slippery, gluey, viscous heterogeneous mixture of a variety of macromolecules – proteins, glycoproteins (mucopolysaccharides), lipids, enzymes, ions and water (Ali-Hassan et al., 1982; Ingram, 1980; Mittal and Nigam, 1986; Sumi et al., 2004; Tkachenko et al., 2006; Nigam et al., 2012a,b). Mucus remains in intimate contact with surrounding aquatic environment and acts as a barrier against mechanical, physical, and chemical stressors, and biological attacks. It is considered to play different biological functions eg., reducing friction during swimming (Uskova et al., 1970; Rosen and Cornford, 1971); osmoregulation (Handy et al., 1989); protection from abrasion during burrowing (Mittal et al., 2002) and diseases (Ellis, 1981, 1999); acceleration of wound healing (Al-Hassan et al., 1989); intra-species communication; and in some fishes, cocoon formation and feeding (Mittal and Banerjee, 1980; Shepherd, 1994). A review of literature reveals that the biochemical composition of fish skin mucus has not been adequately explored. Uskova (1970, 1975) analysed the chemical nature of protein components in the skin mucus of several marine fish species. Ali-Hassan et al. (1982, 1987) and Ali et al. (1989) reported proteins, lipids, carbohydrates and activities of several enzymes in the skin mucus of Arius bilineatus, A. tenuispinis and A. thalaasrius. Venkiah and Lakshmipathi (2000) made a comparative analysis of proteins, carbohydrates, and lipids in the skin mucus secretions of two freshwater catfishes - Clarias batrachus and Heteropneustes fossilis. Chong et al. (2005) compared protein contents in the skin mucus of adult and juvenile, Symphysodon spp. Manivasagan et al. (2009) reported proteins, lipids and carbohydrates in the skin mucus of Arius maculatus. Proteins in the skin mucus, while analysing antibacterial activity, have also been profiled in Cyprinus carpio (Lemaitre et al., 1996); and in Anguilla anguilla, Cyprinus carpio, Tinca tinca, Onchorhyncus mykiss and Scophthalmus maximus (Ebran et al., 1999).

Materials and Methods

Fish collection and mucus sampling: Live specimens of Cirrhinus mrigala (Mean±SD, standard length, Ls 160±5 mm; n=25), Labeo rohita (Mean±SD, standard length, Ls 154±5 mm; n=25), Catla catla (Mean±SD, standard length, Ls 160±8 mm; n=25) and Channa punctata (Mean±SD, standard length, Ls 145 ± 8 mm; n=25) were retrieved from Integrated Taxonomic Information System (http://www.itis.gov). These freshwater fish species inhabit different ecological niches and are considered valuable source of food from both consumers and human beings. This study may provide better understanding of the distribution of proteins with respect to their molecular weight in fish skin mucus. Increased knowledge of these biochemical parameters could be of great importance in understanding the role of mucus in different fish species in relation to their diverse habit and habitat. C. mrigala is bottom feeder and is exposed to heavy accumulation of detritus at the muddy bottom of water bodies. L. rohita is column feeder and C. catla is surface feeder. Both these species live in relatively clean water. C. punctata is an active predator, and inhabits muddy bottom of rivers and ponds. The fish is able to survive droughts and in semi-fluid mud. R. rita, widely distributed in large rivers of Indian subcontinent, is a sluggish, bottom-dwelling carnivorous fish. It inhabits the regions of the river with accumulations of dirty water, often with sewage effluents (Hora and Pillay, 1982).
collected from the fish farm at Banaras Hindu University and from the local ponds at Varanasi. Rita rita (Mean ± SD, standard length, Ls 145 ± 6 mm; n = 25) was collected from the river Ganges, at Varanasi, Uttar Pradesh. Fish were cold anaesthetised following the method given by Mittal and Whitear (1978). At a time, skin mucus was collected from five specimens of each fish species, by gently wiping the dorso-lateral surface of fish with the help of blunt edge of a plastic scraper. During mucus collection, skin surface was kept moist using fish saline (0.67% NaCl) and care was taken to avoid contamination from urino-genital and intestinal excreta. Mucus samples thus obtained were pooled separately for each fish species. Each mucus sample was lyophilized and stored separately at –20°C. The process of collection was repeated to get five different samples of mucus from each fish species.

**Quantitative analysis of proteins, carbohydrates and lipids:**
Quantitative estimations of proteins and carbohydrates were carried out by reconstituting known amount of the lyophilized mucus in phosphate buffered saline (PBS, pH 7.4), and homogenized for 10 minutes at 4°C. The homogenate was centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was collected and the pellet was re-suspended in PBS buffer, homogenized and centrifuged. The pellet was re-extracted twice using the same process. The supernatants were pooled and designated aqueous phase of the mucus. The pellet thus obtained was then suspended in Triton X-100 (0.3%, v/v), a non-ionic non-denaturing detergent, homogenized for 10 minutes at 4°C and then centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was collected and the pellet was re-extracted again using the same process. The supernatants were pooled and designated detergent soluble phase of the mucus. Both the aqueous and the detergent soluble phases of the mucus were used for the estimation of protein by Folin-Ciocalteu method (Lowry et al., 1951) and carbohydrates (neutral sugars) by Phenol-Sulphuric acid method (Dubois et al., 1956).

Quantitative estimation of lipids in fish skin mucus was carried out by the method of Bligh and Dyer (1959), modified by Kates (1972). Known amount of the lyophilized skin mucus was reconstituted and homogenized at 4°C in a mixture of chloroform/methanol/water in the ratio of 1:2:0.8, resulting in a mono-phasic mixture. The homogenate was centrifuged at 5,000 x g for 10 minutes at 4°C, supernatant was collected and the pellet was re-extracted in the above solvent system twice. All the supernatants were pooled and diluted with equal volumes of a mixture of chloroform and water (1:1) to make chloroform/methanol/water in the ratio of 2.2:1.8 forming a biphasic mixture in a separatory funnel. The lower chloroform phase was withdrawn and solvent was evaporated using lyophilizer (Christ Beta -A, Germany). After removal of solvent, the obtained lipid was weighed and total lipid was determined in the terms of µg/mg of lyophilized mucus.

**Qualitative analysis of proteins:** Qualitative analysis of proteins in the aqueous and detergent soluble phases of the skin mucus was carried out by native polyacrylamide gel electrophoresis (Native PAGE) and SDS-PAGE (Laemmli, 1970) on a discontinuous gel system consisting of the upper 5% stacking gel (pH 6.8) and the lower 10% resolving gel (pH 8.8); and Urea SDS-PAGE (Schägger et al., 2006) using the same gel system having the lower 12% resolving gel in the presence of urea at 6M concentration. For native-PAGE, mucus samples (containing 30 µg proteins) were mixed with the loading buffer, without the ionic detergent, sodium dodecyl sulphate (SDS) and the reducing agent, 2-mercaptoethanol to keep the mucus protein in their native state. For SDS-PAGE and Urea-SDS-PAGE, aqueous phase of the mucus was mixed with protein loading buffer (with SDS and 2-mercaptoethanol) and boiled for 2 minutes. Subsequently, mucus samples and marker proteins of high molecular weight range - 205 kDa myosin, 97.4 kDa phosphorylase b, 66 kDa bovine serum albumin, 43 kDa ovalbumin and 29 kDa carbonic anhydrase (PMW-H-105977, GeNei® , Bangalore, India) for 10% SDS-PAGE; and medium range marker - 118 kDa â-galactosidase, 90 kDa bovine serum albumin, 50 kDa ovalbumin, 34 kDa carbonic anhydrase, 26 kDa â-lactoglobulin and 19 kDa lysozyme (pre-coloured, SM0441, Fermentas, India) for 12% urea SDS-PAGE; were run in separate lanes on the same gel and electrophoresis was carried out at 100 Volts. After the electrophoresis, gels were stained with solution of 0.1% (w/v) Coomassie brilliant blue R-250 (CBBR-250) in methanol/acetic acid/distilled water (5:1:4). The stained gels were then treated with the mixture of methanol/acetic acid/distilled water (1:1:8) to destain the background staining to clearly visualize protein bands.

**Statistical analysis:** The results were expressed throughout as mean ± S.D. Each set of experiment was repeated five times to determine the reproducibility. Statistical comparisons were made between data obtained from each fish species using one-way ANOVA followed by Tukey’s post hoc multiple range test. Level of significance was tested at the level of p < 0.05. Level of significance was tested at the level of p < 0.05 was accepted as levels of statistical significance.

**Results**
Skin mucous secretions from fish species investigated showed significant differences in their appearance. Mucus is watery, thin fluid like in Channa punctata, relatively dense in Cirrhinus mrigala, Labeo rohita and Catla catla, and is highly dense sticky gel like in Rita rita. In general, the mucus samples collected from the skin surface are in the form of sticky strands and have characteristic visco-elastic properties. The mucus samples, when allowed to stand, get separated into two
layers, a lower layer consisting of a compact mass, settled at the bottom and an upper fluid like moiety.

Quantitative analysis: The quantitative analysis of skin mucus secretions showed significant differences in the total soluble protein, carbohydrate (neutral sugars) and lipid contents in the fish species investigated (Fig. 1). Protein contents in the aqueous phase of the mucus was significantly higher ($p < 0.05$) in *L. rohita* (54.77%) and *R. rita* (50.21%), than in *C. catla* (39.33%), *C. punctata* (20.64%) and *C. mrigala* (19.99%). In the detergent soluble phase of the mucus, proteins were present less than 3% of the dried weight of mucus in all the fish species investigated. Carbohydrate (neutral sugars) contents in the aqueous phases of the mucus were low in contrast to the protein contents. The carbohydrate moiety in the mucus, in general, were low compared to that of the protein contents and were higher than the carbohydrate moieties. The concentration of lipids extracted were significantly high ($p < 0.05$) in *C. mrigala* (8.60%) followed by in *R. rita* (6.09%), *C. catla* (5.27%), *L. rohita* (4.16%) and *C. punctata* (2.51%).

Qualitative analysis: In the aqueous phase of the skin mucus, qualitative analysis of the proteins by native-PAGE revealed distinct protein bands, six in each of the three fish species, *C. mrigala, L. rohita* and *C. catla,* five in *R. rita* and four in *C. punctata* (Fig. 2a). In all the fish species investigated, except in *C. punctata, a darkly stained zone at the top of the gel could represent merged dense protein bands, which stand for high molecular weight proteins and could not be resolved by this method. In contrast, SDS-PAGE, revealed several distinct protein bands, 31 in *C. mrigala, 29 in L. rohita, 26 in C. catla, 20 in C. punctata and 17 in R. rita* (Fig. 2b). Most of the bands in the molecular weight range from 29 kDa to 205 kDa were clearly resolved. Nevertheless,
protein bands, which were in the molecular weight range below 29 kDa, were poorly resolved. In C. mrigala, two major protein bands (with higher density than other bands), of the molecular weights, 40 kDa and 45kDa were observed. In L. rohita and C. catla two major protein bands of the molecular weights, 35 kDa and 43 kDa were also observed. In R. rita four major protein bands of the molecular weights of 40 kDa, 44 kDa, 50 kDa and 250 kDa were observed. Nevertheless, in C. punctata, major protein band could not be observed. Urea SDS-PAGE resolved the protein bands with a molecular weight below 29 kDa (Fig. 3). With this method, distinct protein bands, 10 in C. mrigala, 12 in L. rohita, 13 in C. catla, 7 in C. punctata and 5 in R. rita, of the molecular weight in the range from 17 kDa to 29 kDa were observed (Fig. 3). In R. rita, three major protein bands, of the molecular weights 17, 21 and 22 kDa were observed. In C. punctata, one major protein band of the molecular weights 18 kDa was observed. Nevertheless, in C. mrigala, L. rohita, C. catla, major protein bands below the molecular weight 29 kDa could not be observed. With native PAGE, SDS-PAGE and urea SDS-PAGE methods, protein bands could not be detected in detergent soluble phases of skin mucus of the fish species investigated.

Discussion

The present study reveals that, in all five fish species investigated, the protein contents in the aqueous phase of the skin mucus are high (19.9 to 54.7%), compared to those in detergent (0.3% Triton X-100) soluble phase, which have small amounts (less than 3%) of proteins. Al-Hassan et al. (1982, 1987), Ali et al. (1989), Chong et al. (2005), and Manivasagan et al. (2009) have also reported predominantly proteinaceous nature of the skin mucus secretions of different fish species. Al-Hassan et al. (1987), after the extraction of proteins in the aqueous phase of the skin mucus of catfish-Arius thalassinus treated the insoluble material left, with different concentrations of Triton X-100 and reported that the amount of the protein in the detergent soluble phase remained less than 5% even if the concentration of Triton X-100 increased to 10%. Protein profile of the aqueous phase of skin mucus was analyzed by Native PAGE, SDS-PAGE and Urea-SDS-PAGE. Native PAGE revealed very few protein bands while in SDS PAGE, several distinct protein bands were observed in the mucus of all the fish species investigated. This suggests that most mucus proteins are of high molecular weight and are engaged in disulphide bonding and non-covalent interactions with each other. Urea SDS-PAGE, in addition, revealed the presence of few to several low molecular weight proteins as well.

It is interesting that the skin mucus of the fish species investigated showed remarkable differences in the number of protein bands and their distribution by molecular weights in the skin mucus. Protein bands in the range from 29 kDa to 205 kDa molecular weight were 26 to 31 (approx.) in C. mrigala, L. rohita and C. catla, 20 in C. punctata, and 17 in R. rita. Nevertheless, in R. rita, the major portion of mucous proteins is distributed between 17 kDa and 50 kDa. In addition, 5-13 protein bands of low molecular weight, below 29 kDa were also present in the skin mucus of the fish species investigated. Differences in the number of protein bands and their molecular weights in different fish species have also been reported previously. More than 14 protein components in the catfish—A. thalassinus (Al-Hassan et al., 1982), two major protein bands at 31 kDa and 27 kDa along with one large band around 50 kDa in common carp – Cyprinus carpio (Lemaître et al., 1996), several protein bands between 20.1 kDa and 94 kDa in eel—Anguilla anguilla, carp – Cyprinus carpio, tench – Tinca tinca, trout – Onchorhynchus mykiss and turbot – Scophthalmus maximus (Ebran et al., 1999), 3-4 protein bands between 24 kDa and 66 kDa molecular weight in freshwater catfish – Clarias batrachus and Heteropneustes fossilis (Venkaiah and Lakshmpathi, 2000), several protein bands with molecular weight varying between 14.4 kDa and 200 kDa in Symphysodon spp. (Chong et al., 2005). Recently, Manivasagan et al. (2009) reported six protein bands (18.4 kDa to 97.4 kDa) in aqueous saline (0.9%) soluble phase, and seven (15.4 kDa to 97.4 kDa) in insoluble phase of proteinaceous gel secretions from skin of catfish – Arius maculatus along with a major protein band of 35kDa. These data suggest that fish secret several proteins with varying from lower to higher molecular weight in their skin mucus. The protein patterns vary with species, and depend upon the method and type of buffer/solvent used to extract mucus proteins. Protein patterns of the skin mucus of Indian major carps, C. mrigala, L. rohita and C. catla suggest that these species secrete similar types of proteins in their skin mucus layer and are closely related fish species belonging to Order – Cypriniformes and Family – Cyprinidae inhabiting similar habitat. On the other hand, catfish, R. rita (Order – Siluriformes; Family – Bagridae) and the murrel, C. punctata (Order – Channiformes; Family – Chanidae) have mucus protein profiles entirely different with each other and from the ICSs.

Presence of several high molecular weight protein bands in the range 50 kDa to 205 kDa in free swimming C. mrigala, L. rohita and C. catla, relatively few in C. punctata, showing intermediate swimming behaviour, and lack of these in the bottom dweller sluggish R. rita is interesting. This difference in the protein bands could be associated with habit, habitat and swimming behaviour of the fish species following USkova et al. (1970) who suggested that higher the amount of high molecular weight proteins in mucus, the greater would be the swimming speed of the fish. They have shown highest content of high molecular weight proteins in the fast swimmer blue runner –Trachurus trachurus, lower in the Correina umbra (at intermediate position from speed viewpoint), and zero in the slow moving bottom dweller placae – Scophthalmus maeoticus. They, further, suggested that the hydrodynamic properties of fish skin mucus are determined by the presence of proteins with a molecular weight of more than 50 kDa. Present study shows the presence of major protein bands in the range 35 kDa and 50 kDa in skin mucus. Such bands are highly dense in R. rita, comparatively less in C. mrigala, L. rohita and C. catla, and absent in C. punctata. This difference of these protein moieties could be associated with the consistency of skin mucus, being thick, dense and sticky gel like in R. rita; compared to that of C. mrigala, L. rohita, C. catla, and watery, thin fluid like in C. punctata. This investigation suggests that group of proteins having molecular weight between 35 kDa to 50 kDa could be one of the major factors governing the appearance, consistency and nature of mucus. Lemalite et al. (1996) observed a large undefined protein band around 50 kDa in SDS-PAGE profile of the skin mucus of Cyprinus carpio. Ebran et al. (1999) also reported a high density of proteins between 43 and 67 kDa in the skin mucus of five fish species, A. anguilla, C. carpio, T. tinca, O. mykiss and S. maximus. However, they have not correlated the presence of major protein bands with the thickness and appearance of the mucus.

In addition, the appearance (thickness) and nature of fish skin mucus may be correlated with their peculiar mode of life. R. rita is a bottom dwelling sluggish and scale-less fish, which usually lives in very dirty areas of rivers. In general, scale-less fishes produce higher...
amount of skin mucus for protection than those with scales (Negus, 1963). This could be a reason that R. rita produces thick mucus layer on the body surface for the protection from physical damage and for the coagulation of dirt and microbes, thereby cleaning the body. C. punctata is a bottom dwelling fish with well-developed scales on its body. It can survive for months without water when buried in moist soil or semi-fluid mud (Hora and Pillay, 1962). Watery, thin fluid like mucus helps the fish to move freely in the mud and prevents the skin from drying during drought conditions. Present study shows the substantially low amounts of carbohydrates (neutral sugars) in a range of 1.2 - 4.5% (w/w) compared to protein contents in the skin mucus secretions of the fish species investigated. Low concentrations of carbohydrates were also reported in skin mucus secretions of A. thalassinus (Al-Hassan et al., 1982), C. batrachus and H. fossilis (Venkaiah and Lakshmipathi, 2000) and A. maculates (Manivasagan et al., 2009).

In the skin mucus secretions of C. migala, L. rohita, C. catla, C. punctata and R. rita, lipids, though presence in low concentration (2.5 - 8.6%, w/w) than the protein moieties, could be derived from the membranous constituentsof the exfoliated dead cell and membrane bound vesicles. Small quantities of lipids have also been reported in the mucus secretions of an eel (Müller and Reinbach, 1914), three species of marine teleosts (Lewis, 1970), and Arabian Gulf catfish (Al-Hassan et al., 1982). Mittal and Nigam (1986) reported very low lipids-protein ratio in C. catla (0.076), Clarias batrachus (0.058), R. rita (0.068) and C. punctata (0.110). Venkaiah and Lakshmipathi (2000), however, reported that the epidermal secretions of H. fossilis and C. batrachus are rich in proteins as well as in lipids. Lipids in skin mucus have been considered to provide a barrier between the internal and external environment, acting as water repellent, and limiting the entry of water into the body of fish species (Mittal and Nigam, 1986). Presence of lipids together with protein molecules has been associated with antibacterial and antifungal properties; and to determine the viscosity of fish skin mucus (Lewis, 1970, Simkiss and Wilbur, 1977; Ebран, et al., 1999).

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