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Effect of alkaline pH on bioactive molecules of epidermal mucus from *Labeo rohita* (Rahu)

[Alkali pH'nın *Labeo rohita* (Rahu) epidermal mukusundaki biyoaktif moleküllere olan etkisi]

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ABSTRACT

Objectives: The present study was carried out to asses the role of bioactive molecules in the epidermal mucus of *Labeo rohita* and their antimicrobial and lysozyme activities.

Materials and Method: The fish mucus was collected by anaesthesia and alkali treatments. Total protein contents in mucus were estimated by Bradford assay and SDS-PAGE was performed for the protein profiling. Antimicrobial and lysozyme activities of mucus were measured.

Findings: Extraction by anaesthesia and alkali treatments produced 100±9.7 ml and 350±38.4 ml of mucus respectively. Significantly higher protein concentration (4.3 mg/ml) was found in anaesthesia treated samples than alkali treated (1.5 mg/ml) and neutralized mucus samples (0.55 mg/ml).The highest antibacterial activity was seen against *Escherichia coli* where as the mucus supported the growth of *Candida albicans*. Lysozyme activity was significantly higher in anaesthesia treated mucus (74.3 units/ml) in comparison to alkali treated (32 units/ml) and neutralized mucus (14 units/ml). Prominent peptide bands ranged between 13KD to 100KD were observed in anaesthesia treated mucus as compared to alkali treatment that denatured the most of the bands.

Conclusion: Alkaline pH reduced the activity of bioactive components of fish mucus, although the volume of fish mucus increased but actual immune parameters such as total protein contents, lysozyme and antimicrobial activity were decreased. Further detailed studies are required to understand the role and nature of the bio molecules found in the mucus.

Key words: mucus, proteins, antimicrobial activity, lysozyme activity, Labeo *rohita*, Rahu

ÖZET

Amaç: Bu çalışmada *Labeo rohita* epidermal mukusunda bulunan biyoaktif moleküllerin rolünün açığa çıkarılması ve bu moleküllerin antimikrobiyal ve lizozim aktivitelerinin anlaşılması amaçlanmıştır.

Gereç ve Yöntemler: Balık mukusu anestezi ile ve alkali muamele ile toplamıştır. Mukusun total protein içeriği Bradford yöntemi ile saptanmış ve protein profillemesi için SDS-PAGE yapılmıştır. Mukusta antimikrobiyal ve lizozim aktiviteleri ölçülmüştür.

Bulgular: Anestezi ve alkali muamele ile sırasıyla 100±9.7 ml ve 350±38.4 ml mukus elde edilmiştir. Anestezi yapılan örneklerde (4.3 mg/ml) alkali ile muamele edilen (1.5 mg/ml) ve nötralize edilen mukus örneklerine (0.55 mg/ml) kıyasla belirgin oranda yüksek protein derişimi saptanmıştır. En yüksek antimikrobiyal aktivite *Escherichia coli*'ye karşı görülmüştür. Lizozim aktivitesinin anestezi yapılan örneklerde (74.3 units/ml) alkali ile muamele edilen (32 units/ml) ve nötralize edilen mukus örneklerine (14 units/ml) göre daha yüksek olduğu saptanmıştır. Ayrıca anestezi ile elde edilen örneklerde büyüklükleri 13KD ile 100 KD arasında değişen peptid bantları görülmüştür.

Sonuçlar: Alkali pH, balık mukus hacminde artmaya neden olsa da biyoaktif bileşenlerin aktivitesini azaltmaktadır. Total protein içeriği, lizozim ve antimikrobiyal aktivite düşmektedir. Balık mukusunda bulunan biyomoleküllerin rolünün ve yapısının anlaşılması için daha ileri çalışmalara gerek bulunmaktadır.

Anahtar Kelimeler: Mukus, proteinler, antimikrobiyal aktivite, lizozim aktivitesi, *Labeo rohita*, Rahu

Introduction

The fish mucus layer contains different bioactive molecules act as a first line of defence against infections [1,2]. Fish live in a microbe-rich environment and are vulnerable to invasion by pathogens [3]. Mucus layer acts as a lubricant as well as offer protective functions against infectious agents and play a possible immunophysiological role [4, 5]. Mucus is continually produced, sloughed off from the fish epidermal surface and protects the fish from pathogens [6].

The physical barriers against infection in fish are its scales and mucosal surfaces of the skin, gills and the epidermis [7]. Mucus plays a role in the prevention of bacterial and fungal colonization [8] and the mucus layer also has a variety of biologically active molecules. These components include immunoglobulins, protease inhibitors, complement proteins, antibacterial peptides and lysozyme which may play a role in defence mechanism [7, 9]. Antimicrobial proteins in the epidermal mucus of fish can have a possible role as novel active therapeutic agents and are also present in a number of different flatfish. Some proteins form pores that cause the cell contents to leak out, and others simply disrupt the ionic gradients of membrane, with the consequent collapse of the cell. A number of antimicrobial proteins reported to kill the microbes without any detectable initial lyses, thus indicating they must have critical cellular targets such as protein and nucleic acid synthesis [8, 10].

Antimicrobial activity in skin secretions of fish is reported against both Gram negative and Gram positive bacteria [11]. The most striking role played by the mucus proteins is the killing of pathogens which interact with the epidermal surface of fish [12]. Mucus proteins are important and widespread bioactive component of defence against diseases in many fish species [12, 13].

Labeo rohita is a native fish of Pakistan, India, Bangladesh, Myanmar and Nepal. It is cultured on large scale in commercial farms [14]. Its consumption rate is high in local market. Large scale aquaculture breeding in Pakistan results in increasing mortality rates in fish farms due to poor management and non hygienic conditions. The aquaculture industry of Pakistan is facing problems due to the infectious diseases. The pH of water in natural and commercial reservoirs is towards alkaline side. The higher pH may affect the epidermal mucus in a way that it reduces the activity of mucus components involved in primary immune response to pathogens. The study was carried out to evaluate the effect of higher pH on bioactive compounds of epidermal mucus of *Labeo rohita* that act as a protective barrier between fish and its surrounding environment.

This study will be helpful in understanding of physiological role of the mucus as a first line of defence in protection mechanism against pathogens and would be beneficial for aquaculture industry to reduce economic loses by improving managemental practices by better understanding.

Materials and Methods

Experimental animal

Labeo rohita was taken from Fisheries Research and Training Institute, Manawan, Lahore, Pakistan of average weight 113.47±4.87 g. The fish were kept in large aerated concrete ponds containing potable tap water (pH 7.5 ± 0.5) facilitated with water and air pumps. The ponds were treated with disinfectant sodium hypochlorite, with the concentration of 200 ppm for 1 hour and washed three times with fresh water [15] prior to the introduction of the fish in the water. The fish were divided in two groups containing fifteen fish for each treatment (alkaline and anaesthesia stress).

Fish experiments

The first group was anesthetized (3-aminobenzoic acid ethyl ester 0.6 g\L) for five minutes, treated by 2 M NaOH solution (pH 11.5) and kept for 25 minutes. The second group was kept in anaesthetic bath for 4 hours [16].

Mucus collection

The mucus was collected from the dorsal surface of the fish after separate treatment of each group with alkaline solution or anaesthesia. The mucus was gently scrapped off with a plastic spatula. The sample was divided into two equal halves. One half was set aside while the other was neutralized to normal pH (7.5) by adding 2 N Tris Hydrochloride buffer. The collected samples were centrifuged at 12,000 x *g* at 4 ˚C for 10 minutes in a refrigerated centrifuge, labelled and stored at -40 ˚C (Biomedical freezer).

Total protein contents

Protein contents were measured by using Bradford micro assay [17]. Protein estimation kit (Bio-Rad USA) was used and absorbance of the samples was recorded at 595 nm. Bovine Serum Albumin was used as a standard.

Antimicrobial activity

The antimicrobial activity of the fish mucus was evaluated against *Sarcinia lutea* (ATCC 9341), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633) and *Candida albicans*. Fresh microbial cultures were plated out on Nutrient agar (SIGMA). Wells were formed by gel borer and 25 µl of samples were poured in each well. Culture plates were incubated at 37 ºC for 24 hours and results were recorded [18].

Lysozyme activity

Mucus was diluted into two-fold serial dilutions in phosphate buffer (0.05 M, pH 6.2). One hundred microliter of each dilution was mixed with 100 µl of a 0.4 mg/ml suspension of *Micrococcus lysodicticus* (SIGMA) in phosphate buffer in a microassay plate. Phosphate buffer

was used as a negative control while egg white lysozyme was treated as a positive control. Absorbance was recorded at 450 nm after the regular intervals of 0, 15, 30 and 60 minutes. A unit of lysozyme activity was defined as the amount of mucus causing a decrease in the optical density of 0.001/min [19].

Pepsin digestion

Antimicrobial and lysozyme activity was determined by digesting the mucus with pepsin [16].

Extraction of proteins with Trizol reagent

Proteins were extracted by Trizol reagent (MRC, U.S.A), briefly 0.4 ml of mucus and 0.8 ml Trizol reagent were added in 1.5 ml tube and incubated for 5 min. Then 0.2 ml chloroform was added and centrifuged at 12,000 x *g* for 10 min. Two upper phases were removed and 1 ml isopropyl alcohol was added in the remaining third phase used for extraction. Sample was gently mixed and stored for 10 min at 22°C. The sediment proteins were pelleted by centrifugation at 12,000 x *g* for 10 min at 4°C. The proteins pellet was washed 3 times in a solution containing 0.3 M Guanidine hydrochloride and stored for 20 min at 22°C. After the final wash, pellet was centrifuged at 7,500 x *g* for 5 min, and stored in 10% SDS buffer (0.05 M, pH 7.2).

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis (non-reducing) was performed as described by Laemmli [20] with slight modifications; briefly 4% stacking and 15% separating gel was used. Electrophoresis was performed under the constant voltage of 120 V. The gel was stained with PageBlue (Fermantas).

Statistical analysis

The samples were analysed in triplicates and the statistical analysis by the T-test and one way Analysis of Variance at α level 0.05.

Results

Appearance and quantity of mucus

The mucus collected after the alkali treatment was much viscous and yellowish in colour in comparison to the mucus obtained through the anaesthetic treatment. The fish under the effect of anaesthesia produced 100±9.7 ml of mucus where as alkali treatment produced a larger amount of mucus 350±38.4 ml.

Protein contents

Anaesthesia treated sample exhibited significantly higher protein concentration (4.3 mg/ml) than alkali treated sample (1.5 mg/ml) and the neutralized alkali sample (0.55 mg/ml) (Figure 1).

Antimicrobial activity

All mucus samples exhibited antimicrobial activity (Figure 2). Significantly higher antimicrobial activity was seen in the mucus extracted by anaesthesia in compa-

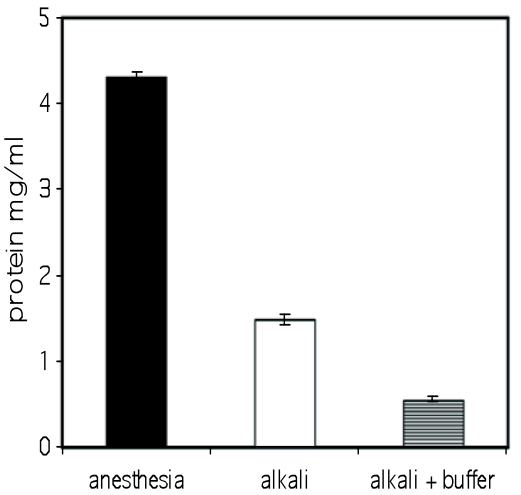

Figure 1. Protein concentration

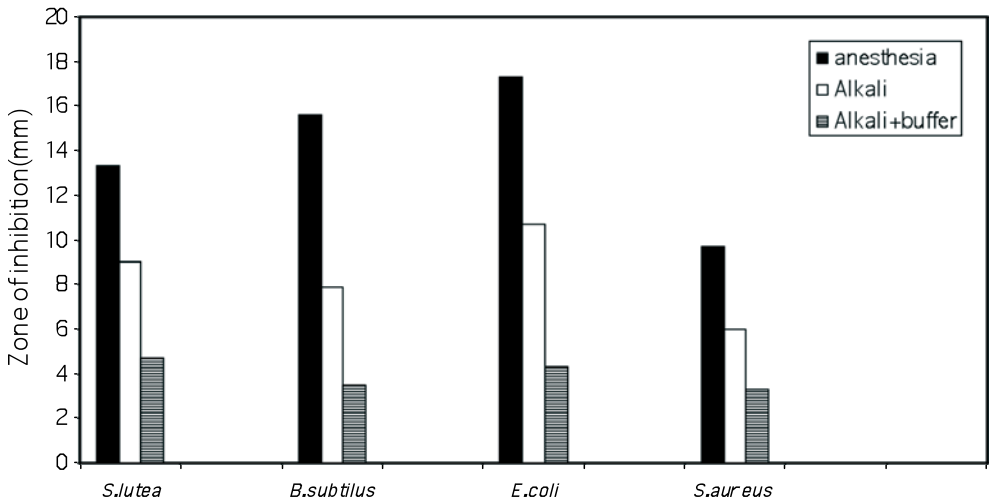

Figure 2. Antimicrobial activity

rison to alkali stress mucus. Maximum antimicrobial activity was observed against *E. coli* where as the larger growth zones of *C. albicans* were seen in all mucus samples.

Lysozyme activity

Lysozyme activity was higher in anaesthesia stress mucus (74.3 units/ml) in comparison to mucus extracted by alkali stress (32 units/ml) and alkali stress sample neutralized to normal pH (14 units/ml) (Figure 3).

Pepsin digestion

No antimicrobial and lysozyme activity was observed in pepsin digested samples.

Trizol extracts

No antimicrobial and lysozyme activity was exhibited in samples extracted with Trizol reagent.

PAGE analysis

The PAGE analysis (Figure 4) depicted the presence of intense protein bands of various molecular weights ranged between 13KD to 100KD and Sharp bands of 50 KD, 35 KD and 13 KD were seen in anaesthesia treated sample where as less no of protein bands were observed in alkali treated mucus. A little no of bands were seen in trizol extracted sample, where as no remarkable bands were observed in pepsin digested samples.

Discussion

The epidermal mucus of fish acts as a protective agent against microbes. Epidermal mucus of fish was extracted by anaesthesia and alkali stress suggested that the alkali treatment enhanced the secretion of mucus, which depicted the active fish response to alkaline conditions by secreting large quantity of mucus [16]. The comparison of anaesthetic and alkali treatments revealed deterioration of proteins in the mucus sample obtained through alkali stress, which in turn directly influenced the antimicrobial activity of the mucus samples. Significant difference was found in physical appearance and viscosity of the mucus in both groups.

Anaesthesia treated samples depicted significantly higher protein contents in comparison to the alkali treated samples (Figure 1). The alkaline conditions in environment can denature the protein structures and functions. The results are not in line with the study of Mozumder [21], reported protein contents 38.28 mg/ml and 9.84 mg/ml in Cod and Salmon skin mucus respectively. The presence of less proteins contents in our study as compared to Cod and Salmon might be due to the species difference and environmental factors such as water quality (dissolved oxygen, CO_2, ammonia, pH) and the presence of contaminants like organic and inorganic pollutants.

Mucus samples by anaesthesia and alkali treatments exhibited antibacterial activity against both Gram-negative and Gram-positive bacteria (Figure 2). The mucus samples were most active against *Escherichia coli* and *Bacillus subtilis* and least active against *Staphylococcus aureus*. Cho *et al* reported the similar findings in crude mucus extracts from the skin of the *Raja kenojei* against *Escherichia coli* and *Bacillus subtilis* [22]. Antimicrobial activity was also reported in mucus of different species, such as rainbow trout (*Oncorhynchus mykiss*), Winter flounder (*Pseudopleuronectes americanus*), Atlantic cod (*Gadus morhua*) and rockfish against Gram-positive, Gram-negative bacteria and the yeast *Candida*

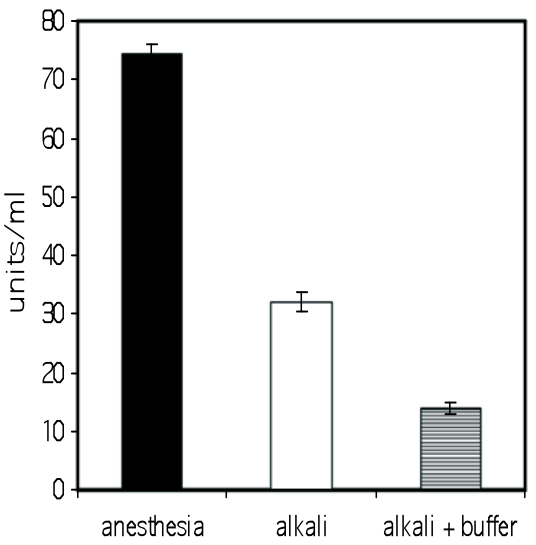

Figure 3. Lysozyme activity

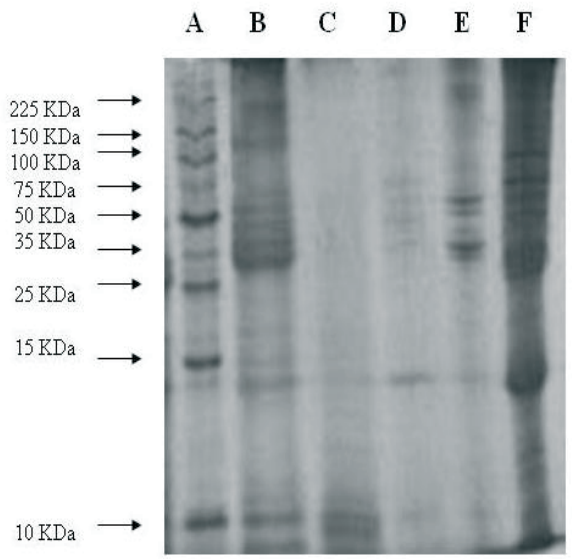

Figure 4. SDS-PAGE presenting protein fragments of labeled biomarker (Novagen USA) (A), mucus extracted through neutralized alkaline (B), digested by pepsin (C), extraction with trizol (D), through alkali stress (E) and through anaesthesia (F)

albicans [10, 17, 24, 25]; Whereas antimicrobial activity was reported in mucus extracts from six separate fish species; Arctic char (*Salvelinus alpinus*), Brook trout (*S. fontinalis*), *Cyprinus carpio* sub sp. koi (Koi carp), Striped bass (*Morone saxatilis*), Haddock (*Melanogrammus aeglefinus*) and Hagfish (*Myxine glutinosa*) [26]. Antimicrobial assay (Figure 2) against the yeast *Candida albicans* resulted in enhanced fungal growth indicated the usage of *Labeo rohita* mucus as a favourable substrate of growth for this particular strain of *Candida*. The diameter of fungal growth was largest in the mucus sample extracted through anaesthesia, which may be caused by the presence of concentrated amount of favourable substrate. The mucus extracted by alkali stress exhibited a slightly smaller growth diameter. In contrast to these findings, previous studies reported inhibitory role of skin exudates of rainbow trout against the yeast *Saccharomyces cerevisiae* [24] and antimicrobial activity against *Candida albicans* was also reported in the epidermal mucus extracts of the Atlantic cod (*Gadus morhua*) [16].

SDS-PAGE analysis of the mucus samples neutralized by buffer, digested by pepsin, extraction with trizol, by alkali treatment and by anaesthesia treatment (Figure 4) illustrated the presence of different protein bands. The most of these protein bands were lost in the alkaline mucus. The neutralized alkaline mucus retained some of the protein bands. Clear protein bands were seen at 50 KD, 35 KD and 13 KD in anaesthesia treated sample. These results indicated the adverse effects of alkali on the protein quality of mucus resulted in less protein contents, reduced antimicrobial and lysozyme activity. These results are in line to other studies where peptides of less molecular weight were reported with antimicrobial activity against the pathogens [25,27]. Most of the bands were lost by trizol extraction method. Pepsin digestion of the mucus samples eliminated the antimicrobial activity and corresponding protein bands. Similar findings were reported in the Atlantic cod (*Gadus morhua*) mucus where the antimicrobial activity was completely lost by pepsin digestion [16]. The sample collected by anaesthesia treatment depicted the significantly higher lysozyme activity (Figure 3). Lysozyme activity in mucus of different species was found [27], whereas no lysozyme activity was detected in mucus of Atlantic cod [16]. This contradiction may correspond to the difference in physiological and immunological actions against environmental pathogens.

This study provides an evident that epidermal mucus of fish serves as a barrier to its environment and acts as an important component of fish immune system. Alkaline pH deteriorates the composition of biologically active molecules of mucus that leads to poor immune responses to pathogens**.** Further characterization of mucus proteins could be helpful to understand specific roles for these factors.

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