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EFFECTS OF ALKALINE PH ON PROTEIN AND FATTY ACID PROFILES OF EPIDERMAL MUCUS FROM *LABEO ROHITA*.

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ABSTRACT

Fish culture is dependent upon ecological parameters such as light, temperature, dissolved oxygen, pH and total alkalinity. The pH of soil in Pakistan is alkaline which subsequently influences the pH of the water used for fish farming. Present study was designed to determine the effects of alkaline pH on the composition of fish epidermal mucus. Mucus was extracted from anaesthetized and alkali treated groups of *Labeo rohita*. Total volume of mucus collected from anaesthesia treated group was 150±11.78ml and 480±24.25ml from alkali treated group. Difference was observed in the mucus appearance, viscosity and odor. HPLC analysis of mucus obtained from anesthetized group showed nine peaks at 280nm and 360nm whereas the mucus collected from alkali treated group depicted only two peaks at 280nm and five peaks at 360nm. Four units of lectin activity were observed in the mucus from the anesthetized group but were altogether absent in the mucus from the alkali treated group. Fatty acid profile by GCMS depicted the presence of acetic acid and 6-8 carbon chain fatty acids in the mucus extracted from both anesthetized and alkali treated groups. These results indicated that higher pH has denatured or deteriorated proteins in the epidermal mucus of fish and caused lectin activity to cease completely, however alkaline pH has no adverse effect on fish mucosal fatty acids. Further studies are needed to evaluate the effects of higher pH on fish immune parameters such as non-specific immunity, microbial resistance and disease susceptibility.

Key words: Mucus, proteins, fatty acids, lectins, *Labeo rohita*

INTRODUCTION

There is no keratin layer on fish epidermis which makes it vulnerable to abrasions or infectious agents (Fernandes *et al*., 2003); therefore the primary interference between fish and its environment is through the mucus layer which covers its entire body (Hancock and Lehrer, 1998). Mucus not only provides a mechanical protective function by preventing the attachment of parasites and colonization of pathogens (Fletcher, 1978 and Ellis, 2001) but also has a variety of biologically active immune components which function together as a chemical shield (Fernandes *et al*., 2002; Maganadottir, 2006 and Rakers *et al*., 2013). Major components of fish mucus are proteins and fatty acids (Lewis, 1970 and Bergsson *et al*., 2005

Fifteen different types of fatty acids have been discovered in the epidermal mucus and skin of *Labroides dimidiatus* (Ariffin *et al*., 2009). Fatty acids present in the fish epidermal mucus have exhibited antimicrobial activity (Bergsson *et al*., 1998 and Bergsson *et al*., 2001) as well as wound healing through rapid cell proliferation (Jais *et al*., 1998 and Ariffin *et al*., 2009). Kuppulakshmi *et al*., (2008) have reported strong bacterial inhibition in mucus isolated from *Channa punctatus* and *Cirrhinus mrigala*, similar results have been observed in epidermal mucus isolated from *Labeo rohita* (al-Arifa *et al*., 2011). Lectins have been associated with a number of important biological functions such as the promotion or inhibition of cell adhesion (Barondes *et al*., 1994), activation of mast cells and basophils (Frigeri *et al*., 1993) and inflammation (Liu, 1993). The lectin-coating of bacteria makes the pathogens more susceptible to macrophages, furthermore the supernatants obtained from such lectin-stimulated macrophages exhibit potent bactericidal activity (Fock *et al*., 2001).

Water quality has a direct impact on the health of fish. Fish epidermal mucus is the first defense barrier and stressed aquatic conditions such as temperature (Quiniou *et al*., 1998), dissolved oxygen (Vatsos *et al*., 2010) and pH (Zuchelkowski *et al*., 1986 and Schäperclaus *et al*., 1992) greatly affect the fish's ability to produce mucus.

Labeo rohita is native to Pakistan and is found commonly throughout rivers and fresh water lakes as well as being commercially produced in fish farms (Khan and Jhingran, 1975 and Hussain *et al*., 2011). In Pakistan the soil pH is on higher side (7.8-8.5) (Leghari *et al*., 2003). Limnological studies of River Soan (Iqbal *et al*., 2006) have reported high water pH, 8.0-9.0 and 7.20-8.20 respectively. Altaf *et al*., (2008) observed alkaline pH in river Chenab at Head Qadirabad (7.9) and Head Khanki (8.1). Water pH of earthen ponds at University of Agriculture Faisalabad was recorded in the range of 7.0-8.5 (Nazish and Mateen, 2011). According to Randall and

Wright (1989) ammonia excreted by the fish is also a cause of the alkalinisation of water. Higher pH affects the appearance, viscosity, composition and production of fish mucus in a way which results in reduced activity of mucosal components such as their antimicrobial properties (al-Arifa *et al*., 2011).

The purpose of present study is to determine effects of pH on quality, quantity and biochemical composition (fatty acids and proteins) of the epidermal mucus of *Labeo rohita* in order to understand the physiological importance of these biomolecules in the mucosal secretions and adaptability of fish to survive at relatively high pH.

MATERIALS AND METHODS

Experimental animal: Healthy yearlings of *L. rohita*, average weight (150-200g) were obtained from Fisheries Research and Training Institute, Manawan, Lahore, Pakistan. Large concrete ponds facilitated with water and air pumps were cleaned and treated with disinfectant (200ppm of Sodium Hypochlorite) for 1 hour and then washed with clean water according to Bergsson *et al*., (2005). The ponds were filled with potable tap water (pH 7.5±0.5) and aerated for several hours prior to introduction of fish.

Mucus collection: The fish were divided into two groups (control and alkali treated), each containing twenty fish for separate treatments. The first group (control) was kept in an anesthetic bath (0.6 g\L of 3-aminobenzoic acid ethyl ester) for 4 hours (Bergsson *et al*., 2005). Whereas the second group (alkali treated) of fish was anesthetized then treated with alkaline solution (pH 11.5) for 25 minutes (Schaperclaus *et al*., 1992) (Figure 1). Plastic spatulas were used to gently scrape off the mucus from the dorsal surface of the fish and then placed immediately on ice. Half of the mucus sample obtained from the alkali treated group was neutralized to normal pH (7.5) by the addition of 2N Tris Hydrochloride buffer. All of the mucus samples were centrifuged (12,000 × g at 4˚C) for 10 minutes in a refrigerated centrifuge, labeled and stored at -40˚C.

Protein profile: For the separation of various proteins, a 4.6 × 250mm, C18 Hypersil Bakerbond BDC column having 5 micrometer pore size (Mallincrodt Baker Inc., Phillipsburg, NJ, USA) was used through high-performance liquid chromatography (Agilent, U.S.A) at 280nm and 330nm. 20µl mucus sample was injected into the HPLC at a flow rate of 1 ml/min. Gradient elution was carried out with 0.1% TFA in water (solvent A) and 0.1 % TFA in 40% (v/v) acetonitrile (solvent B). The solvent B was employed from 0 to 30 minute for 0 to 100% concentration. Temperature was maintained at 25°C (Kosinska *et al*, 2006). Retention time for the elution of protein peaks was 14 minutes.

Lectin activity: Lectin activity was determined through hemagglutination inhibition assay. Serial dilutions of mucus samples were prepared in microtiter plates with 25µl of PBS buffer. The dilutions were then mixed with 25µl of 5% rabbit red blood cell suspension and incubated at room temperature for an hour. Hemagglutination observed in the most diluted sample was expressed as the Lectin titer of that sample (Okamoto, 2005).

Fatty acid profile: The mucus samples were incubated for 45 minutes at 110°C in 14% boron trifluoride/methanol and then analyzed by high-resolution gas-liquid chromatography equipped with a flame detector and a polyethylene glycol column. Temperature of the injector was maintained at 235°C and that of the detector at 250°C. The initial temperature was kept at 90°C for two minutes, then raised to 165°C, first at the rate of 30°C/min, then 3°C/min, and finally held isothermal for 6 minutes. The carrier gas was hydrogen at 31.8Kpka. Identification and calibration of resultant peaks was carried out against relevant standards (Bergsson *et al*, 2005).

Statistical analysis: All the samples were analyzed in triplicate. Statistical analysis was performed by T-test and one way analysis of variance at α level 0.05 using SPSS.

RESULTS

Appearance and quantity of mucus: The mucus obtained from the group treated with higher pH was viscous, yellowish in color and had an augmented fish odor whereas the mucus obtained from the control group was slightly viscous, clear and with only mild fishy odor. The anesthetized group produced 150±11.78ml of mucus where as the alkali treated group produced a larger amount of mucus 480±24.25ml (Figure 2).

Protein profile: Protein profile of the mucus sample extracted from the control group showed a rich array of proteins, a total of 9 peaks were observed at 280nm and 330nm (Figure 3). However there was a marked reduction of proteins in the remaining two mucus samples. The mucus sample obtained from the alkali treated group showed only 2 peaks at 280nm and 5 peaks at 330nm (Figure 4), whereas the neutralized mucus sample presented 2 peaks at 280nm and just a single peak at 330nm (Figure 5).

Lectin activity: Lectins (4 units) were found in the mucus sample extracted from the control group however, lectin activity completely ceased in both of the mucus samples obtained from the group treated with higher pH.

Fatty acid profile: Fatty acid analysis of the mucus sample collected from the control group confirmed the presence of hexane, cyclohexane, acetic acid, heptane,

toluene, ethylbenzene, carbamohydroximic acid, o-xylene, p-xylene and 8-carbon chain fatty acids (Table 1 and Figure 6.1). Carbamohydroximic acid was absent in the mucus sample collected from the alkali treated group as well as from the alkaline mucus sample neutralized by the addition of buffer (Table 1 and Figures 6.2 & 6.3). 6-carbon chain fatty acids were only found in the mucus sample collected from the alkali treated group and were absent in the remaining two samples (Table 1 and Figure 6).

Figure 1: Epidermal mucus production after the alkali treatment.

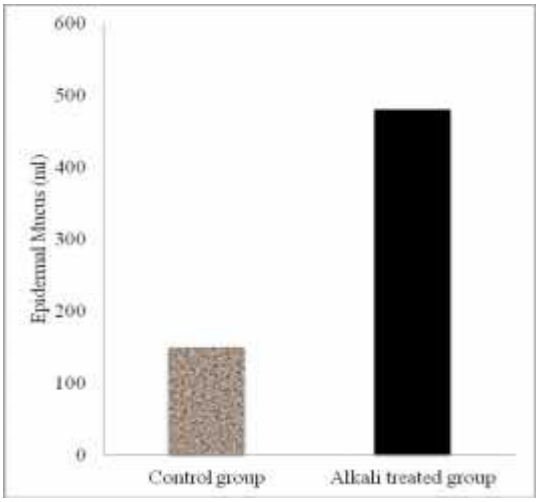

Figure 2: Quantity of epidermal mucus obtained from control and alkali treated groups.

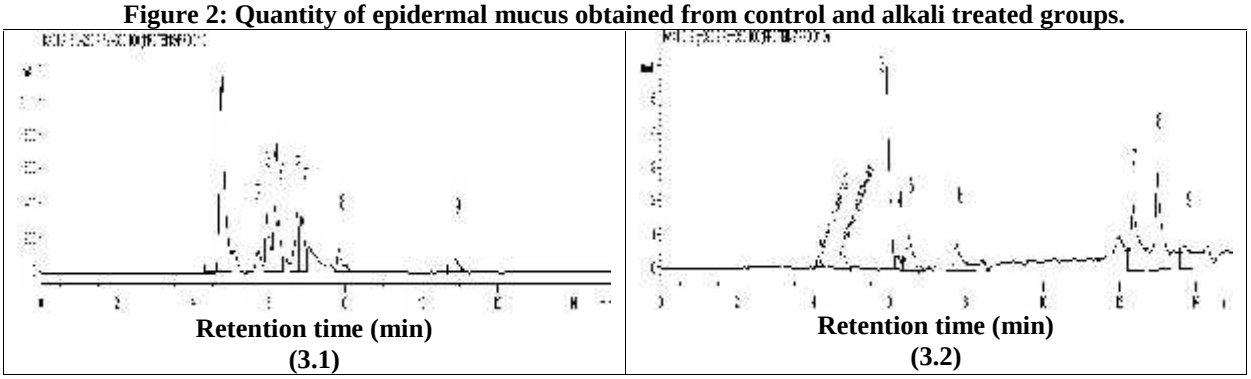

Figure 3: HPLC analysis of mucus obtained from control group. The chromatographs at 280nm (3.1) and 330nm (3.2).

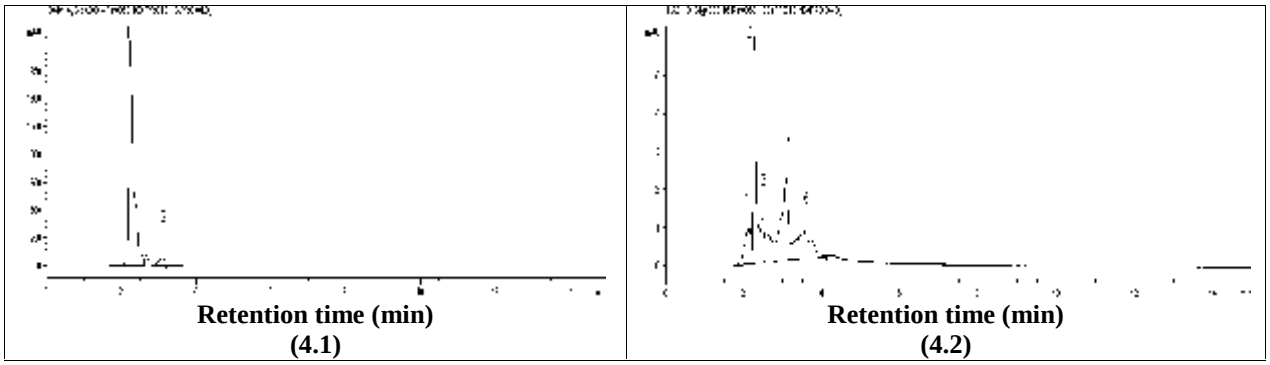

Figure 4: HPLC analysis of mucus obtained through alkali-treatment. Chromatograph at 280nm (4.1) and 330nm (4.2).

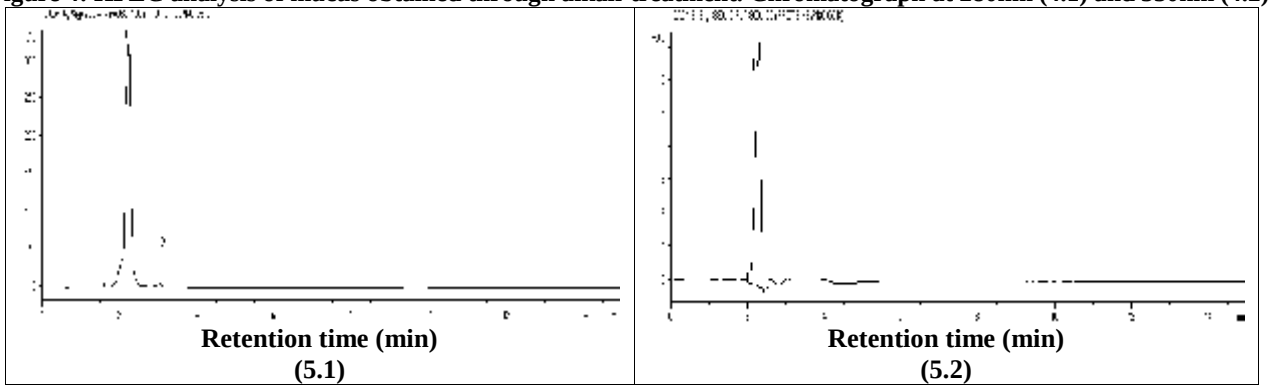

Figure 5: HPLC analysis of alkaline mucus neutralized with buffer. Chromatograph was obtained at 280nm (5.1) and 330nm (5.2).

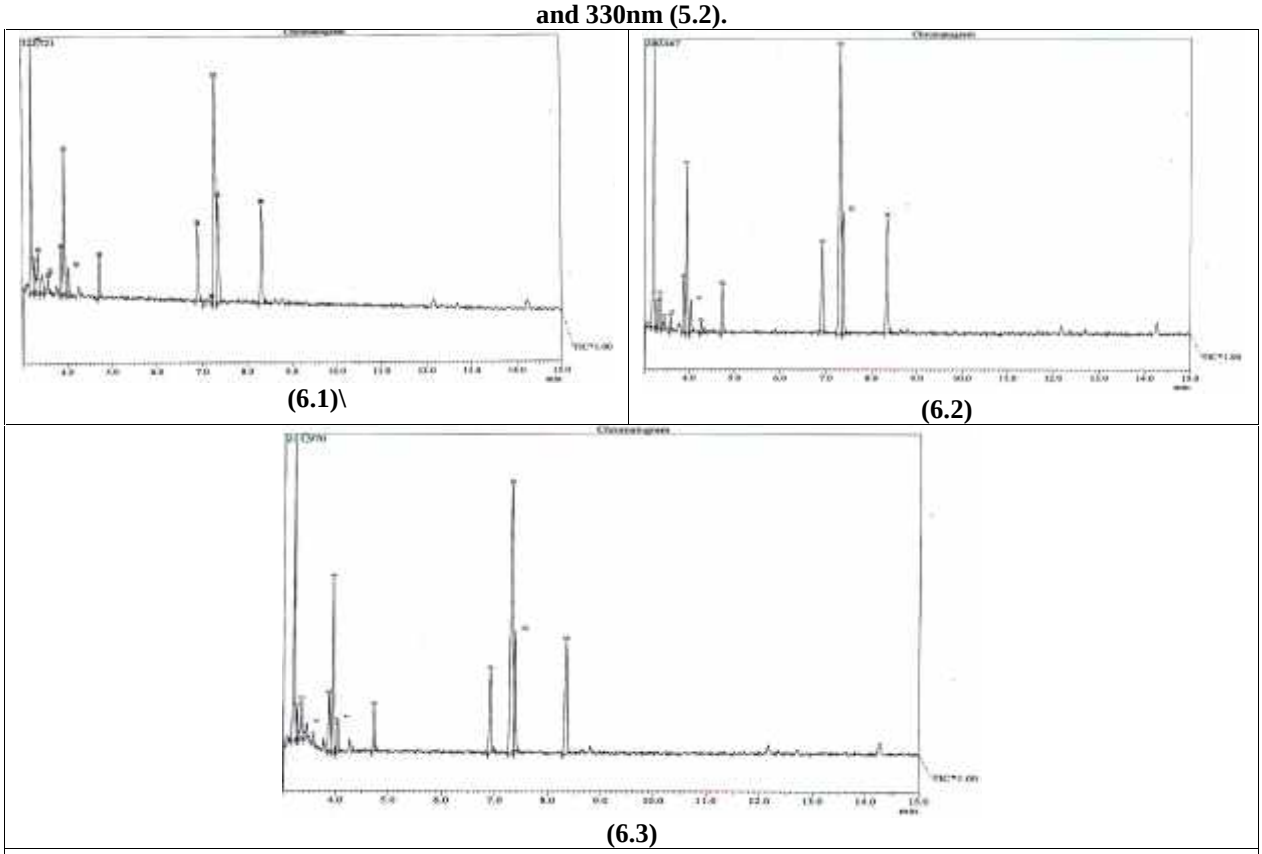

Figure 6: GC/MS of epidermal mucus of *Labeo rohita*. (6.1) Mucus of control group. (6.2) Mucus obtained through alkali treatment. (6.3) Alkaline mucus neutralized with buffer.

Table 1: Fatty acid profile of control and alkali-treated mucus obtained through GC/MS

Fatty acids	Anesthesia samples	Alkali sample	Neutralized buffer
Cyclohexane	+	+	+
Hexane	+	+	+
Acetic acid	+	+	+
8-Carbon chain fatty acids	+	+	+
Heptane	+	+	+
Toluene	+	+	+
Ethylbenzene	+	+	+
Carbamohydroximic acid	+	–	–
O-xylene	+	+	+
P-xylene	+	+	+
6-Carbon chain fatty acid	–	+	–

DISCUSSION

Fish inhabit a microbe rich environment which makes it vulnerable to pathogenic infections. Fish epidermal mucus prevents microbial colonization and contributes to non-specific innate immunity (Shephard, 1993). Results obtained from this study suggest that alkaline pH enhanced mucus production but also impacted its quality in terms of protein concentration and lectin activity as well as altering its general appearance and odor. Change in pH creates stress for the fish in its aquatic environment which directly influences the production of epidermal mucus (Segner *et al*., 1987 and Schaperclaus *et al*., 1992).

Labeo rohita mucus (control group) contains a number of proteins which were reduced or completely absent in the mucus samples obtained from the group of fish exposed to alkaline stress. Several studies concur that proteins found in the fish epidermal mucus play an important role in fish innate immunity (Chong *et al*., 2005; Fagan *et al*., 2003; Subramanian *et al*., 2007 and Rakers *et al*., 2013). The Epidermal Mucus *Mystus gulio* has also exhibited remarkable antibacterial and antifungal activity (Anbuchezhian *et al*., 2011). Antimicrobial glycoproteins from the mucus of *Tinca tinca* and *Oncorhynchus mykiss* exhibited strong antibacterial activity (Ebran *et al*., 2000). Epidermal mucus of *Labeo rohita* exhibits strong antimicrobial activity against *Sarcinia lutea*, *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtili* which was significantly reduced in the mucus obtained from the fish exposed to alkaline pH (al-Arifa *et al*., 2011). Non-specific immunity is dependent upon the quality of mucosal proteins hence the degraded proteins from the alkaline treatment render the fish susceptible to infection.

Four units of lectin activity were detected in the epidermal mucus of the control group but were altogether absent in both of the mucus samples extracted after the alkali treatment. Lectins are a group of Ca^{2+}-dependent proteins which have a role as defense molecules in both vertebrates and invertebrates and exhibit different specificities for binding carbohydrates (Arason, 1996). Lectins can agglutinate a number of bacterial pathogens by binding with the surface carbohydrates and inhibiting invasion of the fish host (Ellis, 2001). Molecularly diverse lectins have been reported in the skin mucus of *Takifugu rubripes* and *Leiognathus nuchalis* (Kilpatrick, 2000 and Suzuki *et al*., 2003). Mannose-binding lectins isolated from the serum of *Salmo salar* have been reported to act as opsonising agents against *Aeromonas salmonicida* (Ottinger *et al*., 1999). Okamoto *et al*., (2005) identified two lactose-binding lectins from the skin mucus of *Leiognathus nuchalis*. The absence of lectins from the mucus may result in reduced immunity and subsequent pathogenic infection.

The epidermal mucus of *Labeo rohita* was found to be rich in fatty acids. Most of the fatty acids were short chain fatty acids (6-8 carbon chain) such as acetic acid and carbamohydroximic acid which is in accordance with C6-C18 chain fatty acids reported in fish epidermal mucus by Bergsson *et al*., (2005). Long chain fatty acids with C18 and C20 and C22 carbon chains have also been reported in the epidermal mucus of fish (Jais *et al*., 1998 and Sato *et al*., 2008) but were not observed in this study. The variation in carbon chain fatty acids may be due to difference in fish species. Both of the mucus samples extracted from the experimental group had the same fatty acid composition. However, it was noted that carbamohydroximic acid was found only in the control group sample, which indicates a slight deteriorating effect of alkaline pH.

Conclusion: Alkaline pH increased the quantity of the fish mucus but also reduced the concentration of epidermal mucosal proteins and terminated all lectin activity. However, alkaline pH had no adverse effects on the fatty acids of the mucus and all but one fatty acid were present in the epidermal mucus of both the control and experimental groups. It is hence concluded that high pH has a direct effect on the susceptibility of the fish to pathogens and may result in an increased mortality rate.

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