Measurement of Alkaline Phosphatase and Lysozyme Enzymes in Epidermal Mucus of Different Weights of *Cyprinus carpio*

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Abstract: Fish epidermal mucus provides the first line of defense against pathogens. Little is known about the role of epidermal mucus enzymes in the innate immune system of fish species such as Common carp (*Cyprinus carpio*) with different weights. The aim of this study was measuring the specific alkaline phosphatase activity and lysozyme in mucus of three weights of common carp, 20, 50 and 200g and the enzyme levels were compared among the three weights. Alkaline phosphatase activity was determined by using the spectrophotometer set and laboratory kits. Lysozyme activity was determined using a turbidometric assay and the *Micrococcus lyzodeikticus* bacterium was substrate. This results indicated that alkaline phosphatase and lyzosyme levels of 200g fish weight is higher than another weights ($p<0.05$) and provide preliminary information for a better understanding of the role of epidermal mucus and its components in the fish innate immune system.

Key words: *Cyprinus carpio* % Mucus % Alkaline Phosphatase and Lysozyme Enzymes

INTRODUCTION

The biological interface between fish and their aqueous environment consists of a mucous layer composed of biochemically diverse secretions from epidermal goblet cells and epithelial cells [1]. The epidermal layer of the skin is also important in this aspect, as it secretes the mucus in addition to providing physical protection [2]. This layer is thought to act as a lubricant [3], to be involved in osmoregulation and locomotion [4], to play a possible immunological role [5] and to have some function in intraspecies chemical communication [6].

An intricate array of both specific and innate immune components have been identified and characterized in fish. Key innate immune components include the mucus layer on the skin, gills and gastrointestinal tract and constituents of the blood such as phagocytes and natural killer cells [7]. Most mucus in animals is secreted by goblet cells, though other cells including those in the submucosal glands produce mucus [8-10]. The main structural proteins of mucus are high molecular mass (~106 kDa) glycoproteins called mucins [11]. Fish serum and skin mucus are known to contain a number of antipathogenic substances such as lysozyme, complement, alkaline phosphatase, C-reactive protein, lectins and other substances [12]. Lysozyme, also referred to as N-acetylmuramide glycanohydrolase or muramidase, is a well-studied bacteriolytic enzyme identified in a wide range of organisms including fish [13]. It functions by hydrolyzing the â-(1>4) linkage between N-acetylmuramic acid and 3-acetyl amino-2-deoxy-D-glucose residues of the mucopolysaccharide found in bacterial cell walls. Lysozyme cleaves the glycosidic bonds of the peptidoglycan layer and, in fish, is an effective agent against both Gram-negative and Gram-positive bacteria by causing lysis of its outermost peptidoglycan layer [14, 15]. Another mucus enzyme, alkaline phosphatase (ALP) has been demonstrated as a potential stress indicator in the epidermal mucus of Atlantic salmon (*S. salar*) [16].

Alkaline phosphatase is a lysosomal enzyme suggested to have a protective role in fish during the first stages of wound healing [17]. The focus in this study was to investigate the specific activities of lysozyme and alkaline phosphatase in the epidermal mucus and compare these enzyme activities over the different of fish weights of 20, 50 and 200g in *C. carpio.*

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MATERIALS AND METHODS

The fish used for this study maintained from Voshmgir barrier of Golestan Province. 15 fish of each weight of 20, 50g were sampled and reared separately in 750L and weight of 200g were placed in 1000L capacity flow-through tanks, with water temperature ranging from 27±1 °C.

Fish were acclimatized to seasonal light regimes, for three weeks (A 12-hour-light: 12-hour-dark cycle was maintained). The health of fish was observed daily and any dead fish was removed directly from tanks. Fish were fed a pelleted diet formulated for twice daily.

After acclimatization, fish were starved for 24 h and the mucus was collected non-lethally by anaesthetizing the fish with a sublethal dose (5 mg/l) of clove oil. Mucus collection was performed using the method of Ross *et al.* [16]. Mucus samples were obtained from 15 fish representing each of the weights. Individual anaesthetized fish were transferred into a polyethylene bags containing 10 ml of 50 mM NaCl, The bags were gently shaken by hand for 2 min to collect mucus and following this treatment fish were returned to recovery tanks. The mucus was immediately were centrifuged at 1500 rpm for 10 min. at 4 °C and the obtained supernatant was stored at-80 °C.

The solution protein concentration of mucus samples was determined using the method of Lowry *et al.* [18] with bovine serum albumin solution (BSA) as standard. The solution protein concentration determined was then used to calculate the specific activity for the respective assays. The absorbance for the various assays was read using a spectrophotometer (Biochrom, Libra, S12, England). Alkaline phosphatase activity was determined by the use of kit (Pars Azmoon Company, Iran) and absorption was read at 405 nm in spectrophotometer.

For measuring of lysozyme activity was used a turbidometric method [16]. 50 µL of the mucus sample were diluted in 40 mM sodium phosphate buffer, pH 6.5 and transferred to a 96 well plate and incubated at 30 °C for 15 min. Lyophilised *Micrococcus lysodeikticus* cells

(50 µL) (0.3 mg ml^1 in 40 mM sodium phosphate buffer, pH 6.5, Sigma) were then added and the absorbance was measured continuously for 50 min at 30°C. The initial rate of reaction was used to calculate the activity. One unit of activity was defined as the amount of enzyme that catalyzed a decrease in absorbance at 450 nm of 0.001 min^1.

All assays were carried out a 25 °C. To evaluate the significance of the differences among enzyme levels in different weights of Common carp were performed by One-Way-ANOVA with Duncan test using SPSS 17, with significant differences being considered as those where p# 0.05 with ± standard deviation (SD).

RESULTS

The presence of lysozyme and alkaline phosphatase were confirmed in the mucus of *C. carpio.* Lysozyme activity was expressed as specific activity (unit per milligram of soluble protein). A pronounced variation in the levels of enzyme activities (Table 1) was observed among the different weights of Common carp. The specific activity of lyzosyme was highest in weight of 200g mucus followed by that of the 50g and 20g. The analysis of mucus alkaline phosphatase levels (Table 1) showed that weight of 200g mucus had the highest alkaline phosphatase activity (IU/l). Weight of 50g had the second highest alkaline phosphatase levels followed by weight of 20g.

DISCUSSION

Fish have adapted to survive in a variety of aquatic environments. The first line of defense against pathogens is the mucus layer, which has evolved a variety of innate immune factors [19]. Differences in activities of antimicrobial enzymes, such as lysozyme and alkaline phosphatase and how they relate to the structure and composition of mucus and epidermal layers, may also relate to the differences observed in disease resistance [2].

Table 1: Comparison of alkaline phosphates and lysozyme activity (mean ± SD) in different weights of common carp (*n* = 15)

Parameter	20g	50g	200g
Solution protein U/mg sample	1.64 ± 0.57[a]	3.46 ± 0.08[b]	4.87 ± 0.17[c]
Lysozyme activity (U/mg protein)	2.94 ± 0.07[a]	4.22 ± 0.19[b]	13.09 ± 1.84[c]
Alkaline phosphatase activity (IU/L)	13.95 ± 3.6[a]	37.67 ± 3.21[b]	69.57 ± 11.16[c]

-Data are represented as mean±SD.

-Means with different superscript letters at the same row are significantly different (P >0.05).

The protective role of the surface mucus layer was previously investigated by Fouz *et al*. [20] showed that the mucus of the turbot has an antibacterial action against different pathogenic bacteria such as *A. hydrophila*, *P. fluorescens*, *S. aureus* and others. This study examined changes in two hydrolytic enzymes activity among the three weights of Common carp. Lysozyme, an enzyme that cleaves the glycosidic bonds of the peptidoglycan layer of bacteria, was present in the mucus of all studied fish weights, Lysozyme has been primarily studied from serum, plasma, lymph, kidney, spleen, stomach, gills, gastrointestinal tract and other organs or tissues in various fish species [21, 22].

In a few number of researches have noted that 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 [23].

In case of lysozyme, a pattern of differences in the enzyme levels was observed between the seawater and freshwater-reared fish species. However, an inverse variation in lysozyme specific activity has been reported in rainbow trout, coho salmon and Atlantic salmon [2].

The variation in lysozyme activity could also be related to several factors such as responses to handling stress, maturity, diet, sex, species variation and genetic variation [24]. The higher lysozyme activities observed in seawater species, that could be related to the species specific evolutionary adaptation to different environmental conditions or genetic adaptation of these species to the environmental factors [25].

Lysozyme has been found in fish mucus, serum and ova [7]. Fish lysozyme occurs in 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 characterized by a high pH optima and a broad substrate specificity [26, 27]. alkaline phosphates is a lysosomal enzyme suggested to have a protective role in fish during the first stages of wound healing [17].

The precise function of alkaline phosphates in the innate immune mechanisms has yet to be elucidated. However it is considered as a potential stress indicator. Alkaline phosphates activity has been detected in the saliva of other arthropod parasites [28].

Subramanian *et al.* [25] in comparative study on innate immune parameters in the epidermal mucus of various fish species (sea water and fresh water species), observed that *C. carpio* mucus had the highest alkaline phosphatase specific activity among the fresh water species.

In another study, about Atlantic salmon (*Salmo salar*) during smoltification, changes in the levels of tissue and blood alkaline phosphatase isoenzymes were associated with smolting and gonadal development [29].

Iger and Abraham [17] observed a large increase in the production of alkaline phosphatase in pavement and mucous cells in the epidermis of carp following wounding. Mucous cells may therefore provide a significant source of alkaline phosphatase, lysozyme and proteases in the mucous layer. Marked increases in lysozyme and alkaline phosphatase have been reported in the mucus of Atlantic salmon infected with sea lice [2, 16]. With increasing weight, alkaline phosphates and lysozyme activities increases, its means that fish with age-related factors also increased the innate immune system.

Overall, the bacteriocidal properties of mucous lysozyme, particular mucous proteins and proteases may have a direct effect on the innate immune response of these species toward certain pathogens. Further examination of proteins and enzymes in mucus could lead to more definitive roles for these various factors.

REFERENCES

1. Pickering, A., 1974. The distribution of mucus cells in the epidermis of the brown trout *Salmo trutta* (L.) and the char *Salvelinus alpinus* (L). J. Fish Biol., 6: 111-118.

2. Fast, M.D., D.E. Sims, J.F. Burka, A. Mustafa and N.W. Ross, 2002. Skin morphology and humoral non-specific defense parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. Comp. Biochem. Physiol., 132(A): 645-657.

3. Rosen, M.W. and N.E. Conford, 1971. Fluid friction of fish slimes. Nature, 234: 49-51.

4. Cameron, A. and R. Endean, 1973. Epidermal secretion and the evolution of venum gland in fishes. Toxicon., 11: 401-410.

5. Fletcher, T.C. and P.T. Grant, 1969. Immunoglobulins in the serum and mucus of plaice, *Pleurowrtes platessa*, J. Biochmi., (Tokyo), 115: 1-65.

6. Saglio, P. and J.M. Blanc, 1989. Intraspecific chemocommunication in immature goldfish, *Carassius auratus* (L): attraction in olfactometer to free amino acid fractions from skin extracts, Biol. Behav., 14: 132-147.

7. Ellis, A.E., 1981. Non-specific defense mechanisms in fish and their role in disease processes. Dev. Biol. Stand., 49: 337-352.

8. Shephard, K.L., 1994. Functions for fish mucus. Rev. Fish Biol. Fish, 4: 401-429.

9. Thornton, D.J. and J.K. Sheehan, 2004. From mucins to mucus: toward a more coherent understanding of this essential barrier. Proc. Am. Thor. Soc., 1: 54-61.

10. Martinez-Antün, A., C. de Bolüs, M. Garrido, J. Roca-Ferrer, C. Barranco, A. Xaubet, C. Picado and J. Mullol, 2006. Mucin genes have different expression patterns in healthy and diseased upper airway mucosa. Clin. Exp. Allergy., 36: 448-457.

11. Tabak, L.A., 1995. In defense of the oral cavity: structure biosynthesis and function of salivary mucins. Annu. Rev. Physiol., 57: 547-564.

12. Yano, T., 1996. The non-specific immune system: humoral defense. Fish Physiol., 15: 105-157.

13. Alexander, J.B. and G.I. Ingram, 1992. Non-cellular non-specific defense mechanisms of fish. Annu. Rev. Fish Dis., pp: 249-279.

14. Chipman, D.M. and N. Sharon, 1969. Mechanism of lysozyme action. Science, 165: 454-465.

15. Grinde, B., 1989. Lysozyme from rainbow trout, *Salmo gairdneri* Richardson, as an antibacterial agent against fish pathogens. J. Fish Biol., 12: 95-104.

16. Ross, N.W., K.J. Firth, A. Wang, J.F. Burka and S.C. Johnson, 2000. Changes in hydrolytic enzyme activities of naïve Atlantic salmon *Salmo salar* skin mucus due to infection with the salmon louse *Lepeophtheirus salmonis* and cortisol implantation. Dis. Aquat. Org., 41: 43-51.

17. Iger, Y. and M. Abraham, 1994. The process of skin healing in experimentally wounded carp. J. Fish Biol., 36: 421-437.

18. Lowry, O.H., A.L. Rosebrough, R.J. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, 193: 265-27.

19. Ingram, G.I., 1980. Substances involved in the natural resistance of fish to infection-a review. J. Fish Biol., 16: 23-60.

20. Fouz, B., S. Devesa, K. Gravningen, J.L. Barja and A.E. Toranzo, 1990. Antibacterial action of the mucus of the turbot, Bull. Eur: Ass. Fish Pathol., 10: 56-59.

21. Fletcher, T.C. and A. White, 1973. Lysozyme activity in the plaice, *Pleuronectes plaessa* (L). Experientia, 29: 1283-1285.

22. Fänge, R., G. Lundblad and J. Lind, 1976. Lysozyme and chitinase in blood and lymphomyeloid tissues of marine fish. Mar. Biol., 36: 277-282.

23. Yamamoto, A. and T. Iida, 1995. Non-specific defense activities of triploid rainbow trout. Fish Pathol., 30: 107-110.

24. Balfry, S.K. and G.K. Iwama, 2004. Observation on the inherent variability of measuring lysozyme activity in coho salmon (*Oncorhynchus kisutch*). Comp. Biochem. Physiol., 138: 207-211.

25. Subramanian, S., S.L.M. MacKinnon and N.W. Ross, 2007. A comparative study on innate immune parameters in the epidermal mucus of various fish species. Comparative Biochemistry and Physiology, 148: 256-263.

26. Fernley, H.N., 1971. Mammalian alkaline phosphates the enzymes (edited by Boyer, P. D) 3rd, edn., 5: 417-445.

27. McComb, R.B., G.N. Boweras and S. Posen, 1979. Alkaline phosphates. Plenum Press, New York.

28. Kerlin, R.L. and S. Hughes, 1992. Enzymes in saliva from four parasitic arthropods. Med. Vet. Entomol., 6: 121-126.

29. Johnston, C.E., B.S. Horney, S. Deluca, A. MacKenzie, J.G. Eales and R. Angus, 1994. Changes in alkaline phosphatase isoenzyme activity in tissues and plasma of Atlantic salmon (*Salmo salar*) before and during smoltification and gonadal maturation. Fish Physiol. Biochem., 12: 485-497.