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RESEARCH ARTICLE

Comparative Immunological and Biochemical Properties of the Epidermal Mucus from Three Brackishwater Fishes

Prem Kumar[1,2] · T. Rajeshwaran[2] · P. Priya[2] · M. Kailasam[2] · G. Biswas[1] · T. K. Ghoshal[1] · K. K. Vijayan[2] · A. R. T. Arasu[2]

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Abstract Immunological and biochemical properties of skin mucus of different fish species may have mutual beneficial effects on health management when the fishes are farmed together in same system. Present experiment was conducted to investigate and compare the immunological and biochemical properties of epidermal mucus of three brackishwater fishes, namely *Lates calcarifer*, *Chanos chanos* and *Mugil cephalus*. Mucus was collected from the dorso-lateral surface of six individual fish of each species and used for analysis of immunological properties and biochemical composition. Innate immune parameters such as lysozyme, haemolytic, phagocytic activities and lectin were significantly ($p < 0.05$) more prominent in the mucus of *C. chanos* followed by that of *L. calcarifer* and *M. cephalus*. Similarly, mucus of *C. chanos* exhibited maximum protease, alkaline phosphatase, and antibacterial activities. UV spectral analysis showed the presence of toluene, isoquinoline, 2-furaldehyde, octadecenoic acid, biphenyl, thymidine, and cinnamic acid in the mucus of *L. calcarifer*, whereas these were absent in other two species. Fourier transform-infrared spectrum analysis revealed that isothiocyanate, aldehyde and alkene were common functional groups in the mucus of all three fishes. The results indicated that mucus of *C. chanos* has stronger innate immune properties as compared to that of other two fishes and therefore, polyculture of this fish with other fish or shrimp species may have beneficial effects for disease prevention.

Keywords Alkaline phosphatase · Innate immunity · Lysozyme · Fish mucus · Polyculture · Spectral · Protease · Haemolytic

Significance statement Finding of the study showed that the mucosal immunity of *C. chanos* is relatively stronger than that of *L. calcarifer* and *M. cephalus*, which will help in developing health management strategies in polyculture of milkfish with other fish or shrimp.

✉ Prem Kumar
prem.cife@gmail.com

[1] Kakdwip Research Centre of ICAR-Central Institute of Brackishwater Aquaculture (CIBA), South 24 Parganas, West Bengal 743 347, India

[2] ICAR-Central Institute of Brackishwater Aquaculture (CIBA), 75-Santhome High Road, R.A. Puram, Chennai 600028, India

Introduction

The mucus on skin of fish is secreted by epidermal goblet cells and composed of water and glycoproteins, which have a wide range of functions in disease resistance, respiration, osmotic regulation, reproduction, excretion, communication, feeding and nest building [1]. Epidermal mucus of fishes such as *Pollachius virens*, *Labrus berglta*, *Platichthys flesus*, *Solea solea* and *Scophthalmus rhombus* [2], *Cynoglossus arel* and *Arius caelatus* [3] has antibacterial, antifungal, and cytotoxic activities. Mucosal immunity which is a part of innate immune system in fish acts as a barrier against pathogen adherence through its continuous production and sloughing off. Moreover, mucus also contains several immune factors such as lysozyme, immunoglobulins, complement proteins, lectins, C-reactive protein, proteolytic enzymes and various antibacterial proteins and peptides [4]. Lysozyme, also referred to as *N*-acetylmuramide glycanohydrolase or muramidase, is a well-studied bacteriolytic enzyme identified in mucus of

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fish [5]. Alkaline phosphatase is a potential stress indicator enzyme present in epidermal mucus of fish [6]. It has a protective role in the initial stage of wound healing in carp (*Cyprinus carpio*) as an antibacterial agent because of its hydrolytic activity [7]. In brackishwater polyculture, shrimps with other fish species such as milkfish, *Chanos* [8], red tilapia [9], milkfish, *Mugil cephalus* [10] and mullet [11] are used. Polyculture is also practiced involving seabass with tilapia [12]. This culture system creates a conducive environment in earthen pond as fish can scavenge algae, excreta of shrimp, and uneaten feed and thereby, improve water quality and reduce the chance of disease outbreaks [13]. Moreover, occurrences of diseases in polyculture may be reduced due to mutual health benefits shared by the cultured species resulted from their mucosal immunity. Several studies have been conducted on beneficial effects of co-culturing fish with shrimp in ponds [14–16]; however, the scientific reason behind these beneficial effects has not been elucidated with factual information. There are limited reports on comparative immunological and biochemical properties of mucus of brackishwater fishes commonly used in polyculture. In this context, the present experiment was carried out to investigate and compare specific activities of enzymes (lysozyme, alkaline phosphatase, and proteases), important innate immune components, ionic composition and chemical constituents of the epidermal mucus of three commercially important brackishwater fishes, namely Asian seabass, *Lates calcarifer*, milkfish, *C. chanos* and grey mullet, *M. cephalus.*

Material and Methods

Experimental Fish

Healthy live 2-year old adult specimens of *L. calcarifer*, *C. chanos* and *M. cephalus* with mean weight of 1.60 ± 0.11, 1.50 ± 0.10 and 1.00 ± 0.12 kg, respectively were collected from the Fish Hatchery of ICAR-Central Institute of Brackishwater Aquaculture, Chennai, India. Physico-chemical parameters such as salinity, pH, dissolve oxygen, temperature and ammonia–nitrogen in water of fish holding tank were 25 ppt, 8.4, 5.8 ppm, 28 °C and 0.05 ppm, respectively.

Mucus Collection

A total of 18 fishes (six from each species; n = 6) were used for mucus collection. Mucus samples were collected following the method described by Schultz et al. [17]. In brief, the mucus was swabbed off from the dorso-lateral surface of fish using polyester sponge and then it was driven into a sterile test tube using a syringe. Then the collected mucus was centrifuged at 15,000 rpm for 15 min, and supernatant was collected and lyophilized. The lyophilized mucus sample was stored at − 4 °C for further analysis. The lyophilized mucus samples were re-suspended in phosphate buffer saline (PBS; pH 7.4) at 1 mg/mL to obtain aqueous extract.

Biochemical Composition

Proximate Composition

Protein, carbohydrate and lipid contents of mucus samples were estimated following the methods described by Lowry method, Dubois method and Folch method, respectively.

Ionic Composition (Na^+, Ca^{2+}, K^+)

The concentration of ions such as Na^+, Ca^{2+} and K^+ in mucus samples was estimated using Inductively Coupled Plasma Optical Emission Spectroscopy (Perkin Elmer Optima 5300 DV ICP-OES).

Fourier Transform-Infrared Spectrum Analysis (FT-IR)

FT-IR spectroscopy of lyophilized mucus samples was performed using Thermo Nicolet, Avatar 370 (Instrumentation laboratories, Anna University, Chennai, India). The mucus sample (10 mg) was mixed with 100 mg of dried potassium bromide (KBr) and compressed into a pellet for reading the spectrum in the range of 4000–400 cm^{-1}.

UV Spectral Analysis

The UV absorbance curve of mucus samples was determined by Shimadzu UV-1800 spectrophotometer (Japan) and recorded by scanning the wave length between 200 and 1000 nm. Maximum absorbance and peak reading were plotted using Graph pad PRISM 5.0 software.

Immunological Parameters

Haemolytic Activity

Haemolytic activity of mucus samples was assessed in the blood of chicken, sheep and human as described by Loganathan et al. [18]. The presence of uniform red colour suspension in the wells indicates haemolysis, whereas button formation at the bottom of the well reveals absence of haemolysis. The reciprocal of the highest dilution of mucus showing haemolysis was considered as one haemolytic unit which was divided by protein content of blood

samples to get the specific haemolytic unit. Protein content in blood samples was estimated by Lowry method.

Antibacterial Activity

Antibacterial activity of mucus extract was determined against a bacterial fish pathogen, *Vibrio parahaemolyticus* using the standard well diffusion method of Bauer et al. [19].

Phagocytosis Assay

Peripheral blood mononuclear cells (PBMC), containing monocytes, macrophages and neutophls were prepared in histopaque solution (Sigma, India). Ammonium chloride potassium (ACK) lysis buffer (ammonium chloride, 8024 mg/L; potassium bicarbonate, 1001 mg/L and EDTA.Na.H_2O, 3.722 mg/L) was used to lyse the erythrocytes. *Bacillus subtilis* bacterial suspension was prepared in Hank's balanced salt solution (HBSS) and blood serum of human (in the ratio of 4:1). Final cell suspension at the concentration 1×10^8 cells/mL was used for the assay. The phagocytic cells were covered with *B. subtilis* suspension on sterile glass slide at 37 °C for 15 min. The mucus sample was added to the suspension and incubated at 37 °C for 1 h, for phagocytosis. The smear was air dried, thereafter, fixed with methanol and stained with Giemsa. The phagocytosed cells were observed under light microscope and their number was counted. This number was taken as the phagocytic index (PI) and was compared with that of the test samples. Immunostimulation (%) was calculated using the following equation.

$$\text{Immunostimulation } (\%) = \{\text{PI (test)} - \text{PI (control)}\} \times 100/\text{PI}$$

Lectin Activity

Lectin activity in mucus was determined through the agglutination of red blood cells as described by Bing et al. [20]. A uniform distribution or clumping of red blood cells in wells was considered as positive haemagglutination and a button formation at the bottom of wells was considered as negative. The haemagglutination titre of lectin expressed as the reciprocal of the highest dilution exhibiting visible agglutination of erythrocytes was reckoned as one haemagglutination unit. Specific activity is the number of haemagglutination units per milligram (mg) protein.

Lysozyme Activity

Lysozyme activity of mucus was determined using the turbidometric method as described by Ross et al. [6] with a slight modification. One unit of activity was defined as the amount of enzyme that catalyzed a decrease in absorbance at 450 nm (1 unit of lysozyme activity = A 0.001 point decrease in OD value at 450 nm).

Protease Activity

Protease activity in mucus sample was determined through the casein hydrolysis assay as described by Carrie [21].

Alkaline Phosphatase Activity

Alkaline phosphatase activity of mucus was determined following the p-nitrophenol phosphate assay as described by Ross et al. [6]. It catalyzes hydrolysis of p-nitrophenol phosphate (pNPP) to p-nitrophenol. The pNPP is colourless, but p-nitrophenol has absorbance at 405 nm.

Ultrastructure and Elemental Composition of Scale

Scanning Electron Microscope (SEM)

Dried and cleaned scales were mounted on aluminium stubs with the help of a double adhesive tape, keeping the dorsal surface upward and ventral surface sticking to the tape, and then sputter coated with gold in gold coating unit (100 Å thicknesses). Then these scales were studied under scanning electron microscope (SEM) (JEOL JSM-6610LV, Instrumentation laboratories, Anna University, Chennai, India) at an accelerating current of 15/20 kV at low probe current.

Energy Dispersive X-Ray Micro-analysis (EDX)

Elemental compositions of scales were determined by energy dispersive X-ray microanalysis (EDX) by adjusting the scanner of INCAx–act analyzer (Instrumentation laboratories, Anna University, Chennai, India) on the area of interest of the scale. This scanner was attached to JEOL JSM-6610LV Scanning electron microscope.

Statistical Analysis

Mean values of all the tested variables were subjected to one way analysis of variance (ANOVA) and post hoc test followed by Duncan's multiple range tests to determine any difference among them. Comparisons were made at 5% probability level. All the statistical analyses were performed using SPSS for Windows v.17.0 programme (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Biochemical Composition of Mucus

Proximate and Ionic Composition

Mucus of all three species showed a significant variation in nutrient and ionic composition (Table 1). Mucus of fish is comprised of salts, free proteins, glycoproteins, lipids and water [1]. In the present study, it was observed that the mucus of milkfish had significantly ($p < 0.05$) higher lipid content and antimicrobial activity than that of other two species that indicate the role of mucosal lipid in innate immune system, which is similar to the report of Wel et al. [22].

Functional Groups and Organic Compounds

FT-IR spectrum of the mucus of all three species, seabass, milkfish and grey mullet revealed the characteristic functional groups that are depicted in Figs. 1, 2 and 3, respectively. *L. calcarifer* mucus contains aromatic primary amines N–H stretch in 3433 cm^{-1}, isothiocyanate R–N=C=S, aldehyde R–CHO stretch in 2059 cm^{-1}, alkenes C=C stretch in 1635 cm^{-1} and alkyl halide C–Br stretch in 534 cm^{-1}. Milkfish mucus sample has aromatic primary amines N–H stretch in 3453 cm^{-1}, isothiocyanate R–N=C=S, aldehyde R–CHO in 2057 cm^{-1}, alkenes C=C in 1636 cm^{-1} and alkyl halide C–Cl stretch in 553 cm^{-1}. Grey mullet mucus sample contains alcohol and phenol O–H in 3395 cm^{-1}, isothiocyanate R–N=C=S, aldehyde R–CHO in 2058 cm^{-1} and alkenes C=C in 1636 cm^{-1}. Infrared spectrum analysis showed that the isothiocynate (R–N=C=S), aldehyde (R–CHO) and alkene (C=C) were common functional groups in the mucus of all three species, whereas, aromatic primary amines (N–H) and alkyl halide were present in mucus of milkfish and Asian seabass, but absent in grey mullet. UV–Vis spectral analysis from 200 to 1000 nm indicates that the majority of the peaks were in between 200 and 400 nm. UV–Vis spectral

Table 1 Proximate, ionic (Ca^{+2}, Na^{-} and K^{+}) composition and immunological parameters of epidermal mucus of brackishwater fish, *Lates calcarifer*, *Chanos chanos* and *Mugil cephalus*

Parameters	*L. calcarifer*	*C. chanos*	*M. cephalus*
Proximate composition (mg/mL)			
Protein	$1.83^{a} \pm 0.01$	$1.47^{b} \pm 0.01$	$1.05^{c} \pm 0.01$
Carbohydrate	$0.86^{c} \pm 0.01$	$1.20^{a} \pm 0.01$	$0.93^{b} \pm 0.01$
Lipid	$24.07^{b} \pm 1.63$	$33.80^{a} \pm 1.40$	$8.73^{c} \pm 0.83$
Ionic composition (mg/mL)			
Ca^{+2}	$0.36^{a} \pm 0.01$	$0.23^{b} \pm 0.01$	$0.36^{a} \pm 0.01$
Na^{+}	$858.20^{b} \pm 0.12$	$557^{c} \pm 0.29$	$868.50^{a} \pm 0.17$
K^{+}	$0.33^{b} \pm 0.01$	$0.21^{c} \pm 0.01$	$0.35^{a} \pm 0.01$
Specific haemolytic activity			
Chicken blood	$0.90^{b} \pm 0.00231$	$7.24^{a} \pm 0.012$	$0.11^{c} \pm 0.0056$
Sheep blood	$0.45^{b} \pm 0.0029$	$3.61^{a} \pm 0.017$	$0.22^{c} \pm 0.012$
Human blood	0.00	$0.44^{a} \pm 0.023$	0.00
Antibacterial activity (zone of inhibition in mm)			
V. Parahaemolyticus	$8.33^{b} \pm 0.33$	$10.33^{a} \pm 0.88$	$5.33^{b} \pm 2.66$
Phagocytosis immunostimulation (%)			
PBMC	$15.38^{b} \pm 3.0$	$35.90^{a} \pm 2.5$	$17.95^{b} \pm 2.6$
Neutrophil	20 ± 0.01	20 ± 0.01	20 ± 0.01
Lectin specific activity			
Chicken blood	$0.45^{b} \pm 0.01$	$3.62^{a} \pm 0.01$	$0.11^{c} \pm 0.01$
Sheep blood	$0.22^{b} \pm 0.01$	$0.90^{a} \pm 0.01$	0.00
Human blood	0.00	$0.43^{a} \pm 0.01$	0.00
Enzyme activities			
Lysozyme activity (U/mg protein)	$83.30^{b} \pm 1.76$	$168.30^{a} \pm 0.33$	$65^{b} \pm 1.52$
Protease activity (U/mg protein)	$0.38^{b} \pm 0.01$	$0.90^{a} \pm 0.09$	$0.31^{c} \pm 0.03$
Alkaline phosphatase (U/mg protein)	$2.46^{c} \pm 0.09$	$5.8^{a} \pm 0.12$	$4.28^{b} \pm 0.01$

The values are expressed as mean ± SE and different superscripts in a row indicate significantly different values ($n = 6$, $p < 0.05$)

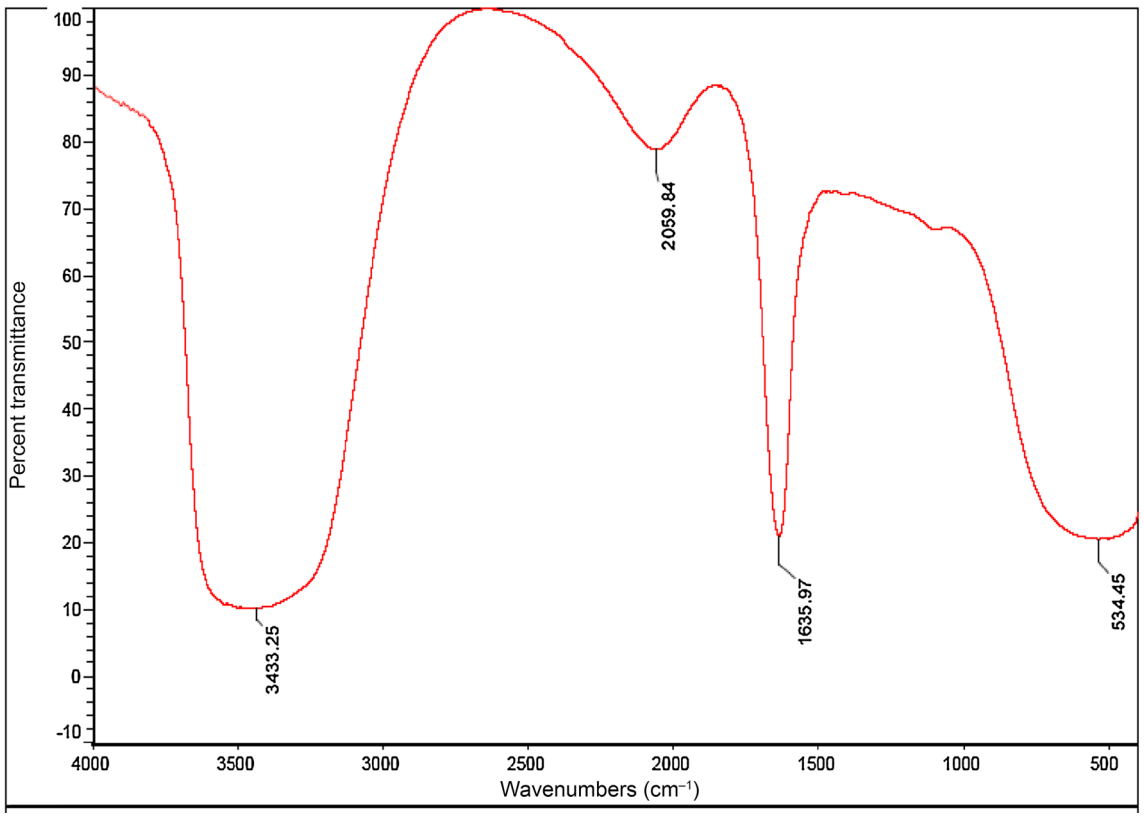

Fig. 1 FTIR analysis of seabass, *Lates calcarifer* epidermal mucus

analysis also showed that the mucus of *L. calcarifer* contains toluene, isoquinoline, 2-furaldehyde, octadecenoic acid, biphenyl, thymidine, cinnamic acid and other organic compounds (Fig. 4). Mucus of milkfish has benzene, bromobenzene, benzaldehyde, acetophenone and uridine, furaldehyde (Fig. 5). Mucus of grey mullet contains benzene, bromobenzene, acetylthiophene, guanosine, 2-picoline, cytidine, 1-3 butadiene and acetophenone (Fig. 6). In this study, mucus of all the three fishes exhibited more than one peak in spectral analysis which is comparable to the finding of Jill et al. [23]. The absorbance of all the mucus samples was high in this range, which might be due to the presence of structural components such as nucleic acid, proteins and other organic compounds as reported by Douglas and Hawryshyn [24]. Herbivorous fishes viz. *C. chanos* and *M. cephalus* have few common organic compounds such as benzene, bromobenzene, and acetophenone that are absent in *L. calcarifer*.

Innate Immune Components of Mucus

Haemolytic Activity

Significantly higher ($p < 0.05$) haemolytic activity was noticed in the epidermal mucus of *C. chanos* followed by that of *L. calcarifer* and *M. cephalus* in blood of chicken (7.24 ± 0.012), sheep (3.61 ± 0.017) and human (0.44 ± 0.023) (Table 1). This haemolysis would have caused by proteinous substance and antimicrobial agents [25] present in the mucus of fishes.

Antibacterial Activity

Although, antimicrobial activity of mucus has been demonstrated in several fish species, it seems to vary from one fish species to another and can be specific towards certain bacteria [26]. In the present study, a significantly higher antimicrobial activity against *V. parahaemolyticus* was caused by mucus of *C. chanos*, which might be due to relatively higher activity of mucus enzymes such as lysozyme, alkaline phosphatase and proteases (Table 1).

Phagocytosis Evaluation

Phagocytic stimulation of macrophage and neutrophil to kill *Bacillus subtilis* by epidermal mucus is given in Table 1. The mucus of milkfish showed a higher percentage of phagocytosis (35.90 ± 2.5) by macrophage followed by grey mullet (17.95 ± 2.6) and seabass

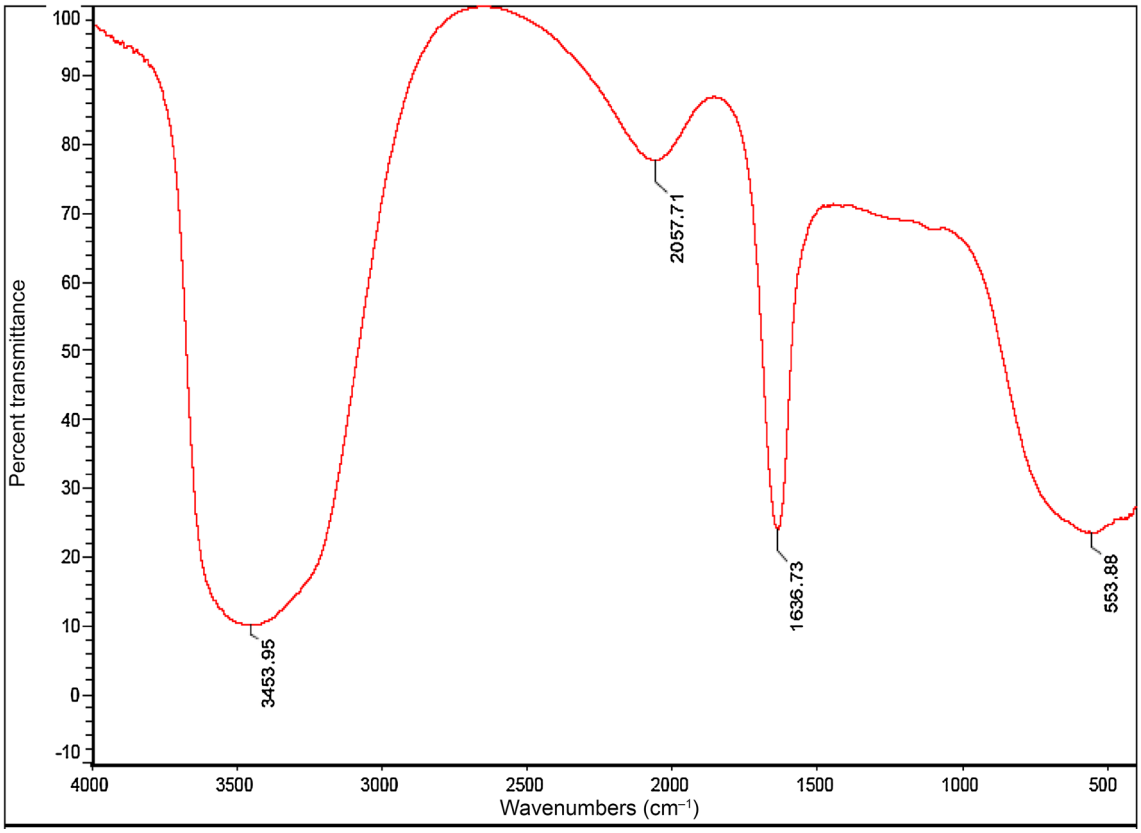

Fig. 2 FTIR analysis of milkfish, *Chanis chanos* epidermal mucus

(15.38 ± 3.0). However, phagocytic stimulation of neutrophil was similar in all the three species.

Lectin Activity

Lectins are elements of the innate immune system and they exhibit affinity towards carbohydrate moieties, as well as cell agglutination and/or precipitation of glycoconjugates. Due to these properties lectin is a potential antimicrobial factor in the skin mucus [27]. There was a significant difference ($p < 0.05$) in lectin activity of brackishwater fish mucus in blood of chicken, sheep and human (Table 1). Highest lectin activity by mucus of milkfish in blood of chicken, sheep and human indicates that mucus of this fish possesses maximum antimicrobial activity as compared to other two species.

Enzyme Activities (Lysozyme, Protease, Alkaline Phosphatase)

Acid and alkaline phosphatases are important lysosomal enzymes, associated with the innate immune system of fishes [28]. Mucus proteases 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 sloughing off mucus and thereby removal of pathogens from body surfaces [29]. Proteases also activate and increase production of other innate immune components present in fish mucus such as complement, immunoglobulins, or antibacterial peptides [30]. Significantly higher ($p < 0.05$) lysozyme (168.30 ± 0.33 U/mg protein), protease (0.90 ± 0.09 U/mg protein) and alkaline phosphatase (5.8 ± 0.12 U/mg protein) activities were noticed in the epidermal mucus of *C. chanos*, followed by that of *L. calcarifer* and *M. cephalus* (Table 1) indicating their probable direct effects on the innate immune response to pathogenic organisms.

Scanning Electron Microscopy (SEM) of Scale and SEM–EDX

Scanning electron microscopy of *L. calcarifer*, *C. chanos* and *M. cephalus* scale is shown in Figs. 7, 8 and 9, respectively. The scales of Asian seabass and grey mullet were ctenoid type, whereas scales of *C. chanos* were cycloid (circular). Ctenoid scales have a rough texture due to the presence of teeth called ctenii on outer edge. Cycloid scales have a smooth texture and are uniform, with a smooth outer edge or margin. In the present study, number of ctenii was more in *L. calcarifer* scale than

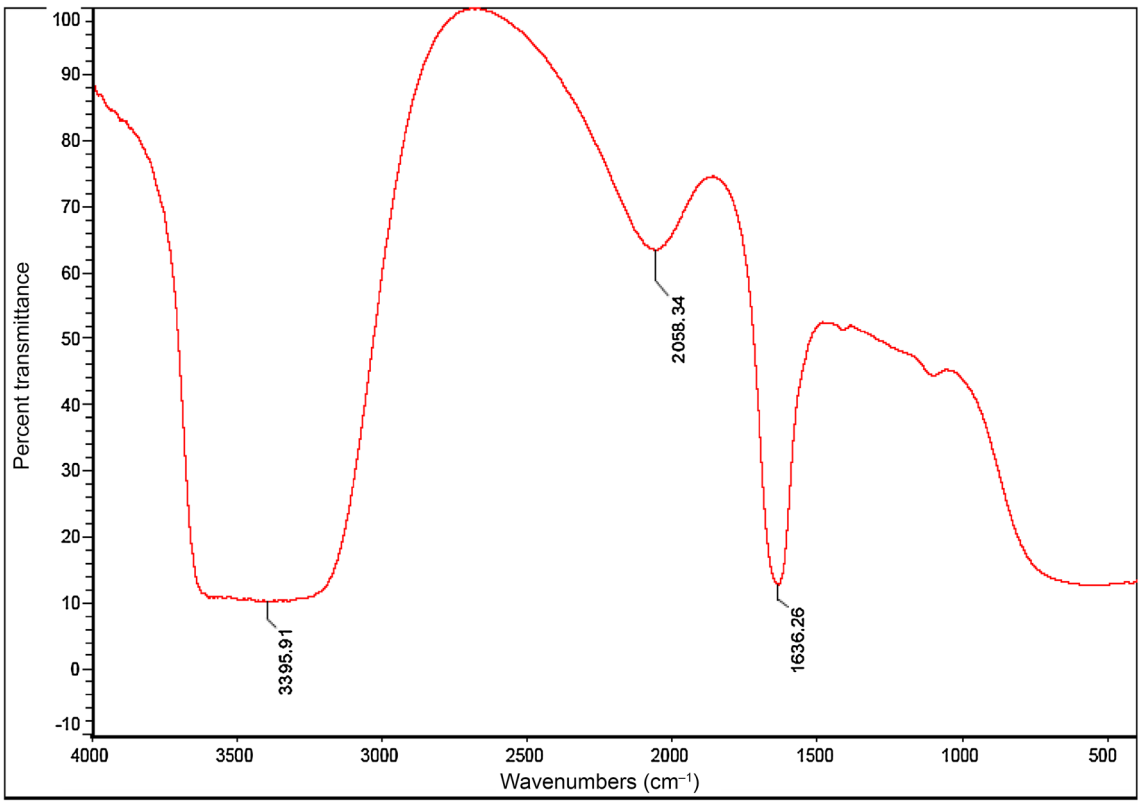

Fig. 3 FTIR analysis of mullet, *Mugil cephalus* epidermal mucus

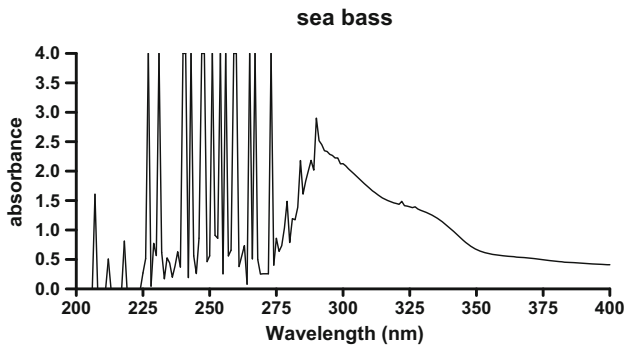

Fig. 4 Showing UV spectral analysis (100–1000 nm) of *Lates calcarifer* mucus having majority of the peaks between 200 and 400 nm

those in *M. cephalus*. Elemental composition of C, O, Na, P and Ca were 18, 15, 1.8, 22 and 40 wt%, respectively in *L. calcarifer*. Elemental composition of C, O, P and Ca were 2.3, 5, 28, and 62 wt%, respectively in *C. chanos*. Sodium was not detected in the scale of *C. chanos*. Elemental composition of C, O, Na, P and Ca were 15.2, 14.6, 2.8, 27 and 40 wt%, respectively in *M. cephalus*. Significantly higher calcium and, lower carbon and oxygen levels were noticed in scale of *C. chanos*, however sodium was not detected.

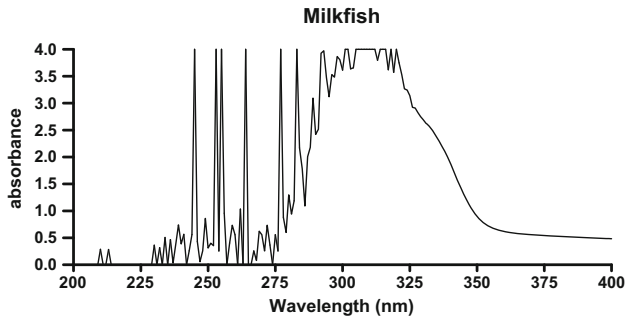

Fig. 5 Showing UV spectral analysis (100–1000 nm) of *Chanos chanos* mucus having majority of the peaks between 200 and 400 nm

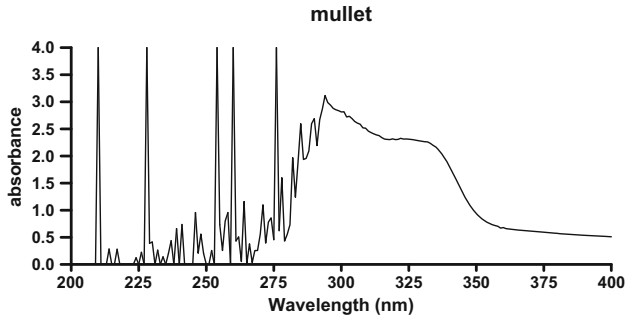

Fig. 6 Showing UV spectral analysis (100–1000 nm) of *Mugil cephalus* mucus having majority of the peaks between 200 and 400 nm

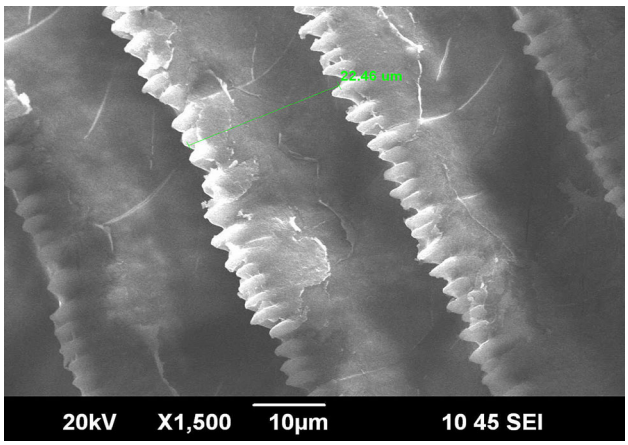

Fig. 7 Scanning electron micrograph of *L. calcarifer* scale that indicates ctenoid type scales with many ctenii

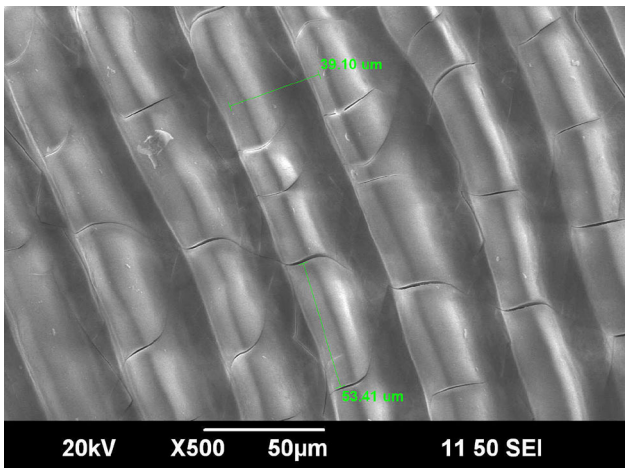

Fig. 8 Scanning electron micrograph of *Chanos chanos* scale that indicates cycloid type scales without ctenii

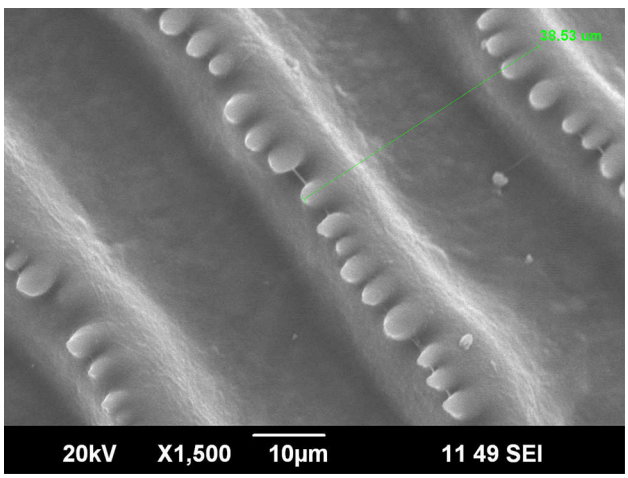

Fig. 9 Scanning electron micrograph of *Mugil cephalus* scale that indicates ctenoid type scales with few ctenii

Conclusion

Innate immune components such as lysozyme, haemolytic, phagocytic activities and lectin have relatively better mucosal immune response in *C. chanos* as compared to that of *L. calcarifer* and *M. cephalus*. *C. chanos* mucus also showed significantly higher protease, alkaline phosphatase, and antibacterial activities than other two species. This finding will help in developing disease prevention strategies in polyculture involving milkfish and other fish or shrimp species.

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Compliance with Ethical Standards

Conflict of interest There is no conflict among the authors regarding the research data presented in the paper.

References

1. Salinas I (2015) The mucosal immune system of teleost fish. Biology 4:525–539
2. Hellio C, Pons AM, Beaupoil C, Bourgougnon N, Gal YL (2002) Antibacterial, antifungal and cytotoxic activities of extracts from fish epidermis and epidermal mucus. Int J Antimicrob Agents 20(3):214–219
3. Bragadeeswaran S, Thangaraj S (2011) Haemolytic and antibacterial studies on skin mucus of eel fish, *Anguilla linnaues* 1758. Asian J Biol Sci 4(3):272–276
4. Cole AM, Weis P, Diamond G (1997) Isolation and characterization of pleurocidin, antimicrobial peptides in the skin secretions of winter flounder. J Biol Chem 272:12008–12013
5. Alexander JB, Ingram GA (1992) Noncellular nonspecific defence mechanisms of fish. Annu Rev Fish Dis 2:249–279
6. Ross NW, Firth KJ, Wang A, Burka JF, Johnson SC (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 Organ 41(1):43–51
7. Iger Y, Abraham M (1997) Rodlet cells in the epidermis of fish exposed to stressors. Tissue Cell 29(4):431–438
8. Eldani A, Primavera JH (1981) Effect of different stocking combinations on growth, production and survival of milkfish (*Chanos chanos* Forskal) and prawn *(Penaeus monodon* Fab.) in polyculture in brackishwater ponds. Aquaculture 23:59–72
9. Yuan D, Yi Y, Yakupitiyage A, Fitzimmons K, Diana JS (2010) Effects of addition of red tilapia (*Oreochromis* spp.) at different densities and sizes on production, water quality and nutrient recovery of intensive culture of white shrimp (*Litopenaeus vannamei*) in cement tanks. Aquaculture 298:226–238
10. Biswas G, Ananda Raja R, De D, Sundaray JK, Ghoshal TK, Anand S, Kumar S, Panigrahi A, Thirunavukkarasu AR, Ponniah AG (2012) Evaluation of productions and economic returns from two brackishwater polyculture systems in tide-fed ponds. J Appl Ichthyol 28:116–122
11. Aghuzbeni SHH, Hajirezaee Saeed, Matinfar Abbas, Khara Hossein, Ghobadi Mahmoud (2017) A preliminary study on

polyculture of western white shrimp (*Litopenaeus vannamei*) with mullet (*Mugil cephalus*): an assessment of water quality, growth parameters, feed intake efficiency and survival. J Appl Anim Res 45(1):247–251
12. Monwar Md, Mostafa Ruhul Amin, Sarker AKM, Das Nani Gopal (2013) Polyculture of seabass with tilapia for the utilization of brown fields in the coastal areas of Cox's Bazar. Bangladesh 5(6):104–109
13. Martinez-Porchas M, Martinez-Cordova LR, Porchas-Cornejo MA, EliaJA Lopez (2010) Shrimp polyculture: apotentially profitable, sustainable, but uncommon aquacultural practice. Rev Aquac 2:73–85
14. Cruz PS, Andalecio MN, Bolivar RB, Fitzsimmons K (2008) Tilapia–Shrimp polyculture in Negros Island, Philippines: a review. J World Aquac Soc 39:713–725
15. Priyadarsani L, Abraham TJ (2013) Ecology of antibiotic resistant vibrios in traditional shrimp farming system (bhery) of West Bengal, India. J Coast Life Med 1(4):265–272
16. Wibowo AM, Maftuch Fadjar (2015) Utilization of Tilapia Mucus to inhibit Vibrio harveyi on Vannamei (*Litopenaeus vannamei*). J Life Sci Biomed 5(5):141–148
17. Schultz DR, Perez N, Mendez AJ, Snodgrass D, Serafy JE, Prince ED, Crow WA, Capo TR (2007) Tracking gender factors in fish surface mucus: temporal patterns in individual koi (*Cyprinus carpio*). J Appl Ichthyol 23:184–188
18. Loganathan K, Arulprakash A, Prakash M, Senthilraja P (2013) Lysozyme, protease, alkaline phosphatase and esterase activity of epidermal skin mucus of freshwater snake head fish *Channa striatus*. Int J Res Pharm Biosci 3(1):17–20
19. Bauer AW, Kirby WMM, Sherris JC, Turck M (1996) Antibiotic susceptibility testing by a nstandardized single disc method. Am J Clin Pathol 45:493–496
20. Bing D, Weyand J, Satvitsky A (1967) Hemagglutination with aldehyde-fixed erythrocytes for assay of antigens and antibodies. Proc Soc Exp Biol Med 1244:1166–1170
21. Cupp-Enyard Carrie (2008) Sigma's Non-specific protease activity assay—casein as a substrate. JOVE 18:889
22. Wel OY, Xavier R, Marimuthu K (2010) Screening of antibacterial activity of mucus extract of Snakehead fish, *Channa striatus* (Bloch). Eur Rev Med Pharmacol Sci 14:675–681
23. Jill P, Losey Zamzow GS (2002) Ultraviolet radiation absorbance by coral reef fish mucus: photo-protection and visual communication. Environ Biol Fishes 63:41–47
24. Douglas RH, Hawryshyn CW (1990) Behavioral studies of fish vision: an analysis of visual capabilities. In: Douglas RH, Djamgoz MBA (eds) The visual system of fish. Chapman and Hall, London, pp 373–418
25. Hellio C, Bremer G, Pons AM, Le Gal Y, Bour Gougnon N (2005) Inhibition of the development of microorganisms (bacteria and fungi) by extracts of marine algae from Brittany (France). Appl Microbiol Biotechnol 54:543–549
26. Noga M, Magarinos B, Toranzo AE, Lamas J (1995) Sequential pathology of experimental pasteurellosis in gilthead seabream *Sparus aurata* a light microscopic and electron microscopic study. Dis Aquat Organ 21:177–186
27. Matsushita M, Matsushita A, Endo Y, Nkata M, Kojima N, Fujita Mizuochi T (2004) Origin of the classical complement pathway: lamprey orthologue of mammalian C1q acts as a lectin. Proc Natl Acad Sci USA 101(27):10127–10131
28. Nigam AK, Kumari U, Mittal S, Mittal AK (2012) Comparative analysis of innate immune parameters of the skin mucous secretions from certain freshwater teleosts, inhabiting different ecological niches. Fish Physiol Biochem 38(5):1245–1256
29. Aranishi F, Mano N, Hirose H (1998) Fluorescence localization of epidermal cathepsins L and B in the Japanese eel. Fish Physiol Biochem 19(3):205–209
30. Cho JH, Park IY, Kim HS, Lee WT, Kim MS, Kim SC (2002) Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. FASEB J 16(3):429–431