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Comparative analysis of the humoral immunity of skin mucus from several marine teleost fish

Q1 Francisco A. Guardiola [a], Alberto Cuesta [a], Emilia Abellán [b], José Meseguer [a], María A. Esteban [a, *]

[a] Department of Cell Biology and Histology, Faculty of Biology, Campus Regional de Excelencia Internacional "Campus Mare Nostrum", University of Murcia, 30100 Murcia, Spain

[b] Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO), Carretera de la Azohía s/n, Puerto de Mazarrón, 30860 Murcia, Spain

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ABSTRACT

Fish skin mucus contains several immune substances that provide the first line of defence against a broad spectrum of pathogens although they are poorly studied to date. Terminal carbohydrate composition and levels of total IgM antibodies, several immune-related enzymes (lysozyme, peroxidase, alkaline phosphatase, esterases, proteases and antiproteases) as well as the bactericidal activity (against fish pathogenic *Vibrio harveyi*, *Vibrio angillarum*, *Photobacterium damselae* and non-pathogenic bacteria *Escherichia coli*, *Bacillus subtilis*, *Shewanella putrefaciens*) were identified and measured in the skin mucus of five marine teleosts: gilthead seabream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), shi drum (*Umbrina cirrosa*), common dentex (*Dentex dentex*) and dusky grouper (*Epinephelus marginatus*). First, lectin binding results suggests that skin mucus contain, in order of abundance, N-acetylneuraminic acid, glucose, N-acetyl-glucosamine, N-acetyl-galactosamine, galactose and fucose residues. Second, results showed that while some immune activities were very similar in the studied fish (e.g. IgM and lysozyme activity) other such as protease, antiprotease, alkaline phosphatase, esterase and peroxidase activities varied depending on the fish species. High levels of peroxidase and protease activity were found in *U. cirrosa* respect to the values obtained in the other species while *E. marginatus* and *S. aurata* showed the highest levels of alkaline phosphatase and esterase activities, respectively. Moreover, skin mucus of *S. aurata* revealed higher bactericidal activity against pathogenic bacteria, contrarily, to what happened with non-pathogenic bacteria (*E. coli*, *B. subtilis*). Thus, study of the variations in the carbohydrate profile and immune-related components of the fish skin mucus could help to understand the fish resistance as well as the presence and distribution of pathogens and magnitude of infections, aspects that are of major importance for the aquaculture industry.

1. Introduction

MALT (mucosa-associated lymphoid tissue) constitutes a very large area for the possible invasion of pathogens and contains defence mechanisms (both innate and adaptive) that constitute the first line of defence against a broad spectrum of pathogens present in the environment. In the case of fish, MALT is present in skin, gill and gastrointestinal tract but its composition and functional characterization has received little research interest till recent years [1]. As part of this MALT, fish skin plays a critical role in the defence mechanisms acting as the first biological barrier [2–5]. The external constituent of this barrier is a mucous gel that forms a layer of adherent mucus covering the epithelial cells (living cells) [6] and is secreted by various epidermal or epithelial mucus cells such as goblet cells [7,8]. This mucus acts as a natural, physical, biochemical, dynamic, and semipermeable barrier that allows the exchange of nutrients, water, gases, odorants, hormones, and gametes [9]. The skin mucus is mainly composed of water and glycoproteins [10,11], containing a large content of high–molecular-weight oligosaccharides, and called mucins [12–16]. Among its functions, skin mucus is involved in fish respiration, osmoregulation, reproduction, locomotion, defence against microbial infections, disease resistance and protection, excretion or communication [7,17]. Perhaps, one of the most interesting and known functions has been its relation with the immune response and disease resistance but deeper characterization is awaiting.

* Corresponding author. Tel.: +34 868887665; fax: +34 868883963.
E-mail address: aesteban@um.es (M.A. Esteban).

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The immunological or protective function of epidermal mucus is the result of its mechanical and biochemical properties. Epidermal mucus is continuously replaced and the its thickness and composition prevents the pathogen adherence to the underlying tissues and provides a medium in which antibacterial mechanisms may act [18–20]. At this respect, mucin carbohydrates may act as microorganism receptors playing a decisive role in either pathogen expulsion or settlement and invasion [21,22]. Secondly, fish epidermal mucus serves as a repository of numerous innate immune components of such as glycoproteins, lysozyme, complement proteins, lectins, C-reactive protein, flavoenzymes, proteolytic enzymes and antimicrobial peptides as well as immunoglobulins (IgM and IgT) [7,20,23–25] which exert inhibitory or lytic activity against different type of pathogens [4,26]. Among them, the most characterized ones are lysozyme and proteases. First, lysozyme is likely the most powerful bacteriolytic protein since it has the ability to cleave the bacterial peptidoglycan. Its bacteriolytic activity in fish epidermal mucus and other tissues contributes to host defence against bacterial infections [2,27–30]. Moreover, lysozyme activity in the mucus greatly varied among the fish species studied and could reflect the differential fish resistance to bacterial pathogens or the bacterial abundance/diversity in the fish environments [2,20,31]. Second, fish mucus also contains a variety of proteases which have a significant role in the innate immune mechanisms by hampering pathogen invasion and viability [11,20]. Added to this, they also activate and enhance the production of various immunological components such as complement, immunoglobulins and antimicrobial peptides [32–35]. Lastly, other innate immune-related molecules present in fish skin mucus such as esterases, phosphatases or peroxidases have received less attention. Few works have shown great variability of these immune parameters in different fish species [2,20,25,31]. Thus, we have already demonstrated the presence of IgM, lysozyme, protease, peroxidase, esterase, alkaline phosphatase, antiprotease and bactericidal activities in gilthead seabream [25]. However, available data seem to indicate that there is no relationship between skin mucus immunity and fresh/marine fish or water cleanness. This needs further investigation at deeper level but also with the study of more fish species.

Taking in consideration the importance of the skin mucus in fish immunity and the poor characterization of the immune molecules present in it we carried out this work. Thus, we aimed to identify, measure and compare the terminal carbohydrate profile and some of the main innate immune parameters (lysozyme, protease, antiprotease, alkaline phosphatase, esterase, peroxidase and bactericidal activities) in the skin mucus of 5 marine fish species: gilthead seabream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), shi drum (*Umbrina cirrosa*), common dentex (*Dentex dentex*) and dusky grouper (*Epinephelus marginatus*). This information will help to understand the mucosal immunity in marine fish and the importance it may have in several aquaculture-relevant marine species.

2. Materials and methods

2.1. Animals

Thirty adult specimens of each one of the following species were sampled: gilthead seabream (*Sparus aurata*) (125 ± 25 g body weight), European sea bass (*Dicentrarchus labrax*) (100 ± 18 g body weight), shi drum (*Umbrina cirrosa*) (565.5 ± 51 g body weight), common dentex (*Dentex dentex*) (1600 ± 210 g body weight) and dusky grouper (*Epinephelus marginatus*) (803 ± 106 g body weight). All fish species were bred and kept at the *Instituto Español de Oceanografía* (IEO, Mazarrón, Spain) facilities except the groupers that were caught at the juvenile stage from the wild and reared in the same facilities for more than 3 years. The fish were kept in 2 m^3 tanks with a flow-through circuit (density 5–10 kg biomass m^{-3}), suitable aeration, filtration system, natural photoperiod and water temperature (14.6–17.8 °C). All the rearing conditions were the same for all species and fish were sampled at the same time (June 2013) to avoid changes due to different salinity, temperature, handling, feeding, photoperiod, etc. The environmental parameters, mortality and food intake were recorded daily.

2.2. Skin mucus collection

Fish were anesthetized prior to sampling with 100 mg l^{-1} MS222 (Sandoz). Skin mucus samples were collected according to the method of Palaksha et al. [30] with some modifications. Briefly, skin mucus was collected by gentle scraping the dorso-lateral surface of naïve five specimens using a cell scraper with enough care to avoid contamination with blood and/or urino-genital and intestinal excretions. In order to get sufficient mucus to all the assays, equal samples of mucus were pooled (3 pools of 10 fish each) and homogenized with 1 volume of Tris-buffered saline (TBS, 50 mM Tris–HCl, 150 mM NaCl, pH 8.0). The homogenates were vigorously shaken and centrifuged (500 g, 10 min, 4 °C) being the supernatant lyophilized following freezing at −80 °C. Lyophilized skin mucus powder was dissolved in Milli-Q water, being the undissolved mucus portion isolated by centrifugation (500 g, 10 min, 4 °C). Protein concentration in each sample was determined by the Bradford method (1976) and skin mucus samples were adjusted to 500 μg protein ml^{-1}. Samples were then aliquoted and stored at −20 °C until use.

2.3. Determination of the terminal glycosylation pattern

Glycosylation pattern in the skin mucus was determined by lectin ELISA as described previously [36]. Thus, 10 μg $well^{-1}$ of skin mucus samples were placed in flat-bottomed 96-well plates in triplicate and coated overnight at 4 °C with the use of 50 mM carbonate-bicarbonate buffer, pH 9.6. Samples were rinsed 3 times with PBS-T (20 mM phosphate buffer (PBS) and 0.05% Tween 20, pH 7.3), blocked for 2 h at room temperature with blocking buffer (3% BSA in PBS-T) and rinsed again. Samples were then incubated for 1 h with 20 μg per well of biotinylated lectins (Table 1), washed and incubated with streptavidin horseradish-peroxidase (1:1000; Life Technologies) for 1 h. After exhaustive rinsing with PBS-T the samples were developed using 100 μl of a 0.42 mM solution of 3,3',5,5'- tetramethylbenzidine hydrochloride (TMB, Sigma), prepared daily in a 100 mM citric acid/sodium acetate buffer (pH 5.4) containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min, stopped by the addition of 50 μl of 2 M H_2SO_4 and the plates read at 450 nm in a plate reader (FLUOstar Omega, BMG Labtech). Negative controls consisted of samples without skin mucus or

Table 1
Lectins used in ELISA, their acronym, and sugar binding.

Acronym	Lectin source	Sugar binding specificity
BSL I	*Bandeiraea simplicifolia*	α-D-galactose, N-acetyl-α-D-galactosamine
PNA	*Arachis hypogaea*	β-D-galactose
UEA I	*Ulex europeaus*	α-L-Fucose
Con A	*Canavalia ensiformis*	α-D-mannose, α-D-glucose
WFA	*Wisteria floribunda*	N-acetyl-D-galactosamine
WGA	*Triticum vulgaris*	N-acetyl-β-D-glucosamine, N-acetylneuraminic acid
LEA	*Lycopersicon esculentum*	N-acetyl-β-D-glucosamine

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