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Changes in the levels of humoral immune activities after storage of gilthead seabream (*Sparus aurata*) skin mucus

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ABSTRACT

Skin mucus is increasingly used as a source for determining immunity-related proteins and enzymes. However, the ability to accurately measure some activities may be modified by inadequate handling and storage of the samples. This study aims to measure the effect of freezing and lyophilization at the time of collection on such activities. Fresh, frozen (immediately after collection at −20 °C and −80 °C) and lyophilized skin mucus samples obtained from the same groups of fish specimens of gilthead seabream (*Sparus aurata* L.) were analysed in the assays. The amount of total proteins and sugar residues (determined by lectin binding) present in skin mucus samples fell after both freezing and lyophilization of the samples. While no significant differences were exhibited in the levels of some proteins or enzymes (immunoglobulin M, antiprotease, peroxidase, esterase and alkaline phosphatase) determined in fresh or frozen mucus samples, protease and lysozyme activities were lower in frozen mucus samples than in fresh samples. Lyophilization of the mucus samples drastically decreased the total level of proteins obtained, as well as of protease, peroxidase, lysozyme and alkaline phosphatase activities. The results suggest that freezing skin mucus samples is more suitable than lyophilization if samples are stored before determining enzymatic activities.

1. Introduction

Skin mucus has become a hot topic in recent years. According to the NCBI database (http://www.ncbi.nlm.nih.gov/), "skin mucus of fish" has 425 entries in May 2016, of which more than half (228 entries) are from 2006 onward. The importance of skin mucus arises from the fact that it is the first barrier of defense, protecting the skin against external aggressions, including those from viruses and bacteria present in aquatic environments [1,2].

Skin mucus is studied for its capacity to adhere to fish pathogens [3–5] and also as a valuable source for the purification and/or identification of several immune molecules such as antimicrobial peptides [6–8], lectins [9,10] and immunoglobulins (Ig) [11,12]. Beside the recent proteomic mapping of skin mucus to find new molecules which may be involved in mucosal immunity [13–15], most available research results concern the changes that take place in humoral immune activities, such as IgM, lysozyme or alkaline phosphatase in the skin mucus of multiple fish species [16,17]. Furthermore, changes in these and other humoral immune activities are typically studied in relation with immunostimulants and/or host resistance to infection [18–25].

There is also a considerable amount of information available on how humoral immune activities are influenced by seasonal variations such as photoperiod and/or temperature [26–31]. But curiously there are no studies on the effects of storage conditions on the humoral immune activities of fish skin mucus. The aim of this paper therefore was to study the changes that take place in different humoral activities (including total protein levels, specific lectin binding, IgM, protease, antiprotease, peroxidase, lysozyme, esterase and alkaline phosphatase) in gilthead seabream (*Sparus aurata*) skin mucus after storage following freezing or lyophilization.

2. Materials and methods

2.1. Animal maintenance

Sixty specimens of the hermaphroditic protandrous teleost gilthead seabream (*S. aurata* L.) with 88.4 ± 10.2 g of weight were obtained from a local farm in Murcia (Spain). Fish were kept in re-

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circulating seawater aquaria (500 l) with a flow rate of 900 l h^{-1} in the Marine Fish Facility at the University of Murcia. The temperature and salinity were 22 ± 2 °C and 28‰, respectively and a photoperiod of 12 h light:12 h dark was followed. A commercial diet (Optibream D4, Skretting) was administered at a rate of 2% body weight day^{-1}. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.2. Mucus collection

Fish were anesthetized prior to sampling with 10 mg l^{-1} MS222 (Sandoz). Skin mucus was gently collected with a cell scraper (Sigma–Aldrich) from the skin surface, avoiding blood, urine and feces during collection [32].

2.3. Experimental design

Three pooled samples from 20 different fish each pool were used in this study in order to get enough skin mucus to carry out all the test. Each pool (with around 10 ml of obtained mucus) was later divided into four groups: fresh mucus, which was analysed immediately, mucus frozen at −20 °C, mucus frozen at −80 °C and lyophilized mucus. Frozen and lyophilized mucus samples were stored for one month before being analysed. For the analysis of lyophilized samples, they were first dissolved in Milli-Q water by vortexing for 10 min before the supernatant was used.

2.4. Total protein levels

The total protein concentration was estimated using the Coomassie Brilliant Blue G-250 method [33] with Bradford reagent (Sigma–Aldrich). Briefly, 5 μl of skin mucus samples in triplicate were incubated with 250 μl of Bradford reagent in flat-bottomed 96-well plates (Nunc), and similar volumes of bovine serum albumin (BSA, Sigma–Aldrich) serial dilutions were used as standard. After incubating for 10 min at room temperature and in darkness, the absorbance was read in a plate reader (BMG, Fluostar Omega) at 550 nm. The total protein concentration was expressed as mg ml^{-1}.

2.5. Specific lectin binding levels

Specific lectin binding to skin mucus was determined by lectin ELISA. Briefly, skin mucus was dissolved 1:4 in 50 mM carbonate-bicarbonate buffer (pH 9.6) and mucus samples were placed in flat-bottomed 96-well plates in triplicate and coated before leaving overnight at 4 °C. Samples were rinsed three times with phosphate buffered saline (PBS; Sigma–Aldrich) containing 0.05% Tween 20 (PBT, pH 7.3), blocked for 2 h at room temperature with blocking buffer (3% BSA in PBT) and rinsed again. Next, samples were incubated for 1 h with 2 μg per well of biotinylated lectin (Table 1), washed and incubated with streptavidin-HRP (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 (TMB, Sigma–Aldrich), prepared daily in Milli-Q water 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 were read at 450 nm in a plate reader. Negative controls consisted of samples without skin mucus or without lectins, whose optical density (OD) values were subtracted from each sample value.

2.6. Total immunoglobulin M levels

Total skin mucus IgM levels were analysed using the ELISA method [34] with some modifications. Thus, 100 μl per well of 1:4 fold diluted mucus were placed in flat-bottomed 96-well plates and the proteins were coated by overnight incubation at 4 °C with 200 μl carbonate-bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6). After three rinses with phosphate buffered saline (PBS; Sigma–Aldrich) containing 0.05% Tween 20 (PBT, pH 7.3), the plates were blocked for 1 h at room temperature with blocking buffer containing 3% bovine serum albumin (BSA; Sigma–Aldrich) in PBT, followed by three rinses with PBT. The plates were then incubated for 1 h with 100 μl per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with secondary antibody anti-mouse IgG-HRP (1/1000 in blocking buffer). After exhaustive rinsing with PBT, the plates were developed using 100 μl of 0.42 mM TMB solution, prepared daily in Milli-Q water containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 μl of 2 M H_2SO_4 and the plates were read at 450 nm in a plate reader. Negative controls consisted of triplicate samples without skin mucus or without primary antibody, whose OD values were subtracted for each sample value.

2.7. Protease activity

Protease activity was quantified using the azocasein hydrolysis assay [35]. Briefly, an equal volume of skin mucus was incubated with 100 mM ammonium bicarbonate buffer containing 0.7% azocasein (Sigma–Aldrich) for 19 h at 25 °C in triplicate. The reaction was stopped by adding 4.6% trichloroacetic acid (TCA, Sigma–Aldrich) and the mixture centrifuged at 13,000×g, for 5 min. The supernatants were transferred to a 96-well plate containing 100 μl $well^{-1}$ of 0.5 N NaOH. Then, the OD was read at 450 nm using a plate reader. Skin mucus was replaced by trypsin solution (5 mg ml^{-1}) as positive control (100% of protease activity), or by buffer, as negative control (0% activity).

2.8. Antiprotease activity

Total antiprotease activity was determined as indicated by the

Table 1
Information related to each lectin used in ELISA.

Name	Symbol	Species of lectin source	Affinity for
Bandeiraea simplicifolia agglutinin	BS-I	*Bandeiraea simplicifolia*	α-D-galactose (primary) *N*-acetyl-α-D-galactosamine (secondary)
Peanut agglutinin	PNA	*Arachis hypogaea*	β-D-galactose
Ulex europaeus agglutinin I	UEA-I	*Ulex europaeus*	L-fucose
Concanavalin A	ConA	*Canavalia ensiformis*	α-D-mannose α-D-glucose
Wisteria floribunda agglutinin	WFA	*Wisteria floribunda*	*N*-acetyl-D-galactosamine
Wheat germ agglutinin	WGA	*Triticum vulgaris*	*N*-acetyl-β-D-glucosamine *N*-acetylneuraminic acid