Fish & Shellfish Immunology 47 (2015) 352–359

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Terminal carbohydrate composition, IgM level and enzymatic and bacteriostatic activity of European sea bass (*Dicentrarchus labrax*) skin epidermis extracts

Francisco A. Guardiola [a], Juan P. de Haro [a], Francisco Guillermo Díaz-Baños [b], José Meseguer [a], Alberto Cuesta [a], M. Ángeles 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] Department of Physical Chemistry, Faculty of Chemistry, Campus Regional de Excelencia Internacional "Campus Mare Nostrum", University of Murcia, 30100, Murcia, Spain

ARTICLE INFO

Article history:
Received 27 May 2015
Received in revised form
6 September 2015
Accepted 14 September 2015
Available online 16 September 2015

Keywords:
Skin
Epidermal extracts
Mucosal immunity
European sea bass (*Dicentrarchus labrax*)
Teleosts

ABSTRACT

Although the skin is one of the main defense barriers of fish to date, very little is known about the immune implications and the properties of the numerous substances present in skin cells. In the present study, terminal carbohydrate composition and some components of the skin immunity (total IgM level, and several enzymatic and bacteriostatic activities) present on aqueous and organic epidermal extracts of European sea bass (*Dicentrarchus labrax*) were determined. Most of the parameters measured followed a protein concentration dose-response. Curiously, both skin extracts have similar levels of total IgM. However, aqueous extracts showed higher presence of some terminal carbohydrates, alkaline phosphatase and esterase activities and lower proteases and ceruloplasmin activities than epidermal organic extracts. Regarding the bacteriostatic activity, the growth of all the bacterial strains tested was reduced when cultivated in presence of organic extracts, being the observed reduction correlated to the protein concentration present in the extract sample. On the contrary, skin aqueous extracts have no significant effect on bacterial growth or even allow bacteria to overgrow, suggesting that the bacteria could use the extracts as a nutrient source. The results are discussed and compared with the same activities studied on fish skin mucus in order to understand their possible implications on mucosal immunity.

1. Introduction

Body surfaces of fish are defended by epithelia, which provide a physical barrier between the internal milieu and the external environment. In the case of skin, from an immunological and metabolic point of view, this epithelium forms an active structure that covers the whole body and protects it, as a primary line of defence [1]. It is well known, that one of the most leading characteristics of fish skin is secretion of mucus, which plays a critical role in the animal defence acting as a natural, semipermeable, dynamic, physical, chemical and biological barrier [2]. In particular, teleost skin is exclusive and histologically diverse [3], features that made this organ crucial for the correct adaptation of the organism to the aquatic environment [1]. In addition, fish skin is a multifunctional organ, and its components play important roles in protection, communication, sensory perception, locomotion, respiration, ion regulation, excretion, and thermal regulation [4,5]. In fact, all these functions are possible due to complex structure and to the cell composition of skin [5].

In general, the layers of the tegument of adult teleosts are the cuticle or mucus layer, the epidermis (a squamous stratified epithelium with goblet cells) and the dermis [6]. Research on fish mucus started in the 80–90 s and demonstrated that it is one of the most important layers of the skin since it acts as barrier where the protective (or immunological) function is the result of the sum of its mechanical and biochemical properties [2]. Recently, the fish skin mucus has attracted again the interest of some researchers due to the importance of the mucosal immunity. Studies on fish mucus have focused on the identification and/or the study of the composition of different molecules present on it by using classical

* Corresponding author.
E-mail address: aesteban@um.es (M.Á. Esteban).

http://dx.doi.org/10.1016/j.fsi.2015.09.026
1050-4648/

histological or biochemical techniques [7–9]. More recently, more precise and sophisticated techniques have been also used in order to better known the fish skin mucus composition, the possible functions of their components or the effect of some environmental factors on its secretion or changes in its components [8,10,11].

Underneath the mucus layer, the non-keratinized epidermis (5–10 cells thick) of fish consists fully of live cells, which are classified in different cell populations (e.g. epithelial cells, mucus cells, club cells) and each of them plays important functions in maintaining the cutaneous immunological barrier and on the adaptation of the organism to its aquatic environment [12,13]. In addition, some immune cells (such as neutrophils, macrophages or T and B lymphocytes) infiltrate in the skin when an injury occurs or during microbial infection [14]. Therefore, fish skin (including both mucus and epidermis) acts as a repository of numerous immune components (of both innate and acquired immune system) such as glycoproteins, lysozyme, immunoglobulins, complement proteins, lectins, C-reactive protein, flavoenzymes, proteolytic enzymes and antimicrobial peptides [7,15–19]. All these molecules exert important inhibitory or lytic activity against different type of pathogens [20,21] contributing to fish well-being. Curiously, studies on the immune properties of epidermal cells of fish are still very limited as well as data on the fish skin-associated lymphoid tissue (SALT). Taken into account all these considerations, the aim of the present work was to study the presence of several terminal carbohydrates and some parameters related to immunity (total IgM, and some enzymatic and bacteriostatic activities) present on aqueous and organic epidermal extracts of European sea bass (*Dicentrarchus labrax*). The better knowledge of the innate immune mechanism present on fish skin will help to understand the mucosal immune responses in order to make advances on fish disease and/or stressor resistances.

2. Materials and methods

2.1. Animals

Six juveniles specimens (104 ± 26 g weight and 18 ± 1.5 cm length) of European sea bass (*D. labrax* L.) obtained from a local farm (Murcia, Spain) were kept in re-circulating seawater aquaria (250 L) in the Marine Fish Facilities at the University of Murcia. The water temperature was maintained at 20 ± 2 °C with a flow rate of 900 L h^{-1} and 28‰ salinity. The photoperiod was of 12 h light: 12 h dark and fish were fed with a commercial pellet diet (Skretting) at a rate of 2% body weight day^{-1}. Fish were allowed to acclimatise for 15 days before sampling. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.2. Skin extracts

Fish skin aqueous and organic extracts were prepared as described by Hellio et al. [22] with slightly modifications. After complete mucus removal from the fish body, epidermis from the dorsal-lateral part of the body was dissected and chopped into small fragments (2–3 mm^2) with the aid of a magnifying glass. The skin fragments were then washed with 5% ethanol for 5 min and immediately frozen in liquid nitrogen. Skin samples were grinded using a coffee grinder placed in a 4 °C cold room. The grinding process was completed using mortar and pestle pre-chilled in liquid nitrogen. Samples were then lyophilised and stored at −80 °C. The aqueous extracts of fish skin were prepared by stirring the grounded material in distilled water (1 mg ml^{-1} of dry weight) for 10 min at 4 °C. After filtration (Millex-GV unit 0.22 μm pore size, Millipore) the sample was lyophilised and stored at −20 °C. For organic extracts, grounded skin fragments were stirred in 95% cold ethanol (1 mg ml^{-1} of dry weight) for 10 min and then filtered using 0.22 μm filters. The ethanol was evaporated using a vacuum pump overnight and 50 ml of distilled water were then added. The extract was partitioned four times with 200 ml (4 × 50 ml) of dichloromethane. The organic phases were pooled, dried for 24 h under anhydrous Na_2SO_4, filtered using 0.22 μm filters and concentrated using a vacuum pump at temperature below 30 °C.

Protein concentration in both aqueous and organic extracts were determined by the dye binding kit (Biorad), based on the method of Bradford [23], using bovine serum albumin (BSA, Sigma) as the standard. Protein concentration was then adjusted to 6, 12, 24, 48 and 96 μg protein ml^{-1} of epidermal extracts with 5% DMSO (dimethyl sulphoxide). Samples were aliquot and stored at −20 °C until use.

2.3. Determination of the terminal glycosylation pattern

Glycosylation pattern was determined by lectin enzyme-linked immunosorbent assay (ELISA) in both epidermal extracts (aqueous and organic) as described previously [24]. Thus, 10 μg $well^{-1}$ of epidermal extracts 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 saline 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 with PBS-T. Samples were then incubated for 1 h with 20 μg per well of biotinylated lectins (Table 1; Sigma), washed and incubated streptavidin conjugated to horseradish-peroxidase (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 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 and stopped by the addition of 50 μl of 2 M H_2SO_4. The plates were read at 450 nm in a plate reader (FLUOstar Omega, BMG Labtech). Negative controls consisted of samples without epidermal extracts or without lectins, whose optical density (OD) values were subtracted for each sample value. Data are presented as the OD at 450 nm for each extracts and lectin used.

2.4. Total immunoglobulin M levels

Total immunoglobulin M (IgM) levels were analyzed for European sea bass by ELISA [25]. Thus, proteins of epidermal extracts were coated to wells, washed and blocked as described previously in section 2.3. The plates were then incubated for 1 h with 100 μl per well of mouse anti-European sea bass IgM monoclonal antibody (1/100 in blocking buffer; Aquatic Diagnostics Ltd.), washed and incubated with the secondary antibody anti-mouse IgG-conjugated to HRP (1/1000 in blocking buffer; Sigma). Washing, development and reading was carried out as above. Negative controls consisted of samples without epidermal extracts or without primary antibody, whose optical density (OD 450 nm) values were subtracted for each sample value.

2.5. Enzymatic activities

2.5.1. Protease activity

Protease activity present in epidermal extracts was quantified using the azocasein hydrolysis assay according to the method of Ross et al. [26]. Briefly, equal volumes of epidermal extracts and 100 mM ammonium bicarbonate buffer containing 0.7% azocasein (Sigma) were incubated for 19 h at 30 °C. The reaction was stopped