# Skin mucus proteome of gilthead sea bream: A non-invasive method to screen for welfare indicators

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**Abstract**

In teleosts, the skin mucus is the first physical barrier against physical and chemical attacks. It contains components related to metabolism, environmental influences and nutritional status. Here, we study mucus and composition based on a proteome map of soluble epidermal mucus proteins obtained by 2Delectrophoresis in gilthead sea bream, Sparus aurata. Over 1300 spots were recorded and the 100 most abundant were further analysed by LC-MS/MS and identified by database retrieval; we also established the related specific biological processes by Gene Ontology enrichment. Sixty-two different proteins were identified and classified in 12 GO-groups and into three main functions: structural, metabolic and protection-related. Several of the proteins can be used as targets to determine fish physiological status: actins and keratins, and especially their catabolic products, in the structural functional group; glycolytic enzymes and ubiquitin/proteasome-related proteins in the metabolic functional group; and heat shock proteins, transferrin and hemopexins, in the protection-related group. This study analyses fish mucus, a potential non-invasive tool for characterising fish status, beyond defence capacities, and we postulate some putative candidates for future studies along similar lines.

**Keywords**

Biomarker; epidermal mucus; gilthead sea bream; mucosal immunity; proteome

## Introduction

Epithelia are the physical barriers of body surfaces of multicel- lular organisms that separate internal and external environments. The vertebrate integument, skin, is a conserved cellular structure organised into stratified cellular sheets: epidermis, dermis and hypodermis [26]. In fish and aquatic larval forms of amphibian, mucus, a complex fluid, covers the skin surface and forms the outermost barrier; whereas for all other vertebrates (adult am- phibians, reptiles, birds and mammals) the external skin consists of a cornified multi-sheet cellular layer. Cutaneous mucus or skin mucus is thus considered the first line of defence against infection through skin epidermis in those animals [40]. Moreover, mucus is a dynamic and semipermeable barrier that performs a number of functions in fish osmoregulation, respiration, nutrition or locomotion [15,22,29,39,42,43].

External mucus is secreted by epidermal goblet cells. It is composed of water and glycoproteins [16,22], and its composition varies between different fish species. Furthermore, both endogenous factors, such as developmental stage, and exogenous factors, such as stress, acid environment and infections [5,47] can influence its composition. Mucins are the most common molecules in mucus. They are glycoproteins densely coated with O-linked oligosaccharides that makes them both large and heavy. Along with mucins, lipids, ions and a mixture of other proteins determine the physical characteristics of mucus including: water content, adherence, viscoelasticity and its capacity to provide both transport and protection. Although the current knowledge is limited, studies of mucus proteins have focussed on the mechanisms of constitutive and inducible innate immune response (reviewed in Refs. [15] and [19]). Thus, the molecules in different mucosal gels (epidermic, branchial and intestinal) that have been most studied to date are mucins, as the major constituent of the defensive matrix [24,30,32,36] and enzymes with biostatic or biocidal activities such as lysozyme, phosphatases, proteases, cathepsins and esterases [15]. To extend the characterisation of fish skin mucus, a few studies have addressed the general mucosa proteome: in discus (Symphysodom sp.), for which parental care effects have been demonstrated [9]; in Atlantic salmon (Salmo salar), for which a response to sea lice infection has been shown [12]; and in Atlantic cod (Gadus morhoa) for which immune competent molecules have been revealed [34]. Less attention has been paid to other proteins with no

direct relationship to the immune system (e.g. proteins involved in carbohydrate and protein metabolism, cytoskeletal proteins or heat shock proteins; HSPs) [33,34]. The presence, amount and role of those proteins may also be important and links to internal tissues and animal status could probably be established. In fact, recent findings indicate the need to study the relevance of feeding, envi- ronment or other stressors on mucus composition [15,19,33].

Conventional 1D or 2D polyacrylamide gel-based proteomic approaches with accurate protein purification allow heavy mucins (with MW of 200 kDae2 MDa) to be discarded, together with other large glycoconjugates, such as proteoglycans, glycoproteins, and glycolipids [25] and allows research to focus on the protein mixture that constitutes mucus. The aim of a proteomic approach is to look for putative proteins which could act as “biomarkers” or status indicators. To classify a molecule as a biomarker, its study and measurement should preferably also be non-invasive or non- destructive, thus allowing or facilitating the monitoring of environmental effects in protected or endangered species [17]. Briefly [4], sets the following criteria for high-quality biomarkers: quantifiable; inducible or repressible; highly accurate; reproducible among experiments; and with a sufficiently sensitive response for routine detection.

This first attempt at soluble protein characterisation of the mucus of gilthead sea bream ethe most important marine species for aquaculture in the Mediterranean e pursued two main objectives: 1) to provide a reference map of mucus proteins by LC-MS/MS analysis and to identify the 100 most abundant proteins, along with mucins; and 2) to identify the proteins, attributing a putative role in mucus to them. The proteome map of epidermal mucus could serve as a starting point for a better understanding of mucus functionality, related to differential expression under environment conditions, stressors or even feeding; and could be used to compare the mucus compositions of other marine and freshwater species.

## Material and Methods

Gilthead sea bream, 145 g average body weight, from a local fish farm were acclimated and reared indoors at the Centre d'Aquicultura (CA-IRTA, Sant Carles de la Ràpita, Tarragona, Spain) at 22ºC for several weeks, and fed a standard commercial fish feed. They were kept in 500 L fibreglass tanks with IRTAmar$^{TM}$ water recirculating systems and monitored via solid and biological filters, water temperature, and oxygen concentration. During the weeks of the

maintenance period, nitrite, nitrate and ammonia concentrations were maintained at their initial values. Twenty randomly captured fish were lightly anaesthetised with 2-phenoxyethanol (0.001%, Sigma-Aldrich) and skin mucus was immediately collected. Sterile glass slides were used to remove mucus carefully from the skin, avoiding bleeding and faecal contamination. The collected mucus was immediately frozen with liquid nitrogen and stored at -80ºC until analysis.

The experiment complied with the Guidelines of the European Union (86/609/EU), the Spanish Government (RD 1201/2005) and the University of Barcelona (Spain) for the use of laboratory animals.

Protein extraction

Mucus samples were solubilised in an equal volume of ice-cold lysis buffer (4 ml/g tissue; 7 M urea, 2 M thiourea, 2% w/v CHAPS and 1% protease inhibitor mixture) and centrifuged at 20,000g for 15 min at 4ºC, with the resultant supernatant aliquoted avoiding the surface lipid layer while the pellet was resuspended. Subsequently, the protein concentration was determined using the Bradford assay (Bradford, 1976) with bovine serum albumin as the standard (BioRad). After various tests, we decided to perform a clean-up procedure to enhance protein extraction before applying the Isoelectric focusing (IEF) and 2D-gel separation protocols, using selective precipitation to remove ionic contaminants such as detergents, lipids, and phenolic compounds from the protein samples (ReadyPrep 2-D clean-up kit, Bio-Rad). Such contaminants may interfere with separation, particularly during IEF and 2-D separations. Cleaned and purified protein extracts were resuspended in the appropriate final volume of lysis buffer.

2-Dimensional electrophoresis separation

Two or three protein mucus extracts were pooled to provide 450 µg dissolved in 450 µL of rehydration solution containing 7 M urea, 2 M thiourea, 2% w/v CHAPS, and 0.5% v/v IPG buffer, pH 3−10 NL (Amersham Biosciences Europe, now GE Healthcare, Madrid, Spain), 80 mM DTT and 0.002% bromophenol blue. Thus, five pooled samples (replicates) of gilthead sea bream epidermal mucus were obtained. The solution was then loaded onto 24 cm, pH 3−10 NL IPG strips. Isoelectric focusing was performed using an IPGphor instrument (Amersham Biosciences), following the manufacturer's instructions (active rehydration at 50 V for 12 h

119 followed by a linear gradient from 500 to 8,000 V until 48,000 V/h). The focused strips were
120 equilibrated in two steps as follows: 15 min with equilibration buffer I (65 mM DTT, 50 mM
121 Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue) and then 15 min with
122 equilibration buffer II (135 mM iodoacetamide, 50 mM Tris-HCl, 6 M urea, 30% glycerol, 2%
123 SDS, bromophenol blue). The equilibrated strips were applied directly onto 12.5%
124 polyacrylamide gels, sealed with 0.5% w/v agarose, and separated at a constant voltage of 50
125 V for 30 min followed by 200 V for about 6 h, until the blue dye reached the bottom of an
126 Ettan DALT II system (Amersham Biosciences, Stockholm, Sweden). The resolved proteins were
127 fixed for 1 h in 40% v/v methanol containing 10% v/v acetic acid and stained overnight using
128 colloidal Coomassie blue G-250. Gel staining was removed by consecutive washing steps with
129 distilled water until the best visualisation was achieved.

130 Gel image analysis

131 The Coomassie blue stained gels were scanned in a calibrated Imagescanner (Bio-Rad, Spain)
132 and digital images captured using Quantity-One software (Bio-Rad, Spain). The images were
133 saved as uncompressed TIFF files. Five replicate gels from five corresponding two- or three-fish
134 mucus pools were used. The gel images were analysed using the software package
135 ImageMaster 2D, version 6.01 (GE Healthcare, Spain). Proteins were detected using the
136 automated routine of the ImageMaster 2.0 software, combined with manual editing when
137 necessary to remove artefacts. The background was removed and normalised volumes were
138 calculated as follows: the volume of each protein spot was divided by the total volume of all
139 the protein spots included in the analysis. The normalised protein spot values were used to
140 select the 100 most abundant proteins in the mucus proteome, which were to be further
141 identified.

142 In-gel digestion

143 In-gel tryptic digestion was performed in an InvestigatorTM Progest (Genomic Solution)
144 automatic protein digestion system. Briefly, the selected spots were washed with ammonium
145 bicarbonate (25 mM $NH_4HCO_3$) and acetonitrile (ACN). Immediately, the proteins were
146 reduced (DTT 10 mM; 30 min, 56ºC) and alkylated (iodoacetamide 55 mM; 21ºC, 30 min, in the
147 dark). Afterwards, the proteins were digested with porcine trypsin (sequence grade modified
148 trypsin, Promega; 80 ng trypsin/sample; 37ºC 12 h overnight). Finally, the resulting peptide

mixture was extracted from the gel matrix with 10% formic acid (FA) and ACN, and dried with a speed vac system. The trypsin digested peptide samples were analysed by LC-MS/MS (CapLC-ESI-Q-TOFl, Micromass-Waters, Manchester, UK).

LC-MS/MS analysis

The dried peptide mixture from the tryptic digestion was resuspended in 100 µL 1% FA and separated by nanoflow chromatography using a nano-LC Ultra TM AS2 system (Eksigent-Applied Biosystems), injecting an aliquot. The injected peptides were trapped in a NanoEase™ trap column (Symmetry 300TM C18 5 µm; Waters), and were separated by reverse-phase chromatography using a C18 reverse-phase capillary column (75 µm Ø, 1.7 µm particle, 10 cm, nanoAcquity UPLC® column; Waters). The elution gradient was 5−65% B in 30 min (A: 2%MeCN/98% water, 0.1% FA; B: 90% MeCN, 0.1% FA). The eluted peptides were subjected to electrospray ionisation in an emitter needle (emitter nano-ES PicoTipTM, New Objective) with an applied voltage of 2100 V to the capillary/needle and of 60 V to cone, and analysed in a Quadrupole-TOF (Q-TOF) mass spectrometer (Micromass, Waters). The Q-TOF mass spectrometer performed a full MS scan ranging from 400 to 1800 m/z with 10.000 FWHM resolution. Simultaneously, as many as the 5 most abundant peptides (minimum intensity of 22 counts/s) from each MS scan were selected and fragmented using CID fragmentation (collision induced dissociation; applied collision energy by charge state recognition; argon gas) to perform MS/MS analysis (scan time 1 s; scan delay 0.1 s; range 100-1700 m/z). The associated instrument software, Masslynx, generated from the MS scans and fragmented spectra a PKL format data file to perform a search against protein/peptide sequence database.

Database search

For MALDI data, the mgf archives were submitted for database searching using a MASCOT search engine and PEAKS Studio v.3.1 against the NCBInr/all database. The following parameters were permitted for the searches: 2 missed cleavage sites as well as fixed and variable modifications; carbamidomethyl of cysteine and oxidation of methionine, respectively. Peptide tolerance was 100 ppm and 0.25 Da (respectively, for MS and MS/MS spectra). Protein identification was accepted when “individual ions scores” > “score number” with $P<0.05$ (provided by the MASCOT search results). The “score number” indicates the

178 identity or extensive homology and P is the probability that the observed match is a random
179 event.

180 **Results and discussion**

181 Mucus proteome of gilthead sea bream

182 Skin mucus is a physical innate immune barrier and a critical component of the piscine
183 immune system. To our knowledge, this is the first report of a broad study of the skin mucus
184 proteome of gilthead sea bream. Herein, the 100 most abundant proteins (Top- 100) after 2D
185 gel analysis were selected and identified using LC-MS/MS and database retrieval. From 5
186 replicated gels (each a pool of 2-3 mucus samples), more than 1300 spots were detected and
187 matched within the broad range of pI (3-10) and molecular weight (200 kDae5 kDa for 12.5%
188 PAGE). Initial evidence was that the clean-up process allowed high-quality resolution and a
189 useful proteome reference map (representative 2D gel profile is shown in Fig. 1), avoiding lipid
190 and ion interference and streaking on in-run 2D gels. A similar conclusion was also reported by
191 protein solubilisation and extraction from skin mucus of Atlantic cod [34]. Few studies report
192 large proteome maps of fish skin mucus; for discus fish [9], for Atlantic salmon [12] and for
193 turbot, Scophthalmus maximus [2], the pI range analysed comprised mainly the acidic zone (pI:
194 5e8; pI: 4e7 and pI: 4e7, respectively), whereas for Atlantic cod the authors screened pI 3e10,
195 but suggested a predominance of acidic proteins in mucus [34,35]. For gilthead sea bream
196 mucus, the soluble proteins were distributed throughout the pI range 3e10 with half of the
197 proteins located at neutral and alkaline pIs. This distribution differed from the highly acidic
198 skin proteome map reported for this species [21] but was more in keeping with the marine
199 water environment, showing pH neutrality or slight alkalinity.

200 The Top-100 spots in intensity are highlighted in Fig. 1 and their identities are listed in
201 Table 1 together with the details retrieved from databases along with physical characteristics
202 inferred from the gel. Table 1 includes the mean intensity for each individual spot from 5
203 replicates, gI or EST accession numbers, theoretical and observed MW and pI, matched and
204 unique peptides, scores, sequence coverage and species. Most of the spots were identified by
205 protein sequences deduced from genes already described in teleost species (except 3 proteins
206 in elasmobranchs). Six spots were identified from mammal sequences, 2 spots by sponge
207 species, 1 spot by a reptile species and 1 spot from insects. It was not possible to assign a

putative identity to eight spots (spots 34, 43, 46, 60, 74, 78, 84, 92) despite a considerable number of database searches. Sixty-two different proteins were identified, evidencing the presence of several isoforms for some of them. The identified proteins were subsequently submitted to the Genecards and AmiGO (Gene Ontology term enrichment processes) databases to establish their involvement in specific biological processes and attribute them to Gene Ontology (GO) groupings. Accordingly, Fig. 2 shows 12 different groups with the GO annotation and significance; only 9 proteins could not be directly grouped. The groups were not exclusive: one protein may belong to different GO annotations according to its possible roles (see details in Table 2). The GO groups were themselves grouped into three main functions: 1) structural, including 2 GO groups (S1: “actin filament-based process” and S2: “keratinisation”); 2) metabolic, including 4 groups (M1: “glucose metabolic process”; M2: “nucleoside biosynthetic process”; M3: “cellular amino acid metabolic process” and M4: “translational process”); and 3) protective function, including 6 groups (P1: “response to stress”; P2: “wound healing”; P3: “immune system process”; P4: “defence response”; P5: “viral process” and P6: “cellular response to chemical stimulus”).

The proteins were also analysed in the GO for their specific location. Thus, “cellular component” clusters from GO referred to the place in the cell where a gene product is active [3]. Forty-nine proteins (Fig. 2) corresponded to the “extracellular region part” (GO: 0044421, p = 5.61e-56). Moreover, all of them also belong to the “extracellular vesicular exosome” location (GO: 0070062, p = 1.53e78) (not shown in Fig. 2). Both location clusters would seem to indicate that these proteins were the product of cell secretion and the explanation of their presence in mucus needs to be analysed further to locate the secretory origin of the cellular exosome vesicles, either in goblet cells, skin cells or even blood cells. For the rest of the proteins not clustered as extracellular (13 proteins), their presence in mucus demonstrated that for fish species a secretory form of the proteins exists. In mammals, there is growing interest in the clinical applications of exosomes, using their protein contents as potential biomarkers for health and disease, or for prognosis and therapy; e.g. in cancer immunotherapy [28], in human breast milk composition [1], or in the study of extracellular vesicles as drug delivery vehicles [14]. However, for fish species, this field merits further study and one of the firsts steps is to characterise mucosal proteins, either in epidermal mucus, digestive mucus or gill mucus.

Structural proteins

Table 2 lists the proteins belonging to the GO biological process groups and highlights the main function proposed when one protein is classified into more than one group. Sixteen different proteins (30 spots) were directly related to structural functions and were grouped into the "actin filament-based process" group (S1) which includes 9 proteins and the "keratinisation" group (S2) which includes 4 proteins. Moreover, three proteins are also related to these biological processes, such as intermediate filament ON3-like protein (ION3, spot 13), a non-neuronal predominant intermediate filament protein, and two keratins (KRT13, spots 22 and 48; and KRT14, spot 7). All of these structural proteins, except ION3, could be located in the extracellular region (indicated in Table 2 as EX: belonging to the "extracellular region part").

Together with mucins, the presence in epidermal mucus of structural cellular proteins must contribute to the formation of the mucus matrix, which supports mucus functions. The soluble proteome map for gilthead sea bream mucus reveals a high abundance of b- actin forms (spots 4, 14, 23, 32, 37 and 51), with molecular masses of approximately 41 kDa and observed Isoelectric points ranging from 4.2 to 5.0 (Table 1). The sum of these isoforms (see data for normalised intensity, INT, for each individual spot from the 5 replicate gels provided in Table 1) reveals that actinwas the most abundant soluble protein in the sea bream epidermal mucus (resulting in a total of 1.5% ± 0.1%). The intracellular role of actin in the formation of structural filaments is highly conserved and its presence in mucus is attributed not only to structural processes but in favouring mucus secretion from goblets cells, for wound repair and immune response [23]. Accordingly, the GO classification for actin also places it in several protection-related clusters (P1, P2, P3 and P4). Recently, mucosal actin and in particular actin fragments generated by mucus protease activities were suggested as putative indicators of handling stress in Atlantic salmon [12,13]. Moreover, significant increases of several actin isoforms were observed in liceinfected salmon [33]. Thus, as an abundant protein in mucus, easily detectable and inducible by modified conditions, actin forms would meet the criteria as a target for further study in sea bream and other fish species as a bioindicator of fish capacity to generate or secrete mucus.

Related to the dynamic nature of actin filaments, skin mucus exuded regulatory proteins of actin de/polymerisation: profilin-1 and -2 (spots 8, 17 and 77), cofilin-2 (spot 12), gelsolin (spots 20, 36 and 89), macrophage-capping protein (spots 40 and 72) and actin-based

motor proteins: tropomyosin (spots 28 and 71) and myosin light chain 6 (spot 91). All of these were clustered in the GO:"actin filament-based process" (S1). Cofilin-2 and tropomyosin forms were reported in Atlantic cod mucus [34]; with both profiling and tropomyosin being up-regulated in fish mucus due to infection [35], and cofilin-2 being up-regulated in fish skin due to wound healing [21]. The role of these proteins, which have also been found in human secretomes [7,10,31], in fish mucus is still unknown. The actin capping protein regulates actin filament assembly and organization by capping the barbed (fast growing) end of the actin filament, and increased expression in epidermal mucus of cichlids has been related to the regulation of mucous cells and mucus production during parental care [8].

Keratins are other structural proteins repeatedly identified from fish 2-DE mucus. Several forms of both Type I and Type II keratins were identified in the present study in the 100 most abundant proteins: KRT1 (spots 76 and 98), KRT2 (spot 78), KRT13 (spots 22 and 48), KRT14 (spot 7), KRT17 (spots 93 and 100) and KRT19 (spot 82). Their location and physical characteristics are shown in Fig. 1 and Table 1. The keratin forms 1, 2 and 17, together with periplakin protein (PPL, spots 44 and 54), a component of desmosomes and of keratinocytes, were grouped in the biological process of "keratinisation" and KRT14 was also included. KRT19 seems to be more related with actin and KRT13 was not directly linked with any other structural GO. Their presence in mucus could be attributed to the dynamic surface cellular layer of the skin. It has recently been reported that some mucus keratins increase following infection with sea lice in Atlantic salmon [12] or upon a natural infection of Vibrio anguillarum in Atlantic cod [35] and decrease in response to thermal stress in turbot [2]. Interestingly, in mammals, epithelial cytokeratins have innate defensive properties and produce cytoprotective antimicrobial peptides, called "keratin-derived antimicrobial peptides" (KDAMPs). These peptides are produced by proteolysis via extracellular proteases and have a bactericidal function [45]. Further studies in this field should focus on the relevance of keratin-derived peptides in piscine species and on their putative protective function in mucus.

Metabolic proteins

The GO charts displaying putative biological processes for the proteins identified resulted in four main groups comprising metabolic proteins. Belonging to one cluster do not exclude a specific protein from also being included in other clusters. This is especially relevant for those groups of metabolic proteins, as most of them were also shown to play protective roles, as

discussed below. The “glucose metabolic process” group (M1) included 11 different proteins (14 spots), the “nucleoside biosynthetic process” group (M2) included 5 proteins, the “cellular amino acid metabolic process” group (M3) included 8 proteins and the “translational elongation” group (M4) included 4 proteins.

Among the most abundant sea bream mucus proteins (detailed intensity values provided in Table 1) are some glycolytic enzymes (clustered as “glucose metabolic process”, M1) such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH, spots 9, 65 and 73; accounting for a total intensity of 0.63% ± 0.08%), enolases (ENO1, spot 18 and ENO3, spot 31), transketolase (TKL1, spot 55), malate dehydrogenases (MDH1, spot 87 and MDH2, spots 25 and 69; accounting for a total intensity for MDH2 of 0.41% ± 0.04%) pyruvate kinase (PKLR, spot 57) and fructose biphosphate aldolase (ALDOA, spot 96). It is not still clear whether the release of these enzymes in mucus is related to goblet cell activity or directly to high activity in cell metabolism of epithelial layers. Most of the glucose metabolism-related proteins that we report in sea bream mucus are ubiquitous enzymes that take part in the constitutive expression required for the maintenance of the basal cellular function. In fact, their presence in human olfactory cleft mucus has simply been related to the scavenger role of the exuded products [11]. In fish, carbohydrate metabolism-related proteins have also been found in Atlantic cod mucus [34] and at least one of them, the mitochondrial malate dehydrogenase, is up-regulated after Vibrio infection [35]. In the same way, increased glycolytic activity has been reported in mucus during parental care and mouthbrooding of cichlids species [8,23]; or resulting from epidermal infections in Atlantic salmon [33]. This last study establishes a relationship between glucoserelated enzymes in mucus and fish diet; reporting altered expression levels for fish fed health diets containing immunostimulants and other functional ingredients. That was the first work to relate changes in some specific enzymes in fish mucus with diet and the authors proposed them as putative biomarkers for strategic validation experiments with selected functional feeds. Nevertheless, the few studies and the scarce attention that the metabolic functions of fish mucus have received to date, make the choice of candidate markers of physiological processes difficult.

As far as we are aware, no information exists for the other proteins detected clustered in M2 “nucleoside biosynthetic process” and M4 “translational regulation”; and only some proteins in M3 are previously referenced in fish mucosas. However, glutathione-S-transferase (GSTA1,

spot 52; normalised intensity of 0.19% ± 0.04%) and proteasome subunits (PSMA4, spot 49 and PSMC3, spot 67; normalised intensities of 0.19% ± 0.02% and 0.17% ± 0.03%, respectively) were already reported to be inducible or modified [23,34,35] and have been considered as detoxificants or immune competent molecules in fish mucus. Moreover, the ubiquitin carboxyl-terminal hydrolase (UCHL1, spot 56; normalized intensity of 0.18% ± 0.03%) and elongation factor forms (EF1B, spot 45 and EEF1G, spot 97; normalised intensities of 0.20% ± 0.02% and 0.15% ± 0.02%, respectively) detected in sea bream mucus are also linked to proteasome function. UCHL1 is a key protease of the ubiquitin-proteasome system and elongation factor-1 plays roles in protein translation. However, in mammal cells the former has also been linked to acetylated protein degradation by the proteasome [20]. The presence in fish mucus of a number of proteins belonging to the ubiquitin/proteasome system suggests a high proteolytic activity and its importance in mucus function needs more attention in further studies of the over-expression or under-expression of that system under stress challenges (temperature variations, handling, confinement, infection, etc.).

Protection-related proteins

As expected, most of the Top-100 mucus proteins showed a principal or secondary protective role. The GO enrichment displayed 6 main clusters (P1eP6, see Table 2) which also included, as mentioned above, proteins grouped as structural or metabolic. “Response to stress” (P1) with 26 different proteins (50 spots); “wound healing” (P2) with 15 proteins (27 spots); “immune system process” (P3) with 14 proteins (28 spots); “defence response” (P4) with 9 proteins (22 spots); “viral process” (P5) with 7 proteins (13 spots) and “cellular response to chemical stimulus” with 15 proteins (24 spots). The P1 group, “response to stress”, contained the largest number of identified proteins: 26, which corresponded to 49 spots of the Top-100. ”Response to stress” is a broad term that refers to “any process that results in a change in state or activity of a cell or an organism as a result of a disturbance in organismal homeostasis, usually, but not necessarily, exogenous” (the definition from the AmiGO web page). Thus, some structural proteins such as b-actin, profilin, gelsolin and keratin 1; some metabolic proteins mentioned above such as apolipoprotein-1, calmodulin, GAPDH, ENO3, ALDOA, PSMC3, PSMA4 and UCHL1; and all the protection-related proteins could be classified in this way. Due to the variety of roles that these proteins can play, their main functions were attributed to the other groups: P2, P3, P4 and P5 (highlighted in Table 2).

There is growing interest in the action of the epidermal mucus in fish species as a defensive mechanism. It has been reported that both constitutive and inducible innate defences are involved in mucus (reviewed in Refs. [15,27,46]. Ref. [27] enumerated the main mucus components that can be related to fish immune systems (discounting mucins) as the innate immune components, proteases, antimicrobial peptides, lectins, proteins and immunoglobulins. Directly related to the immune system the proteins grouped within P3 ("immune system process") and P4 ("defence response") included intracellular housekeeping enzyme activities (GAPDH, nucleoside diphosphate kinase, proteasome subunits, disulfide isomerase, superoxide dismutase, esterase D and elastase) and other proteins such as b-actin, keratins, apolipoprotein A-1, calmodulin, 14-3-3 protein zeta/delta, and some HSPs. Most of them were also reported in fish mucus, being quantifiable and inducible or repressible under different culture conditions (see reviews), and so candidates as biomarkers. Recent studies in fish mucus focused on specific enzyme activities such as proteases, antiproteases, phosphatases, esterases or lysozyme [48e51], even comparing their mucus and serum activities [49,50].

The observed high abundance of iron-binding-related proteins such as transferrin (TF, spots 2, 5 and 42; summing an intensity of 0.91% ± 0.03%), and "warm temperature acclimation related protein" (HPX spots 41, 47 and 63; summing an intensity of 0.91% ± 0.03%) and the presence of several isoforms with close MWs and different pIs, would make them candidates. Transferrin withholds iron and makes bacterial survival difficult, and it has plays a role as activator of fish macrophages [41]. They have already been proposed as biomarkers of disease resistance in fish aquaculture [18]. The warm temperature acclimation protein (Wap65) shares much structural similarity with mammalian hemopexins (HPX) and it is involved in temperature acclimation, immune response and development [37,38]. Both kinds of proteins, transferrin and hemopexin, are inducible [21] and indicative of the skin regeneration process in fish.

A new focus of research could be the presence of a number of molecular chaperones or HSP protein forms in gilthead sea bream mucus (HSPA1A, spot 86; HSPA5 spot 70; HSPA8, spots 10, 11 and 38; and HSPA9, spot 59, with MWs of around 60e80 kDa and individual intensities ranging from 0.15% ± 0.02% for HSPA1A to 0.77% ± 0.03% for the sum of HSPA8 isoforms). Chaperones are produced by cells to protect themselves against unfavourable

conditions such as heat shock, mechanical stress, infection, oxidants and cytokine stimulation [44]. Such properties indicate that they could take part in the protection of epithelia. In fish mucus, the presence of chaperones has been related with protein stability [23,34] as well as in mammal secretomes [1,11]. Thus, an easy way to detect changes of expression in the mucus of fish facing stress would make them candidate proteins as non-invasive markers in aquaculture.

## Conclusions

A reference proteome map of gilthead sea bream epidermal mucus was obtained for the first time and the 100 most abundant proteins were identified. The Gene Ontology enrichment process resulted in 12 functional groups of proteins further classified as structural, metabolic and protection-related proteins. The mucus proteome has been revealed as a powerful tool to found putative bioindicators of fish welfare and physiological status, via a non-invasive method. According to the protein role and literature screening, we suggest a reduced list of candidates for further studies to focus on: 1) the presence and modifications of β-actin and keratin fragments; 2) changes in glycolytic enzymes and in the ubiquitin/proteasome system components; and 3) the inducible/repressible presence of HSPs, transferrins and hemopexins.

## Acknowledgments

We thank Dr. Josefina Blasco and Dr. Jaume Fernández from the Department of Physiology and Immunology (University of Barcelona) for valuable help during the trials. The in-gel digestion as well as LC-MS/MS analysis was performed by the Proteomics Platform of the Barcelona Science Park, University of Barcelona; a member of the ProteoRed-ISCIII network. We also thank Antonia Òdena, Ramón Díaz and Eliandre de Oliverira from the Proteomics Platform for excellent technical assistance. This research was funded by a grant from the Spanish Government (AGL2011-29873).

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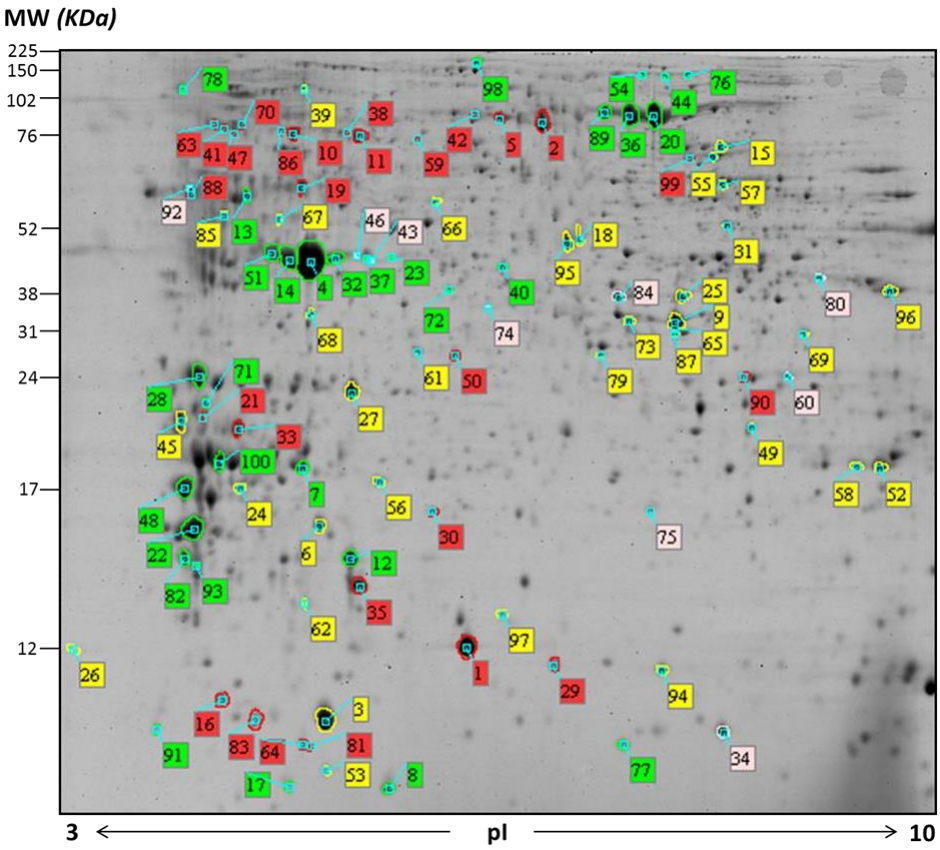


**Figure 1. 2-DE image of gilthead sea bream mucosal proteins.** After a cleaning process, the protein extract was separated on 24 cm non-linear pH 3-10 IPG strips, followed by separation using 12.5% SDS-PAGE. Numbers indicate the order of the Top-100 proteins in normalised intensity (from 5 replicates). Green spots corresponded to “structural proteins”; yellow spots to “metabolic proteins”; and red spots to “protection-related proteins”. Uncoloured spots were not further identified (more details in Figure 2 and Table 2).

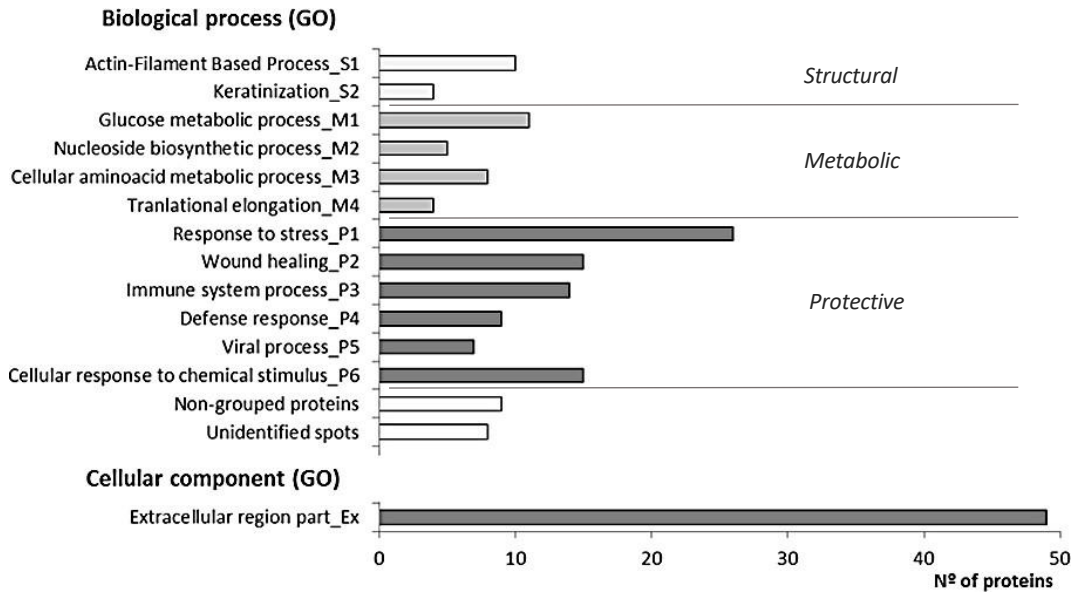


**Figure 2. Classification of protein spots into different categories based on Gene Ontology (GO) categories.** The histogram indicates the number of different proteins included in a GO-biological group. The same protein could be included in more than one cluster (see Table 2). Groups related to structural function: S1 (GO:0030029, p=1.92e-04) and S2 (GO:0031424, p=3.16e-03). Groups related to metabolic function: M1 (GO:0006006, p=7.19e-12), M2 (GO:0009163, p=4.51e-04), M3 (GO:0006520, p= 1.48e-03) and M4 (GO: 0006414, p=6.05e-03). Groups related to protection: P1 (GO:0006950, p=3.37e-11), P2 (GO:0042060, p=1.51e-13), P3 (GO:0002376, p=2.76e-04), P4 (GO:0006952, p=3.76e-02), P5 (GO:0016032, p=3.53e-04) and P6 (GO:0070887, p=4.93e-05). An additional cluster of cellular component categories has been added: extracellular region part: EX (GO: 0044421, p=5.61e-56).

**Table 1. Identification of 100 most abundant proteins in gilthead sea bream epidermal mucus.**

| SPOT ID[1] | INT[2] (%) | SEM | PROTEIN IDENTITY[3] | ACCESSION Nº (gI)[3] | GENE SYMBOL[4] | Theoretical[3] MW | Theoretical[3] pI | Observed[5] MW | Observed[5] pI | PEPTIDES MATCHED[3] | SCORE[3] | SQ[3] (%) | GENE SPECIES[3] | GENE NUMBER[4] | UniProtKB[4] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 0,40 | 0,02 | Complement component 1, q subc. | HS989682 | **C1QC** | 31,5 | 7,2 | 14 | 5,5 | 3/(8) | 111>59 | 14 | *Sparus aurata* | 714 | P02747 |
| **2** | 0,39 | 0,01 | Transferrin | 327243042 | **TF** | 76,1 | 5,9 | 72 | 6,1 | 17/(35) | 907>60 | 30 | *Sparus aurata* | 7018 | Q90YH6 |
| **3** | 0,35 | 0,03 | Deoxycytidylate deaminase-like | FG590567 | **DCTD** | 13,8 | 6,8 | 53 | 8,1 | 1/(1) | 75>60 | 12 | *Sparus aurata* | 1635 | P32321 |
| **4** | 0,33 | 0,03 | Beta-actin | 154818367 | **ACTB** | 42,2 | 5,3 | 40 | 4,5 | 8/(14) | 414>59 | 25 | *Neovison vison* | 60 | P60709 |
| **5** | 0,32 | 0,01 | Transferrin | 327243042 | **TF** | 76,1 | 5,9 | 72 | 5,9 | 18/(27) | 931>60 | 33 | *Sparus aurata* | 7018 | P02787 |
| **6** | 0,31 | 0,03 | Phosphatidylethanolamine-BP | 47221502 | **PEBP1** | 21,1 | 6,9 | 18 | 4,5 | 3/(8) | 230>60 | 27 | *Tetraodon nigroviridis* | 5037 | P30086 |
| **7** | 0,30 | 0,01 | Keratin type I E7 | 185133596 | **KRT14** | 49,2 | 5,5 | 22 | 4,4 | 2/(2) | 88>60 | 4 | *Oncorhynchus mykiss* | 3861 | P02533 |
| **8** | 0,29 | 0,03 | Profilin 1 | FM146227 | **PFN1** | 21,3 | 9,6 | 11 | 4,8 | 7/(14) | 484>60 | 48 | *Sparus aurata* | 5216 | P07737 |
| **9** | 0,29 | 0,03 | Glyceraldehyde-3-P-DH | 15146358 | **GAPDH** | 36,4 | 6,4 | 35 | 7,1 | 10/(26) | 534>59 | 34 | *Pagrus major* | 2597 | P04406 |
| **10** | 0,28 | 0,02 | Stress protein HSC70-1 | 212274295 | **HSPA8** | 71,5 | 5,2 | 66 | 4,4 | 14/(23) | 690>59 | 26 | *Seriola quinqueradiata* | 3312 | P11142 |
| **11** | 0,28 | 0,02 | Heat shock 70kDa protein 8 | 512393038 | **HSPA8** | 71 | 5,4 | 66 | 4,8 | 15/(24) | 699>60 | 28 | *Monopterus albus* | 3312 | P11142 |
| **12** | 0,28 | 0,02 | Cofilin-2-like | FM144266 | **CFL2** | 30,5 | 6,8 | 16 | 4,7 | 5/(13) | 211>58 | 19 | *Sparus aurata* | 1073 | Q9Y281 |
| **13** | 0,28 | 0,02 | Intermediate filament ON3-like | 432864499 | **ION3** | 57,5 | 5,7 | 52 | 4,1 | 2/(2) | 72>60 | 3 | *Oryzias latipes* | N/A | P18520 |
| **14** | 0,27 | 0,02 | Beta-actin | 6693629 | **ACTB** | 42,1 | 5,3 | 41 | 4,3 | 9/(16) | 472>60 | 27 | *Pagrus major* | 60 | P60709 |
| **15** | 0,27 | 0,03 | WD repeat-containing protein 1 | 410920259 | **WDR1** | 66,9 | 6,4 | 65 | 7,4 | 3/(5) | 204>59 | 6 | *Takifugu rubripes* | 9948 | O75083 |
| **16** | 0,27 | 0,01 | Coactosin-like | 47221902 | **COTL1** | 16,2 | 4,9 | 11 | 4,0 | 4/(8) | 178>60 | 22 | *Tetraodon nigroviridis* | 23406 | Q14019 |
| **17** | 0,27 | 0,06 | Profilin 2 | FM146227 | **PFN2** | 21,3 | 9,6 | 11 | 4,3 | 6/(13) | 387>60 | 41 | *Anoplopoma fimbria* | 5217 | P35080 |
| **18** | 0,27 | 0,02 | Enolase 1 (alpha) | 37590349 | **ENO1** | 47,4 | 6,2 | 48 | 6,4 | 8/(12) | 468>60 | 22 | *Danio rerio* | 2023 | P06733 |
| **19** | 0,27 | 0,01 | ER protein precursor | 475653182 | **PDIA3** | 56 | 5,4 | 52 | 4,4 | 7/(9) | 363>59 | 14 | *Dicentrarchus labrax* | 2923 | P30101 |
| **20** | 0,26 | 0,02 | Gelsolin-S1/S2-like | FM026536 | **GSN** | 30 | 5,9 | 77 | 6,9 | 2/(3) | 108>60 | 6 | *Dicentrarchus labrax* | 2934 | P06396 |
| **21** | 0,25 | 0,02 | 14-3-3 protein zeta/delta | 34037589 | **YWHAZ** | 28,2 | 4,7 | 25 | 3,9 | 2/(5) | 120>60 | 7 | *A. queenslandica* | 7534 | P63104 |
| **22** | 0,25 | 0,03 | Keratin type I cytoskeletal 13 | 583991085 | **KRT13** | 48,3 | 5,2 | 18 | 3,9 | 4/(13) | 259>59 | 8 | *N. brichardi* | 3860 | P13646 |
| **23** | 0,25 | 0,03 | Actin 2 | 389744214 | **ACTB** | 42 | 5,5 | 41 | 5,0 | 5/(5) | 187>59 | 13 | *Stereum hirsutum* | 60 | P60709 |
| **24** | 0,24 | 0,03 | Apolipoprotein A-I | 6686379 | **APOA1** | 29,6 | 5,2 | 20 | 4,1 | 9/(16) | 403>59 | 34 | *Sparus aurata* | 335 | P02647 |
| **25** | 0,24 | 0,03 | Malate dehydrogenase-like | 499026334 | **MDH2** | 39,5 | 6,4 | 38 | 7,1 | 4/(11) | 237>61 | 12 | *Maylandia zebra* | 4191 | P40926 |
| **26** | 0,24 | 0,03 | Efh superfamily (Calmodulin) | 71664 | **CALM** | 16,7 | 4,1 | 13 | 3,6 | 1/(1) | 64>60 | 6 | *Oncorhynchus sp.* | 801 | P62158 |
| **27** | 0,24 | 0,02 | Inositol monophosphatase 1-like | 583999941 | **IMPA1** | 27,7 | 5,2 | 27 | 4,7 | 6/(11) | 323>60 | 31 | *N. brichardi* | 3612 | P29218 |
| **28** | 0,24 | 0,02 | Tropomyosin 4-1 | 28557136 | **TPM4** | 28,7 | 4,7 | 29 | 3,8 | 5/(7) | 273>61 | 16 | *Takifugu rubripes* | 7171 | P67936 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **29** | 0,23 | 0,02 | Cu-Zn superoxide dismutase | 409712148 | **SOD1** | 15,9 | 5,8 | 13 | 6,2 | 3/(8) | 167>59 | 32 | *Sparus aurata* | 6647 | P00441 |
| **30** | 0,23 | 0,06 | Peroxiredoxin 2 | 298361172 | **PRDX2** | 21,9 | 5,8 | 18 | 5,3 | 6/(11) | 264>60 | 31 | *Sparus aurata* | 7001 | P32119 |
| **31** | 0,23 | 0,04 | Beta-enolase-like isoform 1 | 348527312 | **ENO3** | 47,9 | 6,3 | 49 | 7,5 | 5/(5) | 210>60 | 10 | *Oreochromis niloticus* | 2027 | P13929 |
| **32** | 0,23 | 0,02 | Beta-actin | 6693629 | **ACTB** | 42,1 | 5,3 | 41 | 4,6 | 4/(4) | 182>59 | 12 | *Pagrus major* | 60 | P60709 |
| **33** | 0,22 | 0,02 | 14-3-3 protein zeta/delta | 10719663 | **YWHAZ** | 28,1 | 4,7 | 24 | 4,1 | 2/(9) | 89>43 | 7 | *Fundulus heteroclitus* | 7534 | P63104 |
| **34** | 0,22 | 0,03 | Trypsin residu | | | | | | | | | | | | |
| **35** | 0,22 | 0,04 | Complement component 1, q subc. | FM156064 | **C1QC** | 23,7 | 5,3 | 15 | 4,8 | 3/(6) | 303>60 | 16 | *Sparus aurata* | 714 | P02747 |
| **36** | 0,21 | 0,03 | Gelsolin | 395505607 | **GSN** | 85,9 | 5,7 | 77 | 6,7 | 2/(2) | 73>61 | 2 | *Sarcophilus harrisii* | 2934 | P06396 |
| **37** | 0,21 | 0,03 | Beta-actin | 6716561 | **ACTB** | 41,9 | 5,4 | 41 | 4,8 | 5/(5) | 228>59 | 15 | *K. marmoratus* | 60 | P60709 |
| **38** | 0,21 | 0,06 | Heat shock cognate 70kDa protein | 209155490 | **HSPA8** | 72,3 | 5,4 | 66 | 4,6 | 5/(5) | 255>59 | 8 | *Salmo salar* | 3312 | P11142 |
| **39** | 0,21 | 0,01 | Cdc48 | 213054513 | **ATAD2B** | 89,8 | 5,2 | 79 | 4,4 | 14/(20) | 626>59 | 17 | *Larimichthys crocea* | 54454 | Q9ULI0 |
| **40** | 0,21 | 0,02 | Macrophage-capping protein-like | 348542563 | **CAPG** | 38,9 | 5,4 | 40 | 5,8 | 3/(4) | 152>59 | 9 | *Oreochromis niloticus* | 822 | P40121 |
| **41** | 0,21 | 0,02 | WT acclimation-related 65KDa protein | 224551742 | **HPX** | 50 | 5,4 | 66 | 3,9 | 5/(9) | 207>60 | 10 | *Sparus aurata* | N/A | C0L788 |
| **42** | 0,20 | 0,03 | Transferrin | 327243042 | **TF** | 76 | 5,9 | 72 | 5,6 | 8/(11) | 326 | 12 | *Sparus aurata* | 7018 | P02787 |
| **43** | 0,20 | 0,04 | Trypsin residu | | | | | | | | | | | | |
| **44** | 0,20 | 0,04 | Periplakin-like | 573898572 | **PPL** | 20,7 | 5,9 | 98 | 6,9 | 2/(2) | 50>42 | 0 | *Lepisosteus oculatus* | 5493 | O60437 |
| **45** | 0,20 | 0,02 | Elongation factor 1-beta-like | 551521377 | **EF1B** | 24,9 | 4,6 | 24 | 3,8 | 3/(4) | 96>59 | 14 | *X. maculatus* | 1933 | P24534 |
| **46** | 0,20 | 0,03 | Trypsin residu | | | | | | | | | | | | |
| **47** | 0,20 | 0,02 | WT acclimation-related 65KDa protein | 224551742 | **HPX** | 49,7 | 5,4 | 65 | 4 | 6/(14) | 267>60 | 12 | *Sparus aurata* | N/A | C0L788 |
| **48** | 0,19 | 0,02 | Keratin, type I cytoskeletal 13 | 348510135 | **KRT13** | 47,3 | 5,4 | 20 | 3,8 | 3/(10) | 219>59 | 7 | *Oreochromis niloticus* | 3860 | P13646 |
| **49** | 0,19 | 0,02 | Proteasome subunit alpha type-4 | 221219640 | **PSMA4** | 29,6 | 6,9 | 25 | 7,7 | 9/(17) | 426>60 | 47 | *Salmo salar* | 5685 | P25789 |
| **50** | 0,19 | 0,03 | Esterase D | 348524078 | **ESD** | 31,6 | 5,9 | 31 | 5,2 | 3/(6) | 114>60 | 14 | *Oreochromis niloticus* | 2098 | P10768 |
| **51** | 0,19 | 0,01 | Actin cytoplasmic 1-like | 348514007 | **ACTB** | 42 | 5,3 | 41 | 4,2 | 9/(20) | 419>60 | 28 | *Oreochromis niloticus* | 60 | P60709 |
| **52** | 0,19 | 0,04 | Glutathione S-transferase | 34014736 | **GSTA1** | 24,7 | 8,5 | 22 | 8,7 | 8/(17) | 385>60 | 41 | *Sparus aurata* | 2938 | P08263 |
| **53** | 0,19 | 0,01 | Gastrotopin (Lipocalin superfamily) | FM146224 | **FABP6** | 25 | 8,9 | 10 | 4,5 | 9/(20) | 467>59 | 47 | *Oreochromis niloticus* | 2172 | P51161 |
| **54** | 0,18 | 0,04 | Periplakin-like | 499048295 | **PPL** | 184 | 5,9 | 98 | 7 | 7/(5) | 138>60 | 4 | *Maylandia zebra* | 5493 | O60437 |
| **55** | 0,18 | 0,03 | Transketolase-like isoform X1 | 551514408 | **TKTL1** | 68 | 6,4 | 64 | 7,3 | 4/(6) | 200>60 | 7 | *Xiphophorus maculatus* | 8277 | P51854 |
| **56** | 0,18 | 0,03 | Ubiquitin carboxyl-terminal hydrolase L1 | AM955423 | **UCHL1** | 28 | 6,2 | 21 | 4,9 | 5/(11) | 293>59 | 21 | *Takifugu rubripes* | 7345 | P09936 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **57** | 0,18 | 0,02 | Pyruvate kinase | 47210667 | **PKLR** | 63 | 7,9 | 57 | 7,4 | 8/(12) | 349>60 | 15 | *Tetraodon nigroviridis* | 5313 | P30613 |
| **58** | 0,18 | 0,03 | Triosephosphate isomerase B | 432908784 | **TPI1** | 26,9 | 6,9 | 22 | 8,5 | 9/(21) | 482>60 | 46 | *Oryzias latipes* | 7167 | P60174 |
| **59** | 0,18 | 0,06 | Glucose regulated protein 75 | 119692141 | **HSPA9** | 69 | 5,6 | 65 | 5,1 | 6/(9) | 330>60 | 12 | *Sparus aurata* | 3313 | P38646 |
| **60** | 0,18 | 0,05 | Trypsin residu | | | | | | | | | | | | |
| **61** | 0,18 | 0,01 | Ribosomal protein large P0-like protein | 48476454 | **RPLP0** | 34 | 5,7 | 32 | 5,1 | 9/(30) | 546>60 | 31 | *Sparus aurata* | 6175 | P05388 |
| **62** | 0,17 | 0,03 | Translation initiation factor 5A | 47209413 | **EIF5A** | 17,5 | 5,2 | 14 | 4,4 | 3/(15) | 188>60 | 13 | *Tetraodon nigroviridis* | 1984 | P63241 |
| **63** | 0,17 | 0,01 | WT acclimation-related 65KDa protein | 224551742 | **HPX** | 49,7 | 5,4 | 67 | 3,9 | 10/(15) | 392>60 | 35 | *Sparus aurata* | N/A | C0L788 |
| **64** | 0,17 | 0,02 | Beta globin | 9126232 | **HBB** | 16,3 | 7,8 | 11 | 4,4 | 2/(2) | 86>60 | 14 | *Sparus aurata* | 3043 | P68871 |
| **65** | 0,17 | 0,04 | Gliceraldehyde 3-P-DH | 15146358 | **GAPDH** | 36,4 | 6,4 | 35 | 7,06 | 3/(3) | 106>52 | 9 | *Pagrus major* | 2597 | P04406 |
| **66** | 0,17 | 0,02 | Antiquitin | 61742178 | **ALDH7A1** | 55,8 | 5,9 | 51 | 5,3 | 7/(14) | 347>60 | 17 | *A. shlegelii* | 501 | P49419 |
| **67** | 0,17 | 0,03 | 26S protease regulatory sub. Unit 6a | 501295933 | **PSMC3** | 48 | 5,2 | 47 | 4,3 | 7/(17) | 390>60 | 19 | *Riptortus pedestris* | 5702 | P17980 |
| **68** | 0,17 | 0,01 | Inorganic pyrophosphatase-like | 432903493 | **PPA1** | 33,4 | 5,1 | 33 | 4,5 | 6/(9) | 231>60 | 23 | *Oryzias latipes* | 5464 | Q15181 |
| **69** | 0,17 | 0,02 | Malate dehydrogenase mitochondrial | 410905057 | **MDH2** | 35,8 | 8,6 | 32 | 8,1 | 10/(16) | 532>61 | 37 | *Takifugu rubripes* | 4191 | P40926 |
| **70** | 0,17 | 0,02 | 78 kDa glucose-regulated protein | 523704370 | **HSPA5** | 72,2 | 5,0 | 67 | 4 | 10/(10) | 328>60 | 17 | *Oryzias latipes* | 3309 | P11021 |
| **71** | 0,17 | 0,02 | Tropomyosin alpha-4 chain isoform 2 | 47085929 | **TPM4** | 28,6 | 4,6 | 26 | 3,8 | 9/(18) | 410>60 | 28 | *Danio rerio* | 7171 | P67936 |
| **72** | 0,17 | 0,03 | Macrophage-capping protein-like | 551506607 | **CAPG** | 38,5 | 5,2 | 40 | 5,4 | 3/(5) | 149>60 | 9 | *X. maculatus* | 822 | P40121 |
| **73** | 0,17 | 0,02 | Glyceraldehyde-3-P DH | 15146358 | **GAPDH** | 36,4 | 6,4 | 35 | 6,7 | 8/(16) | 450>60 | 26 | *Pagrus major* | 2597 | P04406 |
| **74** | 0,17 | 0,07 | Trypsin residu | | | | | | | | | | | | |
| **75** | 0,17 | 0,05 | UMP-CMP kinase-like | 348500565 | **CMPK1** | 24,9 | 8,6 | 20 | 6,8 | 2/(7) | 77>60 | 11 | *Oreochromis niloticus* | 51727 | P30085 |
| **76** | 0,16 | 0,03 | Keratin, type II cytoskeletal 1 | 375314779 | **KRT1** | 66 | 8,2 | 98 | 7 | 11/(16) | 527>60 | 18 | *Homo sapiens* | 3848 | P04264 |
| **77** | 0,16 | 0,02 | Profilin-1-like | FM147922 | **PFN1** | 14 | 8,3 | 11 | 6,6 | 5/(11) | 242>60 | 37 | *Sparus aurata* | 5216 | P07737 |
| **78** | 0,16 | 0,03 | Keratin, type 2 cytoskeletal 2 | 403296725 | **KRT2** | 66,9 | 8,2 | 78 | 3,7 | 4/(4) | 172>60 | 7 | *Saimiri boliviensis* | 3849 | P35908 |
| **79** | 0,16 | 0,02 | Glycine N-methyltransferase | 432950550 | **GNMT** | 33,7 | 6,3 | 32 | 6,5 | 3/(5) | 115>60 | 10 | *Oryzias latipes* | 27232 | Q14749 |
| **80** | 0,16 | 0,02 | Trypsin residu | | | | | | | | | | | | |
| **81** | 0,16 | 0,02 | Beta globin | 91260232 | **HBB** | 16,3 | 7,8 | 11 | 4,5 | 3/(4) | 114>60 | 20 | *Sparus aurata* | 3043 | P68871 |
| **82** | 0,16 | 0,02 | Keratin, type I cytokeratin 19 | 18858423 | **KRT19** | 46,7 | 5,4 | 17 | 3,8 | 4/(7) | 221>60 | 7 | *Danio rerio* | 3880 | P08727 |
| **83** | 0,16 | 0,02 | Coactosin-like 1 | 85719983 | **COTL1** | 10 | 5,5 | 11 | 4,6 | 1/(1) | 43>42 | 10 | *Ictalurus punctatus* | 23406 | Q14019 |
| **84** | 0,16 | 0,04 | Trypsin residu | | | | | | | | | | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **85** | 0,16 | 0,02 | Mitochondrial ATP synthase beta subunit | 387914370 | **ATP5B** | 55,6 | 5,4 | 48 | 3,9 | 11/ |
| **86** | 0,15 | 0,02 | Heat shock protein A1 | 47223819 | **HSPA1A** | 71,4 | 5,2 | 66 | 4,3 | 11/ |
| **87** | 0,15 | 0,02 | Malate dehydrogenase | 551491925 | **MDH1** | 38,4 | 7,6 | 34 | 7,1 | 6/( |
| **88** | 0,15 | 0,01 | Protein disulfide-isomerase-like | 498926878 | **PDIA3** | 57,4 | 4,6 | 52 | 3,6 | 6/( |
| **89** | 0,15 | 0,02 | Gelsolin | 395505607 | **GSN** | 73 | | 78 | 6,5 | 2/( |
| **90** | 0,15 | 0,05 | Elastase | 379317093 | **CELA3B** | 17,2 | 6,1 | 29 | 7,6 | 1/( |
| **91** | 0,15 | 0,01 | Myosin light polypeptide 6 | 229366002 | **MYL6** | 17 | 4,5 | 11 | 3,8 | 5/( |
| **92** | 0,15 | 0,03 | Trypsin residu | | | | | | | |
| **93** | 0,15 | 0,01 | Keratin type I, cytoeskeletal 17 | 334362277 | **KRT17** | 26,6 | 7,8 | 17 | 3,9 | 4/( |
| **94** | 0,15 | 0,02 | Nucleoside diphosphate kinase | 194500331 | **NME1** | 17,1 | 6,4 | 13 | 6,9 | 4/( |
| **95** | 0,15 | 0,03 | Adenosylhomocysteinase | 40363541 | **AHCY** | 48,5 | 6,3 | 45 | 6,3 | 9/( |
| **96** | 0,15 | 0,05 | Fructose-biphosphate aldolase | 221048061 | **ALDOA** | 33,8 | 6,5 | 37 | 8,8 | 2/( |
| **97** | 0,15 | 0,02 | Elongation factor 1-gamma-like | 432877958 | **EEF1G** | 50 | 6,64 | 15 | 5,8 | 3/( |
| **98** | 0,15 | 0,02 | Keratin, type II cytoskeletal 1 | 160961491 | **KRT1** | 65,6 | 7,6 | 102 | 5,5 | 6/( |
| **99** | 0,15 | 0,05 | Protein disulfide-isomerase-like | 498926878 | **PDIA3** | 57,4 | 4,57 | 74 | 7,1 | 9/( |
| **100** | 0,15 | 0,04 | Keratin type I, cytoeskeletal 17 | 334362277 | **KRT17** | 55.2 | 5.3 | 22 | 3,9 | (2)/ |

[1] Spot number from Figure 1 and the corresponding spot ID in Table 1.

[2] Mean and standard error of the mean (SEM) of normalised intensity for each individual spot from 5 replicate gels (pools of soluble protein extract from 2 or 3 fish).

[3] Protein identities, accession number, theoretical MW and pI, peptide matches (unique peptides), score, percentage sequence coverage (SQ) and species identification were supplied by the Mascot Search Results (Matrix science). Further details of search conditions in M&M section.

[4] Gene symbol, gene number (Entrez gene database from NCBI, http://www.ncbi.nlm.nih.gov/) and UniprotKBd (http://www.uniprot.org) of each protein were obtained from the Genecards database search process (http://www.genecards.org).

[4] The UniprotKB number was used for further Gene Ontology enrichment analysis in Table 1.

| SPOT ID[a] | PROTEIN IDENTITY[b] | GENE SYMBOL[c] | BIOLOGICAL PROCESS GROUPS | | | | | | | | | | | | CC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | S1 | S2 | M1 | M2 | M3 | M4 | P1 | P2 | P3 | P4 | P5 | P6 | |
| ***Structural proteins*** | | | | | | | | | | | | | | | |
| 4,14,23,32,37,51 | Beta-actin | ACTB | **X** | | | | | | X | X | X | X | | | EX |
| 8,77 | Profilin 1 | PFN1 | **X** | | | | | | X | X | | | X | X | EX |
| 20,36,89 | Gelsolin | GSN | **X** | | | | | | X | X | | | | X | EX |
| 40,72 | Macrophage-capping protein-like | CAPG | **X** | | | | | | | | | | | | EX |
| 17 | Profilin 2 | PFN2 | **X** | | | | | | | | | | | | EX |
| 12 | Cofilin-2-like | CFL2 | **X** | | | | | | | | | | | | EX |
| 28,71 | Tropomyosin 4-1 | TPM4 | **X** | | | | | | | | | | | | EX |
| 91 | Myosin light polypeptide 6 | MYL6 | **X** | | | | | | | | | | | | EX |
| 13 | Intermediate filament ON3-like | ION3 | **O** | | | | | | | | | | | | |
| 82 | Keratin, type I cytokeratin 19 | KRT19 | **X** | | | | | | | | | | X | | EX |
| 76,98 | Keratin, type II cytoskeletal 1 | KRT1 | | **X** | | | | | X | X | X | X | | | EX |
| 44,54 | Periplakin-like | PPL | | **X** | | | | | | | | | | | EX |
| 78 | Keratin, type II cytoskeletal 2 | KRT2 | | **X** | | | | | | | | | | | EX |
| 93,100 | Keratin, type I cytoskeletal 17 | KRT17 | | **X** | | | | | | | | | | | EX |
| 7 | Keratin, Type I E7 | KRT14 | | **O** | | | | | | | | | | | EX |
| 22,48 | Keratin, type I cytoskeletal 13 | KRT13 | | | | | | | | | | | | X | EX |
| ***Metabolic proteins*** | | | | | | | | | | | | | | | |
| 27 | Inositol monophosphate 1-like | IMPA1 | | | **O** | | | | | | | | | | |
| 9,65,73 | Glyceraldehyde 3-P-DH | GAPDH | | | **X** | | | | X | | X | X | | X | EX |
| 18 | Enolase 1 (alpha) | ENO1 | | | **X** | | | | | | | | | | EX |
| 31 | Beta-enolase-like isoform 1 | ENO3 | | | **X** | | | | X | X | | | | | EX |
| 55 | Transketolase-like isoform X1 | TKTL1 | | | **X** | | | | | | | | | | |
| 87 | Malate dehydrogenase | MDH1 | | | **X** | | | | | | | | | | EX |
| 25,69 | Malate-DH mitochondrial-like | MDH2 | | | **X** | | | | | | | | | | EX |
| 58 | Triosephosphate isomerase B | TPI1 | | | **X** | | | | | | | | | | EX |
| 79 | Glycine N-methyltransferase-like I1 | GNMT | | | X | | **X** | | | | | | | | |
| 57 | Pyruvate kinase | PKLR | | | **X** | X | | | X | | | | | X | EX |
| 96 | Fructose-biphosphate aldolase | ALDOA | X | | **X** | X | | | X | X | | | | | EX |
| 85 | Mitochondrial ATP synthase β subunit | ATP5B | | | | **X** | | | | | | | | | EX |
| 94 | Nucleoside diphosphate kinase | NME1 | | | | **X** | | | | | X | | | X | |
| 3 | Deoxycytidylate deaminase-like | DCTD | | | | **X** | | | | | | | | | |
| 6 | Phosphatidylethanolamine-BP | PEPB1 | | | | **O** | | | | | | | | | EX |
| 39 | Cdc 48 | ATAD2B | | | | **O** | | | | | | | | | |
| 15 | WD repeat-containing protein 1 | WDR1 | | | | | | | X | X | | | | | EX |
| 68 | Inorganic pyrophosphatase-like | PPA1 | | | | | **X** | | | | | | | | EX |
| 66 | Antiquitin | ALDH7A1 | | | | | **X** | | | | | | | | EX |
| 95 | Adenosylhomocysteinase | AHCY | | | | | **X** | | | | | | | X | EX |
| 52 | Glutathione S-transferase | GSTA1 | | | | | **X** | | | | | | | X | EX |
| 67 | 26S protease regulatory 6a | PSMC3 | | | | | **X** | | X | | X | | X | | |
| 49 | Proteasome subunit alpha type-4 | PSMA4 | | | | | **X** | | X | | X | | X | | EX |
| 62 | Translation initiation factor 5A | EIF5A | | | | | X | **X** | | | | | | | |
| 61 | Ribosomal protein large P0 | RPLP0 | | | | | | **X** | | | | | X | | EX |
| 97 | Elongation factor 1-γ-like | EEF1G | | | | | | **X** | | | | | | | |
| 45 | Elongation factor 1-β-like | EF1B | | | | | | **X** | | | | | | | EX |
| 56 | Ubiquitin hydrolase L1 | UCHL1 | | | | | | | X | | | | | | |
| 53 | Gastrotopin (Lipocalin superfamily) | FABP6 | | | | | | | | | | | | | |
| 26 | Calmodulin | CALM1 | | | X | | | | X | X | X | X | | X | EX |
| 24 | Apolipoprotein A-I | APOA1 | | | | | | | X | X | X | X | | X | EX |
| ***Protection-related proteins*** | | | | | | | | | | | | | | | |
| 50 | Esterase D | ESD | | | | | | | O | **O** | | | | | EX |
| 90 | Elastase | CELA3B | | | | | | | O | | O | O | | | |
| 16,83 | Coactosin-like | COTL1 | | | | | | | X | | | **X** | | | EX |
| 21,33 | 14-3-3 protein zeta/delta | YWHAZ | | | | | | | X | X | **X** | X | | | EX |
| 1,35 | Complement component 1q | C1QC | | | | | | | X | | **X** | **X** | | | EX |
| 41,47,63 | WT acclimation-related 65KDa protein | HPX | | | | | | | X | | **X** | X | X | X | EX |
| 86 | Heat shock protein A1 | HSPA1A | | | | | | | X | | **X** | | | | EX |
| 19,88,99 | Protein disulfide-isomerase-like | PDIA3 | | | | | | | X | | **X** | | | | EX |
| 10,11,38 | Heat shock protein 70KDa protein 8 | HSPA8 | | | | | | | X | | | | **X** | | EX |
| 2,5,42 | Transferrin | TF | | | | | | | X | **X** | | | | | EX |
| 70 | 78 kDa glucose-regulated protein | HSPA5 | | | | | | | X | **X** | | | | X | EX |
| 29 | Cu-Zn superoxide dismutase | SOD1 | | | | | | | X | X | **X** | | | X | EX |
| 64,81 | Beta globin | HBB | | | | | | | X | **X** | | | | X | EX |
| 30 | Peroxiredoxin 2 | PRDX2 | | | | | | | X | | | | | X | EX |
| 59 | Glucose regulated protein 75kDa | HSPA9 | | | | | | | O | **O** | | | | | EX |
| | **Total (number of proteins)** | | **10** | **4** | **11** | **5** | **8** | **4** | **26** | **15** | **14** | **9** | **7** | **15** | **49** |