

# Application of proteomic tools to identify changes in the protein composition of physiologically relevant fish tissues

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### Summary

Over the past ten years, proteomic tools have been extensively used in various studies aimed at a better understanding of fish biology and aquaculture. A variety of post-genomic techniques have been developed and adapted for protein profiling of complex biological samples, such as tissues. This review will discuss the applications of expression proteomics in fish health monitoring with regard to nutritional and environmental changes, as well as bacterial or viral infection, in selected tissues relevant to fish physiology, i.e. the kidney, brain, blood plasma, and liver.

**Keywords:** fish, proteomic tools, tissue, proteome

Fish are an important source of protein, averaging 40% of the protein intake for two-thirds of the world population, and is a substantial part of the daily diet in many countries (13). The increasing role of fish for alimentation implies an improvement in culture conditions, the reduction of infections and stress, as well as the augmentation of fertility and body mass to increase the fish yield and profitability. Another challenge to fish consumption is the maintenance of food quality and safety. This is why attempts have been made to identify specific protein biomarkers which may be suitable for verifying the authenticity of food products. Using MALDI-TOF MS (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry) Mazzeo et al. (23) have recently obtained protein profiles from the muscle tissue of 25 distantly and closely related fish species. The authors proved that the signal patterns generated from the muscle proteins with molecular weights of approximately 11 kDa could be considered as species-specific biomarkers (23). The most interesting of the listed protein markers characteristic of different fish species appeared to be the ones of sole ( $m/z$  11975.21), European plaice ( $m/z$  11351.73, 11763.63) and Greenland halibut ( $m/z$  11432.38). The above-mentioned biomarkers

corresponded to parvalbumin, a calcium-binding muscle protein, which is considered as a major fish allergen (17, 23). Additionally, Martinez and Friiz (21) claim that the loss of freshness causes the formation of parvalbumin aggregates which in turn may increase the allergenicity of fish. Other studies dedicated to food quality assessment describe specific allergens (10) and deal with the influence of freezing-thawing cycles (15) on fish meat quality. Fish are also relevant for fundamental biological research. The zebrafish (*Danio rerio*) is typically known as a model organism for embryonic development and organogenesis in vertebrates, but recently has also been used as a model for human neurological diseases (5) and drug discovery (2). Despite the importance of fish for human nutrition, in the monitoring of environmental pollution and as model organisms in fundamental research, the physiology of only a small number of species has been studied, mostly at the molecular level of biochemistry and genomics. Only recently, the proteomic approaches have found their applications in fish physiology studies (22, 25).

Proteomics can be defined as the analysis of the proteome, i.e. of the entire set of proteins expressed at a defined time, under specific conditions that also

includes variants, post-translational modifications and the characterization of protein-protein interactions (21). Proteins perform many cellular functions (12), and the proteome, unlike the genome, changes dynamically and constantly (3, 18). As such, the proteome integrates changes in gene expression, mRNA stability, post-translational modifications and protein turnover (12). By studying the whole proteome, potentially unforeseen responses can be observed (29) and new mechanistic hypotheses can be generated (12).

This review will focus on the the applications of expression proteomics in fish health monitoring with regard to nutritional and environmental changes, as well as bacterial or viral infection in selected organs relevant to fish physiology, i.e. the kidney, brain, plasma, and liver.

### Proteome changes in fish tissues

#### Kidney

The influence of infection with *Moraxella sp.*, a bacteria considered as harmless for fish farming, on the kidney proteome of gilthead sea bream (*Sparus aurata*) was analyzed by Addis et al. (1) using 2-DE (two-dimensional electrophoresis), MALDI-TOF MS and LC-ESI-Q/TOF MS (liquid chromatography-electrospray ionization-quadrupole/time-of-flight mass spectrometry). Mitochondrial enzymes, such as alanine aminotransferase, aldehyde dehydrogenase, dihydridipicolinate synthase, ATP synthase subunit beta, methylmalonate semialdehyde dehydrogenase, acyl-Co A dehydrogenase, which are involved in metabolism and apoptosis, as well as other proteins (e.g. peroxiredoxins, S-adenosyl-homocysteine hydrolase, glutathione S-transferase, Cu/Zn superoxide dismutase) implicated in response to oxidative stress, infection and inflammation, were found to be significantly up-regulated in the kidneys after *Moraxella sp.* infection.

The effect of bacterial and viral infection on the renal proteome was also studied by Booy et al. (6). The authors, using 2-DE, ICAT (isotope-coded affinity tag) and LC-ESI-Q/TOF MS, reported that the infection of Atlantic salmon (*Salmo salar*) by BKD (*Rennibacterium salmoninarum*) provoked an up-regulation of lysozyme, cathepsin L and other proteins related to inflammation and stress response. IHNV (infectious hematopoietic necrosis virus) infection caused a down-regulation of proteins involved in the response to viral infection, like HSP70 (heat shock protein 70) or complement proteins, such as complement components C3 and C5.

#### Brain

**Hypoxia and anoxia.** Smith et al. (31) studied protein expression changes during and after anoxia in the brain of the crucian carp (*Carassius carassius*), a species resistant to anoxia. The authors, using 2-DE and LC-ESI-IT MS (liquid chromatography-electro-

spray ionization-ion trap mass spectrometry), showed a reduced expression of proteins associated with the glycolytic pathway (creatine kinase, fructose biphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase and lactate dehydrogenase), neuronal apoptosis (voltage-dependent anion channel), and neurodegeneration (dihydropyrimidinase-like protein-3 and vesicle amine transport protein-1). Moreover, they found the up-regulation of pre-proependymin, the precursor of ependymin, in response to anoxia. This protein is commonly known as a promoter of neuron growth and regeneration, and it may also play a role in reversing the apoptotic process (31).

A 2D-DIGE (two-dimensional difference gel electrophoresis) and MALDI-TOF/TOF MS (matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry) examination of the medaka (*Oryzias latipes*) brain after episodes of hypoxia revealed subtle changes at the proteome level (24). Several proteins correlated with hypoxia adaptation were found to be up-regulated in medaka brain tissue. These included proteins involved in vascularization (hemoglobin, carbonic anhydrase II), ion transport (calbindin), energy production (aldolase, succinate dehydrogenase), and oxidative stress response (glutathione-S-transferase). The above-mentioned proteins might be considered as potential hypoxia markers in medaka brain tissue (24).

**Model organism.** Damodaran et al. (9) studied the effects of chronic ethanol exposure on the proteome of zebrafish (*Danio rerio*) brain tissue (soluble and pellet fractions), using 2-DIGE and MALDI-TOF MS. Out of the 13 protein spots found to be differentially expressed in the soluble fractions of the brain, 11 were up-regulated and 2 were down-regulated in response to 0.5% (v/v) ethanol exposure when compared with the control tissue. Unfortunately, only 4 overexpressed spots were successfully identified. These included voltage-dependent anion channels 1 and 2, apolipoprotein A-I and glutamic-oxaloacetic acid transaminase-1. The authors also demonstrated that ethanol exposure resulted in significant changes in the expression of 18 protein spots in the pellet fractions of the brain. The expression of 10 spots was increased (e.g. heat shock 70 kDa protein 5, guanine nucleotide binding protein, alpha activating polypeptide O), whereas the expression of 8 (e.g. H<sup>+</sup> transporting ATPase, lysosomal, V1 subunit A; H<sup>+</sup> transporting ATPase, vacuolar, subunit B2) was decreased. Some of these proteins may be considered as novel targets for chronic ethanol exposure and may help in understanding the biological mechanisms explaining alcoholism and alcohol toxicity (9).

In contrast to mammals, the brown ghost knifefish (*Apteronotus leptorhynchus*), which belongs to the teleost fish, has the ability to recover after central nervous system (CNS) injuries. Zupanc et al. (33) used

2-DE and MALDI-TOF/TOF MS in order to find proteins involved in the repair process in the CNS. The authors demonstrated that cerebellar lesions caused changes in 53 protein spots, 30 of which were up-regulated and 23 down-regulated when compared with the control tissue. The expression of the following proteins was found to be increased:  $\beta$ -actin,  $\beta$ -tubulin,  $\beta$ -1-tubulin, chaperonin containing tailless-complex polypeptide 1 subunit epsilon, tropomodulin-3 and -4, bullous pemphigoid antigen 1, myosin heavy chain, B2-lamin, 78 kDa glucose-regulated protein, glutamine synthetase, cytosolic aspartate aminotransferase,  $\alpha$ - and  $\beta$ -enolase, F-ATP synthase  $\beta$ -subunit, vacuolar adenosine triphosphate, 70 kDa heatshock cognate protein, phosphoglycerate kinase, and creatine kinase. Proteins with decreased expression levels were identified as keratin-10, cytosolic aspartate aminotransferase, calcineurin and bone marrow zinc finger 2.

#### Blood plasma

**Acute phase response.** Wu et al. (32) elicited an acute phase response (APR) by four dorsal incisions on loach (*Misgurnus anguillicaudatus*) and investigated acute phase proteins (APPs), using 2-DE, PMF (peptide mass fingerprint) and LC-ESI-Q/TOF MS. Besides well-known APP (e.g. apolipoprotein, cathepsin S and C-reactive protein), the authors also identified new APR-related proteins (e.g. signal recognition particle, parvalbumin and gastrin 71).

Russell et al. (27) studied an APR after the injection of Lipogen triple J vaccine or smooth and rough lipopolysaccharides (LPS) from *A. salmonicida* into rainbow trout (*Oncorhynchus mykiss*), using 2-DE, MALDI-Q/TOF MS (matrix-assisted laser desorption ionisation quadrupole time-of-flight mass spectrometry), LC-ESI-Q/TOF MS and N-terminal Edman sequencing. They observed an increase in apolipoprotein, pre-cerebellin and transferrin. These proteins may be considered as biomarkers of inflammatory processes.

Brunt et al. (7) investigated the effect of probiotics as an alternative to antibiotics on the serum proteins of rainbow trout, using 2-DE and MALDI-TOF MS. An increase in the expression of transferrin commonly known as an APP (4) and a protein with unknown function was observed after the injection of *Bacillus* sp. JB-1.

**Health.** The large yellow croacher (*Pseudosciaea crocea*) is a fish of considerable importance for Chinese farming, but its culture suffers from increasingly frequent viral and bacterial infections. To gain new insights into molecular immunology after bacterial infection, Chen et al. (8) vaccinated fish with formalin-killed *Aeromonas hydrophila* and challenged them with the same bacterial strain. The authors, using 2-DE and PMF, identified eight proteins, among which the most interesting was  $\beta$ 2-microglobulin, a key protein in the MHC (major histocompatibility complex) class I.

Doux fils et al. (11) studied the effect of chronic confinement stress on plasma proteome changes in the Eurasian perch (*Perca fluviatilis*) in the first (F1) and fourth (F4) generations of captive fish, using 2-DE and LC-ESI-Q/TOF MS. They observed a decreased immunity in F1 fish after the application of the confinement stressor (decreased levels of complement C3 and transferrin), whereas the immunity of F4 fish was stimulated by confinement, even if domestication reduced the basal levels of immunity (down-regulation of complement component C3).

#### Liver

**Nutrition.** The effect of short-term starvation (14 days) on the liver proteome in rainbow trout was investigated by Martin et al. (19). The authors, using 2-DE and PMF, found that cathepsin D, a lysosomal endopeptidase, is highly overexpressed in the liver of starved trout in comparison with the control group. This result clearly indicates that the main pathway of protein degradation in the liver of the rainbow trout is through the lysosome.

Jury et al. (16) used 2-DE, LC-ESI-IT MS and LC-ESI-LTQ MS (liquid chromatography-electrospray ionization linear ion trap quadrupole mass spectrometry) to show that a high-calorie treatment in zebra fish decreases oxygen binding and transport activities, peroxid detoxification, and creatine synthesis, as well as induces a rearrangement of the cytoskeleton. Contrary to the hypothesis formulated by the authors, the up-regulation of the pathways of lipid metabolism was not observed and seems not to be the primary response to high-calorie diet in fish species.

Martin et al. (20) studied the influence of a higher proportion of soybean meal in the diet of farmed rainbow trout on their physiology and liver proteome, using 2-DE and PMF. An augmentation in the soybean content down-regulates the expression of structural proteins (keratin II,  $\beta$ -tubulin), heat-shock proteins (HSP70, HSP78) and anabolic proteins (phosphogluconate dehydrogenase, pyruvate kinase, adenosylhomocysteinase, hypoxanthine-guanine phosphoribosyltransferase), while simultaneously up-regulating the expression of HSP108 and the proteins involved in metabolic and energetic pathways, such as adolase B and phosphatase 2A. It should be emphasised, that the authors showed a substantial diminution in the Apo A-I (apolipoprotein A-I) level, which revealed the presence of phytoestrogens – a class of antinutritional factors – in the soybean meal.

Sissener et al. (30) investigated the influence of the genetically modified (GM) soy Roundup Ready in a fish diet inclusion on the liver proteome of Atlantic salmon, using 2-DE LC-ESI-IT. They found only low-fold differences between fish fed with GM soy and those fed with non-GM soy, which indicates minor differences in protein synthesis. They identified three differentially regulated proteins; one was up-regu-

lated (calreticulin) and the other two were down-regulated (triose phosphate isomerase, thymidine phosphorylase), which co-migrated on the gel.

Maslinic acid (MA) is used as a feed additive for its property of increasing growth, protein turnover rates and hyperplasia in fish. Rufino-Palomares et al. (26) investigated cellular mechanisms that are activated in response to the addition of MA to a standard fish diet by analyzing the liver proteome of gilthead sea bream, using 2-DE, PMF and MALDI-TOF/TOF. The results clearly indicate that this diet additive triggers a variety of changes at the protein level. The intake of MA caused an increased expression of proteins involved in glucose metabolism (phosphoglucosylase 1, phosphoglucose isomerase), sterol metabolism (17- $\beta$ -hydroxysteroid dehydrogenase type 4), amino acid metabolism (methylmalonate-semialdehyde dehydrogenase), oxidative stress response (catalase, 6-phosphoglucosyl dehydrogenase), protein synthesis (elongation factor 2 of eukaryotic protein synthesis), protein folding (HSP60, 58-kDa glucose-regulated protein) and cytoskeleton (cytokeratin E7, intermediate filament proteins, keratin II). The down-regulated proteins included those linked to glucose metabolism (S-adenosyl methionine (SAM)-dependent methyltransferase class I), detoxification and xenobiotic metabolism (alcohol dehydrogenase), amino acid metabolism (fumarylacetoacetate hydrolase, 4-hydroxyphenylpyruvic dioxygenase), and growth factor/cell proliferation (kinase suppressor of Ras1).

**Health.** Booy et al. (6) studied the effects of BKD and IHNV infection on the liver proteome of Atlantic salmon. After a bacterial BKD challenge, they found an up-regulation of CNBP (cellular nucleic acid binding protein) and calreticulin, which may play a role in the host's defence by limiting inflammation and tissue injury. Infection by IHNV induced a down-regulation of several ribosomal proteins (indicating a decrease in protein synthesis) and the peroxidase NKEF (natural killer enhancement factor). These proteins are extremely important in cell signalling and immunoregulation.

Gas bubble disease (GBD) can occur in fish under oxygen-supersaturation conditions. It is frequent in outdoor ponds and is mainly caused by high temperature and radiation, which lead to an increase in photosynthetic oxygen. Salas-Leiton et al. (28) investigated changes in the liver proteome of Senegal sole (*Solea senegalensis*) affected by GBD. Gas bubble disease induced changes in five liver proteins. Three of them were significantly up-regulated, including protein engaged in amino acid metabolism – glycine N-methyltransferase, protein involved in the urinate cycle and glucose metabolism – dicarbonyl/L-xylulose reductase, and complement component C3. Fatty acid-binding protein, responsible for the transport of hydrophobic metabolic intermediated and lipid carriers between

membranes, was found to be down-regulated in response to GBD. Moreover, the authors identified one protein –  $\beta$ -globin – that was not present in the control tissue. The main role attributed to this protein is oxygen binding and/or transport (28).

Ibarz et al. (14) investigated the effect of cold stress on the kidney proteome of cold-intolerant gilthead sea bream, using 2-DE, PMF, MALDI-TOF/TOF and LC-ESI-Q/TOF. In general, cold provokes fasting, growth arrest, general metabolic down-regulation and increased hepatic lipid deposition. The proteomic data showed a decreased expression of actin,  $\alpha$ - and  $\beta$ -tubulin, enzymes of amino acid metabolism (glutamate dehydrogenase, aspartate aminotransferase, alanine-glyoxylate aminotransferase, homogentisate 1,2-dioxygenase) and antioxidant enzymes (betaine-homocysteine-methyl transferase, glutathione-S-transferase, catalase). Cold was found to reduce the abundance of proteins involved in proteolysis (peptidase, dipeptidase, proteasome activator protein, trypsinogen-like protein), oxidative stress response and the induction of apoptosis (elongation factor-1 $\alpha$ , oxidative forms of glyceraldehyde-phosphate dehydrogenase and Raf-kinase inhibitor protein).

The results of the studies described above clearly indicate that post-genomic technologies can be successfully applied in various studies of bacterial and viral infections, nutrition or the influence of environmental factors on fish physiology, even if there exist limited genomics data, thus fish are called as a 'non-model' organisms for protein identification.

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