Proteomic approach to investigate alterations, within physiological limits, in serum protein of sea bass (*Dicentrarchus labrax*)

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Introduction

The aquaculture fish production will continue to expand and the perception of aquaculture rearing systems are often associated to high stocking density in tanks. Today, consumer demand for safe and ethically defendable food products is increasing. Production systems must consider animal welfare, which depend on good husbandry practices. Previous studies analyzed the effect of stocking density on biological performance in the growth of fishes. Generally high stocking density is considered as a potential source of stress, with a negative effect. Biological indexes and blood parameters, such as plasma cortisol and glucose level can be used to measure the potential effect of stocking density on fish performance and welfare. Alterations in total serum protein (TSP) concentration are used as a broad clinical indicator of health, stress, and well being of terrestrial and aquatic organisms. TSP was also used to determine crowding stress in trout. Two studies of protein panel modification in sera by chromatography were published. One concerning brown trout injected with PBS and the other, sea bass stressed by ammonia, they permitted to precise modification indiscriminate by TSP. Genomic approach was recently used and shown that increase of stocking densities affect gene level expression in sea bass. Proteomic approaches were recently used on fish tissues by using either 2D electrophoresis and MALDI-TOF3 or SELDI-TOF4 to investigate panel and variation of type of fish tissue proteins. In this experiment, the effects of a high stocking density (100 kg m-3) were monitored on sea bass with all water parameters maintained at non limiting levels. The global results were, alteration of swimming behavior, feed intake, growth rate and respiratory activity. Blood parameter analyses do not change and no conclusive difference in TPS was found. Actually TPS level can discriminate high modifications in rearing conditions, but it cannot detect low alterations, within normal limits (welfare) like high stocking density.
In order to discriminate those situations, serum protein panels were investigated by 2 proteomic approaches: SELDI-TOF on protein lower than 20 kDa and 2D electrophoresis followed by LC-MS-MS identification on proteins from 15 kDa to 250 kDa.

Materiel and method

[3] *MALDI-TOF : matrix assisted laser desorption and ionization - time of flight*

[4] *SELDI-TOF : surface enhanced laser desorption and ionization - time of flight*

Fish sample TSP analysis

Serum used in this study comes from a previous experiment which is still published (Sammouth et al 2008) and concerned sea bass reared with stocking density at 10 kg m-3. TSP was analyzed with A Max Mat Hycel autoanalyzer expressed in mg ml-1

2D-PAGE Electrophoresis

Sera from both groups were analyzed on 18-cm pH NL gd (roughly 10-250 kDa) strips pH 3-10 .

First dimension EF

An aliquot containing 400 µg of sera proteins was mixed for 1 hour with 350 µL of rehydration buffer (8M urea, 4% w/v 3[3-cholaminopropyl diethylammonio]-1-propane sulfonate, ampholyte 1% v/v (Bio-Lyte 3/10; Bio-Rad) and just before use, DTT 20 nM , trace of bromophenol blue added. After 1 hour rehydratation, of 18-cm immobilized pH gradient strips pH 3-10 with samples, focusing was performed overnight in the Protean IEF cell.

Second dimension SDS-PAGE

Strips were in 2 ml of a equilibration solution [Tris 0.375 M, SDS 1% pH 8.8), urea (6M), SDS (1% w/v), glycerol (20% w/v), a trace of bromophenol blue, and DTT (130 mM) for 10min, followed by equilibration for 10 mn in the same buffer containing iodoacetamide (135 mM) instead of DTT.

The strips were transferred to the top of gels [acrylamide 12 %, SDS 1%, APS 0.05%, TEMED 0.03 %] and held in position with molten (0.5% w/v) agarose in running buffer [25 mM Tris pH 8.3; 192 mM glycine; 0.1% w/v SDS and trace of bromophenol blue], kept at 12°C throughout the run till migration front left the gel.

Staining

The gel is washed off gel-fixing solution (TCA 5%) for 6hr, replaced for 6 hr by staining solution [Coomassie blue G-250,0.1%w/v, ethanol 5% v/v H3PO4 8.5 %v/v]. The discoloration solution [ethanol 25%v/v,acetic acid 10% v/v] until background staining of the gel was enough clear.

Scanning and quantification of spots

Gels were scanned with Bio-rad SG 800 calibrated densitometer. Quantification of spots, production of synthetic gels and statistic comparison were performed with software ImageMaster 2D platinium v6.0 GeneBio.

Protein digestion

Proteins were digested in-gel using trypsin by described (Shevchenko et al, 1996)

LC MS-MS

Samples (1 µl) were analyzed online on a ESI quadrupole time-of-flight (Q-TOF) mass spectrometer (QSTAR Pulsar-i, Applied Biosystems, Foster City, CA) coupled with an Ultimate 3000 HPLC (Dionex, Amsterdam, Netherlands).

The Q-TOF was fitted with uncoated silica PicoTip Emitter (NewOjective, Woburn, USA) with an outlet diameter of 8 µm. Spectra were recorded using the Analyst QS 1.1 software (Applied Biosystems). Spectra were acquired with the instrument operating in the information dependent acquisition mode throughout the HPLC gradient. Every 7 s, the instrument cycled through acquisition of a full-scan spectrum (1 s) and two MS/MS spectra (3 s each). Peptides fragmentation was performed using nitrogen as collision gas (CID) on the most abundant doubly or triply charged ions detected in the initial MS scan, with a collision energy profile optimized according to peptide mass (using manufacturer parameters), and an active exclusion time of 0.60 min.

MS/MS Analysis

All MS/MS spectra were searched against the Actinopterygii entries of either SwissProt or Trembl databases (http://www.expasy.ch), or a private database by using the Mascot v 2.1 algorithm (http://www.matrixscience.com). All significant hits were manually inspected. Peptides with a good fragmentation signal that dot not led to identification were manually de novo sequenced according to classical fragmentation rules. The sequences were compared to databases using BLAST algorithm.

SELDI-TOF protein chip system

The SELDI-TOF (Ciphergen PBS II) manufacturer recommendations concern human serum. Trout, salmon and turbot were analyzed with SELDI-TOF but for sea bass serum adaptation were necessary. Two sheep Ciphergen NP20 and IMAC30 were chosen with concentration x2 and contact duration 60mn in place of 5 mn.

Results and comments

Proteinemia and choice of samples

Proteinemia means were 38.99 mg ml-1 for fish reared at 100 kg m-3 and 41.50 mg ml-1 for those reared at 10 kg m-3 with the max at 52 mg ml-1 and mini at 32 mg ml-1 (normal for this seize of sea bass). T-test show a significant difference between means ($p < 005$, $n=30$). We choose, for further investigations, the 15 closest samples from the means of each density.

2D gel comparison

Individual protein panel of 2 groups presents an important difference in number of spots. The spot number varies from 226 to 370 spots for 10 kg m-3 and from 360 to 450 for 100 kg m-3. The gel with the highest number of 10 kg m-3 is close to the smallest of 100 kg m-3.

The most representative gels were processed by Image Master Platinum software to built 2 synthetic gels with 208 spots for 10 kg m-3 and 280 for the 100 kg m-3. Specific spots labeled on the 2 synthetic gels show 17/208 specific spots for 10 kg m-3 and 99/280 specific spots for 100 kg m-3.

The number of protein increased individually in fish of 100 kg m-3 or they were fragmented. The synthetic gel confirmed this increase and permitted to confirm disappearing of 17 proteins or fragments, apparition on 99 new proteins or fragments

This comparison takes into account presence or not of spots; the specific variations in spots parameters should show more important alterations of protein panel.

Statistic analysis

Intra class analysis

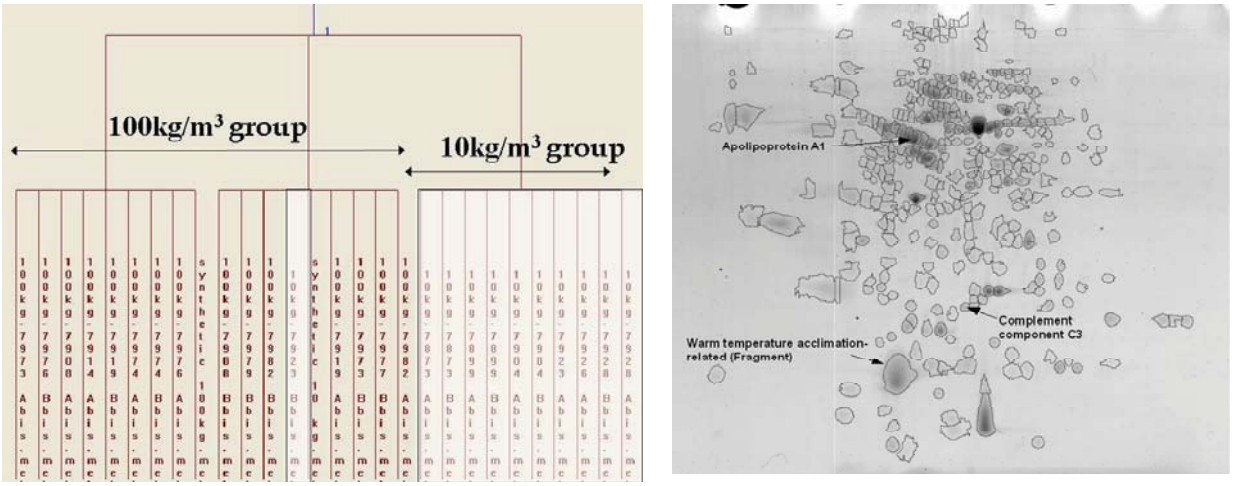
Intra class CR (% volume) shows a good homogeneity, higher in 10 kg m-3 group (0.98>C.R.>0.93 in 10 kg m-3 0.95>C.R.>0.84 in 100 kg m-3).

Analysis was done on spots volume and most of gels have a correlation with reference gel coefficient >0.9 except 3 with coefficient near 0.9 and the 10-6b which is out of its class (0.4). The references gel is a correct representation of real gels

Inter class (fig left)

Heuristic clustering performed on all gels shared them into 3 classes. One including gels from 10 kg m-3 and 2 including 100 kg m-3. Only one 10 kg m-3gel is not in the right class. Kolmogorof Smirnof test ($p<0.05$) show by an other way the protein panel difference and homogeneity.

LC-MS-MS Spot identification (right)



a) b)

Figure 1: Three classes shared by heuristic clustering (a) ; Four spots involved in inflammation identified by LC-MS-MS (b).

20 spots of interest, significantly different between 100 kg m-3and 10 kg m-3, were analyzed. 4 are spots identified are probably involved in inflammation: Inter-alpha (Globulin) inhibitorH3, C3 complement and FBP32 and the Warm temperature acclimatization related protein, (http://www.ncbi.nlm.nih.gov/protein/86610887?report=genpept). Those protein apparitions strengthen the hypothesis of stress by crowding.

SELDI TOF

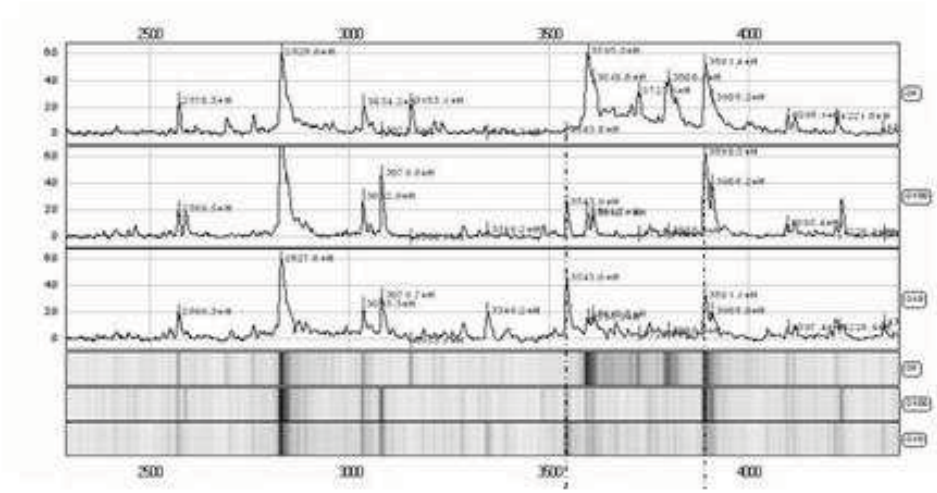


Figure 2: Discriminating picks between 10 and 100 kg m-3

Former (3543) increases in 10 kg m^{-3} and the latter (3899) increase in 100 kg m^{-3} ($P<0.05$) (spotted line).

Conclusion

2D gel electrophoresis show difference between serum proteome of sea bass reared at 100 kg m-3 and 10 m-3 stocking density in number of protein whole or fragments. 17 proteins or fragments disappeared and 99 appeared.
Four proteins probably involved in inflammation were identified at 100 kg m-3 by LC MS-MS strengthen the hypothesis of stress by crowding
SELDITOF permits to show difference in protein panel between the 100 kg m-3 and 10 kg m-3 rearing density
Proteomic analysis can discriminate modifications within normal limits in rearing conditions like high rearing density.

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