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Changes in the soluble bone proteome of reared white seabream (*Diplodus sargus*) with skeletal deformities ☆

Tomé S. Silva, Odete Cordeiro, Nadège Richard, Luís E.C. Conceição, Pedro M. Rodrigues *

Centro de Ciências do Mar do Algarve (CCMar), Universidade do Algarve, FCMA, Campus de Gambelas, 8005-139 Faro, Portugal

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ABSTRACT

One of the main constrains for commercial aquaculture production of white seabream (*Diplodus sargus*) is the high incidence of skeletal malformations in reared fish. The purpose of this study was to obtain a better understanding of the mechanisms involved in the development of these types of skeletal malformations by comparative proteomic analysis of the vertebral column of normal and deformed fish using 2DE for protein separation and MS for protein identification. We observed a 3.2 and 3.4-fold increase in the expression of two tropomyosin isoforms, one of which (tropomyosin-4) is essential for the motility and polarization cycles of osteoclasts. Furthermore, a 1.6, 1.7 and 1.8-fold increase in three parvalbumin spots was detected, suggesting a cellular response to increased intracellular Ca^{2+} levels. These results can be interpreted as signs of increased cellular activity in the bone of white seabream with skeletal deformities coupled to a higher degree of calcium mobilization, which elicits further studies into the use of these proteins as indicators of skeletal metabolic state.

1. Introduction

The white seabream (*Diplodus sargus*) is a recently introduced species of teleost fish in aquaculture production, mainly due to its high market value and signs that its availability in the wild has been decreasing (Ozorio et al., 2006; Santos et al., 2006). One of the main constrains for the commercial production of white seabream is the high incidence of skeletal malformations in reared fish. The more common skeletal deformities present in these fish are characterized by a depression of the dorsal profile accompanied by kyphosis and, in most of the cases, haemal lordosis. Other types of bone pathologies which also occur occasionally include neck and skull malformations, scoliosis and platyspondyly (Sfakianakis et al., 2003). Current knowledge suggests that these deformities can be caused both by neuromuscular effects and by changes in the biological processes responsible for maintaining bone integrity (Lall and Lewis-McCrea, 2007). In the case of white seabream, recent studies indicate nutritional factors (namely, dietary deficiency in specific amino acids) as one of the causes involved in the onset and development of such skeletal deformities (Saavedra et al., 2009), but it is almost certain that these occur as a result of an interaction between nutritional, genetic and environmental factors (Lall and Lewis-McCrea, 2007).

Bone tissue is mainly composed of inorganic hydroxyapatite crystals (about 70% of its total dry weight) embedded in an organic matrix (Salgado et al., 2004). Type I collagen is a fibrous protein and the major component of this organic matrix, providing support for other proteins to bind (Lammi et al., 2006; Rodan, 1992). Non-collagenous proteins present in bone showing biological relevance in its metabolism include osteopontin, bone sialoprotein, osteonectin, several cytokines and growth factors (such as BMPs), alkaline phophatase and Gla proteins (such as osteocalcin) (Lammi et al., 2006; Rodan, 1992; Zhu et al., 2001).

The three main cell types present in bone tissue are osteoblasts (responsible for the production and mineralization of the bone matrix), osteoclasts (bone resorbing cells) and osteocytes (mature osteoblasts that become lodged inside the mineralized bone matrix). The dynamic nature of bone tissue derives from the balance between the matrix forming activity of osteoblasts and the matrix degrading activity of osteoclasts (Rodan, 1992; Teitelbaum, 2000).

The lack of osteocytes in many teleosts (such as members of the *Perciformes* order) is one of the main characteristics that distinguish fish bone tissue from mammalian bone. Despite this difference, both types of bone (cellular and acellular) display continuous growth, as well as regenerative capabilities. Nevertheless, acellular bone is formed by osteoblasts which migrate away from mineralization sites after bone deposition, making them incapable of extensive modeling. (Lall and Lewis-McCrea, 2007; Moss, 1963; Witten et al., 2004).

Abbreviations: CAF, chemically-assisted fragmentation; PFF, peptide fragment fingerprinting; EST, expressed sequence tag; BLAST, basic local alignment search tool.

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* Corresponding author. Tel.: +351 289800100x7855; fax: +351 289818353.
E-mail address: pmrodrig@ualg.pt (P.M. Rodrigues).

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Although some published studies regarding bone proteome and its responses to stressful/pathological states can be found (especially for mammals) (Forlino et al., 2007; Jiang et al., 2007; Lammi et al., 2006; Pastorelli et al., 2005; Schreiweis et al., 2007), there seems to be a lack of research regarding global *in vivo* bone proteome responses in fish. Since the occurrence of skeletal malformations in reared fish results, in many cases, from suboptimal rearing conditions, this type of research is particularly useful in the aquaculture technology context due to the possibility of finding molecular indicators for the assessment of skeletal metabolic state, which can complement existing fish stress predictors, when optimizing rearing conditions of marine teleosts in aquaculture.

Therefore, in order to achieve a better understanding of the metabolic changes that occur in acellular bone associated with the onset and development of skeletal deformities in reared fish, a proteomic screening approach was pursued. We used a previously established protein extraction protocol developed by Pastorelli et al. (2005) to analyze estrogen-regulated proteins in the bone of rats, in order to detect novel indicators of skeletal metabolic state in reared fish, which can be used to further improve our knowledge of the biological processes that occur in acellular bone. The use of a method that does not involve previous demineralization of bone tissue implies a low extraction efficiency for high-abundance proteins associated with the mineralized matrix (such as the thoroughly characterized collagen and osteocalcin), which can be beneficial for the detection of previously uncharacterized variations in low abundance proteins. This comparative analysis of protein expression at the bone tissue level between normal phenotype white seabream and those with skeletal deformities was performed using IPG-IEF/SDS-PAGE two-dimensional electrophoresis and standard mass spectrometric techniques (MALDI-ToF MS and LC/ESI-Ion Trap MS–MS of tryptic peptides) for protein separation and identification, respectively.

2. Materials and methods

2.1. Fish trials and sampling

Fish rearing in the present study was done at the Aquaculture Research Station of IPIMAR, in Olhão, South of Portugal, during 2006. *D. sargus* eggs were obtained from spontaneous spawning of a mixture of wild and farmed origin broodstock consisting of 38 fish with an average mass of 869 g. Eggs were incubated in 200 L fibre glass tanks, with moderated aeration and a water flow of approximately 0.4 L/min and a maximum density of 1 g eggs/L. First-feeding larvae were transferred to 200 L conical cylindrical fibreglass tanks at a density of 80 larvae/L. Three white fibreglass tanks were used. The water inlet was located near the bottom and moderated aeration was used. The water temperature was maintained constant at 19.1 ± 0.7 °C, oxygen at 6.5 ± 0.4 mg/L and salinity at 37 ppt. Water flow started at 0.4 L/min and was slowly increased with larval age until a maximum of 1 L/min, at 12 days after hatching (DAH).The photoperiod was 24-h light, with artificial light (700 lux). The feeding consisted of rotifers, *Brachionus plicatilis*, followed by a microencapsulated diet. Rotifer cultures were done in 600 L tanks at 20 ppt and at a temperature of 26 °C. Rotifers were enriched with Protein Selco (INVE Aquaculture, Belgium) for 24 h before being fed to the larvae. Five rotifers/mL were fed to the larvae in the morning and afternoon from 3 to 14 days after hatching (DAH). Rotifers were then decreased to half in both meals from 15 to 20 DAH. The microencapsulated diet was introduced at 15 DAH. A total of 1.5 g and then 2 g/day of microencapsulated diet was given to the larvae from 15–20 DAH, and after that, respectively. *Tetraselmis spp.* and *Isochrysis galbana* were added to the tanks twice every day in order to create "green-water" conditions (Conceição et al., 2009). The microencapsulated diet consisted of cross-linked casein-walled capsules (Yúfera et al., 1999), using a balanced diet formulation according to Saavedra et al. (2009). At 25 DAH, when fish reached 0.97 ± 0.15 mg dry mass, they were transferred to three 1500 L, 90 cm deep, circular tanks. Rearing temperature was 18–19 °C, water current very mild, but with a flow rate assuring a 25% total volume/hour water renewal, in order to assure oxygen saturation levels above 80% and no accumulation of nitrogenous compounds. Maximum fish density in these tanks never exceeded 1.2 kg/m^3. Fish were fed 7 meals by per day, four by hand feeding to satiation (9.00, 12.00, 14.00, and 17.00 h) and three by automatic feeders (19, 21, and 22 h), using a commercial seabream feed (Dourasoja, SORGAL SA, Ovar, Portugal). At 143 DAH, all three tanks contained a similar ratio of deformed/total juveniles (54/159, 61/203 and 80/205), without any significant differences in terms of length and weight between them (data not shown). Mean individual wet mass was 8 ± 2 g. Classification of individuals as "normal phenotype" or "deformed phenotype" was performed based on macroscopic visual inspection of the fish body (mostly by examining the shape of the lateral line) and confirmed by further macroscopic visual inspection of the vertebral column after dissection. Observed deformities were of kyphotic nature, with a low degree of vertebral rotation, and mainly localized in the caudal region (Fig. 1). These tanks were sampled in order to obtain a sufficient number of normal and deformed specimens for extraction of proteins from bone tissue (between 40 to 60 individuals from each condition, from each tank), having kept them frozen at −20 °C until further processing.

2.2. Protein extraction and preparation

Due to the relatively low amounts of obtained protein *per* fish, bone material from individuals with the same skeletal phenotype from the same tank was pooled. After thawing the whole vertebral column from each individual was removed and cleaned for protein extraction. This procedure avoids introducing "location" as a confounding variable, as would happen if only the affected tissue zones would be used for extraction. Bone material was then fragmented with a pestle, washed at 4 °C with distilled water (3×30 min) and then with acetone (3×2 h). It was left to dry overnight and further reduced to dust with an electric mill and passed through a sieve (1 mm). Protein extracts were prepared by stirring 1.34 g of powdered bone in 10 mL of phosphate buffer (K_2HPO_4 10 mM, KH_2PO_4 10 mM, EDTA PlusOne™ (Amersham), 1 mM, CHAPS PlusOne™ (Amersham) 10 mM) containing a protease inhibitor cocktail (Roche, ref. 11836153001) for 12 h at 4 °C, following centrifugation at 10,000×*g* for 10 min to separate protein extracts from bone material. A final protein precipitation step was done in order to remove non-protein contaminants (ReadyPrep 2-D Cleanup Kit, Bio-Rad, ref. 1632130), using RB buffer (8 M urea, 2% (w/v) CHAPS, 50 mM DTT, 0.2% (w/v) Bio-Lyte® 3/10 ampholytes and traces of bromophenol blue) for sample resolubilization prior to 2DE. All protein quantifications prior to 2DE were done using the Bradford assay (Bio-Rad).

2.3. Two-dimensional gel electrophoresis

In order to accurately estimate the abundance of each protein in each of the 6 pooled samples, 3 technical replicates (i.e. 3 gels) were performed per pooled sample (for a total of 18 gels). 125 μg of protein from each sample was loaded on to 7 cm IPG ReadyPrep pH 4–7 strips (Bio-Rad), by passively re-swelling them overnight in 120 μL of total volume (using RB buffer for sample dilution, as appropriate). This pH gradient 4–7 has been previously established as optimal since major protein content was present within this pH range. Proteins were separated in the first dimension by isoelectric focusing using an Ettan IPGphor (Amersham), at 20 °C, by increasing voltage until 4000 V for 2 h 50 min and then applying a constant 5000 V for 30 min (for a total of about 8150 Vh). Strips were then equilibrated using standard Bio-Rad reduction/alkylation buffers (15 min each step), loaded on to 8×8 cm IPG NuPAGE® 4–12% Bis–Tris gels (Invitrogen) and run at 200 V, using NuPAGE® MES SDS running buffer (Invitrogen). Gels were stained with EZBlue™ colloidal CBB stain (Sigma), according to a manufacturer's instructions.