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Post mortem changes produced in the muscle of sea bream (*Sparus aurata*) during ice storage

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ABSTRACT

The *post mortem* degradation processes that take places in fish muscle gradually modify the initial state of freshness. Tissue degradation is accompanied by drastic myofibrillar proteolysis produced as a consequence of the activation of proteolytic enzymes. In this study, structural changes were correlated with the textural properties of sea bream (*Sparus aurata*) muscle during 14 days of *post mortem* cold storage through the immunohistochemical detection of muscle filament proteins (desmin, actin and dystrophin), as well as endoproteases (μ-calpain, m-calpain) and their endogenous inhibitor (calpastatin). Actin and desmin were detected in fish muscle as late as 10 days *post mortem* (dpm) while dystrophin could not be detected after 4 dpm. In contrast, labelling for both calpains and calpastatin persisted during the entire storage period. Fracturability was clearly reduced after 7 dpm, while greatest changes in hardness, gumminess and chewiness were observed during the first week of storage. Our immunohistochemical observations indicate the disappearance of cytoskeleton proteins at different times *post mortem*. Thus, actin and desmin persist even when the muscle tissue shows a deteriorated appearance and texture, while dystrophin vanishes soon after the death of the fish. Detachment between myofibres and the myocommata was concomitant with the loss of dystrophin and also corresponded to the time when the reduction in flesh hardness observed was most significant. On the contrary, after 4 days of storage, calpain activity remained practically unaltered in refrigerated muscle specimens.

1. Introduction

The changes that take place in fish muscle *post mortem* have a significant impact on the aquaculture industry due to their determining effects on fillet quality and consumer acceptance. Unlike what occurs in mammals, fish muscle rapidly softens during *post mortem* storage due to proteolytic degradation, reducing the quality of the flesh for human consumption. Proteolytic events seem to be common to the different fish species but some species-specific features exist. For instance, during the degradation of desmin measured by electrophoresis of muscle extracts, an intermediate filament that interconnects the myofibrils to the cell membrane at the level of the Z-disk has been detected in turbot (*Scophthalmus maximus*) and sardine (*Sardina pilchardus*) but not in sea bass (*Dicentrarchus labrax*) (Verrez-Bagnis et al., 1999). Further, desmin degradation has been observed early during *post mortem* storage in many fish species (Verrez-Bagnis et al., 1999). Actin is the second most abundant muscle protein. The interaction between thin actin filaments and thick myosin filaments generates the force for muscle contraction. Actin filaments are closely linked to α-actinin, the main linking protein in the Z-disk. The release of α-actinin from the myofibrillar structure results in the loss of Z-disk integrity and subsequent muscle disorganization (Papa et al., 1996; Tsuchiya et al., 1992). In sea bass, 60% of dystrophin (the subsarcolemmal actin-binding protein located in costameric structures that ensures linking between the actin cytoskeleton and the extracellular matrix) is cleaved during the first 24 h and is no longer present after 2–3 days of storage at 4 °C (Papa et al., 1997; Bonnal et al., 2001). The degradation of other muscle protein components such as titin, nebulin, myosin and tropomyosin has also been associated with *post mortem* deterioration (see review by Delbarre-Ladrat et al., 2006). Further, the breakdown of collagen junctions between the myocommata and muscle fibres during ice storage has been correlated with gaping (Bremmer, 1992), a phenomenon that occurs when the myoseptum breaks and muscle blocks become separated, significantly altering fish flesh texture (Johnston et al., 2002). Taylor et al. (2002) showed that softening of salmon texture is related to myofibre–myofibre and myofibre–myocommata splitting. The main groups of

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enzymes involved in fish muscle protein degradation during storage *post mortem* are: calpains, cathepsins and matrix metalloproteases (Kubota et al., 2001; Ladrat et al., 2003; Delbarre-Ladrat et al., 2004a; 2004b; Chéret et al., 2007). Calpains are intracellular endopeptidases that require calcium for their enzymatic activity. Two isoforms are widely present in muscle: μ-calpain and m-calpain. In addition, calpains are regulated by an endogenous specific inhibitor, calpastatin. Cathepsins are acid proteases located in the lysosomes. Several studies have demostrated the susceptibility of numerous myofibrillar proteins to proteolysis by calpains and cathepsins. In sea bass muscle, calpain was able to release α-actinin and tropomyosin from myofibrils *in vitro* (Verrez-Bagnis et al., 2002). Both calpains and cathepsins degrade myosin heavy chain, α-actinin, desmin, actin and tropomyosin (Ladrat et al., 2003; Delbarre-Ladrat et al., 2004a). However, the relative contributions of calpains and cathepsins to protein degradation in fish muscle have not yet been clearly established. Early works suggested that calpains initiated the proteolytic degradation of myofibrils, making them more susceptible to cathepsins (Papa et al., 1996). Notwithstanding, later studies conducted in the same species (Delbarre-Ladrat et al., 2004b) indicated that calpains did not modify muscle protein prior to the hydrolysis undertaken by cathepsin proteases. More recently, Chéret et al. (2007) suggested a secondary role for calpains whereby, despite their abundance in sea bass muscle, they are inhibited by calpastatin (inhibitor), which is present in higher amounts than in other vertebrates. Most experiments, however, have been performed only a few hours *post mortem*. Thus, to improve our understanding of the role of calpains in fish muscle protein degradation and of the complex mechanisms involved, the entire shelf life of fish in cold storage conditions needs to be considered.

The present study was designed to monitor structural and functional changes occurring in sea bream muscle during its shelf life on ice, and to assess the effects of these modifications on flesh texture and the consequent repercussions on quality. Immunohistochemical staining was used as an efficient tool to visualize degradation patterns of myofibrillar proteins and detect the presence of enzymes. The antibodies used for staining were antibodies against desmin, actin and dystrophin, endoproteases (μ-calpain, m-calpain) and their endogenous inhibitor, calpastatin. Calpain activity and flesh texture were also assessed during the *post mortem* period.

2. Materials and methods

2.1. Fish samples

The experiments were carried out at the Instituto Canario de Ciencias Marinas (Canary Islands, Spain). Sixty gilthead sea bream (*Sparus aurata*) (of mean initial body weight 79 ± 8.0 g) were obtained from a local fish farm (ADSA, San Bartolomé de Tirajana, Canary Islands, Spain) and randomly assigned to three fibre-glass tanks of 500 l as groups of twenty fish. Water temperature and dissolved oxygen during the experimental period ranged from 20.0 to 24.2 °C and 5.04 to 8.32 ppm, respectively. The fish were fed a diet containing 45% crude protein and 22% fat supplied by Proaqua S.A. (Dueñas, Palencia, Spain) until apparent satiation three times a day for 4 months. When they attained a weight of 250 g, eighteen fish per tank were killed by immersion in ice cold water (fish:ice ratio 2:1) and packed as whole ungutted fish in polystyrene boxes filled with ice. Fish were stored in a refrigerator at 4 °C for up to 14 days *post mortem* (dpm). During storage, fillets of three fish from each tank were obtained at 0, 2, 4, 7, 10 and 14 dpm. This time period is considered close to the shelf life (from when the fish is removed from the water until it is no longer fit to eat) of whole ungutted sea bream stored between −1 °C and 6 °C (Cakli et al., 2006).

All procedures were approved by the Animal Care Committee of the University of Las Palmas Gran Canaria and conducted according to the guidelines of the Spanish Committee on Animal Care.

2.2. Histology and immunohistochemistry

For histology, 5 mm-thick cross-sections of tissue were obtained from each fish (three fish × time point × tank, $n = 54$) by cutting fillets from the posterior third of the fish body. Tissue samples were fixed in 10% buffered formalin for 1 or 2 days, dehydrated in a graded series of alcohol followed by one of xylene and finally embedded in paraffin wax. Three serial sections (4 μm) were then cut from each paraffin embedded sample and each processed for immunohistochemistry or haematoxylin and eosin (H&E) or Masson's trichrome (MT) staining (Martoja and Martoja-Pierson, 1970).

Sections for immunohistochemistry (Bancroft and Stevens, 1996) were mounted on Vectabon-coated slides (Sigma Diagnostics, St. Louis, MO). These sections were dewaxed in xylene, rehydrated in a graded alcohol series and incubated with 3% hydrogen peroxidase in methanol for 30 min to block endogenous peroxidase activity. Enzyme or microwave treatment was applied according to the primary antibody used, except for the anti-desmin polyclonal antibody (Euro-diagnostica, Malmö, Sweden, diluted 1:150). Sections were incubated with anti-actin (Enzo Diagnostics, USA, clone HHF35, diluted 1:150) or anti-calpastatin (Affinity BioReagents, Golden, USA, clone 2G11D6, diluted 1:6000) monoclonal antibodies in pronase 0.1% in PBS for 10 min at room temperature. When anti-dystrophin (Sigma, Saint Louis, Missouri, USA, clone MANDRA1, diluted 1:350), anti-m-calpain (Affinity BioReagents, Golden, USA, clone 107-82, diluted 1:100) or anti-μ-calpain (Affinity BioReagents, Golden, USA, clone 9A4H8D3, diluted 1:50) monoclonal antibodies were used, the sections were incubated in citric acid 0.01 M, pH 6 in a microwave oven (800 W) at a high setting until boiling point, and then at a low setting (approximately 100 °C) for 7 min. Next, the sections were covered with 10% normal rabbit serum (for monoclonal antibodies) or 10% normal pig serum plus 10% goat serum (for the polyclonal antibody) in PBS for 30 min before incubation with the primary antibody for 18 h at 4 °C. When the primary polyclonal antibody was used, a biotinylated pig anti-rabbit immunoglobulin G diluted 1:200 in PBS was applied for 30 min as the secondary reagent. For the primary monoclonal antibodies, a biotinylated rabbit anti-mouse immunoglobulin G was used diluted 1:20 PBS to detect dystrophin, actin or calpastatin, diluted 1:200 PBS for μ-calpain and 1:100 PBS for m-calpain detection. The different substrates were detected by incubation for 1 h at room temperature with an avidin–biotin–peroxidase complex (ABC, Vector Laboratories, Burlingame, CA) diluted 1:50 in PBS. Sections were then incubated with 3-amino, 9 ethyl-carbazole diluted in 0.1 M sodium acetate-buffer containing 3% hydrogen peroxide, and checked microscopically for adequate chromogen development. Finally, sections were rinsed in tap water, counterstained with Mayer's haematoxylin, dehydrated and mounted. The muscles of mouse, rabbit and rat were used as positive controls. Negative controls were run by replacing each primary antibody with PBS. In each section (3 sections per staining method and time point, 9 × 6), 10 fields were examined under a light microscope separately by two pathologists. The mean proportions of positive cells (red staining of the sarcolemma membrane and/or cytoplasm) were scored as follows: +++, >70%; ++, 30 to 70%; +, <30%; −, negative.

2.3. Preparing muscle extracts for the determination of calpain activity

To determine calpain activity, portions of fillet from fish from each tank were taken from the posterior third of the fish body at 4, 7 and 14 dpm ($n = 9$), minced and then 15 g of tissue was taken and immediately frozen in liquid nitrogen and kept at −80 °C. Frozen samples were homogenized individually (using a polytron homogenizer, Kinematica, Luzern, Switzerland) at 10,000 rpm with intense cooling in an ice–salt mixture in a 5-fold volume of 20 mM Tris–HCl buffer (pH 7.4) containing 5 mM EDTA, 5 mM EGTA and 1 mM dithiothreitol (DTT, Sigma Chemical Company, St. Louis, U.S). 10 μg/ml