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Comparative study of muscle proteins in relation to the development of yake in three tropical tuna species yellowfin (*Thunnus albacares*), big eye (*Thunnus obesus*) and skipjack (*Katsuwonus pelamis*)

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ABSTRACT

Burnt tuna (BT), or *yake-niku*, is a quality flaw of the muscle characterised by a pale colour and grainy and exudative texture. Cathepsin-L, water soluble and total protein components from normal and BT muscles, from three tropical tuna species – yellowfin (YFT, *Thunnus albacares*), bigeye (BET, *Thunnus obesus*) and skipjack (SKJ, *Katsuwonus pelamis*) – were compared by electrophoretic and western blot analyses to identify biomarkers for BT. As expected, SDS–PAGE patterns were species-specific but differences, due to BT, were observed only between some low ionic strength extracts of BET and YFT. Protein oxidation and cell proliferation analysed by immunoblotting did not show differences between BT and normal muscles. Gelatine zymography revealed different gelatinase activity patterns that, although not linked to BT, may affect the final texture of the muscle. A 43 kDa band, identified as creatine kinase by proteomic analysis, showed the potential to be a good indicator for BT in BET and YFT.

1. Introduction

Yellowfin (YFT) *(Thunnus albacares),* big eye (BET) *(Thunnus obesus)* and skipjack (SKJ) *(Katsuwonus pelamis)* are the main target species of the Atlantic, Indian and Pacific Ocean tuna fisheries (Davies, Mees, & Milner-Gulland, 2014). The commercial value of tuna, frozen in brine after capture and intended for the canning industry, is determined by the species and size, SKJ being the most inexpensive (Bartram, Garrod, & Kaneko, 1996). However, very high quality tuna can be destined for the sushi and sashimi market. In this case, the size, species and muscle texture determine the quality and price. One critical quality flaw that makes the muscle unsuitable for sushi and sashimi is the "*burnt tuna*" (BT), or "*yake-niku*" in Japanese, characterised by a change in colour, from bright red to pale pink and by a grainy, softer texture (Cramer, Nakamura, Dizon, & Ikehara, 1981). The condition occurs mainly in large tropical tunas, such as BET and YFT, particularly if they have been subjected to exhausting exercise prior to death (Cramer et al., 1981). Since affected fish are not suitable for the highest priced raw tuna market, it is very important to avoid its development and to identify burnt individuals as fast as possible.

Possible causative factors and several hypotheses have been postulated: the first investigations by Konagaya and Konagaya (1979) on BET and YFT concluded that high temperature and low pH were the main causes for the apparition of the BT. Tuna fish possess a complex network of blood vessels (*rete mirabile*) that acts as a heat exchanger and allows the fish to retain the heat produced by the red muscle. This permits them to reach body temperatures higher than that of the surrounding water, a higher basal metabolic rate and consequently a higher myofibrillar ATPase activity which, in turn, grants them the capability to swim faster (Carey, Teal, Kanwisher, Lawson, & Beckett, 1971). The further increase in muscle temperature caused during struggling in the fishing gear, together with the low *post-mortem* final muscle pH value and the activation of the calcium activated proteases by the *post-mortem* liberation of Ca^{2+} ions from the sarcoplasmic reticulum into the cytosol, were thought to be the cause of myofibrillar protein denaturation leading to BT (Hochachka & Brill, 1987; Watson, Morrow, &

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Brill, 1992). It is generally agreed that the stress generated during the struggle while harvested is determinant to the final quality of tuna flesh (Cramer et al., 1981; Konagaya & Konagaya, 1979; Watson et al., 1992).

In 2010, Ochiai (2010) showed a high degree of protein aggregation and degradation as well as the absence of creatine kinase (CK) in the burnt portion of muscle in wild and farmed tuna, and proposed that this absence of CK might be a good biomarker for BT. In addition to this, Yamashita et al. (2011) found a close inverse relationship between the levels of selenoneine, Se-analogue of ergothioneine discovered in 2010 by Yamashita, Yabu, and Yamashita (2010) and shown to be a powerful antioxidant (Yamashita et al., 2013). The negative influence of oxidative damage on muscle texture has also been supported by Nurilmala, Ushio, Kaneko, and Ochiai (2013) who found that the *post-mortem* accumulation of metmyoglobin (the oxidised form of myoglobin), also keeps an inverse relationship with the quality of the meat.

All these studies indicate that oxidative stress may be one of the main causes for BT. The presence and amount of carbonyl groups in proteins are useful biomarkers of oxidative stress (Chora et al., 2008) and are easy to detect in fish muscle extracts by immunoblotting after dinitrophenyl (DNP) derivatization (Kjærsgård & Jessen, 2004). Degradation of the intracellular myofibrillar proteins also contributes to muscle softening (Bremmer, 1999). The two most relevant intracellular proteolytic pathways, which act in a coordinated manner, are the ubiquitin–proteasome and the lysosomal cathepsins (see review by Sandri, 2010). Cathepsin L is the major contributor of the acidic lysosomal proteases to the total *post-mortem* proteolytic activities in fish (Cheret, Delbarre-Ladrat, Lamballerieanton, & Verrez-Bagnis, 2007; Martinez et al., 2011; Yamashita & Konagaya, 1991). Ubiquitination and cathepsin L were examined in this study by western blot analysis, since both parameters had already been proposed as potential quality markers in farmed salmon (Martinez et al., 2011).

Biomarkers for muscle damage also have the potential to serve as textural quality indicators. Thus, satellites cells are quiescent in adult skeletal muscle, but in response to an injury they can reinitiate the proliferative activity (Yablonka-Reuveni & Rivera, 1994). Gelatinases and matrix metallo proteinases (MMPs) also play clear roles in myoblast differentiation, fusion of satellite cells and myotube formation, essential in order to accomplish the myogenesis necessary for the natural growth of muscle, as well as for muscle repair in cases of injury, regeneration and inflammation (reviewed by Chen and Li (2009)). Unlike most terrestrial species, fish grow through their lives and, therefore, the satellite cells and MMPs can be activated at any time during the fish lifespan, in addition to particular cases of muscle injury and regeneration. The proliferating cell nuclear antigen (PCNA) is an auxiliary protein to DNA polymerase whose levels correlate with DNA synthesis during the cell cycle, becoming maximal during the S phase (Bravo, Frank, Blundell, & Macdonald-Bravo, 1987). Its presence is detectable by a specific and commercially available anti-PCNA antibody, and can be used as a biomarker of muscle proliferation which, in turn might serve as an indicator of textural damage.

The connective tissue is mainly made up of collagen, and fish collagen has a relatively low melting temperature. This already high lability is further increased by the acidic muscle *post-mortem* pH, and both factors, i.e. *post-mortem* high temperature and low pH, contribute to denaturing the collagen into gelatine. Gelatinases are considered to play a role in the softening of the fish muscles and have already been proposed as an indicative parameter for quality flaws in fish (Martinez et al., 2011). In addition to collagen degradation, the proteolytic fragmentation of myosin, the most abundant myofibrillar protein, has traditionally being considered an indication of proteolytic activity contributing to the softening of the muscle (Wang, Martinez, & Olsen, 2009).

The purpose of this work was to evaluate the possibility of identifying and/or confirming the suitability of potential BT biomarkers for the two species which are currently visually tested for BT (BET and YFT), and also on SKJ, since being the one with lowest price it is also the one that shows the highest potential for improvement and value adding.

2. Material and methods

2.1. Sample collection

Samples of muscle and gonads from 23 big eye tuna (BET, *Thunnus obesus*); 70 yellowfin tuna, (YFT, *Thunnus albacores)* and 30 skipjack tuna (SKJ, *Katsuwonus pelamis),* with total weights of 22.6 ± 5.3, 29.1 ± 9.5 and 5.4 ± 1.3 kg; mean core temperatures of 27.5 ± 2.3, 22.7 ± 6.7 and 24.7 ± 1.7 °C and pH values of 6.7 ± 0.2, 6.3 ± 0.2 and 5.4 ± 0.8, respectively, were collected on board of the Basque tuna fishing vessel *Alakrana,* in the West Indian Ocean. The temperature and pH measurements were determined with a thermometer and a pH meter respectively, after 1 h at −2 °C in brine tanks, at the same moment where the visual determination of BTS was also preformed. The samples were taken from the region 5 cm behind the pectoral fin on the lateral line, from the same place where the sashibo was extracted. All the samples used for the extractions described below, were excised from the fish, stored frozen and transported to our lab at −50 °C.

The fish had been harvested in an area between 6° 40′ 00″N–6° 50′ 60″S and 44° 14′ 53″E–59° 52′ 48″E, in January–February 2013. Individuals of the three species were selected in the fish hold according to the species availability, size and boarding time. Immediately after the selection, tunas were cooled down in brine tanks at least for 1 h at −2 °C, prior to visual BST determination in the muscle. To verify the presence of BT a *sashibo* was used. A *sashibo* is a metal hollow cylinder used to obtain a cross-sectional flesh sample of skeletal muscle of approximately 15–20 g in weight, 1.2 cm in diameter, and 8 cm in length. Sashibo samples were taken, as indicated above, on the lateral line, 5 cm behind the pectoral fin. This procedure was applied only to YFT and BET, since SKJ are not considered as valuable species for the raw fish products market. Gonads were extracted *in situ* from the internal cavity of the fish and a section of approximately 3 cm was taken and frozen at −50 °C.

2.2. Cathepsin extraction

Cathepsin extraction was carried out as described by Cheret et al. (2007). For this purpose, portions of 50 mg of white muscle were homogenised in 200 μl of buffer, containing 50 mM Tris HCl (pH 7.5), 10 mM of dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetic acid (EDTA), with 2.8 mm ceramic (zirconium oxide) beads (Bertin Technologies, CK28) at 6000 rpm for 30 s in a Precellys 24-Dual homogeniser (Bertin Technologies), while maintaining a constant temperature of 4 °C, during the whole procedure, with the advanced temperature controller Cryolys (Bertin Technologies). This procedure was performed twice. Homogenised samples were transferred to Eppendorf tubes, centrifuged at 14,000 rpm (Microfuge 22R, Beckman Coulter) for 40 min at 10 °C and the supernatants transferred to new Eppendorf tubes.

2.3. Total and water soluble protein extractions

Portions, of approximately 50 mg, of white muscle were homogenised in 200 μl of buffer containing 62.5 mM Tris HCl (pH 6.8),