Actin as a candidate quality marker for food of animal origin

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I declare that my PhD thesis has been amended to address all the Referee's comments.

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Preface

Most of the relevant meat and meat-product quality characteristics are the results of the chemistry of muscular protein and peptides. Therefore, the inclusion of information on protein differences provided by protein markers can make a significant contribution to the improvement of meat and meat product quality.

Actins are a family of highly conserved proteins, which play fundamental roles in nearly all aspects of eukaryotic cell biology. In the skeletal muscle, in addition to the highly specialized contractile apparatus, there are both actin-associated costameric complexes and functionally distinct cytoskeletal actin-based filaments.

Ante mortem muscle biochemistry strongly influences *post mortem* biochemical processes which, in turn, are linked to final quality attributes, including texture which is one of the most important attributes of muscle foods. However, there is very little knowledge how *ante mortem* biochemistry changes the *post mortem* events and hence the resulting final quality. The understanding of *post mortem* mechanisms is essential for predicting the final quality in terms of texture.

The histochemical and biochemical evidence indicates that much of the tenderization associated with *post mortem* aging is due to the action of the endogenous muscle enzymes. Interestingly, the most abundant proteins of the myofibril, actin and myosin, are not significantly degraded during *post mortem* aging. However, an extensive apoptosis could lead to a progressive degradation of cytoskeletal and thin filaments of actin, resulting in meat tenderization.

The investigation on how actin degradation affects meat and meat product quality is still nascent, but the results reported in this PhD thesis seem promising. Indeed, the main activities have been directed to evaluate the potential use of actin as a biochemical marker for the prediction of muscle food quality in three Italian PDO dry-cured hams and in farmed sea bass, which are two high nutritional value products.

In the first part of this thesis, a brief general introduction on the muscle composition in mammals and fish and on the *post mortem* events occurring in muscle is reported, followed by a description of the characteristics of dry-cured hams and sea bass. Therefore, the two papers related to the above reported topics are attached, accompanied by some conclusive remarks.

1. INTRODUCTION

1.1 MUSCLE ANATOMY AND COMPOSITION

1.1.1 Mammalian muscle

The skeletal muscle is by far the most important in animal agriculture (Du & McCormick, 2009), being the basis for numerous meat products. Skeletal muscles are attached to the skeleton by particular structures, called tendons. Tendons are made by an inextensible connective tissue layer primarily composed by collagen, known as epimysium. The muscle is divided by thin layers called perimysium into fiber bundles (Rogers, 2001). The diameter of the different fibers is between 20 and 100 μm, and the length varies from few millimeters up to several centimeters. Muscle cells are surrounded by two structures: the endomysium, a connective tissue sheath, and the sarcolemma, which is the cell membrane (Figure 1.1).

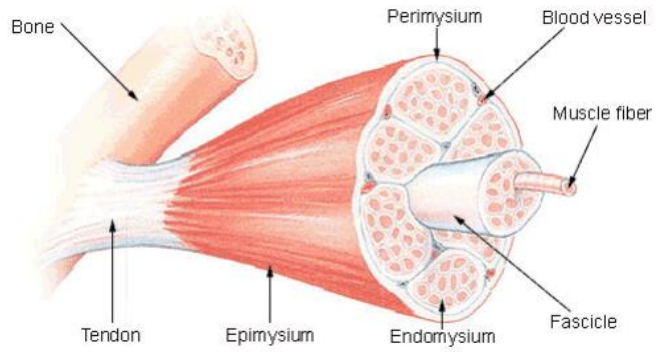

Figure 1.1. Muscle anatomy and composition. *Modified from* Tank & Gest, 2009 .

The striated muscle is composed of muscle fibers (the muscle "cells"), which in turn contain myriad of myofibrils. These are constructed of end-on-end contractile units called sarcomeres, which contain three types of filaments: thick, thin, and connecting. The arrangement of the filaments results in the striated appearance of the muscle under microscope. The dark bands in the central part of the sarcomere form the A-band, while the pale bands at the extremities of the sarcomere form the I-band. In the middle of the A-band, there is the M-line and an optically less dense region that disappears during contraction, called H-band. Each sarcomere is bordered by thin *septa*, defined as Z-disc. The Z-disc forms the structural scaffold for the sarcomere and provides a site for the attachment of a filaments network of that links the individual contractile bundles (myofibrils) to each other and to the plasma membrane (sarcolemma) (Figure 1.2) (Clark, McElhinny, Beckerle, & Gregorio, 2002).

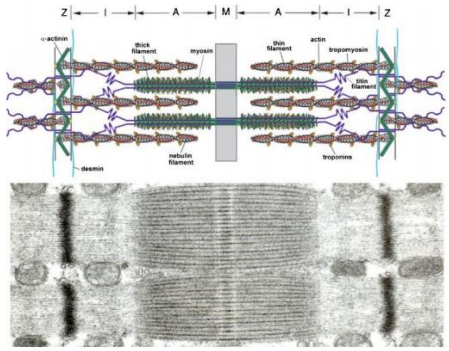

Figure 1.2. Sarcomere structure. *Modified from* Nedergaard, Karsdal, Sun, & Henriksen, 2013.

The disposition of thin and thick filaments in the sarcomere is such that only thin filaments can be found in the I band, in the A-band there are both thin and thick filaments and in the H-band are present only thick filaments. The different filaments form a hexagonal symmetry in which each thick filament is surrounded by six thin filaments. As in all the other cells of the organism, muscle cells also posses intracellular organelles like, Golgi apparatus, mitochondria, nucleus and endoplasmic reticulum. Around the myofibrils the sarcolemma is present, composed of a net of tubules that could be divided in two portions, not communicating to each other. These two portions are the T tubules and the longitudinal reticulum.

The muscle proteins can be divided in three different groups, based on their solubility and muscular collocation: myofibrillar, of which actin and myosin are the most abundant, sarcoplasmic, primarily represented by myoglobin, (a pigment responsible for the red meat color), and stroma proteins, collagen being quantitatively the most important (Goll, Neti, Mares, & Thompson, 2008). In vertebrate muscle, the sarcomere contains twenty-eight different proteins (Craig & Padrón, 2004). Muscle types common feature is that cells are enriched in proteins that interact in a cyclic fashion to produce force and /or shorten the cells.

Muscle fibers can identified by different methods: histochemical staining for the ATPase reaction catalysed by myosin, identification of the isoforms of the myosin heavy chain, and biochemical identification of metabolic enzymes, applicable to humans as well as to animals. Histological analyses showed that there is a correlation between myosin ATPase activity and the speed of muscle shortening, dividing the muscle fibers into type I (slow) and type II (fast). Based on the differences in the acid stability of the myosin ATPase reaction, the fibers are further distinguished in type I (high reactivity), IIa (very low) and IIb (intermediate) (Brooke & Kaiser, 1970). A combination of the histochemical myosin ATPase staining at acidic (4.3-4.6) and

basic (10.4) pH in consecutive serial sections ("two-dimensional approach") led to the identification of four distinct clusters of fibers: types I, IIA, and two subgroups of type IIB, later classified as IIB and IIX (Lind & Kernell, 1991).

According to metabolic enzyme content, three major fiber types have been distinguished: fast twitch glycolytic (white), fast twitch oxidative (intermediate), and slow twitch oxidative (red) (Dubowitz & Pearse, 1960). However, while the comparison with histochemical staining for myosin ATPase confirmed a good correlation between type I and slow twitch oxidative fibers, the correlations between type II and fast twitch fibers were more complex. The most frequently used markers of the aerobic and anaerobic metabolism have been the succinate (SDH) and the lactate (LDH) dehydrogenase, respectively.

Another classification system designate fast twitch fibers as α fibers, and slow twitch fibers as β fibers, with the aerobic metabolic activity designated as strong (R) or weak (W) (Ashmore, Tompkins, & Doerr, 1972). Under this system, fast white fibers are designated αW, and slow red fibers as βR (Table 1.1).

Red (βR) fibers	White (αW) fibers
High myglobin content	Low levels of myoglobin content
Small volume of sarcoplasmic reticulum	Extensive sarcoplasmic reticulum
High triglyceride store	Limited amounts of triglyceride
Limited capacity of anaerobic metabolism	Adapted to anaerobic metabolism
Low phosphocreatine stores	High levels of phosphocreatine
Low glycolytic enzymes activity	High levels of glycolytic enzymes
Slow	Fast

Table 1.1: Differences between red and white fibers in mammalian muscle (Du & McCormick, 2009)**.**

1.1.2 Fish muscle

In fish, skeletal muscle is highly developed and represents the major part of the edible fraction (40% – 60% of body mass depending on species, dimension, maturation and physiological conditions) (Poli, 2006). The lateral muscle of fish differs from mammalian muscle in its myotomal organization and because the two main types of fibers (red and white) are separated into a superficial (red muscle) and a deep (white muscle) layer (Bassani & Dalla Libera, 1991). Typically, 90% of fish skeletal muscle is composed of anaerobic white fibres that can develop power rapidly and almost independently of the circulation, while the red muscle fibers are employed for routine activity, such as migration, using aerobic metabolic pathways to provide sustained energy (Johnson, 1999). The muscle fibres in each myotome insert into a connective tissue sheath or myocommata. The orientation of fibres within the myotome is complex and varies both along the body and with distance from the vertebral column. Right and left muscle are separated by a connective *septum* along the axis of the body that, with an horizontal connective *lamina*, determine the distinction in dorsal epaxial and ventral hypaxial portions (Bremner & Hallet, 1985).

The distinctive properties of fish red and white fibers with respect to the mammalian ones are reported in Table 1.2.

	Red fibers	**White fibers**
Diameter of cell	69-75 µm	35-45 µm
Sarcomere length	2.05 µm	1.8 µm
Colour	Presence of myoglobin	Absence of myoglobin
Enzymes	High activity of enzymes of the oxidative metabolism	High activity of enzymes of the glycolytic metabolism
Mitochondria	Numerous and with a big diameter	Few and with a little diameter
Innervation	Distributed (a single axon innerves different fibrocells)	Focal (a single fibrocell could receive five or more nervous terminations)
Contraction	Slow	Fast

Table 1.2: Differences between red and white fibers in fish muscle (Poli, 2006).

1.2 MUSCLE PROTEINS

1.2.1 Sarcoplasmic proteins

In mammals the 30-35% of total protein in muscle tissue is constituted by sarcoplasmic proteins (Goll, Neti, Mares & Thompson, 2008), while in fish sarcoplasmic proteins account for 20-50% of the total muscle proteins (Delbarre-Ladrat, Chéret, Taylor, & Verrez-Bagnis, 2006). These are cytoplasmic proteins, which are soluble in low salt solutions. This family includes different kind of enzymes, such as myoglobin and glycolytic enzymes, etc. (Goll et al., 2008). The proteolytic enzymes calpains and cathepsins belong to this type of proteins (Delbarre-Ladrat, Verrez-Bagnis, Noel, & Fleurence, 2004) and are involved in cellular changes that take place during *post mortem*.

1.2.2 Stroma proteins

In mammals the 10-15% of the total amount of muscle tissue proteins is given by stroma proteins. These are insoluble in aqueous solvent at neutral pH. Many of them are extracellular, such as different types of collagen. In a different way, fish stroma proteins only account for 3-10% of the muscle proteins (Delbarre-Ladrat et al., 2006).

More than 30 collagen genes have been identified in mammals (Goll et al., 2008; Söderhäll et al., 2007; Veit et al., 2006) and the most abundant muscle collagens are the type I and III. In the perymisium the type I predominates, while in the endomysium type III exceeds type I collagen (Light, Champion, Voyle, & Bailey, 1985). In the fish myocommata, collagen types I, III, IV, V and VI have been identified, type V being much more and type III much less present than in the mammalian muscle (Bruggemann & Lawson, 2005; Sato et al., 1998).

In fish the thickness and stability of the collagen fibrils markedly depend on the proportion between collagen I and V: a higher concentration of collagen V in the muscle forms thinner fibrils (Ando, Nishiyabu, Tsukamasa, & Makinodan, 1999). Moreover, high proportion of collagen V, which is more labile to enzymatic degradation during cold storage, was associated to *post mortem* softening in rainbow trout and sardine (Sato et al., 1997).

Within this group of proteins, it is also important to mention elastin. Elastin is a 70 kDa protein, formed from monomers, and it is normally located in tendons. The presence of crosslinks in elastin structure allows the elongation and shortening of the protein (D'Ancona, 2005).

1.2.3 Myofibrillar proteins

Myofibrillar proteins are a class of skeletal muscle proteins, which are responsible for muscle contraction and most of the muscle functional properties (Goll et al., 2008). In mammals these proteins constitute the 55-60% of the total muscle tissue by weight. The main proteins of this class are myosin and actin, although more than fifteen other proteins are also identified inside this family (Goll et al., 2008). In fish, the myofibrillar protein content is higher than in mammals (60-80% of the total content) (Delbarre-Ladrat et al., 2006).

1.2.3.1 Myosin

Myosin is the predominant protein in the sarcomere, and it is located in the thick filaments. Myosin is a large protein with a molecular weight of about 470 kDa and each molecule is composed by two heavy chains with a molecular weight of about 220 kDa and two pairs of different light chains, ranging from 17 kDa to 22 kDa (Lanier, Yongsawatdigul, & Carvajal-Rondanelli, 2013; Park, Yongsawatdigui, & Park, 2008). Each thick filament is composed by 300 myosin molecules (Miroshnichenko, Balanuk, & Nozdrenko, 2000) which are connected to the M-line, interacting with the actin monomers on the Z-line of the thin filaments. In mammalian muscle, the myosin complex is composed of four light chains comprising the essential light chains MyLC1 and MyLC3 and two regulatory MyLC2. MyLC2 regulates myofilament activation *via* phosporylation by Ca^{2+} dependent myosin light chain kinase. There are two main types of MHC: slow type I and fast type II. This latter group was subdivided in other three groups of myosins: IIA-, IIX- and IIB-MHC, (Pette & Staron, 2000; Schiaffino & Reggiani, 1994), more recently defined as fast2a, fast2x and fast 2b MHC isoforms.

The classification of the myosin heavy chain (MHC) is correlated with the histochemical staining for myosin ATPase reaction. Four MHC isoforms (slow1, fast2a, fast2x and fast2b) have been characterized (Chang et al., 2003) and fibers of types I, IIa, IIx, and IIb (classified by histochemical myosin ATPase) are characterized by the expression of the slow1, fast2a, fast2x and fast 2b MHC isoforms, respectively (Chang et al., 2003). However, few fibers are really pure containing only a single MHC isoform, whereas the majority of fibers contain more than one MHC isoform. Hybrid fibers are classified as types I/IIA, IIA/I, IIA/IIX, IIX/IIA, IIX/IIB, and IIB/IIX) (Pette & Staron, 2000). For example, types I/IIA fibers contain the MHC 1 and 2a isoforms, with type I in excess, whereas types IIA/I fibers also contain the MHC1 and 2a isoforms, but with type IIA in excess (Bottinelli, 2001; Pette & Staron, 2000). In most mammalian muscles the fiber types are mixed even down to the fiber bundle level (Rogers, 2001) (Figure 1.3).

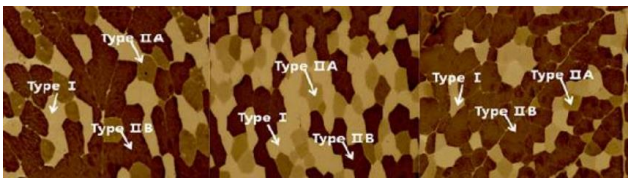

Figure 1.3. Serial sections of three major muscles showing myosin isoforms. From the left, *longissimus dorsi*, *psoas major* and *semimembranosus* (Hwang, Kim, Jeong, Hur, & Joo, 2010).

The structure of the MHCs is composed by an α-helix portion at the C terminal and a globular head at the N terminal. After an enzymatic digestion, the latter part of MHC forms the "subfragment S1" still comprising the actin binding site, and the ATP hydrolysis site (Delbarre-Ladrat et al., 2006; Tammatinna, Benjakul, Visessanguan, & Tanaka, 2007). The myosin rod domain is approximatively 150 nm long, 2 nm in diameter, and it is divided into two main regions, the C-terminal end, comprising two thirds of the rod, termed light meromyosin (LMM) and the N-terminal third, termed heavy meromyosin subfragment 2 (HMM-S2), which connects the globular head to the LMM. It is believed that LMM portion of myosin molecules confers the solubility and the aggregation properties to form the backbone of the thick filament (Koubassova & Tsaturyan, 2011).

Myosin exists in several isoforms also in fish. Specifically, Bassani and Dalla Libera (1991) showed that the myosin in red and white muscle seem to follow the pattern characteristic for slow and fast mammalian myosin. However, fish myosin, in the specific cod myosin, compared with mammalian myosin appears to be markedly unstable (Connell, 1960).

1.2.3.2 Nebulin

Nebulin is a 750/850 kDa protein present in mammals and fish, which is associated to the thin filament (McElhinny, Kolmerer, Fowler, Labeit, & Gregorio, 2001; Stefansson & Hultin, 1994) and it is able to bind other molecules with its N-terminal portion, such as α-actin, desmin and tropomodulin.

1.2.3.3 Tropomyosin

Tropomyosin is a dimer which is present in thin filaments (Perry, 1999), and is a long stiff molecule. Each polypeptidic chain has a molecular weight between 34 kDa and 36 kDa and is able to bind seven F-actin filaments. Tropomyosin has two fundamental functions: it stabilizes the actin filament and regulates its interaction with myosin. In fish muscle, tropomyosins show a marked species-specific thermal stability (Huang & Ochiai, 2005).

1.2.3.4 Troponin and tropomodulin

Troponin is a trimeric protein, which is composed by three subunits: TnI, TnC and TnT. Its principal function is to guarantee the correct calcium-dependent disposition of the tropomyosin on the thin filament (Farah & Reinach, 1995). TnC has four Ca^{2+} binding sites that are exchanged during muscle contraction. Troponin T is composed by two domains: TnT1 and TnT2. The former binds tropomyosin, while the latter binds especially TnI and TnC. Troponin I inhibits the ATPase activity of the actomyosin complex blocking its activation (Chalovich & Eisenberg, 1982; Perry, 1999). Troponin is well conserved in vertebrates, while it shows a low conservation in fish (of about 50%) (O'Brien, Landt, & Ladenson, 1997).

Tropomodulin has a molecular weight of 40 kDa, and consists of two domains. The first domain binds the actin filament blocking the actin extremity (Bao et al., 2012; dos Remedios et al., 2003; Fock & Hinssen, 2002) and consequently its polymerisation and depolymerisation, thus maintaining the length of the filament (McElhinny et al., 2001). Greenfield *et al.* (2005) showed that this part of the molecule is conserved through species, including fish (*Danio rerio* in the specific case). The other N domain binds the N-terminal region of tropomyosin. The binding affinity of tropomodulin for the actin-tropomysin complex is very high, with a Kd lower the 50 pM (Du & McCormick, 2009).

1.2.3.5 CapZ

CapZ is present in the thin filaments in a dimeric form. The dimer is formed by an α (36 kDa) and a β (32 kDa) subunit. This protein was conserved during the evolution, because it is present in all the eukaryotic organisms localized in the Z-band of the skeletal muscle. CapZ binds the α-actin filament, supporting the stabilization of the structure (Clark et al., 2002; Papa et al., 1999).

1.2.3.6 Desmin

Desmin is a protein conserved in mammalian and fish muscle (Verrez-Bagnis, Noel, Sautereau, & Fleurence, 1999). Desmin forms a reticular structure around the myofibrils in the Z-band (Tonino et al., 2010); its molecular weight is of 52 kDa. The structure of desmin has two conserved domains (H1 and H2), two globular domains in the terminal extremities and four linking regions (L1A, L1B, L2A and L2B). In general terms, desmin is proposed to maintain the integrity of the thin filaments (Cohen, Zhai, Gygi, & Goldberg, 2012). The number of desmin isoforms correlates with phylogeny: there is a single isoform in mammalian muscle, two in avian and three in fish. Desmin polymerization is regulated by phosphorylation (Costa, Escaleira, Cataldo, Oliveira, & Mermelstein, 2004). In mammals, a rapid destruction of thin filaments upon fasting was accompanied by an increase of desmin phosphorylation, which promoted desmin ubiquitilation and degradation (Cohen et

al., 2012). To our knowledge, so far no data have been published on a possible correlation between fasting and desmin phosphorylation in fish.

1.2.3.7 α-actinin

α-actinin is an homodimer located in the Z-line (Otey & Carpen, 2004; Papa et al., 1999), in which each monomer of 120 kDa connects the two adjacent sarcomers. α-actinin is of about 3.5 nm in length and has a diameter of 4 nm; each monomer has three principal domains that are able to bind titin, nebulin (Pappas, Bhattacharya, Cooper, & Gregorio, 2008) and the actin of the thin filament, making the structure stable.

1.2.3.8 Titin

Titin is a giant molecule with a monomer size of over $3x10^3$ kDa. A single titin extends from the Z-line (N-term) to the M-line (C-term) in the sarcomere. The structure of the protein is extremely complex, with a large numbers of repeating domains. The structure of titin must be elastic to accommodate the changes in the sarcomere length during contraction and relaxation (Du & McCormick, 2009). From a bionformatic analysis it has been deduced that the rainbow trout titin has a 60% of homology with mammalian titin (EBI, 2015), probably due to the fact that in the trout the titin sequence lacks of the first part.

1.2.3.9 Actin

Actin comprises the 15-30% of the myofibrillar proteins. The monomeric form of actin is globular in shape, being termed “globular actin” (G-actin), and it has a molecular weight of 42 kDa. However, globular actin molecules polymerise to form the actin filament, a form that is referred to as “fibrous actin” (F-actin), which resembles a “string of pearls” in shape (Lanier et al., 2013; Park et al., 2008). Actin represents the principal component of the thin filaments, being a structural element and a co-factor for the activation of the myosinic ATPase. Its structure is composed by four sub-domains: the first two (1 and 2) form the “external domain”, and the other two (3 and 4) constitute the “internal domain” (Holmes, Popp, Gebhard, & Kabsch, 1990; Kabsch, Mannherz, Suck, Pai, & Holmes, 1990). The polymerisation of G-actin monomers to obtain filamentous actin is complex, because it is dependent not only from ATP but also on salts and bivalent cations (Carlier, 1991).

Although actin is often though as a single protein, it actually consists of six different isoforms encoded by separated genes in mammals. These isoforms show differential localisation in the cell reflecting their different roles (Bergeron, Zhu, Thiem, Friderici, & Rubenstein, 2010). Indeed, actin is essential for a great number of cell functions, such as cell division, migration, junction formation, chromatin remodeling, transcriptional regulation, vesicle trafficking, and cell shape regulation.

The isoforms of actin are: muscular ($\alpha_{skeletal}$, $\alpha_{cardiac}$, α_{smooth} and γ_{smooth}) and ubiquitous isoforms (β_{cyto} and γ_{cyto}). Each actin isoform is remarkably similar to other isoforms, with only slight variations in amino acid sequence (Figure 1.4*)* (Perrin & Ervasti, 2010). β- and γ-actin differ by four amino acids in the N-terminus. Actin is well conserved across mammals, fish, and birds.

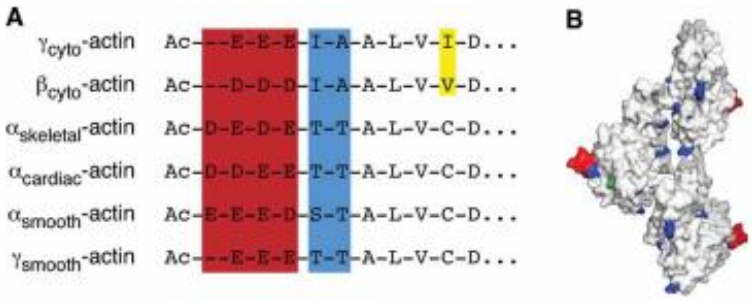

Figure 1.4. Actin isoforms. A) Alignment of the N-terminal ends of the six mammalian actin isoforms. **B)** Aminoacid differences mapped onto F-actin structure approximated by Oda et al. (PDB 2ZWH) (**red**: most variability between muscle and cytoplasmic actins; **blue**: primarily variation between cytoplasmic and muscle isoforms; **yellow**: differences between β- and γ-actin; **green**: substitutions between different muscle isoforms).

In skeletal muscle, α_{sk}-actin is the predominant form in thin filaments, in which α_{ca}-actin is present in low amount (Moutou, Socorro, Power, Mamuris, & Canario, 2001), while γ-actin is absent; this latter has been initially identified in filamentous structures around the mitochondria and near the sarcolemma, but subsequently it has been recognized as an important component of the costameres between the sarcolemma and the Z-disc (Ervasti, 2003).

In non-muscle cells of mammals and fish, the β- and γ-cytoskeletal actin isoforms exist in varying ratios depending on cell type, and these ratios are conserved in a cell and tissue-specific way (Bergeron et al., 2010).

1.3 MUSCLE CONTRACTION

Movement in muscle is generated by the myosin cross-bridges, which interact cyclically with actin filaments and transport them past the myosin filaments. Kinetic, spectroscopy, genetic analyses and *in vitro* motility assays have given new insight into this complex process, which starts at the sarcolemma. When sarcolemma depolarisation reaches the t tubule–sarcoplasmic reticulum junction, the ryanodine receptor (whose name is due to its affinity for the plant alkaloid ryanodine) opens, and Ca^{2+} is released into the cell cytosol. The Ca^{2+} diffuses to the myofibrils and binds to troponin C on the thin filaments. Ca^{2+} causes a change in shape of troponin C, which in turn causes tropomyosin to move deeper into the groove of the actin. With the tropomyosin movement, a binding site for the myosin head on the surface of the actin is exposed, and the myosin binds and pulls or pushes the actin for a small distance (about 10 nm). The myosin head then releases actin, and it can reattach to another actin (Rogers, 2001). In the relaxed state, the cytosolic Ca^{2+} concentration around myofilaments is around 10^{-7} M or lower (Honikel, 2001), while it increases to 10^{-4} M during contraction. A single muscle contraction is called a twitch, and it only requires about 200 milliseconds to occur (Rogers, 2001). Contraction uses adenosine triphosphate (ATP) as fuel (Honikel, 2001; Rogers, 2001). Energy in the form of ATP is required to bind myosin to actin as well as to separate myosin from actin afterwards (Figure 1.5). The Ca^{2+} required for activation is pumped back inside the sarcoplasmic reticulum by an ATP powered process. The cell membrane polarity is re-established by the Na^+/K^+ pump located in the outer cell membrane. It requires ATP to move Na^+ and K^+ against their concentration gradients (Figure 1.6).

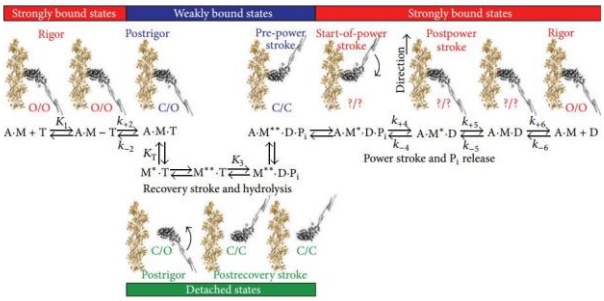

Figure 1.5. Simplified schematic representation of the predominant biochemical and structural states of the actomyosin ATPase cycle. Actin is depicted in orange, the myosin motor domain with the artificial lever arm (X-Ray structure PDB:1G8X) is shown in grey colors (A= actin, M= myosin motor domain, T= ATP_i, D= ADP_i, P= P_i). The open (O) or closed (C) conformation of the active site elements is indicated. The power stroke occurs while the motor domain is bounded to actin. The recovery stroke occurs in the detached state. It is assumed that the two heads of myosin act independently from each other. On the scheme, only one head is shown. Equilibrium constants and rate constants are denoted by upper case and lower case letters, respectively (Månsson, 2014).

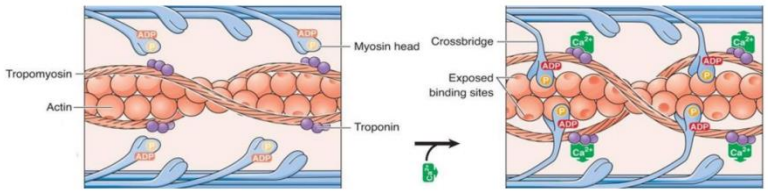

Figure 1.6. Model of contraction mechanism (modified from Tank & Gest, 2009).

1.4 POST MORTEM PROTEOLYSIS

The conversion of muscle into meat occurs through three main steps: the *pre rigor*, the *rigor* and the tenderising step. During these phases muscle proteins do not maintain their original structure/ function and many of them are degraded by endogenous proteases, although at different extent (Koohmaraie, 1996). There has been considerable debate about the specific proteases responsible for these changes. Three main types of enzymes are considered to be involved, at least in red meat species and poultry (Sierra et al., 2012; Warris, 2000), i.e. caspases, cathepsins and calpains.

1.4.1 Cathepsins

Cathepsins are a class of over 30 different enzymes. They are synthesised as inactive proenzymes with N-terminal propeptide regions most of which are finally localized in the lysosomes. There are, however, exceptions such as cathepsin K, which works extracellularly after secretion by osteoclasts in bone resorption. They are classified based on their structure and catalytic type into serine (cathepsins A and G), aspartic (cathepsins D and E), and cysteine cathepsins, the latter constituting the largest cathepsin family. In skeletal muscle, they mediate the protein turnover that is particularly active due to the metabolic, mechanical, and thermal stressors arising from contraction (Bechet, Tassa, Taillander, Combaret, & Attaix, 2005). Impairment of the cathepsin systems leads to the accumulation of unfolded/misfolded proteins and altered organelles leading to muscle cell toxicity. Conversely, excessive activation of proteolytic machinery contributes to muscle loss, weakness, and finally to death (Sandri, 2011). Both in mammalian and fish muscle cathepsins B, D and L are the most abundant members of the cathepsin family (Aoki, Yamashita, & Ueno, 2000; Bechet et al., 2005).

Cathepsins must be released from lysosomes in order to play part in myofibrillar degradation during *post mortem*. This is made possible by the failure of ion pumps in membranes during the development of *rigor*. During *rigor mortis*, as a result of the lactic acid formation of and subsequent decrease in pH value, the walls of lysosomes are destroyed and cathepsins are released (Chéret, Delbarre-Ladrat, Verrez-Bagnis, & de Lamballerie, 2007; Delbarre-Ladrat et al., 2004). Cathepsin release and their capacity to degrade the actomyosin complex *in vitro* (Feiner, 2006) has been suggested that cathepsins can greatly contribute to meat tenderness. In accordance, higher level of cathepsins found in pork and chicken is well correlated with their faster rate of tenderization. Conversely, the ageing of beef requires a significantly longer time than pork and chicken in order to achieve a comparable degree of tenderness as the level of cathepsins is low (Feiner, 2006). Lower tenderness in beef compared to pork and chicken may also be attributed to the other factors, such as collagen content

and solubility (decreasing with age – as pigs and poultry are normally consumed at much lower physiological maturity/age than cattle).

1.4.2 Calpains

Calpains are probably the most extensively studied protease family in the field of meat science. It is widely accepted that calpains contribute to meat tenderization by cutting the long fibers into smaller units along the Z lines, where they are located (Kemp & Parr, 2012). Calpains are a large family of intracellular cystein proteases. In skeletal muscle, the calpain system consists of three proteases, the ubiquitously expressed µ-calpain and m-calpain and the muscle-specific calpain 3. µ-calpain and m-calpain are Ca^{2+}-activated proteases, requiring micro (1–2 µM) and millimolar (50–100 µM) concentration of Ca^{2+}, respectively. For this reason, calpains have been implicated in necrotic cell death (Kohli, Madden, Bentley, & Clavien, 1999), which is associated with massive Ca^{2+} influx. The optimum pH of m-calpain is close to 7.5 (Dayton, Reville, Goll, & Stromer, 1976), whereas µ-calpain was reported to be more active at pH 6.5 than at 7.5 (Pomponio & Ertbjerg, 2012). Fish calpains have still received much less attention compared with their mammalian counterpart (Geesink, Morton, Kent, & Bickerstaffe, 2000). m-calpain has been purified from carp and tilapia muscle (Sakamoto, Yamada & Seki, 1985; Wang, Ma, Su, Chen & Jiang, 1993). µ-Calpain has not yet been purified from fish muscle, but the presence of a Ca^{2+}-dependent caseinolytic peak eluting before m-calpain on an anion-exchange column may indicate its presence in carp muscle (Sakamoto et al., 1985).

Calpains are composed of two subunits; the large subunit has a molecular mass of 80 kDa in m-calpains being slightly larger in µ-calpains, while the molecular mass of the small subunit is of 28 kDa in all calpains. The catalytic site is located in Domain II of the large subunit and contains the catalytic triade consisting of a Cys residue (position 115 or 105 of human µ-calpain or m-calpain, respectively), a His residue (position 272 or 262 of human µ-calpain or m-calpain, respetively) and an Asn residue (position 296 or 286 of human µ-calpain or m-calpain, respectively). However, there is only a marginal sequence homology between calpains and papains or other families of cysteine proteases. Consequently, the calpains have been grouped in a class of cysteine peptidases, CLAN CA, family C2, separated from the other cysteine proteases (Goll, Thompson, Li, Wei, & Cong, 2003). The human genome contains 14 distinct *capn* genes and an examination of other genomes in the Ensemble database (http://www.ensemble.org/index.html) reveals a comparable repertoire in fish (Macqueen et al., 2010). In mammals 11 different paralogous genes encoding calpain large subunits have been identified (Dear & Boehm, 2001).

The relative importance of μ- and m-calpain in promoting tenderization is still debated. Both calpains are located in the region of the Z lines. After exhaustion of ATP and the development of *rigor mortis*, the membrane systems of the sarcoplasmic reticulum and mitochondria no longer take up or sequester calcium ions. The increased Ca^{2+} concentration in cytosol activates the μ-calpains allowing proteolysis to proceed. Normally, calpains are inhibited by being bound to the endogenous protein calpastatin. Ca^{2+} removes this inhibition and calpastatin is itself eventually broken down by calpains and the m-calpain may also be converted to μ-calpains by hydrolysis. Considerable evidence indicates that μ-calpain, but not m-calpain, plays an important role in *post mortem* degradation of myofibrillar proteins and tenderization of muscle during refrigerated storage (Geesink, Kuchay, Chishti, & Koohmaraie, 2006; Hopkins, 2004). Ideed, μ-calpain, but not m-calpain, was found activated in *post mortem* muscle as evidenced by the autolysis of the activated enzyme (Koohmaraie & Geesink, 2006). However, proteolysis pattern in *post mortem* muscles can be mimicked by incubation of myofibrillar proteins with μ- and m-calpain (Geesink et al., 2006). In addition, in bovine and ovine *post mortem* muscle, the extractable activity of μ-calpain declines but the activity of m-calpain is remarkably stable (Veiseth, Shackelford, Wheeler, & Koohmaraie, 2001).

The characterization of the sea bass white muscle showed the presence of three different calpain-like activities: two similar to m-calpain and one to μ–calpain (Delbarre-Ladrat et al., 2006). They share many biochemical properties with those from terrestrial vertebrates, but show a seasonal expression pattern. As an example, in sea bass, the μ–calpain expression increases during the spawning period and this could be related to the higher rate of muscle degradation in *post mortem* (Ladrat, Verrez-Bagnis, Noel, & Fleurence, 2001).

1.4.3 Caspases

Caspases are a family of cysteine proteases with a strict specificity for aspartate residues as cleavage site on target proteins. Their name is a contraction of cysteine dependent aspartate-specific proteases. Mammals contain two biologically distinct caspase sub-families: one of these participates in the processing of pro-inflammatory cytokines, while the other is required to elicit and execute the apoptotic response during programmed cell death (Salvesen, 2002) (Figure 1.7); caspase 1, 4, 5, 11, and 13 appear to be predominantly involved in the inflammatory response system, whilst caspase 3, 6, 7, 8, 9, 10 and 12 are involved in cell death through apoptosis. The apoptotic caspases can be further subdivided into initiator caspase such as 8, 9, 10 and 12, or effector caspases such as 3, 6, and 7, depending in their position in the cell death pathway (Cho, Liu, Gonzales, Zaleska, & Wood, 2003; Kemp & Parr, 2008; Murray et al., 2008; Salvesen, 2002).

In fish, orthologues for caspase-3, -6 and -7 have been identified in several species, such as Rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*), salmon (*Salmo salar* L.) and sea bass (*Dicentrarchus labrax*) (Chakraborty, Nandi, Sinha, & Gera, 2006; Laing, Holland, Bonilla, Cunningham, & Secombes, 2001; Reis, Nascimento, do Vale, Silva, & dos Santos, 2007; Takle, McLeod, & Andersen, 2006; Yabu, Kishi, Okazaki, & Yamashita, 2001).

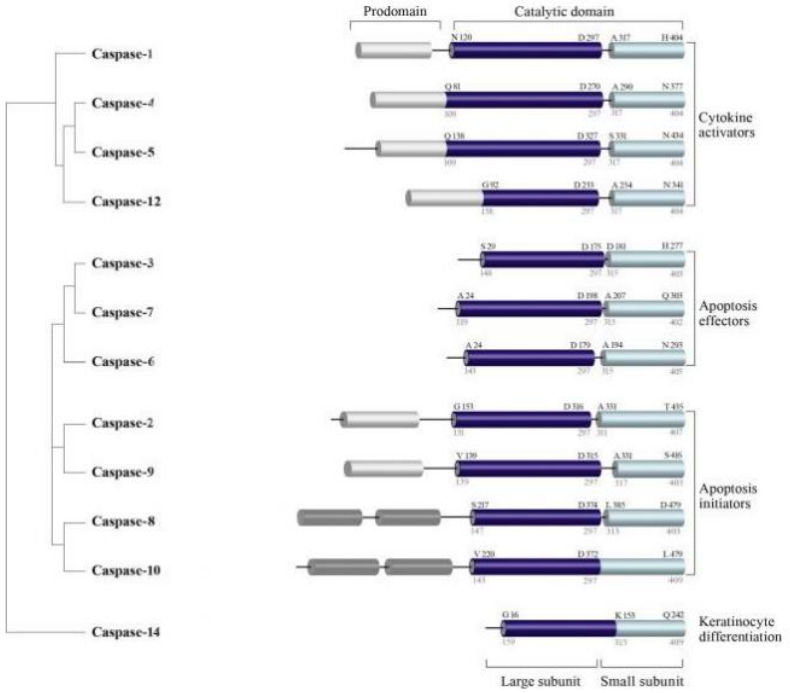

Figure 1.7. Phylogenetic tree of the caspase family. The tree is organized based on the sequence homology and substrate specificity (Roschitzki-Voser et al., 2012).

Caspases are synthesized in the cytosol of cells as inactive zymogens. Whilst initiator caspases are monomeric in their inactive conformation, executioner caspases are dimeric. The zymogens comprise a N-terminal prodomain, a large subunit and a small subunit, which undergo proteolytic processing upon apoptotic stimuli (Cho et al., 2003). In muscle cells caspase 3 is the common downstream apoptosis effector, which exists as proenzyme (pro-caspase 3) and is processed and activated by caspase 9 or caspase 8 to form a heterodimeric form (17 kDa–12 kDa)(Wang, 2000). Numerous studies have shown that caspases are involved in skeletal muscle development and remodeling, with expression being essential for normal muscle differentiation during myogenesis (Fernando, Kelly, Balazsi, Slack, & Megeney, 2002). Caspases are upregulated in pathological conditions such as in sarcopenia

(Dupont-Versteegden, 2005), muscular dystrophies (Sandri et al., 2001), and are activated early in hypoxia/ischemia (Kemp, 2006).

As already mentioned this proteolytic system has been recently proposed to be active in *post mortem*, affecting myofibrillar degradation (Herrera-Mendez, Becila, Boudjellal, & Ouali, 2006; Kemp, Parr, Bardsley, & Buttery, 2006). Animal bleeding induces in all cells a state of anoxia, cells receive no more nutrients and the death biochemical process starts, before the development of *rigor mortis* (Herrera-Mendez et al., 2006). Caspases are specialized in cell de-structuration during apoptosis and a model has been proposed. They are thought to degrade the organization of myofibrils within muscle cells and then other cellular components and organelles with the contribution of other proteolytic systems including cathepsins, calpains and proteasomes (Ouali et al., 2006). Moreover, calpains and caspase systems can potentially influence each other, although their cross-talk appears to be multifaceted. Both have common substrates that are targeted (Wang, 2000) including the proteins actin, actinin, myosin, spectrin, vimentin, and troponin I (Wang, 2000). The similarities between the calpain and caspase systems are not just limited to the substrates cleaved by them. Caspase 12 can be activated by proteolytic cleavage by calpains in response to a disruption in the Ca^{2+} homeostasis within the sarcoplasmic reticulum (ER). The amount of Ca^{2+} required for this cleavage was found to be at millimolar and not micromolar levels, suggesting that m-calpain is responsible for caspase 12 activation. However, in other conditions calpains can also act as negative regulators of apoptosis, cleaving caspases 3, 7, 8 and 9 at distinctive sites to generate inactive isoforms (Wang, 2000). In turn, caspases 3 and 7 can degrade calpastatin, as evidenced by the negative relationship identified between peak caspase 3/7 activity at 8 h after slaughter and calpastatin activity at 0 and 2 d in LD from normal lambs, but not from callipyge lambs, which are characterized by a much higher muscle weight (Kemp, Sensky, Bardsley, Buttery, & Parr, 2010). Intriguingly, caspase activity has been also found in *post rigor* period. In particular Kemp and Parr (Kemp & Parr, 2008) have demonstrated that in *post rigor* meat from pig, caspase-3 activity is stimulated and its activity is negatively associated with Warner−Bratzler shear force, thus suggesting an improvement in tenderness (Underwood, Means, & Du, 2014).

1.5 CELL DEATH MECHANISMS

In muscle tissue, cell death phenomena can be classified on the basis of different criteria, such as morphological, enzymatic (with or without the recruitment of nucleases or proteases, such as caspases, calpains and cathepsins, or transglutaminases), functional (programmed, accidental, physiologic or pathologic cell death) or immunological (Melino, 2001). Based on the morphology, three physiological cell death scenarios are distinguished: apoptosis (type I), autophagy (type II) and necrosis (type III) (Krysko, Vanden Berghe, D'Herde, & Vandenabeele, 2008).

1.5.1 Autophagy

Autophagy is a catabolic enzymatic process, which regulates the cytoplasmic components turnover supporting the degradation of molecules and intracellular complexes. This process is activated during starving conditions (Yabu, Imamura, Mizusawa, Touhata, & Yamashita, 2012) and could be identified as autophagy chaperone-mediates, micro-autophagy and macro-autophagy. The autophagy chaperone-mediated uses proteins (molecular chaperons) which bind the intracellular material addressing it to specific receptors on the lysosomal membrane; the material is then incorporated and degraded. The second type of autophagy internalizes the material creating introflexions on the lysosomal membrane. Macro-autophagy is characterized by the seizure of cytoplasmic material by the autophagosomes, that became phagolysosomes in which the cathepsin-mediated digestion is activated (Levine & Klionsky, 2004). Recent studies did not show active autophagy in the *post mortem*, unlike necrosis or apoptosis (Becila et al., 2010). Indeed, it has been proposed that apoptotic cell death is the first phenomenon in the transformation meat-muscle in mammals (Ouali et al., 2007).

1.5.2 Necrosis

Necrosis consists of morphological alterations of cells that are not able to metabolically and structurally adapt to changes or environmental stresses. When the cellular disorganization is high, the cell loses the membrane integrity, so that cytoplasmic organelles undergo swelling, mainly the mitochondria, and cytoskeleton destabilizes. All these phenomena progressively lead to cell lysis, releasing its content in the extracellular environment and triggering an inflammatory process, which damages the adjacent cells.

1.5.3 Apoptosis

Apoptosis (programmed cell death) is a biological process, which permits the removal of damaged cells from a tissue in the organism, or the elimination of cells that are present in excess, with a well-defined molecular program (Kerr, Wyllie, &

Currie, 1972). It has also been proposed that starving conditions could induce apoptosis (Caro-Maldonado & Muñoz-Pinedo, 2011). From a morphological point of view, the cell undergoes an ordered series of modifications: in the cytoplasm, the endoplasmic reticulum dilates due to the presence of vacuoles and the cytoplasm is reduced; mitochondria undergo different alterations; nuclear DNA is degraded by endonucleases and chromatin is condensed in compact masses that are surrounded by cytoplasmic material, originating the apoptotic bodies; finally, irregular cytoplasmic masses (blebbing) are extruded. For these reasons, apoptosis is defined as a physiological, controlled and programmed process, which consumes ATP. The recognition of apoptotic bodies by the phagocytes avoids the releasing of intracellular components in the external part of the cells, hindering the formation of inflammatory processes (Becila et al., 2010; Lawen, 2003).

Apoptosis could evolve in two different ways, intrinsic and extrinsic, which involves a series of events that facilitate the activation of caspases. The extrinsic way is mediated by membrane receptors that bind extracellular ligands, such as TNF (macrophagic *tumor-necrosis factor*) and Fas-L (present on Natural Killer cells), and have a death domain (DD) on the cytoplasmic side. The DD promotes the formation of DICS (*Death Inducing Signalling Complexes*), by the interaction between DD and a cytoplasmic protein that recruits the initiator caspases (8 or 10), which activate the effector caspases (Figure 1.8). The intrinsic way is activated by different stimuli, included the oxidative stress. This pathway is characterized by the loss of mitochondrial membrane potential and by the fragmentation of the external membrane of mitochondria, which results in releasing of the *cytochrome C* from the mitochondria into the cytosol where, with Apaf-1, forms the apoptosome. The apoptosome is a complex that binds the caspase 9 in the CARD domain (*caspase recruitment domain*) activating it; after that, it happens the proteolytic cleavage on the effector caspase 3 that, when active, cleaves a lot of cellular substrates comprising the actin (Lawen, 2003) (Figure 1.8).

Another important element is calcium ion, which acts as a cellular second messenger both in the apoptotic that in the necrotic pathway. Beside the activation of calpains during necrosis, Ca^{2+} regulates the intrinsic pathway of apoptosis activating the opening of an aspecific pore on mitochondrial inner membrane, defined mitochondrial transition pore (Bernardi, Rasola, Forte, & Lippe, 2015; Giorgio et al., 2013). It has been found that in fish muscle, after the apoptotic stimulus, in addition to caspases also lysosomal proteases are activated, such as cathepsin D, which migrates in the cytosol and contributes to myofibrillar and connective tissue degradation (Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2003; Sato et al., 1997).

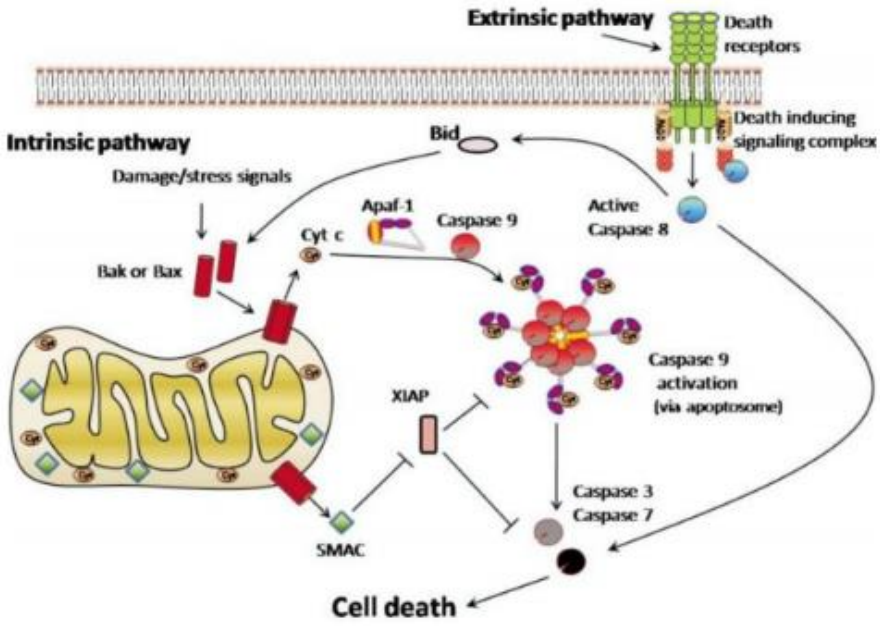

Figure 1.8: activation of the exthrinsic and intrinsic apoptotic pathaways (Fogg *et al.*, 2011)

1.5.3.1 Actin degradation in apoptosis

As previously reported, α-actin mainly forms the thin filaments in the sarcomeres, while β-actin the cytoskeleton. Interestingly, it has been demonstrated that during the apoptotic process caspases degrade β-actin in a selective manner, originating a N-terminal fragment of 32 kDa (*Fr*actin) and a C-terminal fragment of 15 kDa (*t*actin), both used as apoptosis markers (Utsumi, Sakurai, Nakano, & Ishisaka, 2003). More recently, it has been reported the formation of *Fr*actin in rat muscle during *post mortem*, and this evidence has been used to propose that also in the *post mortem* the apoptotic way is activated (Becila et al., 2010).

In addition to be target of the apoptotic process, evidence have been reported that alterations in the polymerization and depolymerization of actin could influence apoptosis. Indeed, stressing conditions could provoke an increase of F-actin in the cytosol, encouraging an increase of oxidative stress and the intrinsic way of apoptosis (Gourlay & Ayscough, 2006); however it has been reported that also depolymerization of F-actin encourages the formation of apoptotic bodies (Levee, Dabrowska, Lelli, & Hinshaw, 1996).

1.6 Sea Bass (Dicentrarchus labrax)

The increasing awareness of healthy and beneficial effects from fish consumption influences the eating habits and an increasing number of consumers requires the presence of fish on the market. Indeed, fish represents a good source of proteins, and is rich of vitamins, minerals, and other nutritive and health-promoting components, such as omega-3 fatty acids (Andrew, 2001). These requests determine an increasing of farmed fish and impose specific requirements of eligibility on the nutritional point of view and on final quality (Burger & Gochfeld, 2009).

1.6.1 Morphological characteristics

Seabass, class *Actinopterygii*, order *Perciformes*, family *Moronidae*, gender *Dicentrarchus*, species *D. labrax* (Linnaeus, 1758) is a marine strictly carnivorous fish, particularly appreciated for its fillet quality, bred in large number in the Mediterranean sea. Sea bass has a long body, slightly compressed and a developed caudal peduncle. The head is rather long and the mouth parts have small sharp teeth on jaws, palate and tongue. The dorsal portion of sea bass has a grey-green colour, the sides are silvery and whitish belly. In the juvenile stage individuals are characterized by the presence of small blackish spots on the body. The operculum has two strong spines and at the top there is a small dark spot. The bass has two dorsal fins well defined and the tail fin is slightly concave (Manzoni & Tepedino, 2008).

This fish is unmistakable for its white and light meat and its low fat content (Body, Green, & Lepors, 1992), that makes it appreciated at worldwide levels (Simitzis et al., 2013). To face the high market request, the number of sea bass farms is increasing (Gòmez-Guillen, Montero, Hurtado, & Borderias, 2000). According to F.E.A.P. (*Federation of European Aquaculture Producers*), sea bass production (*Dicentrarchus labrax*) has reached 88531 tons in the 2006, so it becomes important to maintain the productive quality.

Italian aquaculture is characterized by breeding of different wild species, including the sea bass (*Dicentrarcuhs labrax*). In Friuli Venezia-Giulia modern technologies are utilized, resulting in good quality productions.

In farms, the sea bass can be reared using different modalities, such as extensive ponds, semi-intensive ponds, intensive concrete tanks and intensive sea-cages. According to the FAO directives, cage systems have to be based on an exchange of natural water through the pens. The cages are usually made of steel with areas of four to over 10 m^2, having nets suspended below the walkways up to 6-8m deep (Moretti, Fernadez-Criado, Cittolin, & Guidastri, 1999). Tank systems are usually supplied with seawater (38‰) maintained in a continuous flow-through system at ambient temperature. In tanks, high stocking density is applied (20-35

kg/m^3), meaning that an accurate control of water quality and careful observation of fish health are essential. A recirculating system is used to control the water temperature (13-18°C in autumn/winter). This system is relatively expensive due to the required technology for water control (filters, air stripping, UV treatment, catabolite removal) (Moretti et al., 1999).

Extensive and semi-extensive lagoon systems place special barriers in appropriate lagoon sites. Barriers are made of reeds, nets or cement and in the extensive systems remain open during springtime (in Italy from February to May) allowing natural stocking of lagoon fry. In semi-intensive systems it is present an artificial enrichment of lagoon fry. In both systems, vegetation control is important in order to avoid that fish suffocate (Moretti et al., 1999).

Xiccato et al. (2004) made an evaluation of the effect of the above described rearing conditions on the chemical composition on the seabass fillet and observed that these slightly affected the percentage body water, which was higher in fish reared in extensive pond. The other parameters (crude protein and gross energy) did not show significant differences (Table 1.3).

	Min	*Max*	*Average*	*SD*
Extensive				
Water (%)	71.4	78.2	76.1	1.5
Crude protein (%)	18.0	20.3	19.1	0.6
Ether extract (%)	0.9	8.1	2.8	1.5
Gross energy (MJ/kg)	4.89	7.00	5.72	0.65
Semi-intensive				
Water (%)	64.2	77.0	70.7	3.6
Crude protein (%)	17.1	20.3	19.1	0.6
Ether extract (%)	1.4	16.1	8.5	3.7
Gross energy (MJ/kg)	5.45	10.34	7.86	1.52
Intensive				
Water (%)	62.9	71.9	67.6	2.3
Crude protein (%)	17.2	20.6	19.3	0.7
Ether extract (%)	5.8	16.4	10.8	2.8
Gross energy (MJ/kg)	7.05	10.64	8.53	0.93
Sea-cages				
Water (%)	66.5	73.4	70.3	1.7
Crude protein (%)	18.1	20.8	19.3	0.6
Ether extract (%)	4.2	13.6	8.3	2.1
Gross energy (MJ/kg)	6.44	9.50	7.92	0.76
All samples				
Water (%)	62.9	78.2	70.9	3.8
Crude protein (%)	17.1	20.8	19.2	0.7
Ether extract (%)	0.9	16.4	7.9	3.9
Gross energy (MJ/kg)	4.89	10.64	7.75	1.39

Table 1.3. Chemical composition of sea bass reared in different farm systems (Xiccato, Trocino, Tulli, & Tibaldi, 2004).

It is widely reported that the farmed sea bass is characterized by a higher lipid accumulation in the muscle tissue that the fish caught in open sea, as shown in the Table 1.4 obtained by INRAN (Alasalvar, Taylor, Zubcov, Shahidi, & Alexis, 2002; Lanari et al., 1999; Parisi, Mecatti, Lupi, Scappini, & Poli, 2002). Moreover, the higher lipid content has been associated to a more rapid degradation of the meat after death (Sáez et al., 2013). The same Table also reports a higher protein content of farmed sea bass, but some authors did not observed such a difference (Orban, Nevigato, Di Lena, Casini, & Marzetti, 2003). Interestingly, a differential protein

expression was found in the muscle of farmed sea bass with respect to the wild one (Martinez, Slizyte, & Dauksas, 2007). These data clearly demonstrate that it is fundamental to deeply analyse the fish responses to stresses caused by the aquaculture practices.

Chemical composition	**Farmed sea bass** (100 g)	**Wild sea bass** (100 g)
Water (g)	69.9	79
Proteins (g)	21.3	16.5
Lipids (g)	6.8	1.5
Cholesterol (mg)	75	48
Carbohydrates (g)	0.8	0.6
Sugar (g)	0.8	0.6
Sodium (mg)	61	*n.d.*
Calcium (mg)	30	20
Phosphorus (mg)	1150	202
Fatty acids, total saturated (g)	1.44	*n.d.*
Fatty acids, monounsaturated (g)	2.13	*n.d.*
Fatty acids, Polyunsaturate (g)	2.56	*n.d.*
Energy (Kcal)	149	82

Table 1.4. Comparison between chemical composition of farmed and wild sea bass (INRAN, 2015).

1.6.2 Slaughtering methods

After fish death, as in other animal species, chemical, biochemical and microbiological processes occur that deeply alter muscle structure, encouraging the characteristic tenderizing. These processes may be influenced by the slaughtering techniques, which can induce strong stressing conditions, finally affecting the meat quality (Gjerdrem, 1997). Indeed, the muscular activity during the slaughter could cause consumption of glycogen, increasing the lactic acid production and decreasing the pH during the *post mortem* within muscle cells (Bagni et al., 2007). It has been also observed that these events could reduce the time before *rigor mortis*, and accelerate the subsequent process of meat tenderization (Gòmez-Guillen et al., 2000). Conversely, situations that do not alter the animal welfare affect marginally the anaerobic metabolism of the glycogen after death that is degraded in the *rigor mortis* to create an equilibrium with lactic acid (Bagni et al., 2007).

It is possible to reach a low muscular activity during slaughter with the application of methods that provide a fast sensibility loss in the animal avoiding pain and sufferings to the fish (Ottera, Roth, & Torrissen, 2001). Although some new slaughtering procedures have been proposed (Poli et al., 2004), spiking and water/ice are still the most frequently used techniques in the European fish farms (Simitzis et al., 2013).

Spiking technique is largely used, because fish does not recover the cerebral function. It is difficult to execute, because precision is necessary to give to the fish the mortal hit and a prepared and dedicated staff is requested (Poli et al., 2004). Anaesthetic administration before death could be an alternative, but although these substances seem not to damn the fish quality (Ribas et al., 2007), they could cause bad effects on human health.

Water/ice technique is the most common procedure in Italy (Bagni et al., 2007) and consists in the immersion of the fish on a water/ice slurry, to induce an insensitive state due to anoxic and hypothermic conditions (Ashley, 2007; Bagni et al., 2007; Kestin, Van de Vis, & Robb, 2002). The water/ice method is generally used for slaughtering of the species that are get used in warm water conditions. During the immersion in a water/ice slurry the fish doesn't make excessively violent movements, differently by other slaughtering modalities (such as asphyxia and electric shocks) (Parisi et al., 2002; Poli et al., 2004). However, water/ice method could have drastic effects on muscle cells, because the muscular activity, although not violent, persists for a long time.

1.6.3 Texture of fish muscle

Fish meat is a vulnerable and perishable product and the texture of fish muscle is a main feature used to appreciate the freshness quality (Cheng, Sun, Han, & Zeng, 2014). During the *post mortem* period, the fish muscle undergoes a process of softening, which affects its textural quality due to changes: i) in its chemical composition, such as the content of ATP and its degradation products, and ii) degradation of sarcomeric proteins and collagen by collagenases and other endogenous proteases. As an example, firmness, which is an essential texture attribute closely associated with the human visible acceptability of fish products, depends largely on the structure of connective tissue (Casas, Martinez, Guillen, Pin, & Salmeron, 2006). On the other hand, in fish gaping, which is one of the most serious textural damages, both muscle fibrils and collagen fibers collapse (Andersen, Thomassen, & Bencze Rora, 1997), demonstrating that both structures contribute in maintaining the textural toughness and the integrity of fish muscle (Fuentes, Fernandez-Segovia, Serra, & Barat, 2012).

In addition to structural factors, there are numerous interacting factors affecting fish texture, such as physical factors (species, age and size, feeding ingredients, sample heterogeneity, and gaping), chemical factors (water content and distribution, fat content and distribution, and collagen content), and diverse treatments (storage time and temperature, freezing, salting, chilling, high-pressure processing (HPP) (Cheng et al., 2014).

Texture is commonly measured and presented as a mechanical property, manifesting as performance of hardness/firmness, gumminess, resilience, cohesiveness, springiness, adhesiveness, and viscosity. This latter has been proposed as an indicator of protein denaturation and aggregation (Borderias, Jimenez-Colmenero, & Tejada, 1985), but also as a quality marker for the frozen product (Borderias et al., 1985).

Texture can be evaluated with sensory and instrumental measurements. A sensory method is the “finger method,” which consists in pressing with a finger on the skin or on the fillet. This method gives an indication of the fish suitability for further processing (Sigurgisladottir et al., 1999).

Compared with sensory evaluation, textural measurements by instrumental analysis methods are more precise, reducing the measurement variations arising from human factors. The puncture, compression, shear, and tension are four main instrumental techniques and the methods measure and evaluate the fish texture from

the force–deformation curve, displaying the value of force, deformation, slope, and area (Casas et al., 2006).

The most used instrumental techniques are the Warner-Bratzler and Kramer shear compression cells, which use shearing and cutting devices (Alizadeh, Chapleau, de Lamballerie, & LeBail, 2007; Cavitt, Meullenet, Xiong, & Owens, 2005; Cavitt, Youm, Meullenet, Owens, & Xiong, 2004; Jonsson, Sigurgisladottir, Hafsteinsson, & Kristbergsson, 2001). Another important method is the double-compression method, which is capable of performing a texture profile analysis (TPA) model obtained from a force–time curve, offering a meaningful interpretation to a series of textural parameters (Damez & Clerjon, 2008).

1.7 Italian PDO dry-cured hams

Dry-curing of hams is an ancient method of meat preservation process for times of scarcity and has experienced many improvements that enabled the development of flavourful and attractive meat product that we know today. The processing technology still follows traditional known-how transmitted by manufactures from generation to generation. The production of some types of dry-cured ham is now controlled by a protected designation of origin (PDO) in order to achieve products with high quality sensory characteristics and of reproducible quality

More than 80% of Italy's pig production is destined for the market of traditional Italian dry-cured ham (*Prosciutto*) and the most important PDOs for Italian dry-cured ham are: PDO Prosciutto di Parma (PA), PDO Prosciutto di San Daniele (SD), PDO Prosciutto Toscano (TO). Heavy pig production is subjected to rules fixed by the Consortia, on the basis of the experience of producers and the results from scientific research (Bosi & Russo, 2010). Slaughter animals of at least 9 months of age and 160 kg live weight are required. Pure breeds are limited to Italian Large White and Landrace, selected for specific parameters such as loss at 1st salting of the ham, and their crosses with Duroc and hybrids. Backfat thickness must be "sufficient" to obtain retailed fresh hams with fat cover ranging from 20 to 30 mm, depending on ham weight, and the content of linoleic acid in ham fat cover cannot exceed 15%. Subjects of other breeds can be used for the production of crossed pigs, provided they are obtained by selection with objectives not inconsistent with those of the Italian selection.

The ripening process comprises the following phases, which slightly differ for the three PDOs:

1. Dressing: SD is the only one which retains the trotter. In the dressing phase, upon receipt of the thighs, TO is cut in a "V" shape by removing a greater part of the skin than the other two PDO types, exposing a larger portion of the *Semimembranosus* muscle, in order to increase the penetration of the salt;
2. Salting: the addition of salt to the fresh thigh (moisture 79%, water activity, aw, 1; protein 15-23 %; fat 2.5%; salt 1%) decreases the aw, ensuring the preservation of the dry-cured ham (moisture 57-60%, aw 0.85, protein 9-31%, fat 3-4 %, salt 5-6%), which is 34-36% of the initial weight of the green thigh at the end of maturation, which lasts at least 12 months (Nollet & Toldrà, 2006). The salt can be added to the three PDOs in different forms: rubbed in dry and wet for PA, dry and *ad libitum* for SD, dry and mixed with pepper and vegetable flavourings for TO. The leg is then refrigerated at a temperature ranging from 1°C to 4°C, for two or three weeks for SD and PA and for TO, respectively;

3. Resting: the hams are then hanged in refrigerated, humidity-controlled rooms, at 75% humidity up to 6-10 weeks. Moreover, SD is pressed into the typical guitar shape, in a pre-resting phase, to facilitate the penetration of the salt. In this phase the meat darkens;
4. Washing and drying: the hams are washed with warm water and brushed to remove excess salt and impurities, then hung in drying rooms up to 4-5 months.
5. Mid-curing, after greasing: the exposed lean surface is covered by '*sugna'*, a mixture of leaf fat and wheat or rice flour for drying. For TO, the classic *sugna* is supplemented by pepper and vegetable flavourings. The hams are then hung on frames in well ventilated rooms up to 7-8 months.
6. End of curing: the hams are transferred to rooms with less air and light and hang on racks until the curing is completed.

Prosciutto di San Daniele is produced only in the Friuli Venezia Giulia region, in the hilly area around the town of San Daniele in the province of Udine. Prosciutto di Parma can only be made in the hills around Parma, 5 km south of the via Emilia, limited to the east by the river Enza and on the west by the river Stirone, and up to an altitude of 900m. The area of production of the Toscano PDO ham is in the territory of the Tuscany region.

1.7.1 Proteolysis during dry-cured ham processing

Dry-cured ham production requires long processing times and is associated with proteolytic activity on myofibrillar and sarcoplasmic proteins, resulting in their progressive degradation (Petrova, Bantle, & Eikevik, 2015) and marked ultrastructural changes (Larrea, Hernando, Quiles, Lluch, & Pérez-Munuera, 2006; Monin et al., 1997). The global process appear to be a "cascade process": the initial breakdown of proteins by endopeptidases is followed by the action of exopeptidases, giving rise to the generation of small peptides and free amino acids, that contribute—directly or indirectly—to flavour development (Hinrichsen & Pedersen, 1995). In particular, dry-cured ham flavour seems to be mainly determined by the levels of alanine, leucine, valine, arginine, lysine, glutamic and aspartic acid (Toldrà, Aristoy, & Flores, 2000). Furthermore, it has been proposed that naturally generated small peptides may have additional biological functions, such as antioxidant and antihypertensive activities (Escudero et al., 2013; Mora, Escudero, Fraser, Aristoy, & Toldrá, 2014).

The intensity of proteolysis depends on properties of the raw material (Škrlep et al., 2010) but also on processing conditions, such as NaCl content, water content and ageing temperature that could affect the softness of dry-cured *ham* (Morales,

Serra, Guerrero & gou, 2007). Indeed, the most important factors that could influence proteolysis in dry-cured ham are temperature, humidity, pH, salt concentration and the time of ripening: in the products that are exposed to a longer ripening, proteolysis is more marked (Rentfrow, Chaplin, & Suman, 2012). On the other hand, an increased proteolysis could be responsible for the appearance of textural defects (pastiness and softness) (García-Garrido, Quiles-Zafra, Tapiador, & Luque de Castro, 2000; Virgili, Parolari, Schivazappa, Bordini, & Borri, 1995).

Some authors have studied cathepsin activity during the process and have observed that it is a wide specific for degrading myofibrillar proteins, suggesting an important role during the whole process (Toldrá & Flores, 1998). Although cathepsin activities gradually decrease through processing (García-Garrido et al., 2000), a residual activity of cathepsins B, D and L was reported even at the end of curing (Sárraga, Gil, & Garcia-Regueiro, 1993; Toldra, Rico, & Flores, 1993). The relative contribution of calpains is poor and restricted to the first weeks of processing, due to their low stability even though they can contribute to an initial breakdown of the Z-disk (Larrea et al., 2006; Rosell & Toldrà, 1996).

1.8 Muscular markers

Biomarkers of the meat quality are of prime importance for industry, since they eliminate personal opinions on the product quality. Quality markers would be a reflection of the different metabolic pathways contributing to the final quality. Hence, understanding of these pathways should be a prerequisite for a successful identification of accurate biological/biochemical markers.

For many decades, meat scientists are looking for biomarkers of meat tenderness, which is one of the most important quality characteristics for the consumer (Dransfield, Martin, Bauchart, & Al, 2003), in order to make possible: (1) the meat classification soon after slaughter and (2) the optimization of the genetic selection of meat animals on the basis of this quality. Despite these extensive efforts, a good marker of this quality has not yet identified, possibly because the underlying mechanisms are still unclear (Ouali et al., 2013). An overview has been recently presented, which identified 8 subgroups of potential biomarkers, the three most abundant being enzymes of the glycolytic and oxidative energy supplying pathways together with the family of the Heat Shock Proteins (HSPs) (Ouali et al., 2013). The myofibrillar proteins are also degraded in *post mortem* muscle and the first to be identified was probably troponin T (Verrez-Bagnis, Ladrat, Morzel, Noël, & Fleurence, 2001). In pig and cattle tenderness was correlated to the fragmentation of myofibrillar proteins, including actin, and myosin heavy and light chain (Kim et al., 2008; Lametsch et al., 2003). In particular, the appearance of three actin fragments was correlated with the decrease of the shear force (Lametsch et al., 2003). In the last years, proteomics techniques have been increasingly used in food-producing animals allowing to identify the changes in the global protein profiles during *post mortem* (Bendixen, 2005; Hollung, Veiseth, Jia, Faergestad, & Hildrum, 2007).

A study on the proteome of pigs showed that pre-slaughtering conditions affected the expression levels at slaughter and during the *post mortem* of some stress and glycolytic proteins (Lametsch et al., 2006). Indeed, the farm animals are subjected to many potential stressful factors during the pre-slaughter. These stressors may be of physical origin, related to the transport or food deprivation, or of emotional origin and can activate the muscle metabolism, resulting in higher early *post mortem* muscle temperature, lactic acid content and faster pH decline (D'Souza, Dunshea, Warner, & Leury, 1998; Rosenvold & Andersen, 2003). On the other hand, other studies, which analysed the pig *Longissimus dorsi* (LD) muscle proteome during the *post mortem* storage time (0, 12 and 72 h) after different pre-slaughter treatments, (Morzel et al., 2004) reported that the protein changes associated to the pre-slaughtering treatments did not affected the proteolytic events during the storage period.

Proteomic tools have also played a relevant role in characterizing the intense proteolysis taking place in dry-cured ham and the potential peptidases responsible for

this action (Di Luccia et al., 2005; Mora et al., 2010). Differently from meat aging, curing of ham takes a considerably longer time giving rise to the generation of small peptides, that are involved in the development of the typical and highly appreciated flavour characteristics of these products (Sentandreu & Sentandreu, 2011).

In seafood, the most currently used procedure for the objective measurements of seafood quality is the measure of the level of ATP and its breakdown products by HPLC. Indeed, in *post mortem* fish muscle, degradation of adenosinetriphosphate (ATP) proceeds to adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR) and hypoxantine (Hx). The initial catabolism of ATP normally results in a fast and temporary accumulation of IMP in most postharvest fishes (Massa, Palacios, Paredi, & Crupkin, 2005). Then, the degradation of IMP to form HxR and finally Hx occurs at a slower rate (Özogul & Özogul, 2002). The K-value, defined as the ratio of the sum of HxR and Hx to the sum of the ATP and related catabolites expressed as a percentage, has been widely used as one freshness index to evaluate the quality change of raw fish after catch (Mendes, Quinta, & Nunes, 2001). It is known that IMP contributes to the pleasant, fresh flavor of the meat (Howgate, 2005). On the other hand, Hx accumulation is involved in the progressive loss of desirable fresh fish flavor, such as bitter off-taste (Fletcher & Statham, 1988). Other chemical and biochemical methods for freshness quality have been developed, comprising the detection of the biogenic amines, pH, total volatile basic nitrogen, etc., which are still widely used (Nollet & Toldrá, 2009).

As already discussed, the texture and structure of fish muscle are also important freshness quality attributes that depend on several parameters, including the dismantling of the sarcomeric structure. Application of proteomics, which is still limited in seafood research, appears very promising for seafood management and fish welfare (Forné, Abia, & Cerdà, 2010; Terova et al., 2011).

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Thesis objectives

The research work has been focused to:

- characterize the actin changes in the processing of three Italian PDO dry-cured hams by immunochemical and mass spectrometry approaches;
- describe the actin fragmentation profile in farmed sea bass (*Dicentrarchus labrax*) to provide evidences of muscle structure disassembly following the most commonly adopted slaughtering practices;
- provide the existence of a relationship between the actin changes and the major physical and chemical properties of the edible muscle portion.

2. RESULTS AND DISCUSSION

2.1 The effect of slaughtering methods on actin degradation and on muscle quality attributed of farmed European sea bass (*Dicentrarchus labrax*)

2.1.1 Abstract

In the current study, two different slaughtering procedures, spiking vs immersion in water/ice slurry, were applied on electrically stunned European sea bass (*Dicentrarchus labrax*) and the effects on actin degradation and fillet quality attributes were investigated. *Rigor mortis* index was similar for the two slaughtering techniques, whereas the shear force measurement indicated that *rigor mortis* occurred more quickly and intensely in the water/ice handled fish than in those slaughtered by spiking. The water/ice immersion procedure also resulted in higher amount of actin fragments than spiking. Muscle tissue apparent viscosity and water holding capacity were lower in sea bass treated in water/ice slurry compared with fish handled by spiking, whereas the acidification patterns of the two groups were not easily distinguishable. In conclusion, spiking appeared to preserve actin integrity better that water/ice and it seemed to ensure less muscle damage as indicated by the higher viscosity and water holding capacity values. Finally, a role of actin as a biochemical marker for the prediction of fish quality was suggested.

2.1.2 Introduction

All aquaculture practices, including stunning and slaughtering methods could have a strong effect on fish welfare, affecting the stress response (Ashley, 2007; Conte, 2004; Huntingford et al., 2006; B. M. Poli, Parisi, Scappini, & Zampacavallo, 2005). From a comparison of commercial and experimental killing methods in the European sea bass (*Dicentrarchus labrax*), asphyxia was confirmed to be the most stressful killing method, showing either the most prolonged period prior to death or a remarkable physical activity and affecting the final quality (EFSA 2012). Therefore, stress caused by an improper suppression method can also affect the quality attributes of the fish, which are important for consumer acceptability, as well as for the processing industry. Loss of quality is the result of complex physiological and biochemical processes related to the aquaculture practices and to the *post-mortem* events (Iciar Martinez et al., 2011). However, there is very little knowledge on the way by which ante-mortem biochemistry changes the post-mortem events and hence the resulting final quality (Delbarre-Ladrat et al., 2006). Despite the importance of proteolysis on texture and quality, methods based on monitoring the products of

proteolysis have received little attention, and very little is known about protein changes and fish quality (Verrez-Bagnis et al., 2001). The expanding aquaculture-food market, which requires production efficiency and quality of fillet, could benefit from the detailed knowledge of protein degradation in fish following the most used aquaculture practices and their influence on the fish quality.

Actins are a family of highly conserved proteins with only slight variations in their N-terms, which play fundamental roles in nearly all aspects of eukaryotic cell biology. Four muscle actins predominate in striated (α_{sk} and α_{ca}) and smooth (α_{sm} and γ_{sm}) muscle, while two cytoplasmic non-muscle β-actin and γ-actin isoforms are found in all cells (Rubenstein, 1990). Only subtle sequence variations distinguish the isoactins. In the skeletal muscle, in addition to the highly specialized contractile apparatus, there are both the actin-associated costameric complexes and the functionally distinct cytoskeletal actin-based filaments, which underpin a wide range of functions (Kee,Gunning, & Hardeman, 2009). A progressive degradation of cytoskeletal and thin filaments of actin was proven in mammalian muscle immediately after death and referred to apoptosis (Becila et al., 2010).

The first objective of the present study was to provide evidence of actin degradation as pathognomonic of muscle protein disassembly associated to the early post-mortem periods, following the most commonly adopted slaughtering practices in farmed sea bass (*Dicentrarchus labrax*). Secondly, the existence of a relationship between the actin fragmentation profile and the major physical and chemical properties of the edible muscle portion was also examined to determine the potential use of actin as a biochemical marker for the prediction of fish quality.

2.1.3 Material and methods

2.1.3.1 Animals and experimental design

The experiments were carried out at the indoor facilities of the Department of Food Science of the University of Udine. All procedures of our research protocol involving fish were in accordance with the requirements of the EU Directive 2010/63 (2010) on the protection of animals used for scientific purposes. In this study, 60 European sea bass (*Dicentrarchus labrax*) (344±57 g average body weight and 33.0±2.0 cm average total length) were maintained in cubic fibreglass tanks (500 L) in a partially recirculating marine aquaculture system (total volume 6 m^3, daily water renewal rate 5 %). The system ensured a constant day length of 12 h of artificial light (180 lux) supplied by fluorescent tubes and optimal water quality conditions for sea bass: temperature, 23±2 °C; salinity, 28.6±0.5‰; dissolved oxygen, 7.3±0.13 mg/L (Handy Polaris, Oxyguard, Birkerød, Denmark), pH, 7.89±0.02 (Basic 20, Crison Instruments S.A., Alella, Spain), total ammonia nitrogen concentration<0.12 mg/L

and nitrite-nitrogen concentration<0.015 mg/L (Lasa 20, Hach Lange, Düsseldorf, Germany). Fish were fed 6 days a week a commercial diet (Sketting Optibass, 6 mm) offered in two daily meals to visual satiety, but were deprived of food 36 h before the stunning/slaughtering treatment. Fish were kept under the above described culture conditions over 2 months and then they were exposed to electric stunning (50 V, 50 Hz, direct current; Fishkill EG100, Scubla s.r.l., Remanzacco, Italy) for 5 s just before being slaughtered with two different procedures: immersion in water/ice slurry (ratio 1:1) and spiking (Ikigun, Adept Ltd, New Zealand). For the water/ice procedure fish were left in the slurry for 15 min, then they were removed and manually decapitated (time 0). For the spiking procedure, fish were manually decapitated immediately after spiking (time 0). Except fish used for rigor mortis measurements, which were kept as a whole fish on ice, all fish were filleted.

2.1.3.2 Rigor mortis

Rigor mortis was measured as rigor index (RI %) on 9 fish for each slaughtering treatment using the Cutting's method (as tail drop) (Bito, Yamada, Mikumo, & Amano, 1983). Briefly, the upper half of a whole fish was placed on one side on a horizontal table surface with the other half (tail part) suspended off the edge. At selected time intervals, the vertical distance (L, cm) between the base of the caudal fin and the table surface was measured and RI was calculated from the equation (1):

$$\left[\frac{L_0 - L}{L_0}\right] * 100 \qquad (1)$$

where L_o (cm) is the vertical distance between the base of the caudal fin and the table surface measured immediately after the death.

2.1.3.3 Texture

As a further monitoring of the rigor mortis process, texture of sea bass was measured, according to Fuentes et al. (2010), by a shear force test which incorporates compression of fibers beneath the blade, tension in the adjoining fibers and shearing of the fibers (Bouton et al. 1975). A TA.TX.plus Texture Analyzer (Stable Micro Systems, Godalming, Surrey, U.K.), provided with a 30 kg load cell and a Texture Exponent 32 (Stable Micro Systems) software, was used. The instrument was equipped with a Warner-Bratzler test cell, which sliced the samples perpendicularly to the muscle orientation at a constant speed of 1.0 mm s^{-1}. Textural measurements were performed using 20 × 20 × 10 mm pieces from the epaxial muscle of the fish fillet on at least three samples for each slaughtering method. The maximum peak force (N) required to shear through the sample was recorded as shear force.

2.1.3.4 Protein samples, SDS-PAGE and Western blotting

Possible changes induced in sea bass muscle proteins by the slaughtering methods were investigated by one-dimensional SDS-PAGE on six fish for each slaughtering method. The extraction of proteins from epiaxial muscle tissue was carried out according to Piñeiro et al. (1999) with minor modifications. Briefly, samples (100 mg) were cut in small pieces on ice and suspended in 1 ml of the extraction buffer containing 60 mM Tris/HCl pH 7.5, 2 % wt/vol SDS, 0.1 M dithiothreitol (DTT) and supplemented with 2 µl of antiproteases (Protease Inhibitor, Sigma-Aldrich) to avoid degradation of proteins. After homogenization with UltraTurrax® T25 Digital, the samples were centrifuged for 4 min at 5600 g at room temperature, heated at 98 °C and shaken for 30 min. After heating, samples were centrifuged at room temperature for 10 min at 5600 g. The protein concentration of the supernatant was determined by UV absorbance at 280 nm, after sample dilution with SDS containing solution. Protein extracts were applied to 13 % SDS-PAGE gels (Laemmli, 1970), suitable for separation of 10–200 kDa proteins, using the Mini 2D (Bio-Rad, Richmond, California, USA) electrophoresis equipment. The gels were subsequently stained with NOVEX® Colloidal Blue Staining Kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) or transferred to nitrocellulose membranes using the TE 22 transfer unit (GE Healthcare, Little Buckinghamshire, UK) for Western-Blotting. Western-Blotting was performed with a polyclonal anti actin C-term antibody (1:1000) (SigmaAldrich), suspended in phosphate buffered saline (PBS) containing 0.1 % (vol/vol) Tween-20, or with a monoclonal anti β-actin N-term antibody (1: 1000) (Santa Cruz Biotechnology, Dallas, Texas, USA), suspended in Tris buffered saline (TBS) containing 0.1 % (vol/vol) Tween-20 and 2.5 % (wt/vol) bovine serum albumin (BSA; Sigma-Aldrich). The apparent masses of the protein bands were estimated from a calibration curve obtained by plotting the migration distances of standard proteins (Precision Plus Protein™ Dual Color Standards – Biorad, Hercules, California, USA) versus their known molecular masses. Immunoreactive bands, detected by enhanced chemiluminescence (Pierce, Rockford, Illinois, USA), were analyzed by densitometry with the ImageQuant Software. Bands were expressed as % changes in arbitrary units using the ratio between the optical density of all actin fragments and the optical density of intact actin.

2.1.3.5 Viscosity

Apparent viscosity of fish muscle homogenate was determined, according to Borderias et al. (1985), as an indirect measure of the protein quality of fish muscle. Measurements were performed on at least three samples of epiaxial muscle tissue for each slaughtering method, using a controlled stress rheometer (StressTech Rheometer, Reologica Instruments AB, Sweden), at shear rate of 10^{-1}, at 4 °C±0.2 °C, using bob-cup sensor geometry. Samples (5 g) were subjected to homogenization at 1000 rpm

for 1 min (Polytron PT 3000, Kinematica, Littau, Switzerland) with 5 % NaCl (1:4) and phosphate buffer 50 mM (pH 4.5).

2.1.3.6 Water holding capacity

Water Holding Capacity (WHC) was determined, as liquid loss (LL), after slaughter on at least three samples, for each slaughtering method, of epaxial muscle tissue. Each sample consisted of three sub-portions, of about 5 g, taken in the cephalic, middle and caudal area. Samples (15 g) were transferred to centrifugation tubes and subjected to low speed centrifugation at 210 g for 15 min at 5 °C, as proposed by Olsson et al. (2003). The liquid loss was expressed as a percentage of liquid released as the difference in weight before and after centrifugation, as follows (2):

$$LL\ (\%) = \left[\frac{liquid\ release\ (g)}{total\ weight\ (g)}\right] * 100 \qquad (2)$$

2.1.3.7 Muscle pH

The pH values were measured using a pH meter 213 (Hanna Instruments, Rhode Island, USA) model, with the electrode inserted into the epiaxial muscle after carrying out a lateral incision. Measures were taken in the cephalic, middle and caudal area on at least two samples/fish for each slaughtering method.

2.1.3.8 Statistical analysis

Results were reported as means±mean absolute deviations. In figures, the mean absolute deviations were shown with error bars. For the parameters studied, samples were tested for differences using one-way t-tests and statistical significance was taken as $p<0.05$.

2.1.4 Results and discussion

2.1.4.1 Muscle protein profile and actin degradation

As shown in Fig. 2.1.1, the protein profiles of sea bass muscle tissue samples taken 1 h after slaughtering (time 1) were similar and apparently not affected by the slaughtering method.

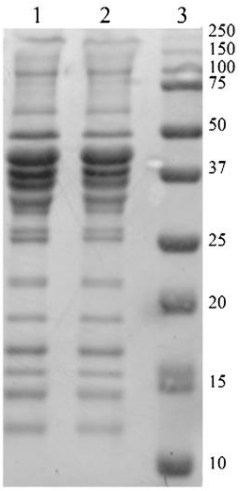

Figure 2.1.1. Electrophoretic pattern of European sea bass muscle samples 1 h after slaughter (time 1) by spiking (*lane 1*) or in water/ice (*lane 2*); molecular weight markers in the range 10-250 kDa are shown in *lane 3*. Panel shows a representative experiment.

When intact actin was subjected to Western-Blotting against actin C-term or β actin N-term, differences in the intact actin bands (42 kDa) between the two slaughtering methods were not evident (Fig. 2.1.2). On the other hand, compared to spiking, the immunodetection of actin fragments with the actin C-term polyclonal antibody showed that the water/ice method produced higher amount of actin C-terminal fragments, notably the ones at apparent molecular weight of 15 kDa. In rat muscle, actin degradation has been often associated to apoptosis considering that its early degradation is ensured by caspases, a family of cysteine proteases responsible of cell dismantling in apoptotic cells (Becila et al., 2010). In particular, *in vitro* studies on simian cells, showed that the C-terminal 15 kDa fragment of the cytoskeletal actin (*t*Actin) is a well characterized product of caspase activity (Utsumi et al., 2003). More recently, cleavage of actin, mediated by the mitochondrial protease HtrA2/Omi was also observed in caspase-independent programmed necrosis in studies *in vitro* on Jurkat and in MEF cells (Sosna et al., 2013; Yamauchi et al., 2014). Anyway, whatever the mechanism is involved, degradation of transversal cytoskeletal actin filaments causes a detachment of sarcolemma from the basal lamina and the extracellular matrix network (Becila et al., 2010; Ahmed Ouali et al., 2013), impairing the structural integrity of the muscle (Kee et al., 2009). Therefore, it was possible to hypothesize that, even there was variability within samples, the effect of a short-term stress, such as that derived from a slaughtering procedure, was significant respect to the development of actin degradation fragments. The appearance of these fragments could likely serve as potential sensitive index of early muscle protein disassembly.

The finding of the 15 kDa fragment, which might correspond to tActin, is intriguing, because the presence of β-actin in sea bass muscle has not yet described, in spite of its expression in other tissues (Scapigliati et al. 2001). Anyway, when tested by monoclonal antibody against the N-term of β-actin, a clear band at the expected 42 kDa apparent molecular weight of the intact protein was found, suggesting its expression in sea bass muscle (Fig. 2.1.2). Conversely, in both kind of samples no band was detected at 15 kDa by monoclonal antibody against the N-term of β-actin (*data not shown*), that is consistent with the sequence of *t*Actin.

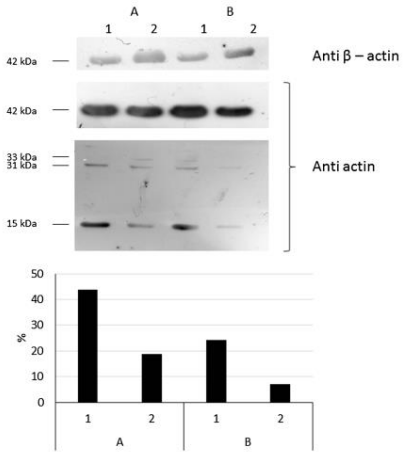

Figure 2.1.2. Immunodetection of actin in four different fish 1h after slaughtering in water/ice slurry (1) and spiking (2) from two representative experiments (A and B) (*upper panel*). Relative percentage of the immunodetected bands is expressed as ratio between the optical density of all actin fragments and the optical density of intact actin (*lower panel*).

2.1.4.2 Quality attributes

Fish quality is influenced by rigor mortis, which is known to affect how firm the flesh becomes or, on the other hand, how the fillet is liable to gape (Borderías & Sánchez-Alonso, 2011). In our study, the development of rigor-mortis was investigated for its relevance to fish quality, and the Rigor Index (Bito et al., 1983) was used as an assessing method for its reported simplicity and effectiveness (Wang, 2000).

Comparing the two slaughtering methods, spiking and water/ice, the onset of rigor mortis, as well as its resolution, resulted similar for the two treatments, although resolution of rigor in some specimens of the water/ice group begun earlier than in the spiking samples (Fig. 2.1.3). Spiking, which involves driving a sharp spike into the brain of the fish, is considered humane slaughter resulting in an immediate loss of

consciousness (Lines and Spence 2014). This technique is relatively slow due to the difficulty in precisely locating the brain with varying fish size. However, when it is done accurately, it reduces physical damage with concurrent quality benefits (FAO 2001). The rate of onset and resolution of rigor is affected by a number of factors, including the condition of the fish, i.e., *rigor mortis* starts immediately or shortly after death if the fish is starved and the glycogen reserves are depleted (FAO 1973). The method used for stunning and slaughtering the fish was also reported to affect the *rigor* onset, with slaughtering in water/ice giving the fastest onset of rigor and a blow on the head producing a delay of up to 18 h (Azam, Mackie, & Smith, 1990; Proctor, Ryan, & McLoughlin, 1992). Significant differences were also observed in the duration of rigor mortis, with percussive stunning producing the highest glycogen reserve and the longest duration of *rigor mortis* in slaughtered rainbow trout in comparison to other slaughtering methods (Sebastio, Ambroggi, & Baldrati, 1996). Therefore, from the results of the present experiment, it could be hypothesized that death by water/ice was not particularly stressful in comparison to spiking or, in alternative, that the Rigor Index was not particularly sensitive to the level of slaughtering stress in European sea bass.

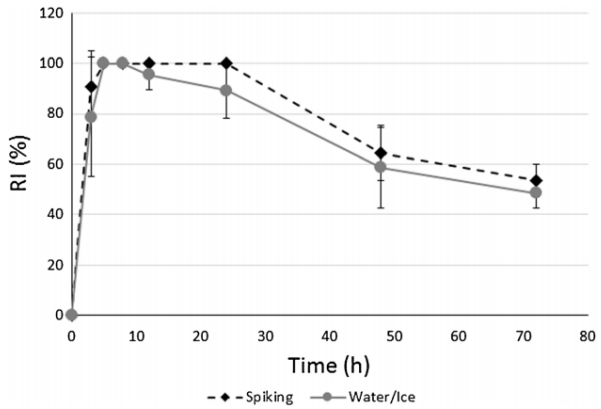

Figure 2.1.3. Evolution of *rigor mortis* as Rigor Index (RI) in whole sea bass slaughtered by spiking or in water/ice. Values are provided as mean (*n* = 9) and mean absolute deviations (*bars*).

To test these hypotheses, a more sensitive approach to monitor the *rigor mortis* process was used, and the texture hardness, expressed in Newtons, was evaluated. With this approach, it was registered not only the time curve but also the strength of the rigor. As shown in Fig. 2.1.4, spiking delayed time to the maximum total load (1–3 h), as compared to the water/ice slaughtering method (1 h). In addition,

a significant difference was observed in the maximum stiffness between fish slaughtered by spiking (0.93–0.95 N) or in water/ice (1.32 N).

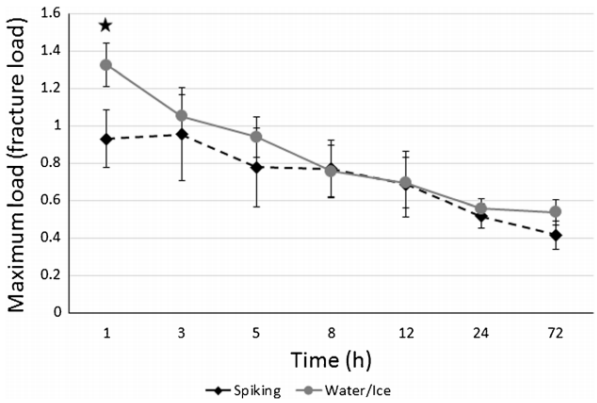

Figure 2.1.4. Evolution of shear force of sea bass measured by Warner-Bratzler shear blade. Values are mean ± mean absolute deviations (*error bars*) of three different samples. * $p < 0.05$, one-way *t*-tests.

These differences in timing and hardness suggested that the water/ice fish entered rigor earlier and generated a greater tension in fish muscle in respect to the fish slaughtered by spiking. Water is the major constituent of muscle foods and the interaction between water and macromolecules determine the water holding capacity, which is affected by the physical and biochemical changes in the muscle (Aursand, Erikson, & Veliyulin, 2010). In our study, the WHC was expressed as the difference in weight before and after centrifugation, i.e., total weight loss. As shown in Fig. 2.1.5, the spiking samples had a significantly lower liquid loss than their water/ice counterpart until 5 h *post-mortem*. These results support the idea that the mobile water was more easily removed from the more disassembled protein structures, such as those found in water/ice samples, which showed a more fragmented actin.

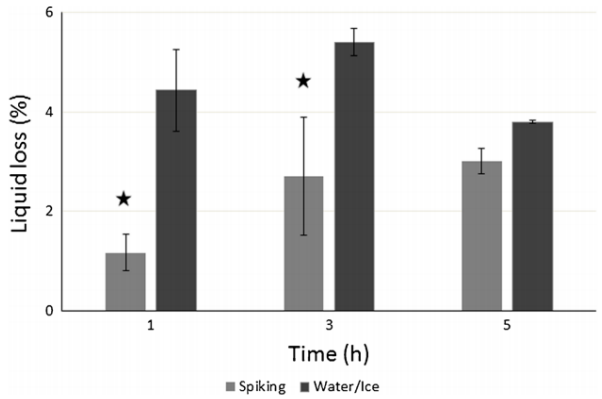

Figure 2.1.5. Evolution of liquid loss of sea bass slaughtered by spiking or in water/ice. Samples were withdrawn at different times after death and values are mean ± mean absolute deviations (*error bars*) of three different samples for each harvesting time. * ***p*** **< 0.05, one-way *t*-tests.**

Muscle *post-mortem* acidification is caused by glycogen conversion to lactate and more hypoxic conditions with an increased anaerobic glycolytic activity are caused by the stress before death (Hultmann, Phu, Tobiassen, Aas-Hansen, & Rustad, 2012). Acidification of muscle decreases protein electrical charge and induces an increase in their hydrophobicity, thereby reducing water content (Ahmed Ouali et al., 2013). On this basis a different pattern of pH values in the sea bass slaughtered by spiking in respect to fish slaughtered in water/ice could be expected. However, as shown in Fig. 2.1.6, the acidification patterns of the two groups were not easily distinguishable and therefore could not provide a clear explanation of the differences in liquid loss. As pointed out by Becila et al. (2010) an early increase in extracellular space, resulting from the expulse of intracellular water, started immediately after slaughter, whereas pH was still very close to neutrality. A cell death, possibly induced by apoptosis, would provide a reasonable explanation of the early change in water loss in post-mortem fish muscle. The markedly different behaviour observed in the first period of post-mortem might be consistent with the higher actin degradation, and in particular with the higher formation of the apoptotic C-terminal 15 kDa actin fragment in water/ice than spiking samples. Apparent viscosity is considered a rheological measure of functional quality of proteins (Borderias et al., 1985), which can eventually affect consistency of the comminuted products.

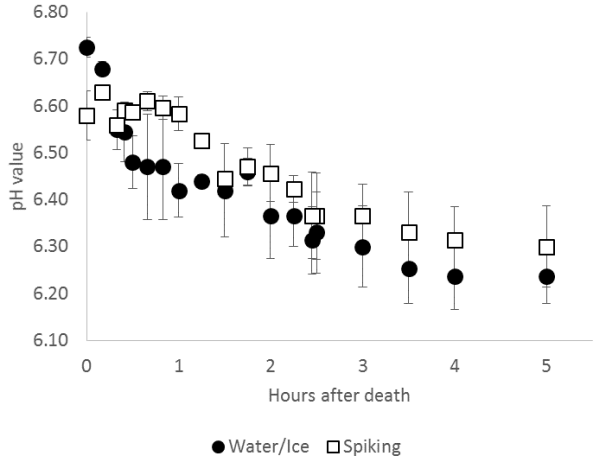

Figure 2.1.6. Evolution of pH in samples of sea bass slaughtered by spiking or in water/ice. Each value is the mean of the measurements taken in three areas (cephalic, middle and caudal) of the epaxial muscle of each fish. At each sampling time, at least two fish were analyzed for each slaughtering method.

As shown in Fig. 2.1.7, at 1 and 3 h post-mortem the water/ice slaughtering procedure resulted in a significant decrease in apparent viscosity, not explained by differences in pH (Fig. 2.1.6). It should be noticed that pH is one of the most important factor that produces strong effects on the structure of myofibrillar proteins (Feng & Hultin, 2001; Liu, Bao, Xi, & Miao, 2014). It was therefore proposed that an initial partial degradation of muscle proteins, such as that reported for actin, may be accountable for differences in the apparent viscosity.

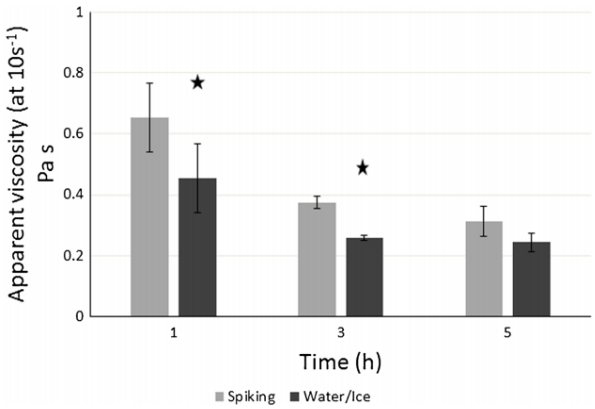

Figure 2.1.7. Evolution of apparent viscosity in samples of sea bass slaughtered by spiking or in water/ice. Samples were withdrawn at different times after death and values are mean ± mean absolute deviation (*error bars*) of three independent samples for each harvesting time. * $p < 0.05$, one-way *t*-tests.

2.1.5 Conclusions

Biomarkers of the fish quality are of primary importance for the fish industry, which has to satisfy the increasing consumer expectations. The biomarker should be enough sensitive and certainly indicative of a variation in texture, which is one of the main quality attribute of fish fillet. A protein of great interest is actin, whose degradation is likely to impair the muscle structural integrity. It appears, therefore, that actin could be a sensitive and reliable predictor of fish quality.

This paper provided the evidence that actin proteolysis increased by employing the water/ice slaughtering method, as compared to spiking. This latter procedure also resulted in a significant variation of some important fish quality attributes, including texture, viscosity and water holding capacity. However, more detailed biochemical investigations will be needed to study the actin cleavage sites and the likely proteolytic enzymes engaged in the actin degradation in the ante/early *post-mortem* period.

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Bibliography

2.2. Proteolytic resistance of actin but not of myosin heavy chain during processing of Italian PDO (protected designation of origin) dry-cured hams

2.2.1 Abstract

Proteomics is the approach of choice to study the fate of specific proteins, and it is very useful in identifying quality molecular markers. A combination of immunochemical and mass spectrometry analysis was used to assess the occurrence of proteolytic changes of actin and myosin heavy chain (MHC) proteins in pig *biceps femoris* skeletal muscle during processing of three Italian PDO dry-cured hams. Early *post-mortem* muscle displayed low levels of actin and myosin fragments. In spite of a high proteolysis index and the presence of active cathepsin D until the final ripening phase of dry-cured ham processing, very low actin proteolysis was found. The identification of the generation fragments were not recognized as α-skeletal muscle isoform fragments, while the identified fragments derived mainly from the cardiac actin isoform. On the other hand, MHC showed a remarkable degradation of its catalytic head, generating a C-terminal 135-kDa fragment. Based on its ability to interact with actin in vitro, this MHC fragment might have a role in stabilisation of actin. In conclusion, these results suggest that maintenance of skeletal muscle α-actin could reflect limited dismantling of the sarcomeric structure and be a useful marker to monitor the events that result in the typical texture of dry-cured ham.

2.2.2 Introduction

The production of dry-cured ham requires quite long processing times and is associated with proteolytic activity on myofibrillar and sarcoplasmic proteins, resulting in their progressive degradation. Characterisation of the main myofibrillar proteins is essential for predicting the final quality of dry-cured hams in terms of structure and texture (Harkouss et al., 2015; Petrova et al., 2015). The initial breakdown of proteins by endopeptidases is followed by the action of exopeptidases, giving rise to the generation of small peptides and free amino acids, that contribute—directly or indirectly—to flavour development (Hinrichsen & Pedersen, 1995). Furthermore, small peptides naturally generated from Spanish dry-cured ham may have additional biological functions, such as antioxidant and antihypertensive activities (Escudero et al., 2013; Leticia Mora et al., 2014). In high-quality Spanish dry-cured ham, proteolysis does occur, although the typical ultrastructural elements that constitute the sarcomere are conserved (Larrea et al., 2007). On the other hand, an increased proteolysis could be responsible for the appearance of textural defects (pastiness and softness) (García-Garrido et al., 2000; Parolari, Virgili, & Schivazappa, 1994). Due to their low stability, the cytosolic endopeptidases calpains and caspases

were reported not to be relevant in the processing, whereas cathepsins cause significant proteolysis until the end of the dry-cured ham ripening (Toldrá & Etherington, 1988; Toldrá & Flores, 1998). Indeed, although cathepsin activities gradually decrease through processing (García-Garrido et al., 2000), a residual activity of cathepsins B, D and L was reported even at the end of curing (Sárraga et al., 1993; Toldra et al., 1993). A number of studies have been published on the use of non-protein nitrogen (NPN) fraction as an indicator of drycured ham proteolysis. More recently, proteomics has been used to study the fate of specific myofibrillar and sarcoplasmic proteins (Di Luccia et al., 2005; Mora, Sentandreu, & Toldrá, 2011; Picariello et al., 2006). This approach is very useful in identifying molecular markers to predict and discriminate quality characteristics (Leticia Mora et al., 2014). During processing, myosin heavy chain (MHC) has been recognised as a target of proteolysis, along with myosin light chain and troponin (Larrea et al., 2006; Luccia et al., 2005; Mora et al., 2011; Škrlep et al., 2011; Théron, Sayd, Pinguet, & Chambon, 2011; Toldra et al., 1993). On the other hand, the influence of dry-cured ham processing on actin and its changes is still debated (Di Luccia et al., 2005; Monin et al., 1997; Santé-Lhoutellier et al., 2012; Sentandreu et al., 2007; Škrlep et al., 2011; Soriano Pèrez, Garcìa Ruiz, Marescal Contreras, & Cabezudo Ibanes, 2003; Théron et al., 2011; Toldra et al., 1993). Whether the actin isoforms, which are structurally and functionally distinct (Perrin & Ervasti, 2010), undergo differential degradation remains to be clarified. In this work, a combination of immunochemical and mass spectrometry analysis was used to assess the occurrence of proteolytic changes of actin and MHC during processing of San Daniele dry-cured ham. The other two main Italian PDO (protected designation of origin) dry-cured hams Parma and Toscano, clearly differentiated for their sensory characteristics (Laureati et al., 2014), were also taken for comparison.

2.2.3 Materials and methods

2.2.3.1 Samples

The samples were obtained from *biceps femoris* (BF) muscle of heavy pig thighs, with morphological characteristics in line with the requirements for conformity of the three Italian PDO dry-cured hams: San Daniele (D), Parma (P) and Toscano (T). The animals, crosses of Large White x Landrace of Italian selection (ANAS), were bred on a single farm, slaughtered in the same abattoir in three batches at 6-week intervals, and the thighs were distributed to the three PDO processing plants. The initial thigh weights varied from 13.59 to 14.53 kg, and the subcutaneous fat thickness was from 33 to 36 mm, in line with the three PDO dressing procedures. BF muscle (pH_{24h} 5.63 ± 0.093) was taken from 5-cm-thick slices, which were transversally cut at about 8 cm from the femoral head, and samples for proteomics were cut from a limited (9 cm^2) BF central area. Three samples of raw meat were collected from three

thighs within 30 min of slaughtering (T0), immediately frozen in liquid nitrogen and stored at −80 °C until the time of analysis. During maturation, three thighs per each PDO were collected from each of the following processing phases, which were done in accordance with the rules of each PDO consortium: T1: out of salting (day 16, 20 and 23 for D, P and T, respectively); T2: introduction to the resting room (day 35, 62 and 77 for D, P and T, respectively); T3: after washing and drying (day 117, 121 and 136 for D, P and T, respectively); T4: mid-curing, after greasing (day 216, 240 and 269 for D, P and T, respectively); T5: end of curing (day 391, 393 and 384 for D, P and T, respectively). All (45) independent samples (3 × 3 × 5) were stored at −80 °C until the time of analysis.

2.2.3.2 Preparation of protein samples

For the electrophoretic analyses, extraction of proteins was carried out according to Piñeiro et al. (Piñeiro et al., 1999) method with minor modifications. Samples of BF (100 mg) at slaughter and at the different processing phases were scraped free of connective and adipose tissue, cut in small pieces on ice and suspended in 1 ml of the extraction buffer containing 60 mM Tris/HCl, pH 7.5, 2 % wt/vol sodium dodecyl sulphate (SDS), 0.1 M DTT and supplemented with antiproteases (protease inhibitor, Sigma-Aldrich). After homogenisation with UltraTurrax® T25 Digital (3 × 30 s), the samples were centrifuged at 5600 g for 4 min at room temperature, and the supernatants were heated to 98 °C, shaken for 30 min and centrifuged as before. Protein concentration was determined by UV absorbance at 280 nm, after sample dilution with an SDS containing solution. To measure the proteolysis index, total proteins were precipitated by 20 % (wt/vol) trichloroacetic acid, and nonprotein nitrogen (NPN) and total nitrogen (TN) were determined according to the Kjeldahl method. The index was expressed as (NPN/TN)x100.

2.2.3.3 SDS-PAGE, Western blotting and blot overlay assay

Protein extracts were applied to 10 or 13 % SDS-PAGE gels (Laemmli, 1970) using the Mini 2D (Bio-Rad) electrophoresis equipment. The separated proteins were subsequently stained with NOVEX® Colloidal Blue Staining Kit (Invitrogen) or transferred to nitrocellulose membranes for Western blotting or blot overlay assay using the TE 22 transfer unit (Amhersham Biosciences). When indicated, bands of interest were excised from the gel and rerun in iterative SDS-PAGE gel. At the end of the second electrophoresis, the bands were excised for mass spectrometry analysis or subjected to Western blotting. Anti-myosin heavy chain antibody (anti-MHC) (1:1000) (Sigma-Aldrich), anti-MHC Fast (1:10,000) (SigmaAldrich), anti-MHC Slow (1:4000) (Sigma-Aldrich), anti-actin C term antibody (1:200) (Sigma-Aldrich) were suspended in phosphate-buffered saline (PBS) containing 0.1 % Tween 20 (Sigma-Aldrich) and 3 % wt/vol nonfat dry milk (Bio-Rad). Anti-cathepsin D (1:500)

(Cell Signalling Technology) was suspended in Tris-buffered saline (TBS) containing 0.1 % Tween 20 (SigmaAldrich) and 5 % (wt/vol) bovine serum albumin (BSA; Sigma-Aldrich) and used to recognise preprocathepsin D (43 kDa), procathepsin D (46 kDa) and cathepsin D heavy chain (28 kDa). Immunoreactive bands were detected by enhanced chemiluminescence (Pierce). When blot overlay assay was performed (Edmondson & Roth, 2001), samples of San Daniele ham at T1 and T5 were subjected to 13 % SDS-PAGE and the separated proteins were transferred to nitrocellulose sheet to be incubated or not with 1 mg/ml of purified actin (Sigma-Aldrich) in PBS for 2 h at room temperature. Nitrocellulose sheet was washed three times with TBS to eliminate aspecific binding and finally immunodetected with actin C term antibody. Bands of interest were quantified by densitometry using ImageQuant software (GE Healthcare). The apparent masses of the protein bands were estimated from a calibration curve obtained by plotting the migration distances of high-range standard proteins (Precision Plus Protein™ Standards, Bio-Rad) versus their known molecular masses.

2.2.3.4 Protein identification by mass spectrometry

Protein gel slices of San Daniele samples were excised and in-gel-digested according to the protocol by Link (Link & Labaer, 2009). Peptide analysis was performed with a Dionex Ultimate 3000 micro-HPLC coupled with the LTQ Orbitrap mass spectrometer equipped with a conventional ESI source. For the chromatography separation, an Acclaim PepMap300 C18 (5 µm, 300 Å, 15 cm × 300 µm) column was used, and the column oven temperature was set to 35 °C; the separation was run for 90 min using a gradient of 99.8/0.2 H_2O/HCOOH (eluent A) and 99.8/0.2 ACN/HCOOH (eluent B) and a flow rate of 4 µL/min. For MS1 scans, the Orbitrap resolution was 30,000 and the selected ion population was 4×10^5, with an m/z window from 250 to 2000. For MS/MS in the LTQ, the selected ion population was 1×10^4 (isolation width of 2 m/z unit). A maximum of four precursor ions (most intense) were selected for activation and subsequent MS/MS analysis. CID was performed at 35 % of the normalised collision energy (NCE) in all cases, and MS/MS spectra were analysed by Xcalibur® software (Thermo Fisher Scientific Inc.). Among all the accessions found by aligning detected peptides in database, actin- and myosin-related proteins were identified and characterised according to their sequence coverage percentage and number of peptides identified.

2.2.3.5 Statistical analysis

Results are reported as means ± mean deviations (MD). In figures, the MDs are shown with error bars. A general linear model (GLM) for repeated measures was used to compare variables at different times of ham processing both within subject and between subjects (PDO hams). SPSS for Windows software, version 18.0 (SPSS

Inc., Chicago), was used to perform all statistical analyses, and statistical significance level was set at $p < 0.05$.

2.2.4 Results and discussion

2.2.4.1 Proteolysis index and content of cathepsin D in dry-cured ham

Proteolysis was initially followed by evolution of the proteolysis index during the different phases of San Daniele processing. Samples were obtained from the *biceps femoris*, which is an internal muscle less exposed to NaCl penetration, allowing for considerable proteolytic activity (Parolari et al., 1994; Toldrá & Flores, 1998). In BF muscle, the proteolysis index was undetectable at slaughter (data not shown) and progressively increased during processing, reaching a percentage value of 25.6 ± 0.30 at the end of ripening (Fig. 2.2.1a). These values, as well as those obtained for Parma (28.6 ± 0.99) and Toscano (22.4 ± 1.44), were in the range of values typical of drycured ham (Ruiz-Ramírez, Arnau, Serra, & Gou, 2006). In San Daniele ham, immunodetection of cathepsin D content was performed (Fig. 2.1.1b), because this protease is potentially involved in skeletal muscle actin degradation, as demonstrated by in vitro incubation of F-actin with cathepsin D (Hughes, Healy, McSweeney, & O'Neill, 2000). The active form of cathepsin D (28-kDa fragment) was present at slaughter and remained well detectable until the end of ripening, in accordance with previous reports showing that cathepsin D activity goes on during Teruel dry-cured ham processing (Larrea et al., 2006), resulting in the generation of small peptides (Sentandreu et al., 2007).

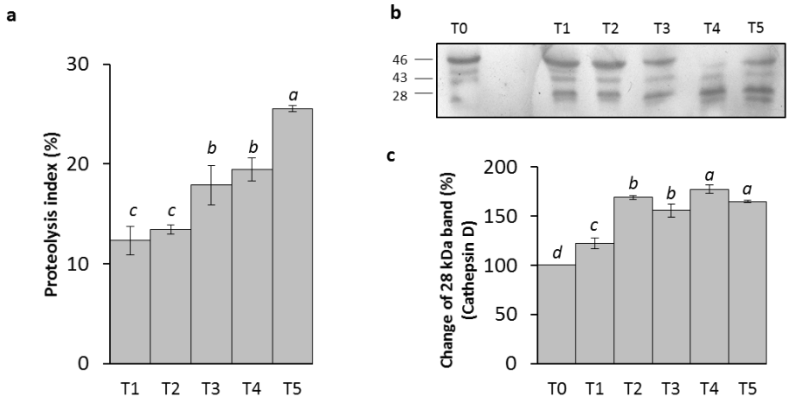


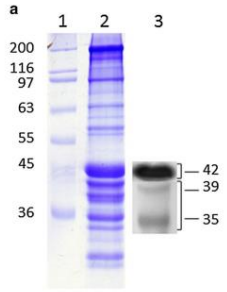
Figure 2.2.1. Proteolysis index and content of cathepsin D of *biceps femoris* muscle during processing of San Daniele dry-cured ham. **a)** Proteolysis index increased significantly during processing (p = 0.007; GLM for repeated measures). **b)** Cathepsin D recognized by Western blotting. Panel show a representative experiment. **c)** Percentage ratio between the active cathepsin D heavy chain (28 kDa) band area and the sum of 28-, 43- and 46-kDa cathepsin D band areas, relative to the ratio at T0, taken as 100%. Values are mean ± MD (*error bars*) of three samples for each processing phase. An increase over time is shown (p = 0.007; GLM for repeated measures). T1 out of salting (day 16), T2 introducing to the resting room (day 35), T3 after washing and drying (day 117), T4 mid-curing, after greasing (day 216), T5 end of curing (day 391).

2.2.4.2 Actin-derived fragments in raw meat and dry-cured ham

Actin proteolysis was first evaluated in raw meat, and the results are shown in Fig. 2.2.2a. Coomassie staining of SDS-PAGE gel obtained from protein extracts evidenced the presence of a thick band at apparent molecular weight of 42 kDa attributable to intact actin. Its identity was confirmed by Western blotting with antibody against actin C term, which is the same for all actin isoforms (Perrin & Ervasti, 2010). Two very thin bands at apparent molecular weight of 39 and 35 kDa attributable to two actin proteolytic fragments were also recognised. These two bands were excised, loaded in separate lanes on an iterative SDS-PAGE gel and subjected to Western blotting, which confirmed their reactivity towards anti-actin antibody. Mass spectrometry analysis (Fig. 2.2.2b) confirmed the identity of the 42-kDa band as α-skeletal muscle actin. In the 35-kDa band, MS/MS identified 11 peptides specific to α-skeletal muscle actin, as well as glyceraldehydes-3-phosphate dehydrogenase (G3P_PIG; 39 % coverage) as the more abundant co-migrated protein. In the 39-kDa band, only one actin peptide was detected and attributed to cytoplasmic β-actin (Q95319_PIG; 12.9 % coverage), along with the co-migrated protein creatine-kinase M type (KCRM_PIG; 42 % coverage). As a whole, these results indicate that in raw pork meat suitable for PDO production actin is minimally degraded. The presence of

actin fragments at slaughter, whose levels changed according to breed and age, has already been reported (Hollung et al., 2007; Lametsch et al., 2003). These fragments produced in *ante/*early *post-mortem* catabolic conditions (Becila et al., 2010; J. Du, Wang, & Miereles, 2004; Goll et al., 2008), unlike the fulllength actin, are particularly sensitive to degradation by μ-calpain (Lametsch, Roepstorff, Møller, & Bendixen, 2004), suggesting that they may be degraded in the early *post-mortem*, when this protease is still active.

a



b

SDS PAGE	MS Analysis			
	Actin identification			
Band (kDa)	Identification	% coverage	Uniprot code	# peptides
42	Alpha skeletal muscle actin	51.0	ACTS_PIG	91
35	Alpha skeletal muscle actin	14.3	ACTS_PIG	11

Figure 2.2.2. Actin and actin fragment identification in *biceps femoris* muscle. **a)** SDS-PAGE and Western blotting against actin. *Lane 1* molecular weight markers; *lane 2* protein extract bands stained by Coomassie Blue; *lane 3* actin and actin fragments recognized by Western blotting. Panel shows a representative experiment. **b)** MS/MS identification of actin and actin fragment. The % coverage and number of peptides identified in 42- and 35-kDa bands of actin are listed with their UniProt entry name.

To analyse the actin fate during ham processing, intact actin content was first evaluated by choosing appropriate conditions for Western blotting to avoid signal saturation, i.e. by loading low quantities of extracts. Interestingly, intact actin content remained rather stable until the end of processing (Fig. 2.2.3a, b) and no difference between the three PDOs was observed. In San Daniele dry-cured ham, Western blotting against actin C term recognised only four thin bands attributable to actin fragments, with an apparent molecular weight comprised between 39 and 29 kDa (Fig. 2.2.3c). Densitometric analyses (data not shown) of these immune reactive bands at the different phases of processing showed that the relative content of the 35- and 29-kDa fragments decreased slowly over time, although immunoreactive bands were still detectable at the end of ripening.

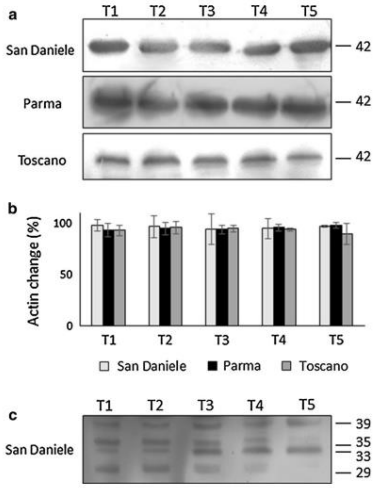


Figure 2.2.3. Actin and actin fragment during processing of Italian PDO dry-cured hams. **a)** Actin recognized by Western blotting during processing of San Daniele (D), Parma (P) and Toscano (T). Panel shows a representative experiment. **b)** Percentage ratio between the intact actin band area at the different processing phases and band area at T0, taken as 100%. Values are mean ± MD (*error bars*) of three independent samples for each processing phase. No significant difference within the group and between groups was obtained ($p = 0.354$ and $p = 0.826$, respectively; GLM for repeated measures). **c)** Actin fragments recognized by Western blotting in San Daniele ham. Panel shows a representative experiment. T1 out of salting (day 16, 20 and 23 for D, P and T, respectively), T2 introducing to the resting room (day 35, 62 and 77 for D, P and T, respectively), T3 after washing and drying (day 117, 121 and 136 for D, P and T, respectively), T4 mid-curing, after greasing (day 216, 240 and 269 for D, P and T, respectively), T5 end of curing (day 391, 3693 and 384 for D, P and T, respectively).

A similar finding of actin fragments was revealed in the other two PDOs (Fig. 2.2.4). In San Daniele, samples collected at T5 MS/MS analyses (Table 2.2.1) confirmed the identity of the full actin as α-skeletal muscle actin. Two peptides of the actin-related protein 2-like that contributes to the structural integrity of the cytoskeleton (Winder, 2002) were also identified (B5APU3_PIG; 1.8 % coverage), along with the co-migrating tropomyosin α-chain (TPM 4_PIG; 28.23 % coverage). In the fragment bands, MS/MS identified peptides belonging to the actin family and, in the 39-kDa band, to the co-migrating tropomyosin α-chain isoforms (TPM1/3/4_PIG; 24.6–41.2 % coverage). The 35-kDa fragment was identified by MS/MS as an actin fragment, which showed a very high sequence identity with the cardiac muscle α-actin 1, while the 39-kDa fragment was unambiguously identified by MS/MS as the cardiac muscle α-actin 1. This latter isoform, which is the prominent one in the embryonic muscle tissue, is still present as a minor component (<5 %) in the adult skeletal muscle (Vandekerckhove, Bugaiskyv, & Buckingham, 1986). Our

finding of fragments from the cardiac isoform in ham samples may reflect its higher tendency to be degraded compared to the α-skeletal actin, suggesting a higher susceptibility of the cardiac filaments to dismantling, that is consistent with their established lower structural stability (Orbán, Lorinczy, Nyitrai, & Hild, 2008). In both 33- and 29-kDa bands, only one peptide was detected and attributed to cytoplasmic β-actin (Q95319_PIG; 12.9 % coverage), which is present in low amounts in skeletal muscle, where it plays a wide range of functions (Kee et al., 2009).

SDS-PAGE	MS analysys			
	Actin identification			
Band (kDa)	Identification	% Coverage	UniProt code	# peptides
42	Alpha skeletal muscle actin	52.5	ACTS_PIG	24
39	Cardiac muscle alpha actin 1	17.2	B6VNT8_PIG	7
35	Actin (fragment)	13.8	B2ZFN7_PIG	2

Table 2.2.1. Actin and actin fragment identification in San Daniele dry-cured ham. **a** MS/MS identification of actin fragments at the end of curing (T5: day 391). The % coverage and number of peptides identified in 42-, 39- and 35-kDa bands are listed with their UniProt entry name.

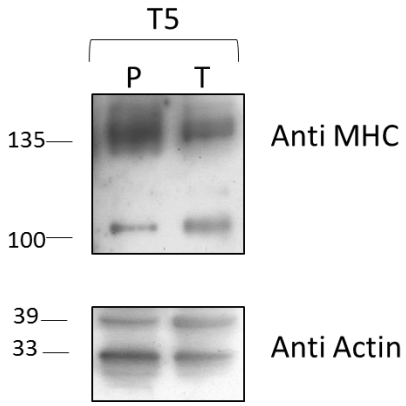


Figure 2.2.4. MHC and actin fragments recognized by Western Blotting in Parma and Toscano ham at the end of curing (*T5*). Figure shows a representative experiment. See legend of Fig. 2.2.3 for processing phase description.

As a whole, our data indicated that, in spite of a high proteolysis index and the presence of active cathepsin D until the final processing phase (Fig. 2.2.1b), the α-skeletal actin does not seem to undergo degradation during dry-cured ham processing, since no identified actin fragment belongs to the skeletal isoform. It should be remembered that actin degradation and formation of actin fragments have been found to be associated with quality defects, such as the pastiness, of dry-cured ham from Slovenia (Škrlep et al., 2011), whereas actin was not hydrolysed in high-quality Spanish Serrano and French Bayonne dry-cured hams (Santé-Lhoutellier et al., 2012; Toldra et al., 1993). The degradation resistance of F-actin in dry-cured ham may be explained by the existence of a steric hindrance to the access of the still-active proteases (Toldrá & Etherington, 1988; Toldrá & Flores, 1998), possibly mediated by the interaction with the co-migrating tropomyosin or with other proteins, which could remain associated with F-actin even at high ionic strength (Forlemu et al., 2011; Rao, Madasu, & Dominguez, 2014; Tanji, Ikeuchi, Yoshizawa, & Suzuki, 1997). A lower solvent accessibility of F-actin rather than MHC at the actin–myosin interface, as revealed by site-directed spectroscopy (Korman, Anderson, Prochniewicz, Titus, & Thomas, 2006), may also limit protease access.

2.2.4.3 Myosin heavy chain (MHC)-derived fragments in dry-cured ham

In addition to actin, the degradation pattern of MHC was analysed in protein extracts of raw meat and San Daniele ham during the different phases of processing (Fig. 2.2.5). In raw meat, immune detection after SDS-PAGE separation with anti-MHC antibodies, which are able to recognise both slow and fast MHC isoforms, showed the presence of a very thick band of intact MHC at apparent molecular weight of 220 kDa, along with a very thin band at apparent molecular weight of 135 kDa

attributable to an MHC fragment. The occurrence of MHC degradation in the early *post-mortem* has been recently observed in beef (Wu, Clerens, & Farouk, 2014), although the underlying mechanisms need to be investigated. No change of these bands was observed until the mid-curing (T4), when the MHC band was markedly reduced, while the 135-kDa band was noticeably increased and a new band of apparent molecular weight of 100 kDa appeared, indicating that, differently than actin, MHC is largely degraded during San Daniele dry-curing process, in accordance with previous reports (Di Luccia et al., 2005). As expected, a remarkable degradation of MHC was also observed in the other two PDOs (Fig. 2.2.4). In San Daniele ham, Western blotting with antibodies against the slow and fast isoforms of MHC showed that at the end of ripening (T5) the slow MHC isoform was predominant in the intact 220-kDa band, less abundant in the 135-kDa band and not detectable in the 100-kDa band, while the fast MHC isoform was well detectable in all the three MHC bands, indicating that only the 135-kDa fragment is a degradation product of both types of MHC isoforms. The two fragments and the intact MHC were further analysed to establish their capacity to bind actin. They were immobilised on a membrane and probed with purified G-actin. After this recognition, Western blotting was normally carried out with anti-actin C term antibody, which recognised the presence of actin at the level of the 135-kDa fragment (Fig. 2.2.5b), but not of the 100-kDa fragment (data not shown). As expected, also the intact MHC was found to bind G-actin (Fig. 2.2.5b). To confirm the identity of the MHC and its fragments, a proteomic approach, as already described before, was applied. Peptides identified in the 220-, 135- and 100-kDa bands fully belonged to the myosin family (data not shown). As far as the 135-kDa band is concerned, peptides were found to belong mainly to the C term of both fast and slow MHC isoforms (Fig. 2.2.4), strongly suggesting that this fragment was formed by the release of the N term catalytic head of MHC. The high number of peptides identified with a very high score indicates that this fragment is quite abundant, suggesting an extensive myosin degradation. Based on the identified peptides, EXPASY software predicted for the MHC fragment a molecular mass of 135 kDa comprising the residues 769–1937, which is consistent with the electrophoretic migration data. The 135-kDa fragment strongly resembles the one found by in vitro incubation of native myosin with musclespecific cathepsin B and/or D (Schwartz & Bird, 1977), with the latter being well detected until the end of the ripening of San Daniele (Fig. 2.2.1b, c). Interestingly, at least 5 potential cleavage sites for cathepsin B (Biniossek, Nägler, Becker-Pauly, & Schilling, 2011) and two for cathepsin D (Sun et al., 2013) are present just above Ala 769, the first identified residue of the 135-kDa fragment. Despite the observed interaction between the 135-kDa fragment and actin by the blot overlay assay, neither the MS/MS recognised peptides in the 135-kDa band, nor the whole predicted 769–1937 sequence comprised the actinbinding sites located in the catalytic head (Behrmann et al., 2012; Milligan,

1996). Nonphysiological conditions have been reported to induce unpredictable interactions between actin and myosin fragments revealed by in vitro motility assay (Guo & Guilford, 2004), making plausible that the 135-kDa fragment interacts with actin through non-native interactions. Such interactions may protect F-actin against denaturation by overcoming the destabilising effect of high salt concentration, as shown for meromyosin (Ikeuchi, Iwamura, Machi, Kakimoto, & Suzuki, 1992), suggesting another possible explanation for the lack of actin proteolysis in dry-cured processing.

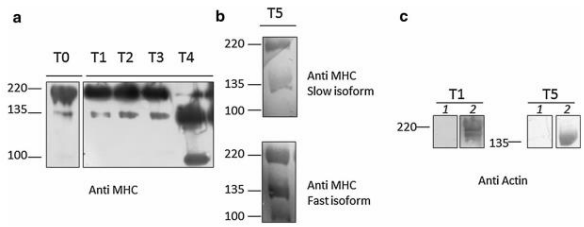


Figure 2.2.5. Myosin heavy chain (MHC) proteolysis and capacity to bind actin in San Daniele dry-cured ham. **a)** Total MHC evaluated by Western blotting during processing. **b)** Slow of fast MHC at the end of curing (T5). **c)** Blot overlay assay at T1 and T5 of the 220- and 135-kDa MHC bands by Western blotting against actin. Only in *lane 2* and *lane 4*, which were pre-incubated with purified actin, actin was immunodetected in the 220- and 135-kDa bands, respectively. In non-preincubated *lane 1* and *3*, actin was not revealed. Panels show representative experiments. See legent of Figure 2.2.3 for processing phase description.

2.2.5 Conclusions

Raw meat displays low levels of actin and MHC fragmentation, which is related to the muscle state *ante*/early *post-mortem*, consistent with limited protein catabolism. Dry-cured hams produced in line with the requirements for conformity with the specifications of the three Italian PDOs show a common proteolytic pattern on the actomyosin complex, characterised by an extensive degradation of only MHC, but not of actin. Since actin may be stabilised at high ionic strength by interaction with other proteins and possibly their fragments, we speculate that PDO dry-cured ham processing may favour those conditions which could limit protease access, resulting in actin resistance. More precisely, during ham ripening, actin degradation gives rise to a limited number of fragments, that are not produced by the cleavage of the α-skeletal actin. Thus, different actin isoforms do not undergo the same changes during dry-cured ham processing, possibly as consequence of non-native interactions with other proteins, including the 135 kDa fragment of MHC. It is well known that

sensory qualities, including texture, benefit when proteolysis is not excessive. α-skeletal actin could be a useful marker of integrity of the sarcomeric structure, which confers the typical texture of dry-cured ham. On the other hand, MHCs and their degradations products could also be appropriate quality markers at least to follow the real time of dry-cured ham maturation (M. Škrlep, personal communication). Our characterisation of fragments from other actin isoforms, each of which performs a specific function, should encourage future investigations of their changes during the ripening process.

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2.2.6 Bibliography

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3. General conclusions

Protein markers are very promising for generating management strategies to govern meat and meat product quality and to solve protein-based problems in muscle food products. This PhD thesis discusses the potential of actin and actin degradation products to interpret molecular mechanisms involved in meat quality. Relevant examples concerning the Italian dry-cured hams and the farmed sea bass (*Dicentrarchus labrax*) fillets are included.

The properties of skeletal muscle as food have been analysed by integration of gel electrophoresis, immunochemistry, MS/MS, and data base search algorithms. In the case of Italian PDO dry-cured hams, actin conservation reflected a limited dismantling of the myofibrillar structure, resulting in the typical texture of dry-cured ham. Indeed, differently from myosin heavy chain, intact actin content remained rather stable until the end of processing, and only four actin fragments were recognized, two of them were identified as $\alpha_{cardiac}$ actin fragments and two were attributed to β-actin, while no fragments of the $\alpha_{skeletal}$ isoform were identified. An increased proteolysis could be responsible for the appearance of textural defects (pastiness and softness). Therefore, our results confirmed the very limited proteolysis of $\alpha_{skeletal}$ actin in dry-cured ham, produced according to PDO specifications.

On the other hand, for the fish fillet, actin degradation showed the impairment of the muscle structural integrity. Indeed, a stressing slaughtering technique, such as the water/ice procedure, was associated to the formation of a 15 kDa actin fragment attributable to β-actin degradation by caspases, which are activated by the apoptotic process. The presence of this fragment thus suggested the cytoskeleton degradation as responsible for the loss of muscle structural integrity. Consistently, rheological analyses revealed that water/ice induced a higher liquid loss until five hours during *post mortem* than a less-stressing procedure, such as spiking. Moreover, at 1 and 3 h during *post mortem* the water/ice slaughtering procedure resulted in a significant decrease in apparent viscosity.

These findings demonstrate the actin ability to indicate a variation of muscle texture, indicating actin as a promising biomarker of quality for meat and fish products.

4. Notes to the reviewer

Comment on page 65 to Figure 2.1.6:

Even if we agree with the reviewer's comment regarding the possible effect of pre-slaughter stress on pH, we cannot support this statement with our scattered data.

5. Publications inherent to the PhD activities

- "Proteolytic resistance of actin but not of myosin heavy chain during processing of Italian PDO (protected designation of origin) dry-cured hams" **Fabbro A**.,Bencivenni M., Piasentier E., Sforza S., Stecchini M. L., Lippe G. European Food Research and Technology DOI 10.1007/s00217-015-2594-8 2015
- "Actin as a quality marker for food of animal origin" **Fabbro A.** In XX Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology, 267-271, ISBN: 978-88-99407-02. Perugia, 2015
- "The effect of slaughtering methods on actin degradation and on muscle quality attributes of farmed European sea bass (*Dicentrachus labrax*)" Tulli F., **Fabbro A**., D'Agaro E., Messina M., Bongiorno T., Venir E., Lippe G., Tibaldi E., Stecchini M.L. Journal of Food Science and Technology DOI 10.1007/s13197-015-1829-9 2015
- "Effects of ozone processing on chemical, structural and functional properties of whey protein isolate" Segat A., Misra N.N., **Fabbro A**., Buchini F., Lippe G., Cullen P.J., Innocente N. Food Research International 66: 365-372 2014
- "Lycopene bioaccessibility and bioavailability from processed foods" Anese M., Mirolo G., **Fabbro A.**, Lippe G. Journal of Scientific & Industrial Research 72: 543-547 2013

6. Annexes

- The effect of slaughtering methods on actin degradation and on muscle quality attributed of farmed European sea bass (*Dicentrarchus labrax*)

- Proteolytic resistance of actin but not of myosin heavy chain during processing of Italian PDO (protected designation of origin) dry-cured hams

J Food Sci Technol
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ORIGINAL ARTICLE

The effect of slaughtering methods on actin degradation and on muscle quality attributes of farmed European sea bass (*Dicentrarchus labrax*)

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Abstract In the current study, two different slaughtering procedures, spiking *vs* immersion in water/ice slurry, were applied on electrically stunned European sea bass (*Dicentrarchus labrax*) and the effects on actin degradation and fillet quality attributes were investigated. *Rigor mortis* index was similar for the two slaughtering techniques, whereas the shear force measurement indicated that *rigor mortis* occurred more quickly and intensely in the water/ice handled fish than in those slaughtered by spiking. The water/ice immersion procedure also resulted in higher amount of actin fragments than spiking. Muscle tissue apparent viscosity and water holding capacity were lower in sea bass treated in water/ice slurry compared with fish handled by spiking, whereas the acidification patterns of the two groups were not easily distinguishable. In conclusion, spiking appeared to preserve actin integrity better that water/ice and it seemed to ensure less muscle damage as indicated by the higher viscosity and water holding capacity values. Finally, a role of actin as a biochemical marker for the prediction of fish quality was suggested.

Keywords Slaughtering · Actin degradation · Fish quality · European sea bass

Highlights
- Sea bass quality attributes resulted sensitive to slaughtering method
- Water/ice slaughtering increased the intensity of actin proteolysis compared to spiking
- Actin is proposed as a sensitive and reliable predictor of sea bass quality.

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Introduction

All aquaculture practices, including stunning and slaughtering methods could have a strong effect on fish welfare, affecting the stress response (Ashley 2007; Conte 2004; Huntingford et al. 2006; Merkin et al. 2014; Poli et al. 2005). From a comparison of commercial and experimental killing methods in the European sea bass (*Dicentrarchus labrax*), asphyxia was confirmed to be the most stressfull killing method, showing either the most prolonged period prior to death or a remarkable physical activity and affecting the final quality (EFSA 2012). Therefore, stress caused by an improper suppression method can also affect the quality attributes of the fish, which are important for consumer acceptability, as well as for the processing industry. Loss of quality is the result of complex physiological and biochemical processes related to the aquaculture practices and to the *post-mortem* events (Martinez et al. 2011). However, there is very little knowledge on the way by which *ante-mortem* biochemistry changes the *post-mortem* events and hence the resulting final quality (Delbarre-Ladrat et al. 2006).

Despite the importance of proteolysis on texture and quality, methods based on monitoring the products of proteolysis have received little attention, and very little is known about protein changes and fish quality (Verrez-Bagnis et al. 2001). The expanding aquaculture-food market, which requires production efficiency and quality of fillet, could benefit from the detailed knowledge of protein degradation in fish following the most used aquaculture practices and their influence on the fish quality.

Actins are a family of highly conserved proteins with only slight variations in their N-terms, which play fundamental roles in nearly all aspects of eukaryotic cell biology. Four muscle actins predominate in striated (α_{sk} and α_{ca}) and smooth (α_{sm} and γ_{sm}) muscle, while two cytoplasmic non-

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muscle β-actin and γ-actin isoforms are found in all cells (Rubenstein 1990). Only subtle sequence variations distinguish the isoactins.

In the skeletal muscle, in addition to the highly specialized contractile apparatus, there are both the actin-associated costameric complexes and the functionally distinct cytoskeletal actin-based filaments, which underpin a wide range of functions (Kee et al. 2009). A progressive degradation of cytoskeletal and thin filaments of actin was proven in mammalian muscle immediately after death and referred to apoptosis (Becila et al. 2010).

The first objective of the present study was to provide evidence of actin degradation as pathognomonic of muscle protein disassembly associated to the early *post-mortem* periods, following the most commonly adopted slaughtering practices in farmed sea bass (*Dicentrarchus labrax*). Secondly, the existence of a relationship between the actin fragmentation profile and the major physical and chemical properties of the edible muscle portion was also examined to determine the potential use of actin as a biochemical marker for the prediction of fish quality.

Material and methods

Animals and experimental design

The experiments were carried out at the indoor facilities of the Department of Food Science of the University of Udine. All procedures of our research protocol involving fish were in accordance with the requirements of the EU Directive 2010/63 (2010) on the protection of animals used for scientific purposes. In this study, 60 European sea bass (*Dicentrarchus labrax*) (344±57 g average body weight and 33.0±2.0 cm average total length) were maintained in cubic fibreglass tanks (500 L) in a partially recirculating marine aquaculture system (total volume 6 m^3, daily water renewal rate 5 %). The system ensured a constant day length of 12 h of artificial light (180 lux) supplied by fluorescent tubes and optimal water quality conditions for sea bass: temperature, 23±2 °C; salinity, 28.6±0.5‰; dissolved oxygen, 7.3±0.13 mg/L (Handy Polaris, Oxyguard, Birkerød, Denmark), pH, 7.89±0.02 (Basic 20, Crison Instruments S.A., Alella, Spain), total ammonia nitrogen concentration<0.12 mg/L and nitrite-nitrogen concentration<0.015 mg/L (Lasa 20, Hach Lange, Düsseldorf, Germany). Fish were fed 6 days a week a commercial diet (Sketting Optibass, 6 mm) offered in two daily meals to visual satiety, but were deprived of food 36 h before the stunning/slaughtering treatment. Fish were kept under the above described culture conditions over 2 months and then they were exposed to electric stunning (50 V, 50 Hz, direct current; Fishkill EG100, Scubla s.r.l., Remanzacco, Italy) for 5 s just before being slaughtered with two different procedures: immersion in water/ice slurry (ratio 1:1) and spiking (Ikigun, Adept Ltd, New Zealand). For the water/ice procedure fish were left in the slurry for 15 min, then they were removed and manually decapitated (time 0). For the spiking procedure, fish were manually decapitated immediately after spiking (time 0). Except fish used for *rigor mortis* measurements, which were kept as a whole fish on ice, all fish were filleted.

Rigor mortis

Rigor mortis was measured as rigor index (RI %) on 9 fish for each slaughtering treatment using the Cutting's method (as tail drop) (Bito et al. 1983). Briefly, the upper half of a whole fish was placed on one side on a horizontal table surface with the other half (tail part) suspended off the edge. At selected time intervals, the vertical distance (L, cm) between the base of the caudal fin and the table surface was measured and RI was calculated from the equation (1):

$$[(L_0 - L)/L_0] \times 100 \quad (1)$$

where L_o (cm) is the vertical distance between the base of the caudal fin and the table surface measured immediately after the death.

Texture

As a further monitoring of the *rigor mortis* process, texture of sea bass was measured, according to Fuentes et al. (2010), by a shear force test which incorporates compression of fibers beneath the blade, tension in the adjoining fibers and shearing of the fibers (Bouton et al. 1975). A TA.TX.plus Texture Analyzer (Stable Micro Systems, Godalming, Surrey, U.K.), provided with a 30 kg load cell and a Texture Exponent 32 (Stable Micro Systems) software, was used. The instrument was equipped with a Warner-Bratzler test cell, which sliced the samples perpendicularly to the muscle orientation at a constant speed of 1.0 mm s^{-1}. Textural measurements were performed using 20 × 20 × 10 mm pieces from the epiaxial muscle of the fish fillet on at least three samples for each slaughtering method. The maximum peak force (N) required to shear through the sample was recorded as shear force.

Protein samples, SDS-PAGE and Western blotting

Possible changes induced in sea bass muscle proteins by the slaughtering methods were investigated by one-dimensional SDS-PAGE on six fish for each slaughtering method. The extraction of proteins from epiaxial muscle tissue was carried out according to Piñeiro et al. (1999) with minor modifications. Briefly, samples (100 mg) were cut in small pieces on

ice and suspended in 1 ml of the extraction buffer containing 60 mM Tris/HCl pH 7.5, 2 % wt/vol SDS, 0.1 M dithiothreitol (DTT) and supplemented with 2 μl of antiproteases (Protease Inhibitor, Sigma-Aldrich) to avoid degradation of proteins. After homogenization with UltraTurrax® T25 Digital, the samples were centrifuged for 4 min at 5600 g at room temperature, heated at 98 °C and shaken for 30 min. After heating, samples were centrifuged at room temperature for 10 min at 5600 g. The protein concentration of the supernatant was determined by UV absorbance at 280 nm, after sample dilution with SDS containing solution.

Protein extracts were applied to 13 % SDS-PAGE gels (Laemmli 1970), suitable for separation of 10–200 kDa proteins, using the Mini 2D (Bio-Rad, Richmond, California, USA) electrophoresis equipment. The gels were subsequently stained with NOVEX® Colloidal Blue Staining Kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) or transferred to nitrocellulose membranes using the TE 22 transfer unit (GE Healthcare, Little Buckinghamshire, UK) for Western-Blotting. Western-Blotting was performed with a polyclonal anti actin C-term antibody (1:1000) (Sigma-Aldrich), suspended in phosphate buffered saline (PBS) containing 0.1 % (vol/vol) Tween-20, or with a monoclonal anti β-actin N-term antibody (1: 1000) (Santa Cruz Biotechnology, Dallas, Texas, USA), suspended in Tris buffered saline (TBS) containing 0.1 % (vol/vol) Tween-20 and 2.5 % (wt/vol) bovine serum albumin (BSA; Sigma-Aldrich). The apparent masses of the protein bands were estimated from a calibration curve obtained by plotting the migration distances of standard proteins (Precision Plus Protein™ Dual Color Standards – Biorad, Hercules, California, USA) versus their known molecular masses. Immunoreactive bands, detected by enhanced chemiluminescence (Pierce, Rockford, Illinois, USA), were analyzed by densitometry with the ImageQuant Software. Bands were expressed as % changes in arbitrary units using the ratio between the optical density of all actin fragments and the optical density of intact actin.

Viscosity

Apparent viscosity of fish muscle homogenate was determined, according to Borderias et al. (1985), as an indirect measure of the protein quality of fish muscle. Measurements were performed on at least three samples of epiaxial muscle tissue for each slaughtering method, using a controlled stress rheometer (StressTech Rheometer, Reologica Instruments AB, Sweden), at shear rate of 10^{-1}, at 4 °C±0.2 °C, using bob-cup sensor geometry. Samples (5 g) were subjected to homogenization at 1000 rpm for 1 min (Polytron PT 3000, Kinematica, Littau, Switzerland) with 5 % NaCl (1:4) and phosphate buffer 50 mM (pH 4.5).

Water holding capacity

Water Holding Capacity (WHC) was determined, as liquid loss (LL), after slaughter on at least three samples, for each slaughtering method, of epiaxial muscle tissue. Each sample consisted of three sub-portions, of about 5 g, taken in the cephalic, middle and caudal area. Samples (15 g) were transferred to centrifugation tubes and subjected to low speed centrifugation at 210 g for 15 min at 5 °C, as proposed by Olsson et al. (2003). The liquid loss was expressed as a percentage of liquid released as the difference in weight before and after centrifugation, as follows (2):

$$\mathrm{LL}(\%) = [\text{liquid released(g)}/\text{total weight(g)}] \times 100 \qquad (2)$$

Muscle pH

The pH values were measured using a pH meter 213 (Hanna Instruments, Rhode Island, USA) model, with the electrode inserted into the epiaxial muscle after carrying out a lateral incision. Measures were taken in the cephalic, middle and caudal area on at least two samples/fish for each slaughtering method.

Statistical analysis

Results were reported as means±mean absolute deviations. In figures, the mean absolute deviations were shown with error bars. For the parameters studied, samples were tested for differences using one-way t-tests and statistical significance was taken as $p<0.05$.

Results and discussion

Muscle protein profile and actin degradation

As shown in Fig. 1, the protein profiles of sea bass muscle tissue samples taken 1 h after slaughtering (time 1) were similar and apparently not affected by the slaughtering method. When intact actin was subjected to Western-Blotting against actin C-term or β actin N-term, differences in the intact actin bands (42 kDa) between the two slaughtering methods were not evident (Fig. 2).

On the other hand, compared to spiking, the immunodetection of actin fragments with the actin C-term polyclonal antibody showed that the water/ice method produced higher amount of actin C-terminal fragments, notably the ones at apparent molecular weight of 15 kDa. Actin degradation has been often associated to apoptosis considering that its early degradation is ensured by caspases, a family of cysteine proteases responsible of cell dismantling in apoptotic cells (Becila

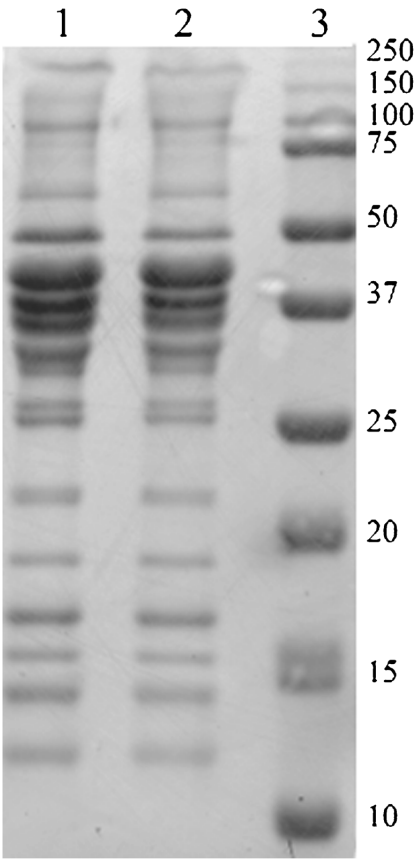

Fig. 1 Electrophoretic patterns of European sea bass muscle samples 1 h after slaughtering (time 1) by spiking (*lane 1*) or in water/ice (*lane 2*); molecular weight markers in the range of 10–250 kDa are shown in *lane 3*. Panel shows a representative experiment

et al. 2010). In particular, the C-terminal 15 kDa fragment of the cytoskeletal actin (tActin) is a well characterized product of caspase activity (Utsumi et al. 2003). More recently, cleavage of actin, mediated by the mitochondrial protease HtrA2/Omi was also observed in caspase-independent programmed necrosis (Sosna et al. 2013; Yamauchi et al. 2014). Anyway, whatever the mechanism is involved, degradation of transversal cytoskeletal actin filaments causes a detachment of sarcolemma from the basal lamina and the extracellular matrix network (Becila et al. 2010; Ouali et al. 2013), impairing the structural integrity of the muscle (Kee et al. 2009). Therefore, it was possible to hypothesize that, even there was variability within samples, the effect of a short-term stress, such as that derived from a slaughtering procedure, was significant respect to the development of actin degradation fragments. The appearance of these fragments could likely serve as potential sensitive index of muscle protein early disassembly.

The finding of the 15 kDa fragment, which might correspond to tActin, is intriguing, because the presence of β-actin in sea bass muscle has not yet described, in spite of its expression in other tissues (Scapigliati et al. 2001). Anyway, when tested by monoclonal antibody against the N-term of β-actin, a clear band at the expected 42 kDa apparent molecular weight of the intact protein was found, suggesting its expression in sea bass muscle (Fig. 2). Conversely, in both kind of samples no band was detected at 15 kDa by monoclonal antibody against the N-term of β-actin (data not shown), that is consistent with the sequence of tActin.

Quality attributes

Fish quality is influenced by *rigor mortis*, which is known to affect how firm the flesh becomes or, on the other hand, how the fillet is liable to gape (Borderías and Sánchez-Alonso 2011). In our study, the development of *rigor-mortis* was investigated for its relevance to fish quality, and the Rigor Index (Bito et al. 1983) was used as an assessing method for its reported simplicity and effectiveness (Wang et al. 2000). Comparing the two slaughtering methods, spiking and water/ice, the onset of *rigor mortis*, as well as its resolution, resulted similar for the two treatments, even if in some specimens of the water/ice group resolution of *rigor* begun earlier than in the spiking samples (Fig. 3).

Spiking, which involves driving a sharp spike into the brain of the fish, is considered humane slaughter in that it results in an immediate loss of consciousness (Lines and Spence 2014). This technique is relatively slow due to the difficulty in precisely locating the brain with varying fish size. However, when it is done accurately, it reduces physical damage with concurrent quality benefits (FAO 2001).

The rate of onset and resolution of *rigor* is affected by a number of factors, including the condition of the fish, i.e., *rigor mortis* starts immediately or shortly after death if the fish is starved and the glycogen reserves are depleted (FAO 1973). The method used for stunning and slaughtering the fish was also reported to affect the *rigor* onset, with slaughtering in water/ice giving the fastest onset of rigor and a blow on the head producing a delay of up to 18 h (Azam et al. 1990; Proctor et al. 1992). Significant differences were also showed in the duration of *rigor mortis*, with percussive stunning producing the highest glycogen reserve and the longest duration of *rigor mortis* in slaughtered rainbow trout in comparison to other slaughtering methods (Sebastio et al. 1996).

Therefore, from the results of the present experiment, it could be hypothesized that death by water/ice was not particularly stressfull in comparison to spiking or, in alternative, that the Rigor Index was not particularly sensitive to the level of slaughtering stress in European sea bass.

To test these hypotheses, a more sensitive approach to monitor the *rigor mortis* process was used, and the texture

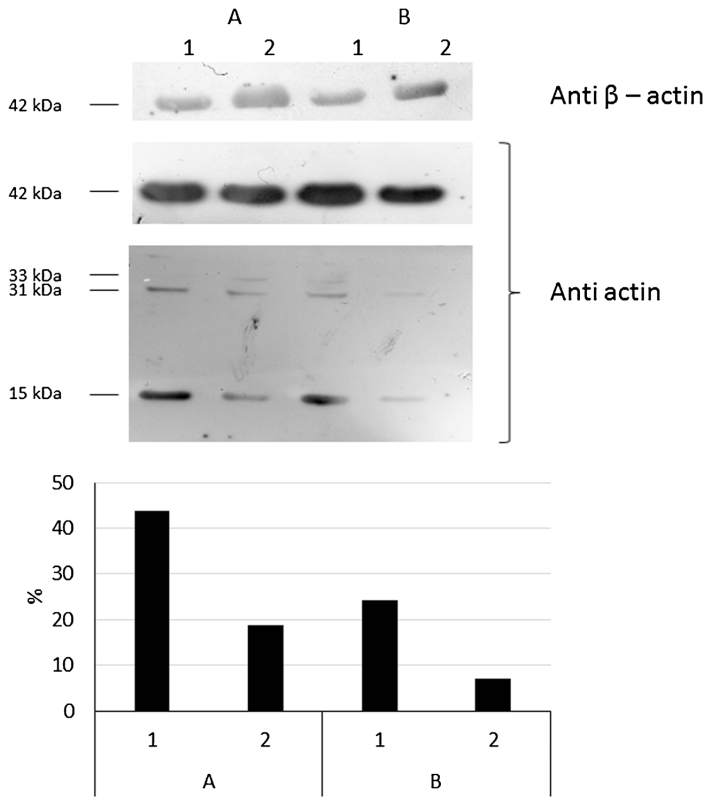

Fig. 2 Immunodetection of actin in four different fish 1 h after slaughtering in water/ice slurry (1) or by spiking (2) from two representative experiments (**a** and **b**) (*upper panel*). Relative percentage of the immunodetected bands is expressed as ratio between the optical density of all actin fragments and the optical density of intact actin (*lower panel*)

hardness, expressed in Newton, was evaluated. With this approach, it was registered not only the time curve but also the strength of the *rigor*. As shown in Fig. 4, spiking delayed time to the maximum total load (1–3 h), as compared to the water/ice slaughtering method (1 h). In addition, a significant difference was observed in the maximum stiffness between fish slaughtered by spiking (0.93–0.95 N) or in water/ice (1.32 N).

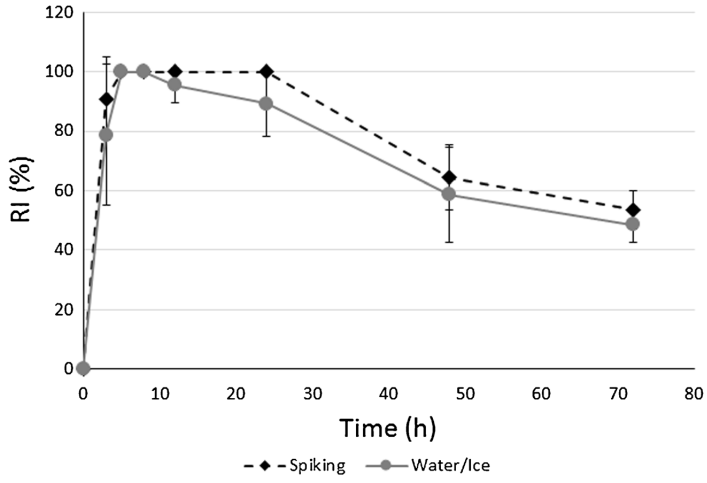

Fig. 3 Evolution of rigor mortis as rigor index (RI) in whole sea bass slaughtered by spiking or in water/ice. Values are provided as mean (n=9) and mean absolute deviation (*bars*)

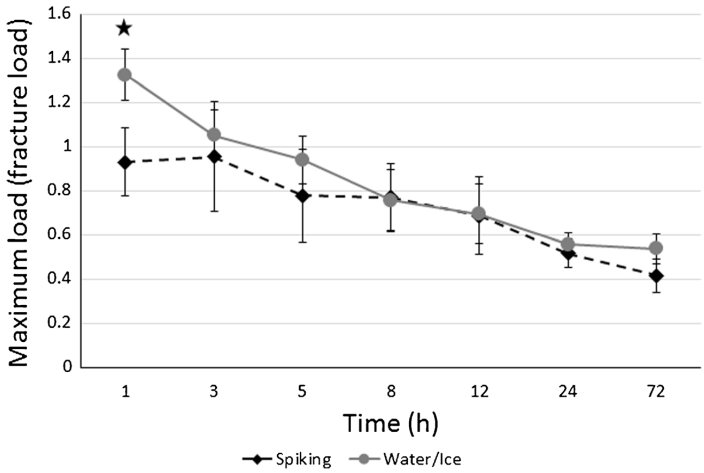

Fig. 4 Evolution of shear force of sea bass fillet measured by Warner-Bratzler shear blade. Values are mean±mean absolute deviation (error bars) of three independent samples

These differences in timing and hardness suggested that the water/ice fish entered rigor quickly and generated a greater tension in fish muscle respect to fish slaughtered by spiking.

Water is the major constituent of muscle foods and the interaction between water and macromolecules determine the water holding capacity, which is affected by the physical and biochemical changes in the muscle (Aursand et al. 2010). In our study, the WHC was expressed as the difference in weight before and after centrifugation, i.e., total weight loss. As shown in Fig. 5, the spiking samples had a significant lower liquid loss than their water/ice counterpart until 5 h *post-mortem*. These results support the idea that the mobile water was more easily removed from the more disassembled protein structures, such as those found in water/ice samples, which showed a more fragmented actin.

Muscle *post-mortem* acidification is caused by glycogen conversion to lactate and more hypoxic conditions with an increased anaerobic glycolytic activity are caused by the stress before death (Hultmann et al. 2012). Acidification of muscle decreases protein electrical charge and induces an increase in their hydrophobicity, thereby reducing water content (Ouali et al. 2013). On these basis a different pattern of pH values in the sea bass slaughtered by spiking respect to fish slaughtered in water/ice could be expected. However, as shown in Fig. 6, the acidification patterns of the two groups were not easily distinguishable and therefore could not provide a clear explanation of the differences in liquid loss. As pointed out by Becila et al. (2010) an early increase in extracellular space, resulting from the expulse of intracellular water, started immediately after slaughter, whereas pH was still very close to neutrality. A cell death, possibly induced by apotosis, would provide a reasonable explanation of the early change in water loss in *post-mortem* fish muscle. The markedly different behaviour observed in the first period of

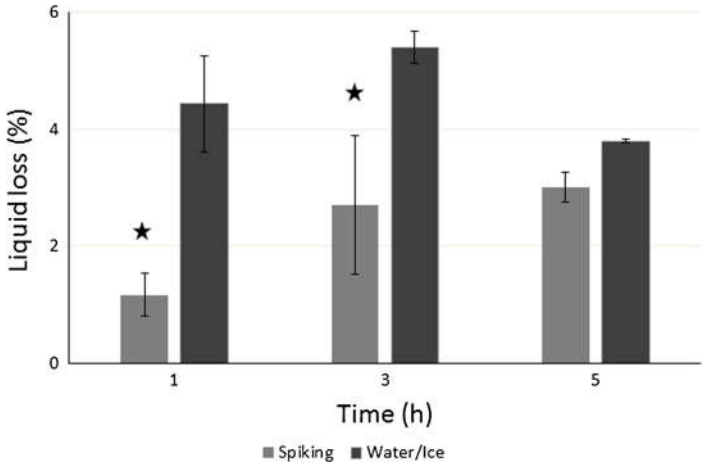

Fig. 5 Evolution of liquid loss in samples of sea bass slaughtered by spiking or in water/ice. Samples were withdrawn at different times after death and values are mean±mean absolute deviation (error bars) of three independent samples for each harvesting time

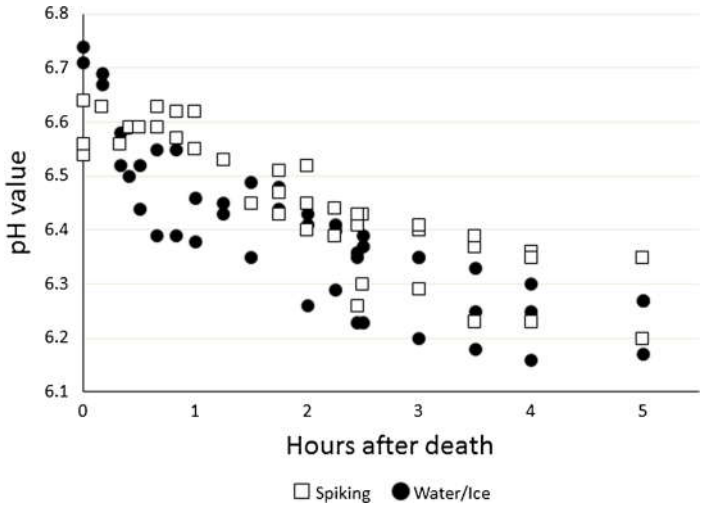

Fig. 6 Evolution of pH in samples of sea bass slaughtered by spiking or in water/ice. Each value is the mean of the measurements taken in three areas (cephalic, middle and caudal) of the epiaxial muscle of each fish. At each sampling time, at least two fish were analysed for each slaughtering method

post-mortem might be consistent with the higher actin degradation, and in particular with the higher formation of the apoptotic C-terminal 15 kDa actin fragment in water/ice than spiking samples.

Apparent viscosity is considered a rheological measure of functional quality of proteins (Borderias et al. 1985), which can eventually affect consistency of the comminuted products. As shown in Fig. 7, at 1 and 3 h *post-mortem* the water/ice slaughtering procedure resulted in a significant decrease in apparent viscosity, not explained by differences in pH (Fig. 6). It should be noticed that pH is one of the most important factor that produces strong effects on the structure of myofibrillar proteins (Feng and Hultin 2001; Liu et al. 2014). It was therefore proposed that an initial partial degradation of muscle proteins, such as that reported for actin, may be accountable for differences in the apparent viscosity.

Conclusions

Biomarkers of the fish quality are of primary importance for the fish industry, which has to satisfy the increasing consumer expectations. The biomarker should be enough sensitive and certainly indicative of a variation in texture, which is one of the main quality attribute of fish fillet. A protein of great interest is actin, whose degradation is likely to impair the muscle structural integrity. It appears, therefore, that actin could be a sensitive and reliable predictor of fish quality.

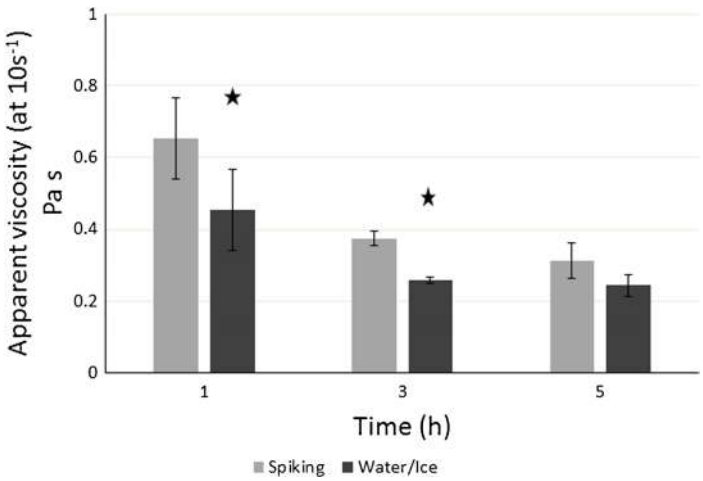

Fig. 7 Evolution of apparent viscosity in samples of sea bass slaughtered by spiking or in water/ice. Samples were withdrawn at different times after death and values are mean±mean absolute deviation (error bars) of three independent samples for each harvesting time

This paper provided the evidence that actin proteolysis increased by employing the water/ice slaughtering method, as compared to spiking. This latter procedure also resulted in a significant variation of some important fish quality attributes, including texture, viscosity and water holding capacity. However, detailed biochemical investigations will be needed to study more in depth the actin cleavage sites and the likely proteolytic enzymes engaged in the actin degradation in the *ante*/early *post-mortem* period.

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ORIGINAL PAPER

Proteolytic resistance of actin but not of myosin heavy chain during processing of Italian PDO (protected designation of origin) dry-cured hams

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Abstract Proteomics is the approach of choice to study the fate of specific proteins, and it is very useful in identifying quality molecular markers. A combination of immunochemical and mass spectrometry analysis was used to assess the occurrence of proteolytic changes of actin and myosin heavy chain (MHC) proteins in pig *biceps femoris* skeletal muscle during processing of three Italian PDO dry-cured hams. Early *post-mortem* muscle displayed low levels of actin and myosin fragments. In spite of a high proteolysis index and the presence of active cathepsin D until the final ripening phase, during dry-cured ham processing, very low actin proteolysis and no generation of fragments from α-skeletal muscle isoform were found, while the identified fragments derived mainly from the cardiac actin isoform. On the other hand, MHC showed a remarkable degradation of its catalytic head, generating a C-terminal 135-kDa fragment. Based on its ability to interact with actin in vitro, this MHC fragment might have a role in stabilisation of actin. In conclusion, these results suggest that maintenance of skeletal muscle α-actin could reflect limited dismantling of the sarcomeric structure and be a useful marker to monitor the events that result in the typical texture of dry-cured ham.

Keywords Dry-cured ham · Italian PDO · Proteolysis · Actin · Myosin · Mass spectrometry

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Introduction

The production of dry-cured ham requires quite long processing times and is associated with proteolytic activity on myofibrillar and sarcoplasmic proteins, resulting in their progressive degradation [1]. Characterisation of the main myofibrillar proteins is essential for predicting the final quality of dry-cured hams in terms of structure and texture [1, 2].

The initial breakdown of proteins by endopeptidases is followed by the action of exopeptidases, giving rise to the generation of small peptides and free amino acids, that contribute—directly or indirectly—to flavour development [3]. Furthermore, small peptides naturally generated from Spanish dry-cured ham may have additional biological functions, such as antioxidant and antihypertensive activities [4, 5].

In high-quality Spanish dry-cured ham, proteolysis does occur, although the typical ultrastructural elements that constitute the sarcomere are conserved [6]. On the other hand, an increased proteolysis could be responsible for the appearance of textural defects (pastiness and softness) [7, 8].

Due to their low stability, the cytosolic endopeptidases calpains and caspases were reported not to be relevant in the processing, whereas cathepsins cause significant proteolysis until the end of the dry-cured ham ripening [9, 10]. Indeed, although cathepsin activities gradually decrease

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through processing [8], a residual activity of cathepsins B, D and L was reported even at the end of curing [11, 12].

A number of studies have been published on the use of non-protein nitrogen (NPN) fraction as an indicator of dry-cured ham proteolysis. More recently, proteomics has been used to study the fate of specific myofibrillar and sarcoplasmic proteins [13–15]. This approach is very useful in identifying molecular markers to predict and discriminate quality characteristics [16].

During processing, myosin heavy chain (MHC) has been recognised as a target of proteolysis, along with myosin light chain and troponin [12–14, 17–19]. On the other hand, the influence of dry-cured ham processing on actin and its changes is still debated [12, 13, 18–23]. Whether the actin isoforms, which are structurally and functionally distinct [24], undergo differential degradation remains to be clarified.

In this work, a combination of immunochemical and mass spectrometry analysis was used to assess the occurrence of proteolytic changes of actin and MHC during processing of San Daniele dry-cured ham. The other two main Italian PDO (protected designation of origin) dry-cured hams Parma and Toscano, clearly differentiated for their sensory characteristics [25], were also taken for comparison.

Materials and methods

Samples

The samples were obtained from *biceps femoris* (BF) muscle of heavy pig thighs, with morphological characteristics in line with the requirements for conformity of the three Italian PDO dry-cured hams: San Daniele (D), Parma (P) and Toscano (T). The animals, crosses of Large White x Landrace of Italian selection (ANAS), were bred on a single farm, slaughtered in the same abattoir in three batches at 6-week intervals, and the thighs were distributed to the three PDO processing plants. The initial thigh weights varied from 13.59 to 14.53 kg, and the subcutaneous fat thickness was from 33 to 36 mm, in line with the three PDO dressing procedures. BF muscle (pH_{24h} 5.63 ± 0.093) was taken from 5-cm-thick slices, which were transversally cut at about 8 cm from the femoral head, and samples for proteomics were cut from a limited (9 cm^2) BF central area. Three samples of raw meat were collected from three thighs within 30 min of slaughtering (T0), immediately frozen in liquid nitrogen and stored at −80 °C until the time of analysis. During maturation, three thighs per each PDO were collected from each of the following processing phases, which were done in accordance with the rules of each PDO consortium: T1: out of salting (day 16, 20 and 23 for D, P and T, respectively); T2: introduction to the resting room (day 35, 62 and 77 for D, P and T, respectively); T3: after washing and drying (day 117, 121 and 136 for D, P and T, respectively); T4: mid-curing, after greasing (day 216, 240 and 269 for D, P and T, respectively); T5: end of curing (day 391, 393 and 384 for D, P and T, respectively). All (45) independent samples (3 × 3 × 5) were stored at −80 °C until the time of analysis.

Preparation of protein samples

For the electrophoretic analyses, extraction of proteins was carried out according to Piñeiro et al. [26] method with minor modifications. Samples of BF (100 mg) at slaughter and at the different processing phases were scraped free of connective and adipose tissue, cut in small pieces on ice and suspended in 1 ml of the extraction buffer containing 60 mM Tris/HCl, pH 7.5, 2 % wt/vol sodium dodecyl sulphate (SDS), 0.1 M DTT and supplemented with antiproteases (protease inhibitor, Sigma-Aldrich). After homogenisation with UltraTurrax® T25 Digital (3 × 30 s), the samples were centrifuged at 5600 g for 4 min at room temperature, and the supernatants were heated to 98 °C, shaken for 30 min and centrifuged as before. Protein concentration was determined by UV absorbance at 280 nm, after sample dilution with an SDS containing solution.

To measure the proteolysis index, total proteins were precipitated by 20 % (wt/vol) trichloroacetic acid, and non-protein nitrogen (NPN) and total nitrogen (TN) were determined according to the Kjeldahl method. The index was expressed as (NPN/TN)x100.

SDS-PAGE, Western blotting and blot overlay assay

Protein extracts were applied to 10 or 13 % SDS-PAGE gels [27] using the Mini 2D (Bio-Rad) electrophoresis equipment. The separated proteins were subsequently stained with NOVEX® Colloidal Blue Staining Kit (Invitrogen) or transferred to nitrocellulose membranes for Western blotting or blot overlay assay using the TE 22 transfer unit (Amhersham Biosciences). When indicated, bands of interest were excised from the gel and rerun in iterative SDS-PAGE gel. At the end of the second electrophoresis, the bands were excised for mass spectrometry analysis or subjected to Western blotting. Anti-myosin heavy chain antibody (anti-MHC) (1:1000) (Sigma-Aldrich), anti-MHC Fast (1:10,000) (Sigma-Aldrich), anti-MHC Slow (1:4000) (Sigma-Aldrich), anti-actin C term antibody (1:200) (Sigma-Aldrich) were suspended in phosphate-buffered saline (PBS) containing 0.1 % Tween 20 (Sigma-Aldrich) and 3 % wt/vol non-fat dry milk (Bio-Rad). Anti-cathepsin D (1:500) (Cell Signalling Technology) was suspended in Tris-buffered

saline (TBS) containing 0.1 % Tween 20 (Sigma-Aldrich) and 5 % (wt/vol) bovine serum albumin (BSA; Sigma-Aldrich) and used to recognise preprocathepsin D (43 kDa), procathepsin D (46 kDa) and cathepsin D heavy chain (28 kDa). Immunoreactive bands were detected by enhanced chemiluminescence (Pierce).

When blot overlay assay was performed [28], samples of San Daniele ham at T1 and T5 were subjected to 13 % SDS-PAGE and the separated proteins were transferred to nitrocellulose sheet to be incubated or not with 1 mg/ml of purified actin (Sigma-Aldrich) in PBS for 2 h at room temperature. Nitrocellulose sheet was washed three times with TBS to eliminate aspecific binding and finally immunodetected with actin C term antibody.

Bands of interest were quantified by densitometry using ImageQuant software (GE Healthcare). The apparent masses of the protein bands were estimated from a calibration curve obtained by plotting the migration distances of high-range standard proteins (Precision Plus Protein™ Standards, Bio-Rad) versus their known molecular masses.

Protein identification by mass spectrometry

Protein gel slices of San Daniele samples were excised and in-gel-digested according to the protocol by Link [29]. Peptide analysis was performed with a Dionex Ultimate 3000 micro-HPLC coupled with the LTQ Orbitrap mass spectrometer equipped with a conventional ESI source. For the chromatography separation, an Acclaim PepMap300 C18 (5 µm, 300 Å, 15 cm × 300 µm) column was used, and the column oven temperature was set to 35 °C; the separation was run for 90 min using a gradient of 99.8/0.2 H_2O/HCOOH (eluent A) and 99.8/0.2 ACN/HCOOH (eluent B) and a flow rate of 4 µL/min. For MS1 scans, the Orbitrap resolution was 30,000 and the selected ion population was 4×10^5, with an m/z window from 250 to 2000. For MS/MS in the LTQ, the selected ion population was 1×10^4 (isolation width of 2 m/z unit). A maximum of four precursor ions (most intense) were selected for activation and subsequent MS/MS analysis. CID was performed at 35 % of the normalised collision energy (NCE) in all cases, and MS/MS spectra were analysed by Xcalibur® software (Thermo Fisher Scientific Inc.). Among all the accessions found by aligning detected peptides in database, actin- and myosin-related proteins were identified and characterised according to their sequence coverage percentage and number of peptides identified.

Statistical analysis

Results are reported as means ± mean deviations (MD). In figures, the MDs are shown with error bars. A general linear model (GLM) for repeated measures was used to compare variables at different times of ham processing both within subject and between subjects (PDO hams). SPSS for Windows software, version 18.0 (SPSS Inc., Chicago), was used to perform all statistical analyses, and statistical significance level was set at $p < 0.05$.

Results and discussion

Proteolysis index and content of cathepsin D in dry-cured ham

Proteolysis was initially followed by evolution of the proteolysis index during the different phases of San Daniele processing. Samples were obtained from the *biceps femoris*, which is an internal muscle less exposed to NaCl penetration, allowing for considerable proteolytic activity [7, 10]. In BF muscle, the proteolysis index was undetectable at slaughter (data not shown) and progressively increased during processing, reaching a percentage value of 25.6 ± 0.30 at the end of ripening (Fig. 1a). These values, as well as those obtained for Parma (28.6 ± 0.99) and Toscano (22.4 ± 1.44), were in the range of values typical of dry-cured ham [30].

In San Daniele ham, immunodetection of cathepsin D content was performed (Fig. 1b), because this protease is potentially involved in skeletal muscle actin degradation, as demonstrated by in vitro incubation of F-actin with cathepsin D [31]. The active form of cathepsin D (28-kDa fragment) was present at slaughter and well detectable until the end of ripening, in accordance with previous reports showing that cathepsin D activity goes on during Teruel dry-cured ham processing [17], resulting in the generation of small peptides [23].

Actin-derived fragments in raw meat and dry-cured ham

Actin proteolysis was first evaluated in raw meat, and the results are shown in Fig. 2a. Coomassie staining of SDS-PAGE gel obtained from protein extracts evidenced the presence of a thick band at apparent molecular weight of 42 kDa attributable to intact actin. Its identity was confirmed by Western blotting with antibody against actin C term, which is the same for all actin isoforms [24]. Two very thin bands at apparent molecular weight of 39 and 35 kDa attributable to two actin proteolytic fragments were also recognised. These two bands were excised, loaded in separate lanes on an iterative SDS-PAGE gel and subjected to Western blotting, which confirmed their reactivity towards anti-actin antibody. Mass spectrometry analysis (Fig. 2b) confirmed the identity of the 42-kDa band as α-skeletal muscle actin. In the 35-kDa band, MS/MS

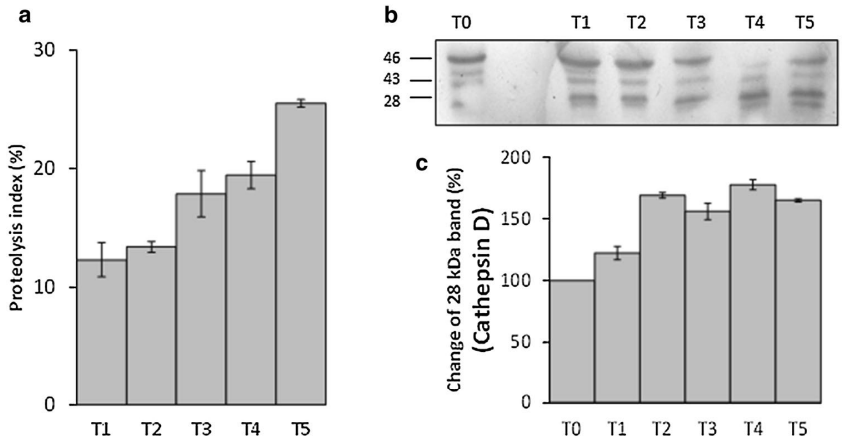
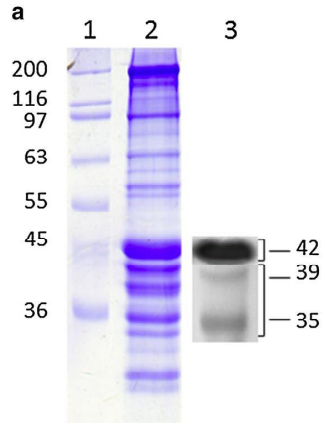


Fig. 1 Proteolysis index and content of cathepsin D of *biceps femoris* muscle during processing of San Daniele dry-cured ham. **a** Proteolysis index increased significantly during processing ($p = 0.007$; GLM for repeated measures). **b** Cathepsin D recognised by Western blotting. Panel shows a representative experiment. **c** Percentage ratio between the active cathepsin D heavy chain (28-kDa) band area and the sum of the 28-, 43- and 46-kDa cathepsin D band areas, relative to the ratio at *T0*, taken as 100 %. Values are mean ± MD (*error bars*) of three samples for each processing phase. An increase over time is shown ($p = 0.007$; GLM for repeated measures). *T1* out of salting (day 16), *T2* introduction to the resting room (day 35), *T3* after washing and drying (day 117), *T4* mid-curing, after greasing (day 216); *T5* end of curing (day 391)



b

SDS PAGE	MS Analysis			
	Actin identification			
Band (kDa)	Identification	% coverage	Uniprot code	# peptides
42	Alpha skeletal muscle actin	51.0	ACTS_PIG	91
35	Alpha skeletal muscle actin	14.3	ACTS_PIG	11

Fig. 2 Actin and actin fragment identification in *biceps femoris* muscle. **a** SDS-PAGE and Western blotting against actin. *Lane 1* molecular weight markers; *lane 2* protein extract bands stained by Coomassie Blue; *lane 3* actin and actin fragments recognised by Western blotting. Panel shows a representative experiment. **b** MS/MS identification of actin and actin fragment. The % coverage and number of peptides identified in the 42- and 35-kDa bands of actin are listed with their UniProt entry name

identified 11 peptides specific to α-skeletal muscle actin, as well as glyceraldehydes-3-phosphate dehydrogenase (G3P_PIG; 39 % coverage) as the more abundant co-migrated protein. In the 39-kDa band, only one actin peptide was detected and attributed to cytoplasmic β-actin (Q95319_PIG; 12.9 % coverage), along with the co-migrated protein creatine-kinase M type (KCRM_PIG; 42 % coverage). As a whole, these results indicate that in raw pork meat suitable for PDO production actin is minimally degraded.

The presence of actin fragments at slaughter, whose levels changed according to breed and age, has already been reported [32, 33]. These fragments produced in *ante*/early *post-mortem* catabolic conditions [34–36], unlike the full-length actin, are particularly sensitive to degradation by

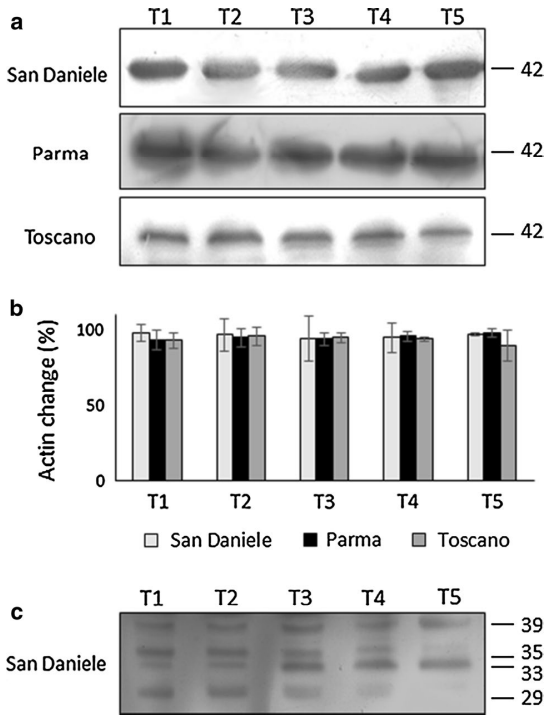


Fig. 3 Actin and actin fragments during processing of Italian PDO dry-cured hams. **a** Actin recognised by Western blotting during processing of San Daniele (D), Parma (P) and Toscano (T). Panel shows a representative experiment. **b** Percentage ratio between the intact actin band area at the different processing phases and band area at *T0*, taken as 100 %. Values are mean ± MD (*error bars*) of three independent samples for each processing phase. No significant difference within group and between groups was obtained ($p = 0.354$ and $p = 0.826$, respectively; GLM for repeated measures). **c** Actin fragments recognised by Western blotting in San Daniele ham. Panel shows a representative experiment. *T1* out of salting (day 16, 20 and 23 for D, P and T, respectively); *T2* introduction to the resting room (day 35, 62 and 77 for D, P and T, respectively); *T3* after washing and drying (day 117, 121 and 136 for D, P and T, respectively); *T4* mid-curing, after greasing (day 216, 240 and 269 for D, P and T, respectively); *T5* end of curing (day 391, 393 and 384 for D, P and T, respectively)

μ-calpain [37], suggesting that they may be degraded in the early *post-mortem*, when this protease is still active.

To analyse the actin fate during ham processing, intact actin content was first evaluated by choosing appropriate conditions for Western blotting to avoid signal saturation, i.e. by loading low quantities of extracts. Interestingly, intact actin content remained rather stable until the end of processing (Fig. 3a, b) and no difference between the three PDOs was observed. In San Daniele dry-cured ham, Western blotting against actin C term recognised only four thin bands attributable to actin fragments, with an apparent molecular weight comprised between 39 and 29 kDa (Fig. 3c). Densitometric analyses (data not shown) of these immune reactive bands at the different phases of processing showed that the relative content of the 35- and 29-kDa fragments decreased slowly over time, although immunoreactive bands were still detectable at the end of ripening. A similar finding of actin fragments was revealed in the other two PDOs (Fig. S1).

In San Daniele, samples collected at T5 MS/MS analyses (Table 1) confirmed the identity of the full actin as α-skeletal muscle actin. Two peptides of the actin-related protein 2-like that contributes to the structural integrity of the cytoskeleton [38] were also identified (B5APU3_PIG; 1.8 % coverage), along with the co-migrating tropomyosin α-chain (TPM 4_PIG; 28.23 % coverage). In the fragment bands, MS/MS identified peptides belonging to the actin family and, in the 39-kDa band, to the co-migrating tropomyosin α-chain isoforms (TPM1/3/4_PIG; 24.6–41.2 % coverage). The 35-kDa fragment was identified by MS/MS as an actin fragment, which showed a very high sequence identity with the cardiac muscle α-actin 1, while the 39-kDa fragment was unambiguously identified by MS/MS as the cardiac muscle α-actin 1. This latter isoform, which is the prominent one in the embryonic muscle tissue, is still present as a minor component (<5 %) in the adult skeletal muscle [39]. Our finding of fragments from the cardiac isoform in ham samples may reflect its higher tendency to be degraded compared to the α-skeletal actin, suggesting a higher susceptibility of the cardiac filaments to

Table 1 Actin and actin fragment identification in San Daniele dry-cured ham

SDS-PAGE	MS analysis			
	Actin identification			
Band (kDa)	Identification	% coverage	UniProt code	# peptides
42	Alpha skeletal muscle action	52.5	ACTS_PIG	24
39	Cardiac muscle alpha actin 1	17.2	B6VNT8_PIG	7
35	Actin (fragment)	13.8	B2ZFN7_PIG	2

MS/MS identification of actin and actin fragments at the end of curing (*T5*: day 391). The % coverage and number of peptides identified in the 42-, 39- and 35-kDa bands are listed with their UniProt entry name

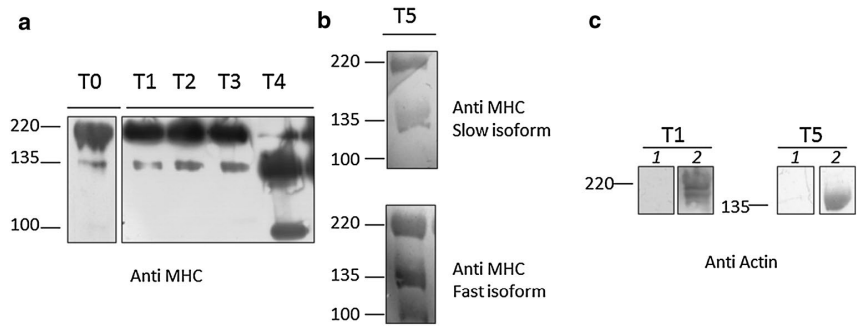


Fig. 4 Myosin heavy chain (MHC) proteolysis and capacity to bind actin in San Daniele dry-cured ham. **a** Total MHC evaluated by Western blotting during processing. **b** Slow or fast MHC at the end of curing (*T5*). **c** Blot overlay assay at *T1* and *T5* of the 220- and 135-kDa MHC bands by Western blotting against actin. Only in *lanes 2* and *4*, which were pre-incubated with purified actin, actin was immunodetected in the 220- and 135-kDa bands, respectively. In non-pre-incubated *lanes 1* and *3*, actin was not revealed. Panels show representative experiments. See legend of Fig. 3 for processing phase description

dismantling, that is consistent with their established lower structural stability [40]. In both 33- and 29-kDa bands, only one peptide was detected and attributed to cytoplasmic β-actin (Q95319_PIG; 12.9 % coverage), which is present in low amounts in skeletal muscle, where it plays a wide range of functions [41].

As a whole, our data indicated that, in spite of a high proteolysis index and the presence of active cathepsin D until the final processing phase (Fig. 1b), the α-skeletal actin does not seem to undergo degradation during dry-cured ham processing, since no identified actin fragment is from the skeletal isoform. It should be remembered that actin degradation and formation of actin fragments have been found to be associated with quality defects, such as the pastiness, of dry-cured ham from Slovenia [18], whereas actin was not hydrolysed in high-quality Spanish Serrano and French Bayonne dry-cured hams [12, 22].

The degradation resistance of F-actin in dry-cured ham may be explained by the existence of a steric hindrance to the access of the still-active proteases [9, 10], possibly mediated by the interaction with the co-migrating tropomyosin or with other proteins, which could remain associated with F-actin even at high ionic strength [42–44]. A lower solvent accessibility of F-actin rather than MHC at the actin–myosin interface, as revealed by site-directed spectroscopy [45], may also limit protease access.

Myosin heavy chain (MHC)-derived fragments in dry-cured ham

In addition to actin, the degradation pattern of MHC was analysed in protein extracts of raw meat and San Daniele ham during the different phases of processing (Fig. 4). In raw meat, immune detection after SDS-PAGE separation with anti-MHC antibodies, which are able to recognise both slow and fast MHC isoforms, showed the presence of a very thick band of intact MHC at apparent molecular weight of 220 kDa, along with a very thin band at apparent molecular weight of 135 kDa attributable to an MHC fragment. The occurrence of MHC degradation in the early *post-mortem* has been recently observed in beef [46], although the underlying mechanisms need to be investigated. No change of these bands was observed until the mid-curing (T4), when the MHC band was markedly reduced, while the 135-kDa band was noticeably increased and a new band of apparent molecular weight of 100 kDa appeared, indicating that, differently than actin, MHC is largely degraded during San Daniele dry-curing process, in accordance with previous reports [13]. As expected, a remarkable degradation of MHC was also observed in the other two PDOs (Fig. S1).

In San Daniele ham, Western blotting with antibodies against the slow and fast isoforms of MHC showed that at the end of ripening (T5) the slow MHC isoform was predominant in the intact 220-kDa band, less abundant in the 135-kDa band and not detectable in the 100-kDa band, while the fast MHC isoform was well detectable in all the three MHC bands, indicating that only the 135-kDa fragment is a degradation product of both types of MHC isoforms. The two fragments and the intact MHC were further analysed to establish their capacity to bind actin. They were immobilised on a membrane and probed with purified G-actin. After this recognition, Western blotting was normally carried out with anti-actin C term antibody, which recognised the presence of actin at the level of the 135-kDa fragment (Fig. 4b), but not of the 100-kDa fragment (data not shown). As expected, also the intact MHC was found to bind G-actin (Fig. 4b).

To confirm the identity of the MHC and its fragments, a proteomic approach, as already described before, was applied. Peptides identified in the 220-, 135- and

100-kDa bands fully belonged to the myosin family (data not shown). As far as the 135-kDa band is concerned, peptides were found to belong mainly to the C term of both fast and slow MHC isoforms (Fig. S1), strongly suggesting that this fragment was formed by the release of the N term catalytic head of MHC. The high number of peptides identified with a very high score indicates that this fragment is quite abundant, suggesting an extensive myosin degradation. Based on the identified peptides, EXPASY software predicted for the MHC fragment a molecular mass of 135 kDa comprising the residues 769–1937, which is consistent with the electrophoretic migration data.

The 135-kDa fragment strongly resembles the one found by in vitro incubation of native myosin with muscle-specific cathepsin B and/or D [47], with the latter being well detected until the end of the ripening of San Daniele (Fig. 1b, c). Interestingly, at least 5 potential cleavage sites for cathepsin B [48] and two for cathepsin D [49] are present just above Ala 769, the first identified residue of the 135-kDa fragment.

Despite the observed interaction between the 135-kDa fragment and actin by the blot overlay assay, neither the MS/MS recognised peptides in the 135-kDa band, nor the whole predicted 769–1937 sequence comprised the actin-binding sites located in the catalytic head [50, 51]. Non-physiological conditions have been reported to induce unpredictable interactions between actin and myosin fragments revealed by in vitro motility assay [52], making plausible that the 135-kDa fragment interacts with actin through non-native interactions. Such interactions may protect F-actin against denaturation by overcoming the destabilising effect of high salt concentration, as shown for meromyosin [53], suggesting another possible explanation for the lack of actin proteolysis in dry-cured processing.

Conclusions

Raw meat displays low levels of actin and MHC fragmentation, which is related to the muscle state *ante*/early *post-mortem*, consistent with limited protein catabolism. Dry-cured hams produced in line with the requirements for conformity with the specifications of the three Italian PDOs show a common proteolytic pattern on the actomyosin complex, characterised by an extensive degradation of only MHC, but not of actin. Since actin may be stabilised at high ionic strength by interaction with other proteins and possibly their fragments, we speculate that PDO dry-cured ham processing may favour those conditions which could limit protease access, resulting in actin resistance. More precisely, during ham ripening, actin gives rise to a limited number of fragments that are not produced by cleavage of α-skeletal actin. Thus, different actin isoforms do not undergo the same changes during dry-cured ham processing. It is well known that sensory qualities, including texture, benefit when proteolysis is not excessive. α-skeletal actin could be a useful marker of integrity of the sarcomeric structure, which confers the typical texture of dry-cured ham. Our characterisation of fragments from other actin isoforms, each of which performs a specific function, should encourage future investigations of their changes during the ripening process.

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Compliance with ethical standards

This article does not contain any studies with human or living animal subjects.

Conflict of interest None.

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