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TESI DI DOTTORATO DI RICERCA

CASPASE AND CALPAIN ACTIVITY IN BEEF TENDERIZATION IN TWO BOVINE SKELETAL MUSCLES

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Summary

The tenderness of beef is one of the most important factors that can influence consumer choices. Among the factors that affect tenderness, post mortem proteolysis has a key role. Calpains and capsases are two families of cytosolic proteases essential for a proper skeletal muscle function and are consider having a key role in the post mortem tenderization process. The goal of this work was to evaluate the activities of μ-calpain and caspase-3 in two different muscle types, *Longissimus Dorsi (*LD) and *Infraspinatus* muscle (IS), of Simmental young bulls at slaughter and during ageing, and their relationship with the beef tenderness. Samples of *Longissimus dorsi* and *Infraspinatus* muscles were collected within 20 minutes (T0), 48h (T48h) and 7 days (T7) from slaughtering of 17 Italian Simmental young bulls, and stored at -80 °C until the analysis. By SDS-PAGE, alpha II spectrin, PARP-1 and their degradation products, and fast and slow myosin heavy chain (MHC) were separated and then quantified in relative terms. In particular, the activity of μ-calpain and caspase-3 was assessed by the quantification of alpha II spectrin cleavage products of 145 kDa (SBDP145) and of 120 kDa (SBDP120) respectively. Moreover the activity of caspase-3 was assessed also by the quantification of poly (ADP-ribose) polymerase-1 (PARP-1) cleavage products of 89 kDa. Warner-Bratzler Share Force (WBSF) analysis was carried out after 7 days of ageing. The analysis of MHC isoforms revealed that LD was mainly composed by fast-MHC (67%) and consequently can be considered a white muscle, while IS was mainly composed by slow-MHC (84%) and can be considered a red muscle. IS showed higher WBSF than LD ($P<0.01$). At slaughter, the alpha II spectrin full length 250 kDa (SBDP250) level was higher in IS than LD ($P<0.01$), it means that, at slaughter, alpha II spectrin was less degraded in IS than LD, also a positive correlation between SBDP250 and WBSF was found ($r=0.810$; $P<0.01$), indicating that a different early proteolysis between muscles may contribute to explain their different tenderness. LD showed higher level of SBDP145 than IS ($P<0.01$), while SBDP120 was similar between

muscles ($P>0.05$). Also the 89 kDa from PARP-1 degradation was found. At T48h SBDP250 and PARP-1 cleavage product were not detected and no differences were found between muscles in the level of SBDP145 and SBDP120. However the level of SBDP145 was higher than those of SBDP120 in both muscles. After 7 days of ageing neither alpha II spectrin nor PARP-1 cleavage products were found. Taking into account the results of this study, the different tenderness observed between LD and IS muscles was greater influenced by μ-calpain than caspase-3 activity. Indeed the activity of μ-calpain was higher than those of caspase-3 in both muscles and detectable at 48h of ageing. Conversely caspase-3 was similar between muscles and seems active only in the early post mortem.

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1. The mechanism of tenderization

Meat quality is a generic term used to describe properties and perceptions of meat and generally comprised colour, flavour, texture, tenderness, and juiciness. The mechanisms responsible for the development of these meat qualities are interdependent, highlighting the complexity of the conversion of muscle into meat. Tenderness of meat is related to breakdown of muscle fibres, predominantly due to the activity of endogenous enzymes, and loosening of connective tissue, specifically collagen.

1.1 Muscle anatomy and composition

Whole muscle is attached to bone by a tendon, nearly inextensible connective tissue layer called the epimysium. This layer is composed primarily of the protein collagen. The muscle is divided into bundles by thinner layers called perimysium and is composed of fibre bundles (Rogers, 2001). Bundles are an aggregation of fibres, the latter representing the muscle cells. The fibres are between 20 and 100 µm in diameter and vary in length from a few millimetres up to several centimetres. The cells are surrounded by a connective tissue sheath, the endomysium, and the cell membrane, the sarcolemma (fig. 1). Within the cell, about a thousand myofibrils are arranged in the direction parallel to the long axis of the cell. The myofibrils have a diameter of 1–2 µm and run the whole length of the muscle fibers which have alternating stripes.

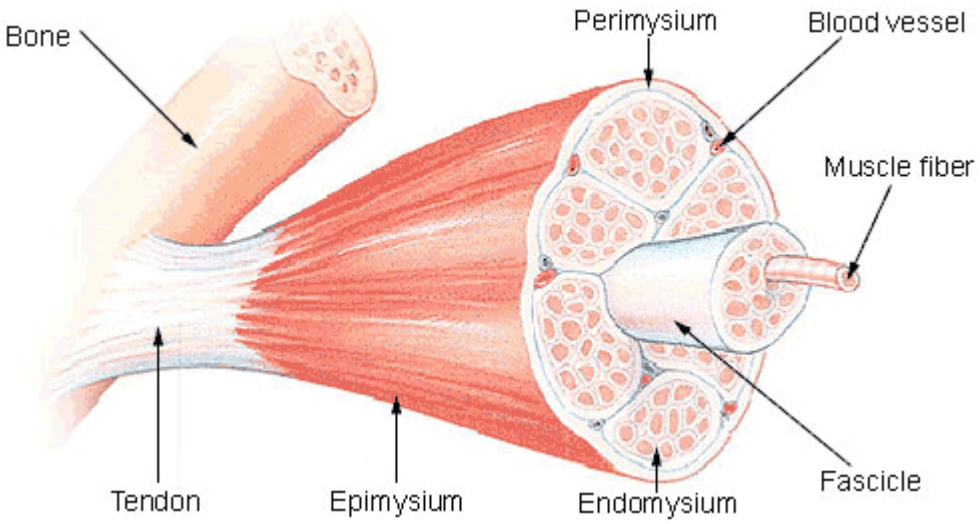


Figure 1 Muscle anatomy and composition modificatied from Tank P. W. & Gest T. R. (2008).

The striations in the fibers occur because the adjacent myofibrils have their respective light and dark bands aligned (fig**.** 2). The dark bands are called the A-bands and the light bands the I-bands. A thin perpendicular line referred to as the Z line bisects the I-bands. The region between successive Z lines is called sarcomere and it is the smallest functional unit of the myofibril.

The fibrils are composed of thick and thin filaments. Thick filaments consist mainly of myosin, a protein with a molecular weight of about 520 kDa and amounting to about 45% of the myofibrillar proteins. Thin filaments are composed of actin (20%), troponin and tropomyosin (5% each). Titin, a giant protein that extends from the middle of the sarcomere to the Z line, is elastic and important for myofibril assembly and for protecting muscle from overstretch. Nebulin is attached to the Z lines and is request to regulate the length of the thin filaments (fig. 2). These latter two proteins are referred to as cytoskeletal proteins (Rogers, 2001; Lawrie, 2006).

The sarcomere and its filaments are highly arranged in cross-section as well as longitudinally. The thick filaments are arranged in a hexagonal pattern. Six thin filaments

surround each thick filament, and each thin filament is centered between three thick filaments.

The length of sarcomeres varies during muscle contraction, as specified just below. The A-bands always have the same length, while the I-bands decrease in length in myofibrils with shorter sarcomeres. Myofibrils with long sarcomeres have a zone in their middle that has somewhat lower intensity; this region is called the H zone (Rogers, 2001).

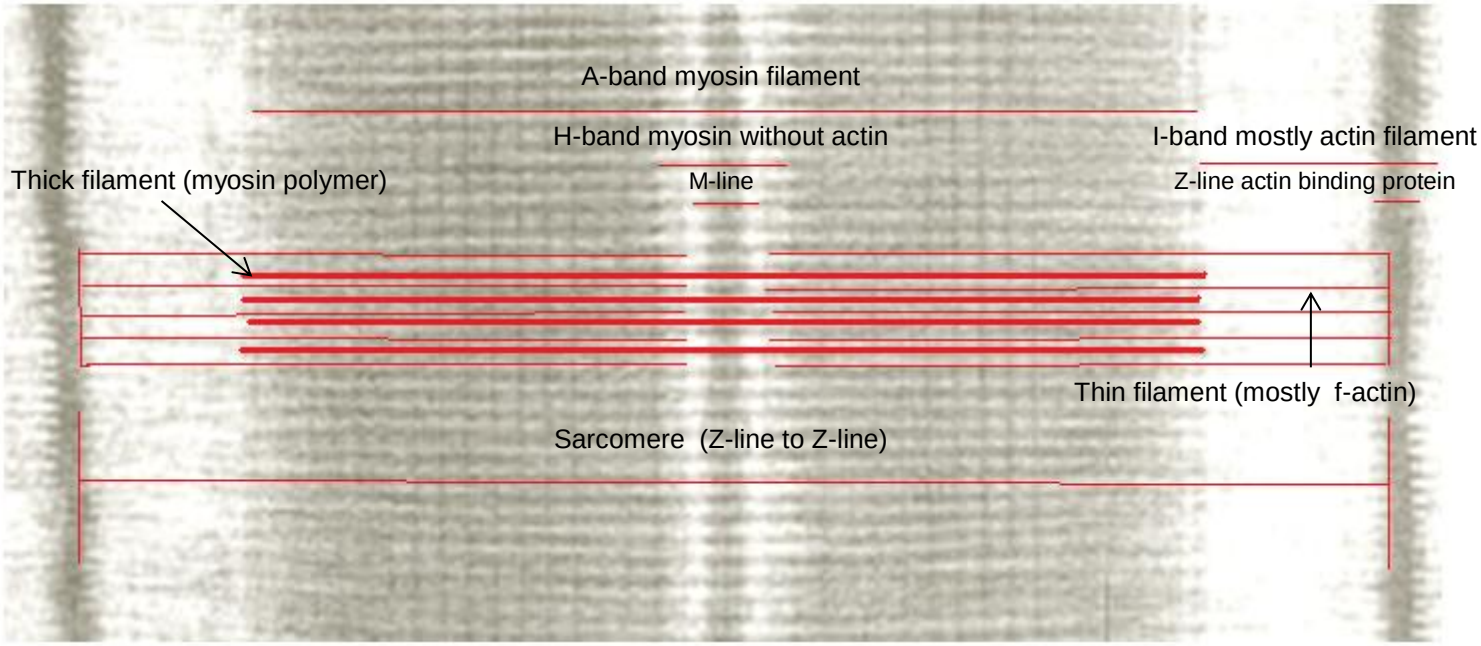


Figure 2 Electron microscope image of a sarcomere. A schematic representation of the myosin and actin filaments and the A and I-bands is reported modificatied from (Picouet et al., 2012).

1.2 Muscle contraction

As long as an animal is alive, muscle contraction takes place when thick and thin filaments slide into each other, thus shortening the length of the sarcomere, which is 2–3 µm long in the resting state of a muscle. One sarcomere can shorten by about

0.7 μm (Honikel, 2001).The thick filaments (about 1.6 μm) in the A-band intertwine with thinner filaments (about 1 μm) that attach to the Z lines (fig.3).

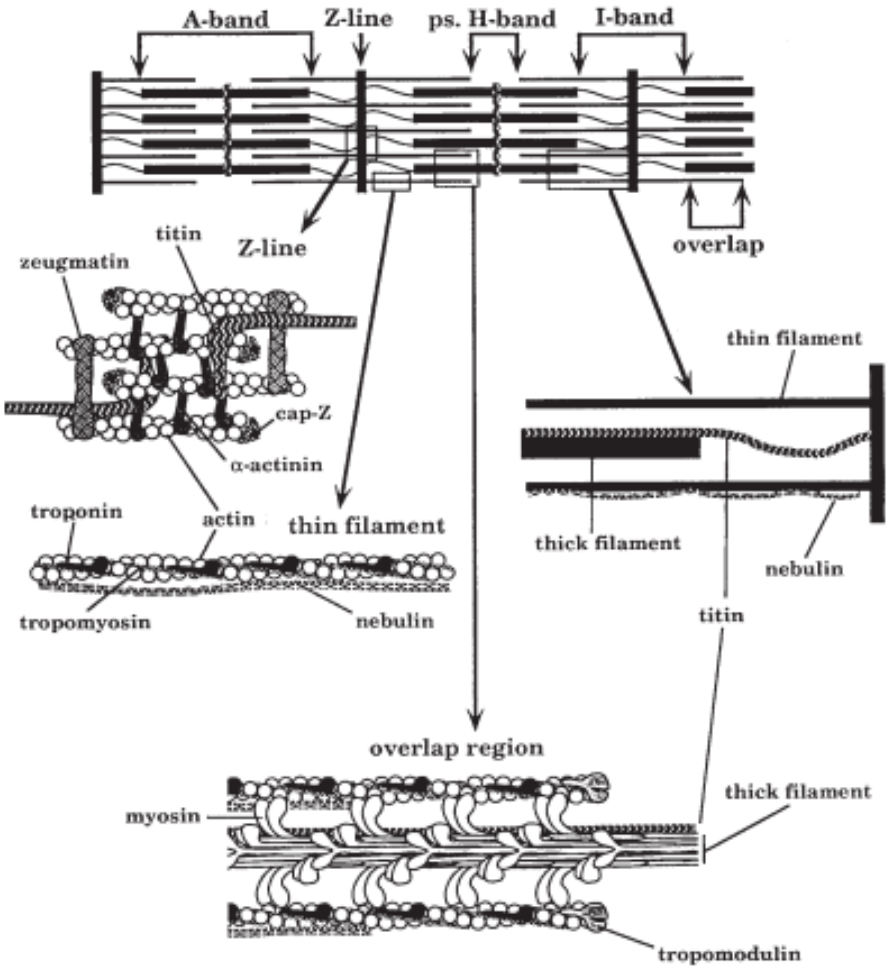


Figure 3 Sarcomeric and cytoskeletal proteins (Lawrie, 2006).

Initially, whole muscles were classified as being fast or slow based on speeds of shortening. This division also corresponded to a morphological difference, with the fast muscles appearing white and the slow muscles appearing red. The redness is due to the high amount of myoglobin and the high content of capillary, which contribute to the oxidative ATP production in the abundant mitochondria (which have abundant cristae). The white muscles have instead an energy metabolism that depends

on glycolysis, being poor in mitochondria (and these have few cristae). In general, the width of the Z-lines in white muscles is about half that in ‘red’ ones.

Histological analyses later showed that there is a correlation between myosin ATPase activity and the speed of muscle shortening, leading to the original division of muscle fibers into type I (slow) and type II (fast). Currently, muscle fibers are typed using three different methods: histochemical staining for myosin ATPase reaction, myosin heavy chain isoform identification, and biochemical identification of metabolic enzymes, which are applicable to humans as well as to animals.

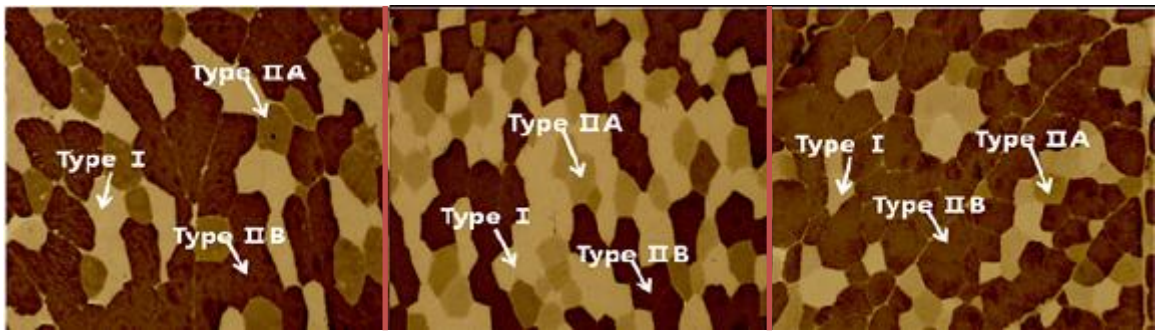
Different myosin ATPase histochemical identification methods for muscle fiber types have been introduced over the years. The first commonly accepted form of classification in muscle biology was based on differences in the acid stability of the myosin ATPase reaction, which distinguished the fibers in type I (high reactivity), IIa (very low) and IIb (intermediate) (Brooke & Kaiser, 1970). Later, 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 A. & Kernell D., 1991). Further advances in the histochemical staining technique have led to recognize 7 muscle fiber types in humans (Wayne , Stevens & Binder-Macleod, 2001).

Another classification scheme has based on a qualitative histochemistry for certain enzymes that reflect the energy metabolism of the fiber, among which the most frequently used have been the succinate (SDH) and the lactate (LDH) dehydrogenase as marker of the aerobic and anaerobic metabolism, respectively. 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 varied. As an example, Lind & Kernell (1991) found that the mean intensity of SDH staining per fiber type was ranked such that IIA greater than I greater than IIB subgroups.

The type classification system based on immunodetection of myosin heavy chain (MHC) isoforms has instead resulted well 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 are pure fibers containing a single MHC isoform and the majority of fibers contain more than one MHC isoforms. 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) (fig. 4).



A B C

Figure 4 Serial sections of 3 major muscles showing myosin isoforms; A: Longissimus dorsi; B: Psoas major; C: Semimembranosus (Hwang, Kim, Jeong, Hur, & Joo, 2010).

Muscles contract in response to a nerve stimulus, that is transmitted into the muscle cell via t tubules (Gerhard Feiner, 2006). The t tubules are attached to a specialized intracellular membrane system, the sarcoplasmic reticulum. The attachment involves a protein called the ryanodine receptor (whose protein name is due to its affinity for the plant alkaloid ryanodine). When sarcolemma depolarization reaches the t tubule–sarcoplasmic reticulum junction, the ryanodine receptor 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 shape change in the troponin C; this 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 a small distance (about 10 nm). The myosin head then releases 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 increase to 10^{-4} M during contraction. A single muscle contraction is called a twitch, and it only requires about 200 milliseconds to complete (Rogers, 2001).

Contraction uses adenosine triphosphate (ATP) as fuel (Honikel, 2001; Rogers, 2001). Energy in the form of ATP is required to bind myosin into actin as well as to separate myosin from actin afterwards. 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 found in the outer cell membrane. It requires ATP to move Na^{+} and K^{+} against their concentration gradients (Fig.5).

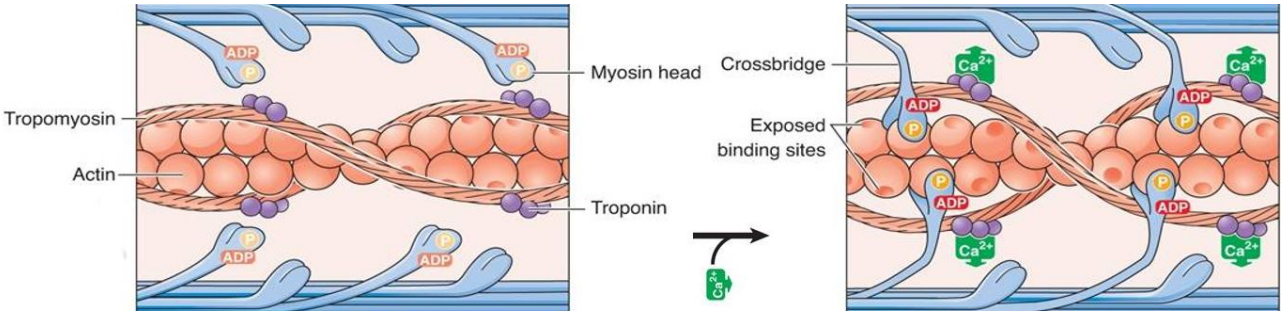


Figure 5 Model of contraction mechanism modified from Tank & Gest (2008).

1.3 Post-slaughter changes in muscle tissue

Once the animal is slaughtered, since respiration ceases, no further oxygen enters the body and the cessation of the blood supply means that there is no longer supply of glucose or fatty acids from the blood- stream. Muscular glycogen, present at slaughter in muscle tissue, is converted anaerobically after slaughter into pyruvate, as this step occurs anaerobically regardless of whether the animal is alive or dead. The major difference between aerobic and anaerobic glycolysis is that the pyruvate obtained from glucose is not converted into acetyl CoA and the citric acid and oxidative phosphorylation don't take place. Instead, pyruvate is reduced predominantly to lactic acid (lactate and H+) under anaerobic circumstances and this is catalyzed by the cytosolic enzyme lactate dehydrogenase. The lactic acid formed is not transport

ed back to the liver, as happens whilst the animal is still alive, and therefore the concentration of lactic acid within muscle tissue increases steadily after slaughter and the pH of muscle tissue declines. Only two molecules of ATP are obtained from one molecule of glucose during post mortem glycolysis, compared with 36 molecules of ATP during aerobic glycolysis.

The effects of the two molecules of ATP obtained in an anaerobic state can be observed on the carcass as there is visible fibre movement even though the animal is already dead. In living muscle, the level of ATP is around 5 mmol per gram of muscle tissue but levels as low as 1.5 mmol per gram of muscle tissue are sufficient for single cycles of muscle contraction and relaxation. The ATP content remains unchanged for a short time after slaughter as ATP is rebuilt anaerobically by creatine phosphate binding to ADP to generate ATP. After a certain period of time (in pork after about 2–4 h, in chickens 1–2 h and in cattle 4–8 h from slaughter), the concentration of ATP in muscle tissue drops below 1 mmol per gram of muscle tissue.

At this point, meat exhibits a pH value of around 6.0 (Honikel, 2001) and actin and myosin do not dissociate any longer and instead remain bound together to form the actomyosin complex, typical of rigor state. Conversely the sarcoplasmic reticulum Ca^{2+} pump and the cell membrane Na^{+}/K^{+} pump continue to function to move their respective ions against the concentration gradients (Rogers, 2001).

During contraction in living animals the myofibrillar space decreases and a part of the water in the fibrils is reversibly traslocated into the sarcoplasmic space. In contrast, in post mortem, the displacement of water from the myofilaments permanently increases the amount of sarcoplasmic water. The effect of displacement is further enhanced after death as the pH falls from 7.0 to a value around 5.5, causing shrinkage of my

ofilaments, but the still intact cellular membrane keeps the water as intracellular water for some time. With ongoing ageing, the water passes through the membrane into the extracellular space collecting between the cells.

In a muscle of al living animal at around pH 7, more than 95% of the water is within cells; some days post mortem, about 15% is in the extracellular space. From these capillary-like channels, the water appears as drip at the surface of the meat (Honikel, 2001). Rigor mortis (latin: rigor "stiffness", mortis "of death") in beef is complete after around 24–40 h. Upon its completion, the pH value has dropped to around 5.3, and actin and myosin are present as the actomyosin complex (Gerhard Feiner, 2006), this causing the limbs of the carcass which become stiff, difficult to move and to manipulate.

1.4 *Post mortem* proteolysis

The conversion of muscle into meat is assumed to occur through three steps: the pre-rigor step which is not well defined yet, the rigor step and the tenderizing 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. Evidence suggests that proteolysis of myofibrillar and associated proteins are the cause of meat tenderization. These latter are involved in inter- (e.g., desmin and vinculin) and intra-myofibril (e.g., titin, nebulin, and possibly troponin-T) linkages or linkage of myofibrils to the sarcolemma by costameres (e.g., vinculin, dystrophin), or the attachment of muscle cells to the basal lamina (e.g., laminin, fibronectin and the newly described 550 kDa protein). Their function is in fact to maintain the structural integrity of myofibrils and their degradation would, therefore, cause weakening of myofibrils by protease action and, thus, tenderization (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.

A protease must meet certain criteria to be considered a possible candidate for involvement in *post mortem* tenderization. The logic for the first criteria is that the protease must be endogenous to skeletal muscle cell. Secondly, the protease must have the ability to reproduce *post mortem* changes in myofibrils in an *in vitro* setting under optimum conditions. Finally, the protease must have access to myofibrils in tissue. If a protease does not have these characteristics, it cannot be considered as a candidate in the *post mortem* tenderization process. Likewise, if a protease meets these criteria, it would be impossible to exclude its involvement in the tenderization process.

Calpains (Ca^{2+}-activated proteases) are the proteases that meet all of the above requirements and are considered to have a main role in meat tenderization. Calpains, localized in the sarcoplasm, are known to work more effectively at higher pH values in meat, such as 6.2–7.0. This led to the assumption that calpains are more important during the early stage of *post mortem* glycolysis (Paredi, Raboni, Bendixen, de Almeida, & Mozzarelli, 2012; Rogers, 2001), together with caspases, another class of neutral proteases which mediate cell apoptosis in living animals and are inactivated by acidic pH (Ouali et al., 2006). Conversely, cathepsins, which are acidic proteases, prefer a lower pH value around 5.4–5.9 and this led to conclude that cathepsins act later in the process.

1. 4.1 Calpains

Calpains are probably the most extensively studied protease family with regard to meat science and is widely accepted that calpains contribute to meat tenderization cutting the long fibres 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, ubiquitously expressed μ-calpain, m-calpain and 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, as further discussed below. 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).

Calpains are composed of two subunits, the large subunit has a molecular mass of 80 kDa in m-calpains and is slightly larger in μ-calpains, while the molecular mass of the small subunit is of 28 kDa in all. The catalytic site is located in Domain II of the large subunit and contains the catalytic Cys residue at position 115 (μ-calpain) or 105 (m-calpain), a His residue at position 272 (μ-calpain) or 262 (m-calpain) and an Asn residue at position 296 (μ-calpain) or 286 (m-calpain), but, it has only marginal sequence homology to papain 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 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 the calpains are inhibited by being bound to the endogenous protein calpastatin. Ca^{2+} removes this inhibition and calpastatin is itself eventually broken down by the 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). In fact, 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), and in bovine and ovine *post mortem* muscle, the extractable activity of μ -calpain declines but the activity of m-calpain is remarkably stable (Geesink & Koohmaraie, 1999; Kretchmar et al., 1990, Veiseth et al., 2001).

1. 4.2 Cathepsins

Cathepsins are a class of over 30 different enzymes, 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, these latter constituting the largest cathepsin family. In skeletal muscle they operate the quality control of proteins that is particularly active due to the metabolic, mechanical, and thermal stressors arising contraction. Impairment of the cathepsins system leads to accumulation of unfolded/misfolded proteins and altered organelles which turns into toxicity for the muscle cells. Conversely, excessive activation of proteolytic machinery contributes to muscle loss, weakness, and finally to death (Bechet, Tassa, Taillandier, Combaret, & Attaix, 2005).

During rigor mortis, as a result of the formation of lactic acid and therefore the decrease in pH value, the walls of lysosomes are destroyed by the impact of lactic acid, and cathepsins are released (Cheret, Delbarreladrat, Lamballerieanton, & Verrezbagnis, 2007). This fact and their capacity to degradate the actomyosin complex *in vitro* (Gerhard Feiner, 2006), suggested that cathepsins can greatly contribute to meat tenderness. In accordance, higher level of cathepsins was found in pork and chicken that 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 and the level of cathepsins is low (Gerhard Feiner, 2006).

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) (fig.6); 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; Earnshaw, Martins, & Kaufmann, 1999; C M Kemp & Parr, 2008; Murray et al., 2008; Salvesen, 2002).

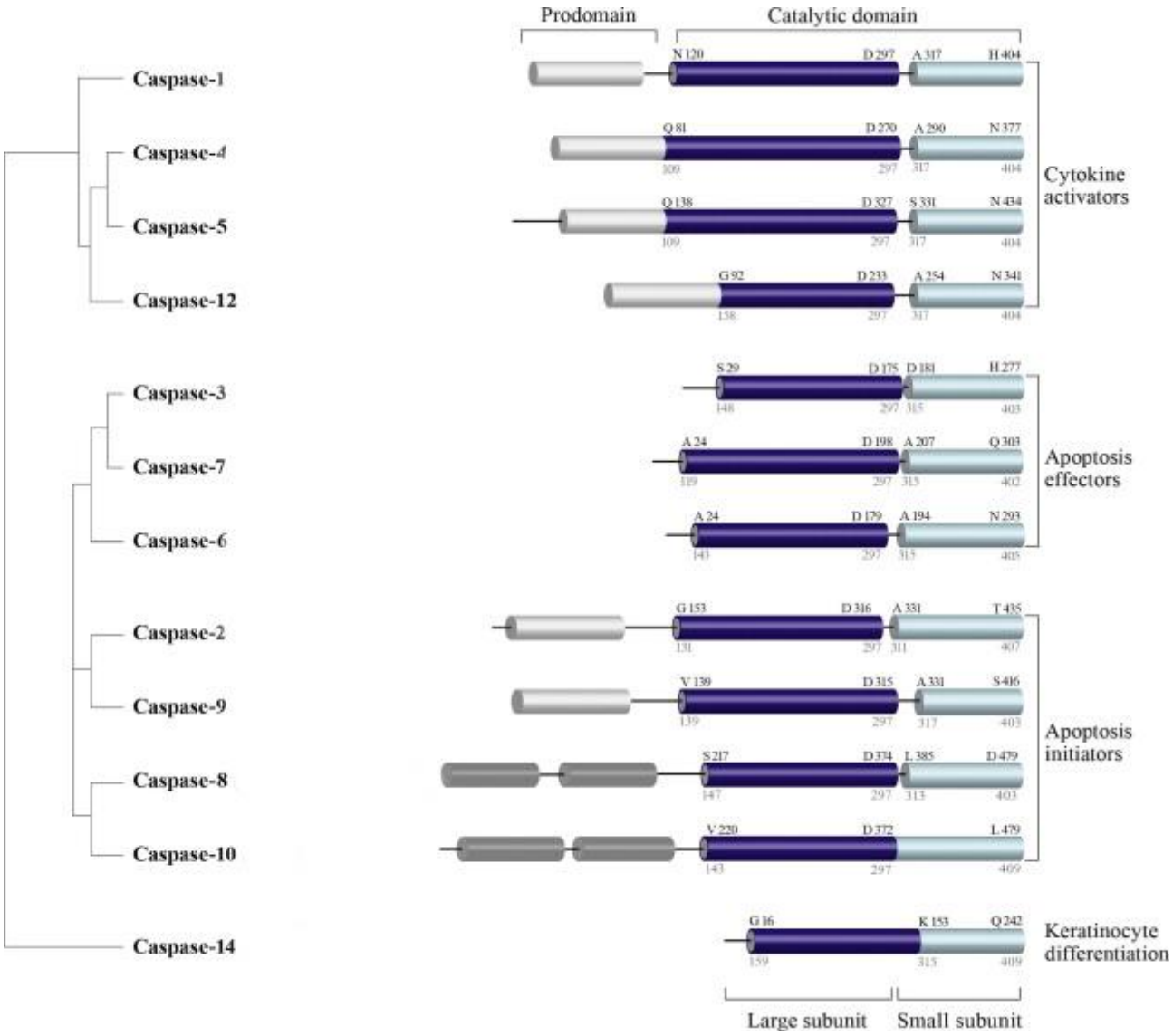


Figure 6 Phylogenetic tree of the caspase family. The tree is organized based on the sequence homology and substrate specificity (Roschitzki-Voser et al., 2012).

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 et al., 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, Bardsley, & Parr, 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. Cell death induce a series of biochemical and structural changes in dying cells which likely occur in *post mortem* muscle, before the development of rigor mortis (Herrera-Mendez et al., 2006). Caspases are specialized in cell destructuration during apoptosis and a model has been proposed they could first 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 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 that they cleave. Caspase 12 can be activated by proteolytic cleavage by calpains in response to ~~a~~ disruption in the Ca^{2+} homeostasis in the sarcoplasmic reticulum (SE).

The amount of Ca^{2+} required for this cleavage was found to be at millimolar 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 degradate 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 callipyge lambs, which are characterized by a much higher muscle weight (Kemp, King, Shackelford, Wheeler, & Koohmaraie, 2009). Interpretation of this negative relationship was that whilst caspase 3/7 activity may contribute to the decrease in calpastatin in normal lambs, in callipyge animals the calpastatin content is overwhelming, and caspase activity is simply not sufficient enough to overcome it. Consequently, in the callipyge phenotype calpastatin activity is the over-riding factor in reducing *post mortem* proteolysis.

Intriguingly, caspase activity has been also found in post-rigor period. In particular 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. A similar study has not been performed in beef, yet (Underwood, Means, & Du, 2008).

1. 4.4 PARP-1

Poly (ADP-ribose) polymerase 1 (PARP-1), a nuclear enzyme of 113 kDa, is one of the major substrates of the executor caspase-3, which cleaves PARP-1 into 25- and 89-kD fragments thus destroying its ability to bind to DNA (Boulares, 1999). PARP-1 is the canonical representative of PARP super family consisting of 17 members and

binds preferentially at single-strand DNA breaks, symmetrically covering 7-8 nucleotide residues on each side of the break and inducing a V-shaped sharp bend with the enzyme located at the kink. The enzyme is formed by two distinct regions: an amino terminal DNA-binding domain containing two unusually long zinc fingers of 28-30 residues and a nuclear localization sequence (Lindahl, Satoh, Poider, & Kiungland, 1995), and a carboxy-terminal catalytic domain (fig.7).

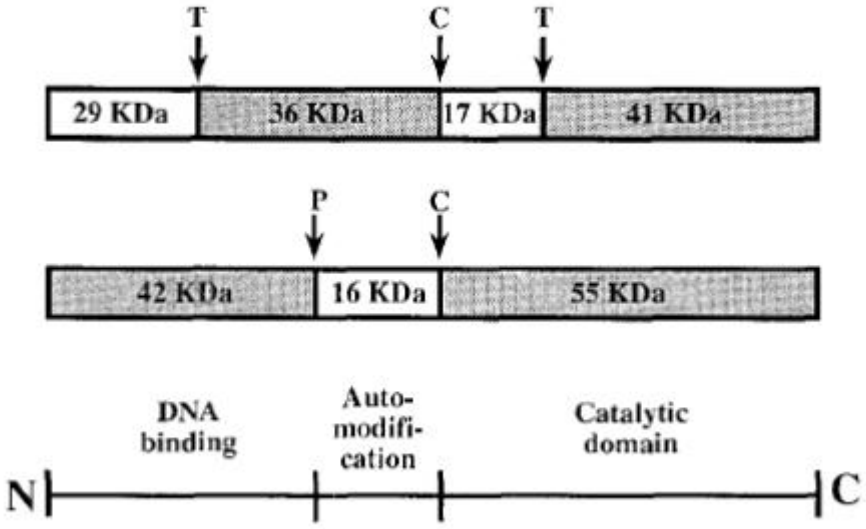


Figure 7 Proteolytic domains of poly (ADP-ribose) polymerase. Letters indicate the cleavage sites of proteases. P, papain; T, trypsin ; C, α-Chymotryspin (Lautier, Lagueux, Thibodeau, & Poirier, 1993)

Once PARP-1 is bound to DNA, the catalytic domain starts to catalyze transfer of ADP-ribose groups from NAD^+ to a variety of nuclear proteins, including histones as well as PARP-1 itself, activating signals for the other DNA-repairing enzymes such as DNA ligase III, DNA polymerase beta (polβ), and scaffolding proteins.

PARP-1 can be activated within minutes by single-stand DNA damage, but poly(ADP-rlbose) synthesis in normal healthy cells Is very low, and after DNA repairing, the PARP-1 chains are rapidly removed by Poly(ADP-ribose) glycohydrolase.

Conversely, during cellular stresses extreme activation of PARP-1 may lead to cell death due to depletion of ATP used to re-synthasize NAD^+. PARP-1 cleavage is an universal phenomenon observed at early phase of apoptosis induced by a variety of apoptotic stimuli and is a critical event for cell to ensure normal apoptosis by preventing NAD^+ and ATP depletion and thereby to inhibit unwanted necrosis and pathological inflammatory response (Herceg & Wang, 1999).

Amino-terminal sequencing of the purified 85 kDa PARP-1fragment (that contains the active site) revealed that cleavage by capsase-3 occurs between Asp216 and Gly217, a site that is conserved in human, bovine and chicken PARP-1, (Boulares, 1999; Le Rhun, Kirkland, & Shah, 1998). However, PARP-1 is also partially degraded in necrotic conditions to a 40 kDa fragment by μ-calpain activity.

1.4.5 Alpha II Spectrin

The cytoskeletal spectrin is an elongated actin-cross linking protein composed of α and β subunits arranged in a tetramer (Bennett, Baines, Lecomte, Maggs, & Pinder, 2004; Bignone & Baines, 2003; Dubreuil, 2006). In mammals, there are two alpha (alpha I spectrin and alpha II spectrin) and five beta (beta I-IV spectrin) genes. Most tissues, including heart, have abundant alpha II, while alpha I is expressed principally in erythrocytes (Bennett et al., 2004). Spectrin is essential for muscle integrity, ensuring muscle cell adhesions and the resilience of both sarcomeres and sarcolpasmaics reticulum to the stress of contraction (Bennett et al., 2004). In skeletal myocites spectrin is localized at sarcomeric Z- disk and I-band, but is concentrated in hexagonal arrays on the cytoplasmatic face of costameres, which connect the extracellular matrix to the internal cytoskeleton: the function of spectrin in these complexes is to stabilize cell adhesions and transmit force from the contractile apparatus to the ex

ternal substratum. In costameres, spectrin molecules interact also with ankyrin, which attaches the spectrin scaffold to a host of different membrane transporters such as Na^+/K^+-ATP ase and Na^+/Ca^{2+}- exchangers (Bennett et al., 2004). This interaction has an important role in modulating the behavior of interacting membrane transporters and is generally conserved among most isoforms (Dubreuil, 2006), e.g. in erythrocytes ankyrin connects spectrin to the anion exchanger band 3. Alpha II spectrinis a major substrate for cystein proteases involved in necrotic (calpain) and apoptotic (caspase-3) cell death. There is considerable evidence that alpha II spectrin is processed to cleavage products also known as spectrin breakdown products (SBDP).

µ-calpain produces a SBDP of 150 kDa (SBDP150) by cleavage between Tyr 1176 and Gly 1177, and subsequently a SBDP of 145 kDa (SBDP145). In addition alpha II spectrinis cleaved to a major cleavage product of 120 kDa (SBDP120) by caspase-3, between Asp 1478 and Ser 1479 (Chen, Feng, Zhang, Xu, & Zhou, 2012; Czogalla & Sikorski, 2005; Herceg & Wang, 1999; Pike et al., 2004; Wang, 2000; Weiss et al., 2009).

2. Thesis objectives

Meat tenderization is considered a multienzymatic process involving not only, the well studied systems of calpains, but also caspases, whose functions in postmortem muscle are less clear (Herrera-Mendez et al., 2006, Nowak et al., 2011; Kemp and Parr, 2008; Cao et al., 2010; Underwood et al., 2008). In particular, it has been suggested that caspases, that are up-regulated early in pathological events associated with hypoxia/ischemia, may be also involved in *post mortem* proteolysis and meat tenderization because they are able to cleave several myofibrillar proteins, such as spectrin (Kemp & Parr, 2008). In general, the *post mortem* proteolysis process are not completely clear (Mohrhouser et al., 2011).Therefore the aim of this study is to determine the activities of μ-calpain and caspase 3 in two muscle types in Italian Simmental young bulls at slaughter and during ageing, and their relationship with the beef tenderness.

3. Materials and methods

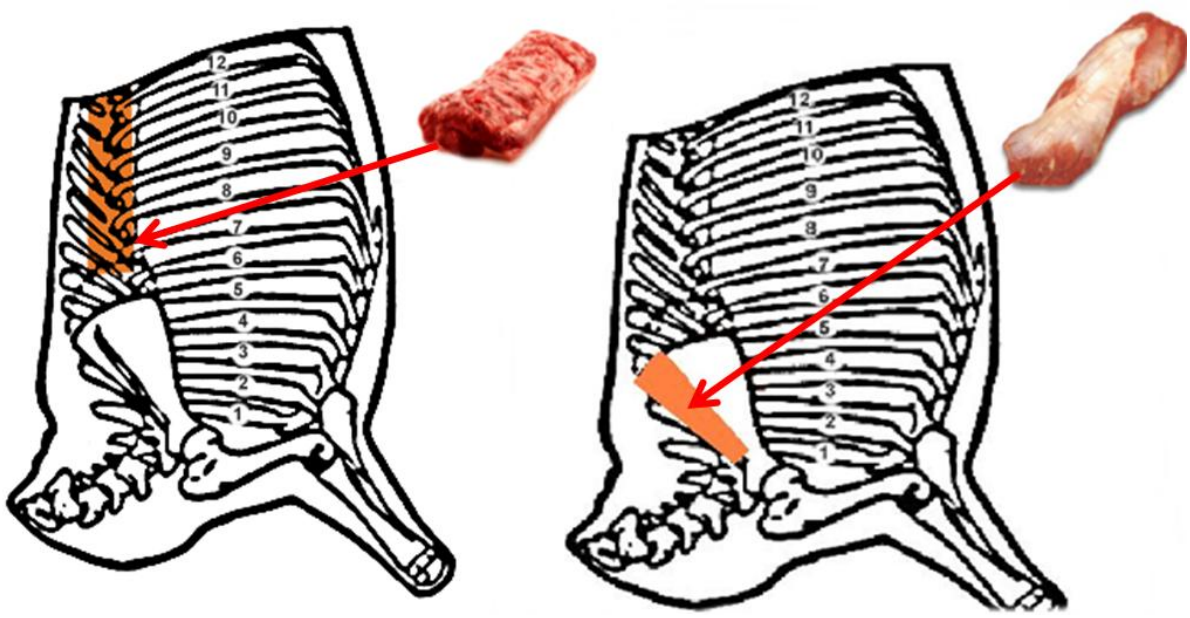
3.1 Experimental animals and samples

All procedures meet the requirements of the European Community Directive, 86-609-EC for Scientific Procedure Establishments.

Seventeen Italian Simmental young bulls were randomly chosen from one farm, the genetic center of A.N.A.P.R.I. in Fiume Veneto (PN, Italy), fed with corn silage-based diets and slaughtered at an average weight of 693.5 ± 11.6 kg (mean ± SE).

The animals were slaughtered at an EU-licensed abattoir following standard procedures.

At slaughter, within 20 min from exsanguination (T0), approximately 10 g of samples of *Longissimus dorsi* muscle (LD; $6^{th}/7^{th}$ rib) and *Infraspinatus* muscle (IS) (fig.8) were collected from the exposed part of muscles after skinning, frozen in liquid nitrogen, and stored at -80 ºC until electrophoretic analyses. LD and IS were chosen for the analysis based on their different expected fibers composition, in particular IS contains more type I fibres compared to LD that contains more of type IIB fibres. In fact, LD is defined as white muscle while as IS red muscle (Kirchofer, Calkins, & Gwartney, 2002).



A B

Figure 8 *Longissimu dorsi* (A) and *Infraspinatus* (B) muscle localisation.

At the same time, the hot carcass weight was recorded and the dressing percentage calculated. The carcasses were graded for carcass conformation and fatness according to the EU Regulation No 1208/81, 1026/91.

After chilling at 4 °C for 48 h from the right side of the carcass a sample joint was removed from the 8th rib position and colour evaluated. On the same sample also the proximate analysis was performed. At the same time, samples of LD ($6^{th}/7^{th}$ rib) and IS were collected, and then divided into two parts each. The first samples, approximately 10 g each, were frozen in N^2 and stored at -80 ºC until electrophoretic anal

yses (T48h); while on the second samples of LD and IS the pH was measured and then they were wrapped in geo-textile and aged at 4 °C for five days. After seven day of aging (T7d) the samples were submitted to WBSF analysis, drip and cooking loss measured and approximately 10 g collected, frozen in N^2, and stored at -80 ºC until electrophoretic analyses.

3.2 Physical and chemical Analyses

The pH was measured with a pH meter Hanna HI 8424, with probe Crison, 52-32. The color was detected using a portable spectrophotometer (Minolta CM 2600 d, Ramsey, NJ, USA) with an aperture of 8 mm, illuminant D65 and observation angle of 10°. The recorded values were lightness (L* value), redness (a* value), yellow (b* value), Chroma (C*) and Hue (H°). Hue angle ($\tan^{-1}(b^*/a^*)$) and chroma ($\sqrt{(a^{*2} + b^{*2})}$) values were derived from a* and b*. Proximate analysis was performed: dry matter (AOAC official method 945.15), protein (AOAC official method 992.15), ether extract (AOAC official method 960.39), ash (AOAC official method 920.153) according to AOAC (2000). The sample joint was dissected into lean, fat and bone, and the carcass composition was estimated as proposed by Andrighetto, Rioni Volpato, Andreoli, and Cozzi (1996). The cooking loss was evaluated in 2 cm slices of muscle; slices have been cooked, in plastic bags until core temperature of 75 °C was reached , using a waterbath, in agreement with the procedure described by Honikel (1998). Each slice of meat was weighed before and after cooking. The total loss of liquid was calculated by difference between the weight before and after cooking and expressed as percentage of the initial weight. Drip loss was evaluated according to Honikel (1998). The Shear Force was determined on raw samples

(Honikel, 1998), using the device "Warner- Bratzler " (test " WBSF Warner - Bratzler Share Force") with a triangular blade (60 °), mounted on a load cell of 100 N and using a Lloyd dynamometer TA Plus (Lloyd , UK). From the meat, cylinder of 20mm diameter were collected in the same orientation of muscle fibers (at least 7 cylinders from steak). The Shear Force of the cylinders was calculated perpendicularly to the fibers. The speed of execution of the test was 100 mm / min. The parameter detected was the maximum load.

3.3 Experimental design for protein immuno analysis

The separation and the quantitative evaluation of the target proteins were carried out by a denaturating electrophoresis followed by Western blot analysis. In order to evaluate the effect of muscle type (IS vs. LD) on the relative amount of target proteins, the samples of both muscles taken at every experimental time, from each animal were analysed on the same gel. In particular the following sets of proteins were separately considered and examined in the muscle pairs:

1. Slow myosin heavy chain (MHC) and actin;
2. Fast MHC and actin;
3. Alfa-II-spectrin and its cleavage products from caspase 3 and μ-calpain activity, at times: T0, T48h and T7d *post mortem*;
4. PARP-1 and its cleavage product from caspase 3 activity, at times: T0, T48h and T7d *post mortem*.

3. 4 Protein extraction and quantification

3.4.1 Extraction

100 mg of muscle were cut with a scalpel in small pieces at 4 °C (to avoid the activation of proteolytic processes and the consequent degradation of proteins) and suspended in 1 ml of the extraction buffer containing 60 mM Tris/HCl pH 7.5, 0.1 M dithiothreitol (DTT), a redox reagent, 2% wt/vol of Sodium-dodecyl-sulphate (SDS), an anionic detergent, and supplemented with 2 µl of antiproteases (Protease Inhibitor, Sigma-Aldrich).

The samples were homogenized by Ultra-Turrax® T25 Digital operating at 13000 rpm for 3x30 seconds and centrifuged 4 minutes at 5600 g at room temperature to remove excess foam due the presence of SDS in the extraction buffer. The samples were then heated at 98 °C and shaken for thirty minutes at room temperature, to facilitate the extraction of proteins. After this incubation, the samples were again centrifuged at 5600 g for 10 minutes at room temperature to eliminate the not solubilized components.

3.4.2 Protein quantification

The evaluation of the protein content of the extracts was performed for a cross-sample standardization of the amount of protein to be loaded on the gel lanes. The protein content of the extracts was determined according to the absorption spectrum at 280 nm. The majority of proteins in fact have a maximum absorption at 280 nm, due to the presence of the aromatic amino acids tryptophan, tyrosine and phenylala

nine (Walker, 2002). This method is easy to do, quickly, and allows determining protein concentrations between 20 and 3000 µg/ml. The samples were diluted 1:10 with water and their absorption was compared with a calibration curve obtained with solutions of bovine serum albumin (BSA) dissolved at known concentrations in 0.2%SDS.

3.5 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

µ-Calpain and caspase-3 activities were assessed through alpha II spectrin and PARP-1 cleavage products, which were separated from protein extracts by SDS-PAGE and recognized by Western blotting with specific antibodies.

SDS-PAGE (Laemmli, 1970) was performed using 10x10x0.1 cm gels. The acrylamide concentration in the separating gel was chosen according to the molecular size of the proteins to be separated, varying from 8% (alpha II spectrin and PARP-1) to 13% (actin and MHC) acrylamide and maintaining an acrylamide:N,N'-methylenebisacrylamide ratio of 37.5:1 (w/w), which were solubilized in 0.25M glycine, 0.025M Tris/HCl, pH 8.8 and 0.1% (w/v) SDS. The stacking gel was prepared using 4% (v/v) acrylamide in stacking buffer (0.125M Tris/HCl, pH 6.8, 0.1% (w/v) SDS). 0.1% ammonium persulfate and 0.0015% (v/v) TEMED were added to start gel polymerization.

Electrophoresis was performed with a limiting voltage of 250 V and a current of 15 mA until the dye front reached the bottom of the gel. The electrophoresis system was cooled using an internal fan to circulate water. Protein molecular weights were esti

mated by running standard proteins of known molecular weight in separated lanes (Bio-Rad).

ACRYLAMIDE 4% STACKING BUFFER (one gel)

Stacking solution*	2 ml
10% SDS	20 µl
10% Ammonium persulfate	20 µl
Temed (N,N,N',N'–Tetramethylethylenediamin	5 µl

*STACKING SOLUTION (25 ml)

40% acrylamide	2.4 ml
2% Bis – acrylamide	1.3 ml
0.5 M Tris/HCl pH 6.8	6.6 ml
H_2O	14.6 ml

ACRYLAMIDE 8% RUNNING BUFFER (one gel)

Running solution**	6 ml
10% SDS	60 µl
10% Ammonium persulfate	60 µl
Temed (N,N,N',N' – Tetramethylethylenediamii	9 µl

**RUNNING SOLUTION (50 ml)

40% acrylamide	9.7 ml
2% bis – acrylamide	4.6 ml
Tris/HCl 1.5 M pH 8.8	12.5 ml
H_2O	23.1 ml

ACRYLAMIDE 13% RUNNING BUFFER (one gel)

Running solution* **	6 ml
10% SDS	60 µl
10% Ammonium persulfate	60 µl
Temed (N,N,N',N' – Tetramethylethylenediamir	9 µl

***RUNNING SOLUTION (50 ml)

40% acrylamide	15.8 ml
2% bis – acrylamide	5.8 ml
Tris/HCl 1.5 M pH 8.8	12.5 ml
H_2O	19.5 ml

ELECTROPHORESIS BUFFER

Glycine	250 mM
Tris/HCl pH 8.8	25 mM
SDS	0.1 %

3.6 Western blot analysis

Proteins separated via SDS-PAGE were transferred to 0.2 µm pore size nitrocellulose membranes (Schleicher & Schuell, Bio Science) using a semi-dry blotting apparatus (TE 22 transfer unit from Amersham Biosciences).

The transfer was performed at 2.5 mA/cm^2 for 1 h in the transfer buffer. After staining with Ponceau S solution (ATX Poceaus S red staining solution Fluka), to verify transfer efficiency, the nitrocellulose sheets were cut at the level of 50 kDa. The two parts were saturated with 3% (w/v) non-fat dry milk in PBS buffer plus 0.1% Tween 20 for 1h at room temperature and then incubated overnight separately with the antibodies, i. e. the lower part with anti-actin antibodies, while the upper part with anti-fast-MHC or anti-slow-MHC, as detailed in the paragraph below.

3.6.1 Actin, Fast and Slow Myosin heavy chain antibody

Antibodies
• Polyclonal Anti-actin (1:5000) (SIGMA, A2066) • Monoclonal Anti-MHC (skeletal, fast) (1:4000) (SIGMA, M1570)

Antibodies
• Polyclonal Anti-actin (1:5000) (SIGMA, A2066) • Monoclonal Anti-MHC (Skeletal, slow)(1:4000) (SIGMA, M8421)

Blots were then rinsed three times with PBS buffer plus 0.1 % Tween 20 and incubated for 1.30 h with peroxidase coupled to anti-mouse-IgG (1/8000) for the both anti-MHC antibodies or anti-rabbit-IgG (1:15000) for the anti-actin antibody, at room temperature.

The membranes were washed again three times in PBS buffer plus Tween 20 and developed with SuperSignal ® West Dura (Thermo Scientific) according to the manufacturer's protocol.

3.6.2 Alpha II spectrin and PARP-1antibody

After the transfer and saturation run as in 3.6.1, the nitrocellulose sheet was incubated overnight with the antibodies detailed in the paragraph below.

Antibodies
• Monoclonal Anti-Spectrin α II (1:250) (Santa Crutz biotechnology Inc., sc-48382)

Antibodies
• Polyclonal Anti-PARP-1 (1:500) (Santa Crutz biotechnology Inc., sc-7150)

Blots were then rinsed three times with PBS buffer plus 0.1 % Tween 20 and incubated for 1.30 h with peroxidase coupled to anti-mouse-IgG (1/8000) for the alpha II spectrinantibody or anti-rabbit-IgG (1:15000) for the anti-PARP-1 antibody, at room temperature. Film development was run as in 3.6.1.

TRANSFER BLOT BUFFER (pH 8.1 – 8.3)

Tris/HCl	25 mM
Glycin	192 mM
Methanol	20% (v/v)

PBS 1x

Na_2HPO_4	8.1 mM
KH_2PO_4	1.9 mM
NaCl	137 mM
KCl pH 7.4	2.7 mM

3.7 Densitometric analyses

High resolution images of the films and gels were acquired using the instrument SF Launcher and processed using the program ImageQuant TL. The option “1D Gel Analysis” allowed the densitometric reading of the bands, which were hand-selected and their intensity was substracted from that of the background.

The percentage area of the slow and fast myosin heavy chain band was calculated taken as 100% the area of the actin band used as load reference in each sample. The percentage areas of alpha II spectrin, PARP-1 and their respective cleavage products were calculated taken as 100% the total area of immuno-detected bands.

4. Statistical Analysis

The statistical analysis was performed using SPSS for Windows (version 7.5.21, Inc 1989–1997). Normality of data distribution and homogeneity of variance were tested using Kolmogorov-Smirnoff and Levene test, respectively. Differences between muscles within time of sampling were carried out using paired samples t-test procedure. Pearson coefficients were used to determine associations between variables

5. Results and Discussion

5.1 Animal characteristics

The carcass characteristics of young bulls were reported in tab. 1. The average dressing percentage was lower than those previously reported 57.4% by Piasentier et al. (2009), which considered Italian Simmental young reared in the same farm and with similar hot carcass weight at slaughter. Another study of our research group conducted on Italian Simmental young bulls, (Corazzin, Bovolenta, Sepulcri, & Piasentier; 2012), highlighted an higher dressing percentage, but similar carcass composition than those reported in the present study. Moreover the carcass composition was also very similar to those reported by Kaufmann, Leuenberger, & Kiinzi (1996) (lean 70.9%, bone 17.3%). Cozzi, Brscic, Contiero, & Gottardo (2009) that considered Italian Simmental young bulls slaughtered at a lower live weight (624 kg), showed a lower dressing percentage, 53.7%, a similar conformation of the carcasses, but a worse state of fatness compared to that shown in the present study.

Table 1 Carcass characteristics of animals, n=17.

	mean	SE
Carcass		
Hot weight (kg)	381.1	8.93
Dressing percent	54.9	0.67
SEUROP conformation[1]	2.8	0.13
SEUROP fat score[2]	3.1	0.12
8th rib sample joint composition		
Meat (%)	64.8	0.58
Fat (%)	16.6	0.75
Bone (%)	18.6	0.64
Carcass composition		
Lean meat (%)	69.3	0.27
Fat (%)	13.7	0.51
Bone (%)	17.0	0.32

[1]S = 5 (superior), E = 4, U = 3,..., P = 0 (poor).
[2]Class 5 = 5 (very fat),..., class 1 = 1 (very lean).

5.2 Instrumental meat parameters

The characteristics of meat (*Longissimus Dorsi* m.) after 48h of ageing were reported in tab. 2. The colour parameters were comparable to the reference values suggested for beef by Frickh et al. (2005) L*=34-40; a* >10, and by Ripoll & Albertí (2012), L* ,<39.5, Chroma >17. The meat composition was similar to those reported by Corazzin et al. (2012) and by Bureš, Bartoň, Zahrádková, Teslík, & Krejčová (2006), in Simmental young bulls.

Table 2 Characteristics of *Longissimus Dorsi* after 48 h of ageing, n=17.

	mean	SE
Colour		
L*	34.8	0.44
a*	10.8	0.80
b*	13.2	0.52
Chroma	17.1	0.90
Hue	51.5	1.04
Proximate composition		
Dry matter (g/kg)	256.9	0.27
Protein (g/kg)	212.4	0.30
Ether extract (g/kg)	28.1	0.26
Ash (g/kg)	10.7	0.09

As reported in tab. 3, all the samples of LD muscle showed an ultimate pH lower than 5.87 which was considered as reasonable threshold for dark cutting meat (Page, Wulf, & Schwotzer, 2010). However IS meat showed higher pH48, WBSF and drip loss than LD meat. As showed in fig. 10 the analysis of MHC isoforms revealed that LD muscle had 67% and 28% of fast-MHC and slow-MHC, respectively. While IS muscle had 37% and 84% of fast-MHC and slow-MHC respectively. Billetter et al. (1980) explained that type I fibers have exclusively slow-MHC, while type IIA and IIB fibers have exclusively fast-MHC.

These results confirm that IS could be classified as a red muscle (rich in type I fiber), while LD could be considered as white muscle (rich in type IIB fiber). It is well known that the morphological and biochemical characteristics of muscle fiber types influence the muscle energy metabolism in live animals. Moreover, some studies have highlighted that fiber composition also affects the *post mortem* conversion of muscle to meat and consequently the meat quality in pork (Gil et al., 2003; Choi et al., 2007), cattle (Olivan et al., 2004; Muroya et al., 2007), rabbit (Ramirez et al., 2004), and sheep (Sazili et al., 2005). In agreement with our results the content of type IIB fibers are considered to be negatively related to muscle pH (Ryu & Kim, 2006; Ryu et al., 2008).

Table 3 effect of muscle type (*Longissimus Dorsi* , LD; *Infraspinatus* ,IS) on meat physical traits n=34.

Item	LD	IS	SEM
pH 48h	5.51^{B}	5.72^{A}	0.037
7 days of ageing			
pH	5.58^{B}	5.77^{A}	0.029
Colour			
L*	35.1	34.9	0.28
a*	11.6	11.7	0.22
b*	13.1^{a}	12.3^{b}	0.25
Chroma	17.6	17.0	0.31
Hue	48.5^{a}	46.2^{b}	0.48
Warner-Bratzler shear force (N)	20.2^{B}	30.3^{A}	1.28
Drip loss (%)	1.84^{b}	2.35^{a}	0.113
Cooking loss (%)	24.15	21.73	0.80

^{A,B}:P<0.01
$^{a,b:}$P<0.05

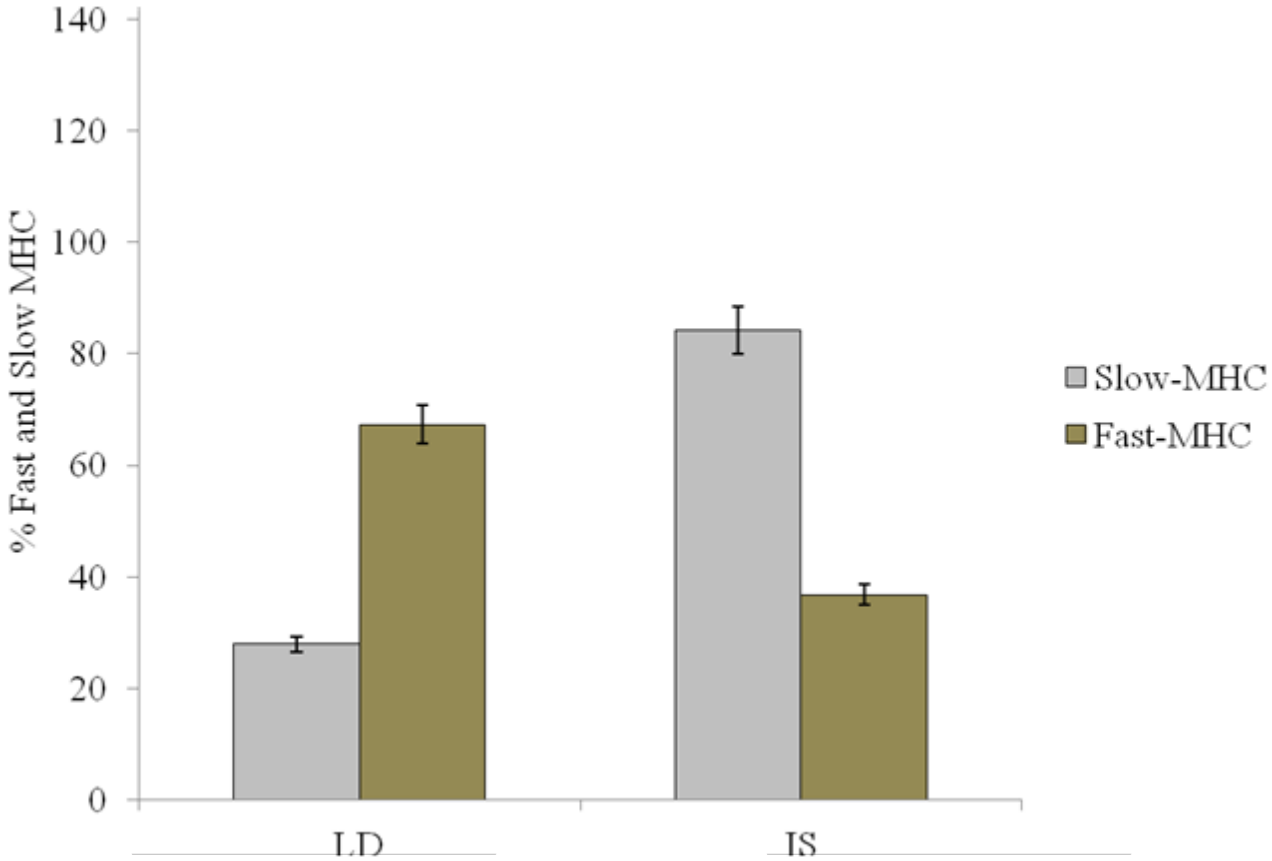


Figure 10 Characterization of LD and IS based on the percentage of fast and slow myosin heavy chain (MHC). Proteins from LD and IS muscles were extracted with 60 mM Tris/HCl pH 7.4, DTT 0.1 M, SDS 2% (w/v), as specified in Methods. 60 µg of each extract was subjected to 8% SDS-PAGE followed by Western-Blotting with anti-actin and anti-slow or anti-fast MHC. Each immuno-detected band was analyzed by densitometry with the ImageQuant software and the % area of the slow and fast MHC was calculated taken as a percentage of the area of the actin band (100%) in each sample. Muscle samples were obtained from seventeen Italian Simmental young bulls.

5.3 Enzymatic contribution to meat quality

Meat tenderization is considered a multienzymatic process (Herrera-Mendez et al., 2006). In particular, as already discussed in the Introduction section, it has been suggested that not only μ-calpain, but also caspases, that are up-regulated early in pathological events associated with hypoxia/ischemia, may be involved in *post mortem* proteolysis and meat tenderization (Kemp & Parr, 2008). The activity of caspases and calpains were assessed by analyzing the changes in the levels of their specific substrates and of the corresponding degradation fragments, i.e. the nuclear enzyme PARP-1 and the cytoskeletal protein alpha II spectrin and their fragments. Alpha II spectrinis cleaved by caspase-3 and μ-calpain producing alpha II spectrin breakdown products (SBDP) of different molecular weight (fig. 11). In particular, the fragment of 150 kDa is produced by both proteases, while the fragments of 145 kDa and of 120 kDa are cleaved exclusively by μ-calpain and caspese-3 respectively.

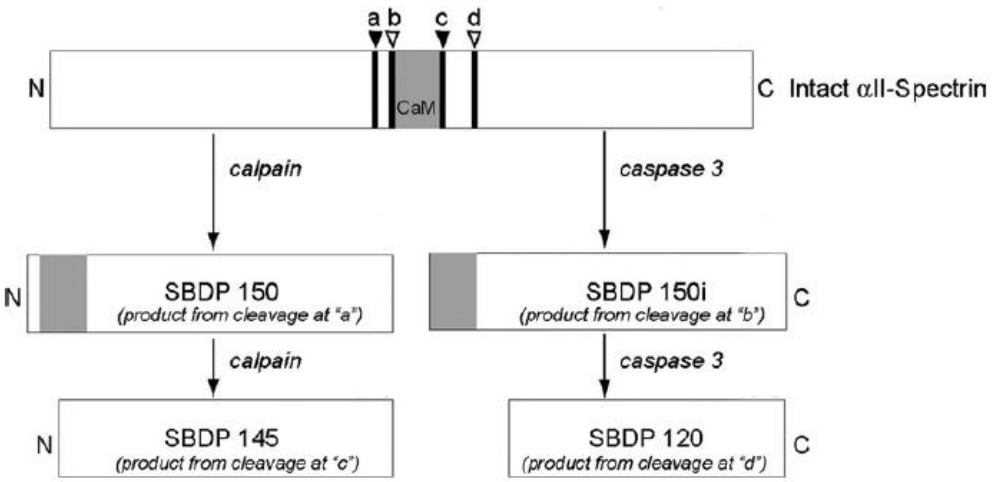


Figure 11 Alpha II-spectrin cleavage products (Weiss et al., 2009).

The caspase-3 activity was also assessed by the quantification of the cleavage products of PARP-1. Indeed, caspase-3 is able to cleave the full-length PARP-1 of 116 kDa producing two fragments of 89 kDa and 24 kDa.

The analysis performed on the samples collected at slaughter (T0) showed that the full-length alpha II spectrin level was much higher in IS than LD ($P < 0.01$); fig. 12, meaning that, at slaughter, alpha II spectrin was less degraded in IS than LD. This result is in agreement with previous studies (Koohmaraie et al., 1988; Monin & Ouali, 1991; Whipple & Koohmaraie, 1992; Joo, Kim, Hwang, & Ryu 2013) who found, in the early *post mortem* period, a lower proteolysis in slow twitch with respect to fast twitch fibers. It might be due to the more extensively developed sarcoplasmic reticulum and transverse-tubule system and the thinner Z-band of the fast twitch fibers, which make proteins located at the Z-band more susceptible to proteolytic degradation than those in slow twitch fibers (Xiong, 2004). Moreover, a positive correlation between full-length alpha II spectrin level and the WBSF measured at the 7th day of ageing was found ($r = 0.810$; $P < 0.01$), indicating that a different early proteolysis between LD and IS muscles may contribute to explain their different tenderness. In fact, alpha II spectrin degradation could significantly compromise the membrane permeability and cytoskeletal integrity (Wang, 2000), a process that is associated with meat tenderization (Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995). After 48h *post mortem* (fig. 13) the presence of the full-length alpha II spectrin has not been detected in both muscles. This means that proteolysis within the muscles continued from 0h to 48 h after death.

When the caspase-3 activity was analysed by immuno-detecting SBDP120, its levels were found similar in the two muscles ($P > 0.05$), suggesting that, although active in the early *post mortem* period, caspase-3 did not play a primary role for explaining the difference in tenderness in the two muscles considered. The activity of caspase-3 in the early *post mortem* was confirmed by the PARP-1 cleavage products analysis. Indeed as showed in fig. 14 the 89 kDa PARP-1 fragment (50.4 % of the total protein detected) was detected and it was similar between muscles (P>0.05). These findings seems to be confirmed by another study of our research group performed on the same animals and aimed to compare the mRNA abundance of enzymes involved in meat tenderization process in LD and IS (Annex I). In particular CAPN1mRNA tended to be higher in LD than IS (P = 0.08), while CASP3 was similarly expressed between muscles ($P > 0.05$). No correlation was found between SBDP120 level and WBSF ($P > 0.05$). This result is not in agreement with the findings of Kemp et al. (2010) who found, in pig, a negative correlation between WBSF and SBDP120 level measured in LD muscle 2h *post mortem*, but is consistent with the study of Underwood et al. (2008), who found no correlation between caspase-3 activity and WBSF in *Longissimus thoracis* muscle of steers. These data suggest a species-specific trend, moreover differences in caspase-3 activity were already observed considering different muscles. Indeed, Cao et al. (2010) reported similar caspase-3 activities in three skeletal muscles of bulls, i.e. *Longissimus dorsi*, *Semitendinosus*, and *Psoas minor,* in the early *post mortem* period. Conversely, in lamb caspase-3 activity was different and decreased with different rate in *Longissimus dorsi*, *Semimembranosus* and *Infraspinatus* during *post mortem* (Kemp, King, Shackelford, Wheeler & Koohmaraie, 2009). At T48h the relative levels of SBDP120 were very low, as those recorded at slaughter, without differences between muscles (P>0.05).

Considering PARP-1 analysis, only the full-length 116 kDa was found at the same time. Taking into account these results, it seems that caspase-3 was not active at 48 h, confirming the study of Mohrhauser Kern, Underwood, & Weaver (2011), who argue that μ-calpain is the primary protease responsible for the myofibrillar degradation during aging. Cao et al. (2013), considering *Longissimus dorsi* muscle of crossbreed bulls, found that caspase-3 increased from 0 h to 24h *post mortem* and then decreased from 24h to 48h *post mortem* reaching a value similar to those recorded at slaughter. Also Huang et al. (2014), in *Longissimus thoracis* muscle of Luxi × Simmental crossbreed bulls, showed an increase of caspase-3 activity from 0 to 6h and then a reduction from 24h to 72h *post mortem*. However, the caspase-3 activity at 72h was lesser than that at 0h *post mortem*, and equal to 70% of the latter. In agreement with our results, these authors concluded that caspase-3 may effectively function in the early *post mortem* (until 24h from slaughter).

Regarding μ-calpain activity analysed by immuno-detection, at T0 (fig. 12) SBDP145 level was higher than SBDP120 both in LD ($P < 0.01$) and IS ($P < 0.01$); it means that the activity of μ-calpain was higher than that of caspase-3 in both muscles at slaughter. However, a different reactivity of the antibody used for Western Blotting against SBDP120 and SBDP145 cannot be excluded. When the SBDP145 was analyzed by Western Blotting to evaluate the μ-calpain activity, its level was significantly higher in LD than in IS muscles ($P < 0.01$), indicating that this protease was more active in LD than in IS. Moreover, a slightly positive correlation between SBDP145 level and WBSF was found ($r = 0.355$; $P = 0.08$). This result is quite interesting, because this correlation strongly suggests that the μ-calpain activity was in

volved in the higher tenderization of LD, which is characterized by a higher content of fast-MHC.

In fact, many studies have already highlighted the role of μ-calpain in *post mortem* degradation of myofibrillar proteins and muscle tenderization (Geesink et al., 2000; Hopkins & Thompson, 2002; Koohmaraie et al., 2002), while Ouali et al. (1990) analyzed calpain activity and meat ageing separately in red and white muscles. In accordance with the data of the present thesis, these authors found that in bovine, pig and sheep the slow twitch red muscles exhibited a lower ageing rate and a lower calpain content with respect to the white ones. However, fig. 12 shows that the level of SBDP150 is similar in both LD and IS muscles. This result is rather surprising and possibly suggests that it is quickly degraded to SBDP145 and SBDP120 by μ-calpain and caspase-3, respectively.

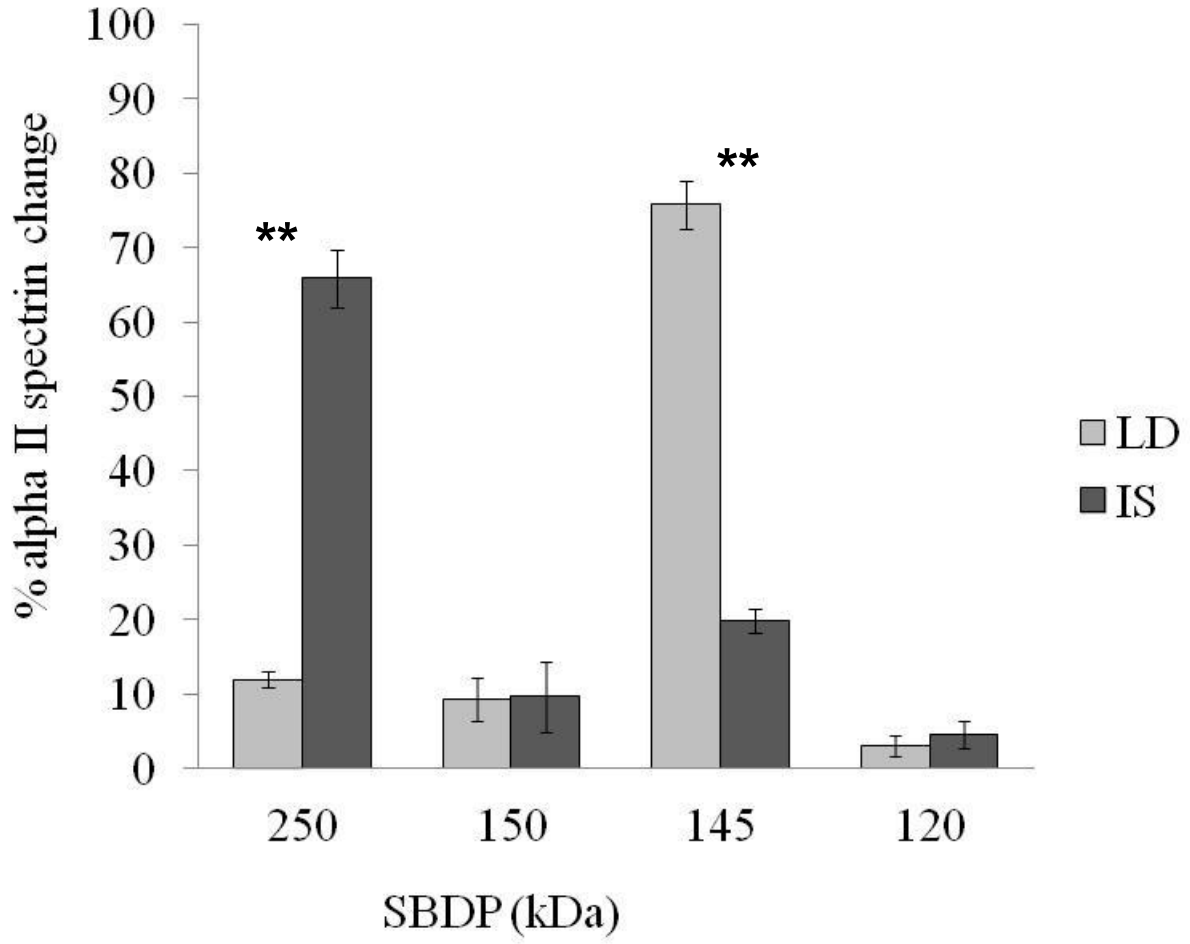


Figure 12 Full-length alpha II spectrin (250 kDa) and its degradation products (SBDP) detected at 20 minutes after slaughter (T0) in LD and IS muscles. The LD and IS muscle samples characterized for their slow- and fast-MHC content were also analysed for alpha II spectrin and its proteolytic products. Proteins were extracted as in figure 10 and 60 µg of protein extracts were subjected to 8% SDS-PAGE followed by Western-Blotting with alpha II spectrin antibody. Each immuno-detected band was analyzed by densitometry with the ImageQuant software and its % area was calculated taken as 100% the total area of immuno-detected bands. SBDP145 and SBDP120 were cleavage products of µ-calpain or caspase-3, respectively, while SBDP150 was a cleavage product of both proteases. .** $P < 0.01$

At T48h the cleavage products SBDP150 and SBDP145 were still present. In particular, SBDP150 in both muscles and SBDP145 in IS were increased with respect to slaughter. However the level of SBDP150 and SBDP145 were similar between muscles at 48h post mortem. Taking into account these results μ-calpain was active at 48h in both muscles, but its activity seems delayed in IS with respect to LD.

An important characteristic of μ-calpain is that they are Ca2+ -activated proteases by autolysis: Ca2+ ions change their structure exposing the enzyme molecules to digestion by calpains themselves as well as by other proteins (Cambell & Davies, 2012; Goll et al., 2003; Goll, Thompson, Taylor, & Ouali, 1998). The concept that the calpains were proenzymes was widely accepted from the 1990s, and numerous papers have described “activation” of the calpains as being synonymous with autolysis (Goll et al., 2003). Calpains autolysin once activated, ultimately leads to loss of activity. Consistently, in bovine muscle, the extractable activity of μ-calpain declines during postmortem (Koohmaraie & Geesink, 2006). Takahashi, 2006, evaluated the Ca2+ concentration in *Longissimus thoracis* from beef during post mortem, showing that Ca2+ was 16 μM at 40 min after slaughter and reached a maximum of 210 μM at 4 days postmortem. These are concentrations compatible for μ-calpain activation, which are 3-50 μM (Goll et al., 2003), supporting that in our samples at T0 μ-calpain was fully activated in LD. On the other hand, it is plausible to hypothesize that in IS Ca2+ increase was less sharp, leading to a lower enzyme activation. Nevertheless, the higher μ-calpain activation in LD would lead to its more rapid autolysis during post mortem that it might be consistent with our observation of similar level of SBDP145 at T48h in both muscles. Kemp et al. (2006) showed an increasing of

SBDP150 level during ageing in pig. In this study, despite the antibody used was not able to distinguish between the μ-calpain and caspase-3, no correlation between SBDP150 levels and caspase-3/7 activity was found, suggesting that the increasing of SDBP150 during ageing could be mainly due to calpain rather than caspase activity.

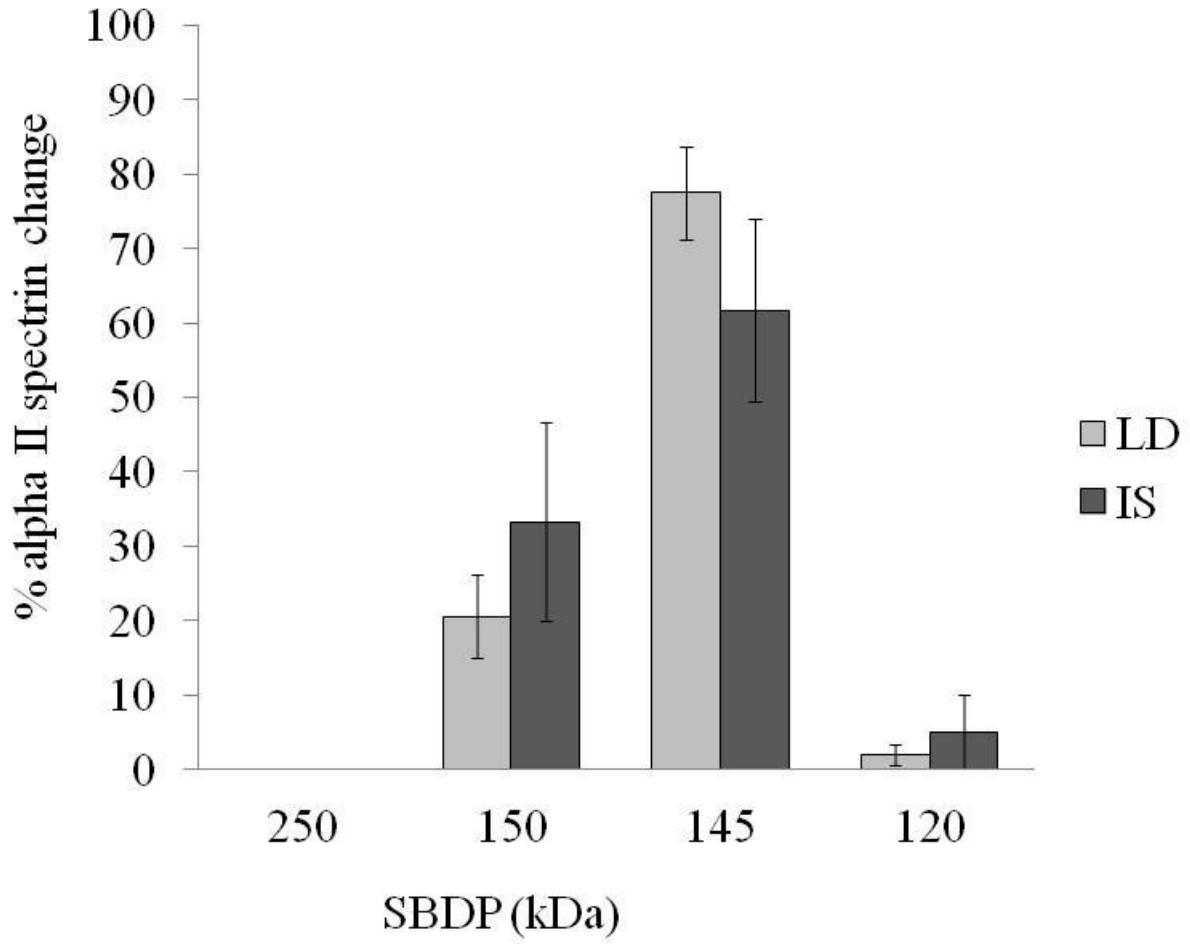


Figure 13 Full-length alpha II spectrin (250 kDa) and its degradation products (SBDP) detected at 48h after slaughter in LD and IS muscles. The samples were obtained from the same animals analysed in figures 10 and 12, as well as the Western Blotting with alpha II spectrin antibody and the histogram were obtained as in figure 12.

As expected, considering that SBDP250 was not already found after 48h (fig.13), after seven days of ageing neither SBDP150, nor SBDP145, nor SBDP120 were detected. Considering these results the activity of calpain cannot be evaluated at seven days of ageing through the alpha II spectrin cleavage products. At this sampling time only the full-length 116 kDa of PARP-1 was found indicating that caspase-3 was not active.

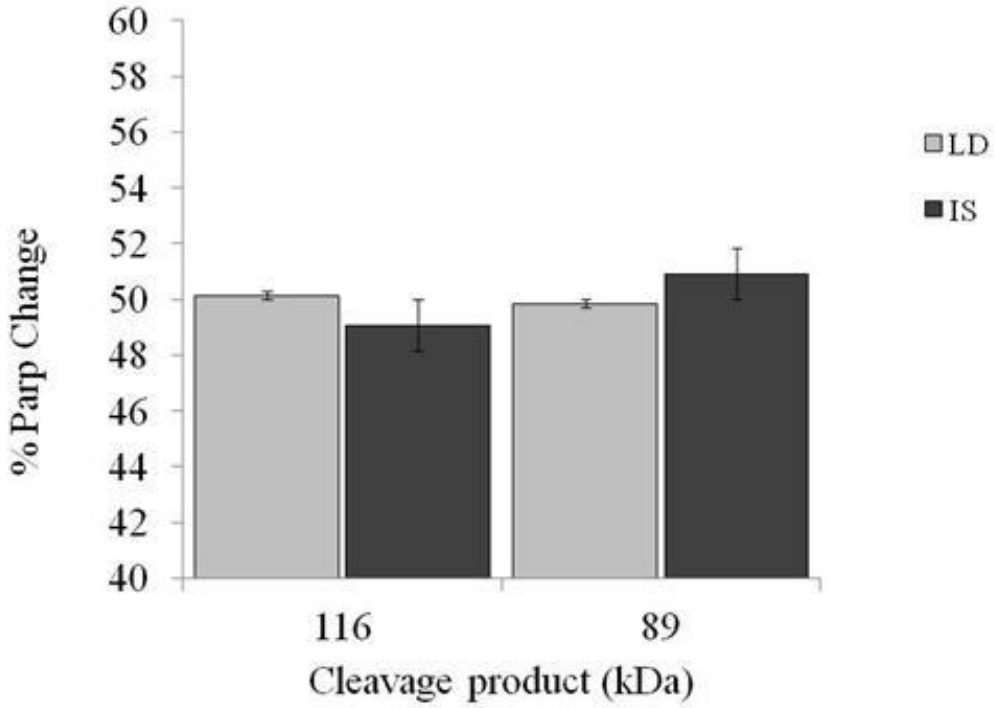


Figure 14 Full-length PARP-1 at 116 kDa and its 89 kDa cleavage product detected at 20 minutes after slaughter. The LD and IS muscle samples characterized for their slow- and fast-MHC content were also analysed for PARP-1 and its proteolytic products. Proteins from LD and IS at slaughter were extracted as in figure 1 and 60 µg of extracts were subjected to 8% SDS-PAGE followed by Western-Blotting with PARP-1 antibody. Each immunodetected band was analyzed by densitometry with the ImageQuant software and its % area was calculated taken as 100% the total band area. $P > 0.05$

6. Conclusion

The method of immune-detection by SDS-PAGE of alpha II spectrin, PARP-1 and their degradation products allows to draw some coclusions about the activities of μ-calpain and caspase-3 in two different bovine muscle types, *Longissimus dorsi* and *Infraspinatus* muscle, during ageing. Both caspases and μ-calpain were found active, having been recognized their target degradation products. More specifically, in each animal no difference in the level of alpha II spectrin fragment, derived from the caspase-3, activity was found between the *Longissimus dorsi* and *Infraspinatus* muscles. On the other hand, the μ-calpain activity appeared to be influenced by the type of muscles. These functional data were confirmed by the gene expression assays carried out in a parallel study. Based on the results of the thesis, the different tenderness between LD and IS muscles in Italian Simmental young bulls underlay a different *post mortem* proteolysis which is greatly influenced by μ-calpain than caspase-3 activity.

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Annex I:

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ACTIVITY OF ENZYMES AND EXPRESSION OF GENES INVOLVED IN BEEF TENDERIZATION IN TWO BOVINE SKELETAL MUSCLES

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Abstract – The objective of this trial was to determine the early *post-mortem* activity of enzymes and expression of some genes involved in tenderization process of beef in two muscle types. Samples of *Longissimus dorsi* (LD) and *Infraspinatus* (IS) muscles were collected within 20min from slaughtering of 17 Italian Simmental young bulls. By SDS-PAGE the αII spectrin and its degradation products were analyzed to assess μ-calpain (CAPN1) and Caspase 3 (CASP3) activities. The mRNA abundances of CAPN1, m-Calpain (CAPN2), CASP3, CASP9, Heat Shock Protein 70 (HSP70), HSP27 and HSP40 were detected by RT-PCR. Also pH_{48h}, and WBSF after 7 days of ageing were measured. LD showed lower WBSF and higher CAPN1 activity than IS, while CASP3 activity was similar between muscles. Within each muscle CAPN1 activity was higher than that of CASP3. LD had lower HSP27 and HSP70 mRNA abundances than IS. No differences between muscles were found in the expression of the other genes. Even if some interesting tendencies were highlighted concerning CAPN1 and CASP9 expression at slaughter, the differences in WBSF between muscles seem to be mainly linked to CAPN1 activity and HSPs early *post-mortem* expression.

Key Words – Calpain, Caspase, Heat Shock Proteins, Tenderness, αII spectrin degradation products

I. INTRODUCTION

From the consumer point of view, tenderness is one of most important factors able to modify its buying decision process for beef [1]. Tenderness is affected by many factors such as: breed, amount and solubility of collagen, intramuscular fat content, pH decline, fiber type characteristics and consequently type of muscle [2], and *post-mortem* proteolysis which have a key role [3]. Koohmaraie [4] explained that 70% of tenderness was explained by environmental and 30% by genetic factors. It is widely recognized that proteolytic enzymes such as calpain and caspase directly contribute to beef tenderness. Moreover Hocquette *et al.* [5] found that the expression of some genes, such as heat shock proteins, are related to beef tenderness in Charolais young bulls, however the same authors highlighted the difficult to extent these results outside the reference population. Aim of this study is to determine different activities of enzymes and expression of some genes involved in tenderization process of beef in two muscle types in Italian Simmental young bulls.

II. MATERIALS AND METHODS

Seventeen Italian Simmental young bulls were randomly chosen from 1 farm, fed corn silage-based diets and slaughtered at 694±11.6kg (mean±se) of weight. At slaughter, samples of *Longissimus dorsi* muscle (LD) and *Infraspinatus* muscle (IS) were obtained within 20min of exsanguination, frozen in N_2, and stored at -80°C until transcriptomic and proteomic analysis. After chilling at 4°C for 48h, samples of LD (6th-7th ribs) and IS were collected, pH measured by a pH-meter (Crison 52-32 electrode), and then aged at 4°C for 7d. Shear force was measured on raw sample, using a Warner-Bratzler device (WBSF), with a triangular hole in the shear blade, mounted on a Lloyd TAPlus texture analyser (ELIS, IT). Measurement was recorded as the peak yield force (N), required to shear, at a 100 mm/min crosshead speed, perpendicular to the direction of fibres, 7 cylindrical cross-section replicates, (15mm diameter×30mm length).
RNA was extracted from 40 mg muscle using RNeasy Fibrous Tissue Mini Kit (Qiagen, DE). Concentration and purity of RNA were assessed using spectrophotometer NanoDrop 2000c

(Thermo Fisher Scientific, MA). To obtain cDNA, the iScript cDNA Synthesis kit (BioRad, IT) was used. Each 20 µl of reaction contained 4 µl of 5X iScript reaction mix, 1µl of RNase H+ MMLV-derived reverse transcriptase, 10µg of RNA, 5µg of nuclease-free water. The mixture was held 5min at 25°C, 1h at 42°C, and 5min at 85°C before being cooled at 16°C. The cDNA was quantified using NanoDrop 2000c. A qualitative PCR was carried out to verify primers (Table 1). The quantitative PCR was performed using the Bio-Rad CFX96 system (BioRad, CA), on a reaction volume of 20µL, containing 0.3µL of each forward and reverse primer (0.3µM), 10µL of iQ SYBR Green Supermix (BioRad, CA), 8.4µL of sterile water, 1µL of cDNA. Amplification conditions included 1 cycle of 3min at 95°C, 40 PCR cycles of 15s at 95°C, 30s at 60°C, and 30s at 72°C, then 1min at 95°C followed to a melt curve of 55-95°C with 0.5°C increments every 5s. Each sample was analysed in triplicate and relative gene expression was calculated according to the efficiency-corrected method [6]. β-actin, cyclophilin, glyceraldehyde-3-P dehydrogenas, and ribosomial protein large P0, were treated as reference genes and used for normalization of RT-qPCR data (M<0.10). Data were presented in fold-change ratio having as reference IS; primer efficiency was calculated and verified using the standard curve obtained by serial dilution of the pooled cDNA [6].

CAPN1 and CASP3 activities were assessed through αII spectrin cleavage products. Total protein was extracted from 100mg of each muscle. Sample were homogenized 3 time for 30s with Ultra-Turrax® T25 in extraction buffer (0,05M dtt, 2% sds, 60mM tris-HCl, 0,2% antiproteases), boiling for 2min at 98°C, mixed 30min at room temperature and centrifuged 10min at 11000rpm. Protein was determinate by the Near UV Absorbance (280nm) method [7]. Equivalent quantity of protein of each sample were subject to 8% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [8]. Proteins separated via SDS-PAGE were transferred to 0.2µm pore size nitrocellulose membranes using a semi-dry blotting apparatus (TE 22 transfer unit, Amersham Biosciences) for 1h in a transfer buffer. After staining with Ponceau S. solution (ATX Poceaus S red staining solution Fluka), to verify transfer efficiency, the nitrocellulose sheets were saturated with 3% (w/v) non-fat dry milk in PBS buffer plus 0.1%, Tween 20 for 1h at room temperature and then incubated overnight with the antibodies spectrin α II (C-3; Santa Cruz Biotechnology, inc.). Blots were then rinsed 3 times with PBS buffer plus Tween 20 and incubated for 1.30h with anti-mouse-IgG (1/8000) at room temperature. Protein bands were acquired using the instrument SF Launcher and processed using ImageQuant TL; data were expressed in percentage on total protein detected. Normality of data distribution was tested by Kolmogorov-Smirnov test. Differences between muscles were carry out using paired samples t-test procedure. Pearson coefficients were used to determine associations between variables.

Table 1. Primer sequences (5' to 3') and amplification products

Gene	Primers Forward-Reverse, Amplicon (A; bp), Accession Number (AN), Efficiency (E), R^2
CAPN1	F:AACCGGATCCGGAATTACCTGTCCATCTTC R:GTAACCACTTAAACAAGTCAAAGGTCACCA A:282; AN:NM_174259; E:0.98; R^2: 0.99
CAPN2	F:CGAGGACATGCACACCATTGGCTTCG R:TCCTCGCTGATGTCAATCTCGTCAATGTTG A:314; AN: NM_001103086.1; E:1.00; R^2: 0.99
CASP3	F:AGAACTGGACTGTGGTATTGAGA R:CACAAAGAGCCTGGATGAAC A:167; AN: NM_001077840.1; E: 1.00; R^2: 0.99
CASP9	F:CCTGTGGTGGAGAGCAGAAAG R:CATCTGGCTCGTCAATGGAA A:134; AN: NM_001205504.1; E: 0.99; R^2: 0.99
HSP70	F:AACAAGATCACCATCACCAACG R:TCCTTCTCCGCCAAGGTGTTG A:274; AN: NM_00174550; E: 0.98; R^2: 0.99
HSP27	F:CGTTGCTTCACTCGCAAATA R:TACTTGTTTCCGGCTGTTCG A:210; AN: NM_001025569.1; E: 0.92; R^2: 0.99
HSP40	F:GGACTGACCATTGCTGCTG R:CAAACCCACCTCTGTAATAGC A:138; AN: NM_001034458.1; E: 1.00; R^2: 0.99
Cycloph	F: GGATTTATGTGCCAGGGTGGTGA R: CAAGATGCCAGGACCTGTATG A: 119; AN: NM _00178320; E: 1.00: R^2: 0.99
β-actin	F: CTCTTCCAGCCTTCCTTCCT R: GGGCAGTGATCTCTTTCTGC A:177; AN: NM_00173979; E: 0.96; R^2: 0.99
GAPDH	F: TCATCCCTGCTTCTACTGGC R: CCTGCTTCACCACCTTCTTG A:177; AN:NM_001034034; E:0.93; R^2: 0.99
RPLP0	F: CAACCCTGAAGTGCTTGACAT R: AGGCAGATGGATCAGCCA A: 226; AN: NM_001012682; E: 0.93; R^2: 0.99

III. RESULTS AND DISCUSSION

IS showed higher pH_{48} (5.72 *vs.* 5.52; P<0.01) and WBSF (30.27 *vs.* 20.20 N; P<0.01; data not tabulated) than LD on raw samples; highlighting a probable different proteolysis between muscles. αII Spectrin is a muscle structural protein that is degraded during proteolysis, its full length is 250 kDa (SBDP250). SBDP250 level was higher in IS than LD (P<0.01; Figure 1), it means that, at slaughter, αII spectrin was less degraded in IS than LD, consistent with WBSF results.

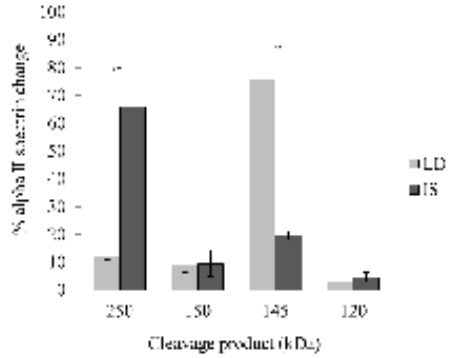

Figure 1. Cleavage products of αII spectrin in *Longissimus dorsi* (LD), *Infraspinatus* (IS) muscle at slaughter. Data expressed in percentage on the total protein detected.**P<0.01.

μ-Calpain (CAPN1) and m-calpain (CAPN2) are responsible for *post-mortem* tenderization being involved in muscle proteolysis in presence of calcium. CAPN1 mRNA abundance was slightly higher in LD than IS (P=0.08), while CAPN2 expression was similar between muscles (P>0.05; Table 2). 150 kDa peptide (SBDP150) and 145 kDa peptide (SBDP145) derived from αII spectrin cleavage. However SBDP145 is an indirect indicator of CAPN1 activity, while SBDP150 can derive both by CAPN1 and caspase activity. As reported in Figure 1, SBDP150 level was similar between muscles (P>0.05), while SBDP145 was higher in LD than IS (P<0.01), suggesting that CAPN1 was more active in LD than in IS, that is consistent with the transcriptomic and WBSF analysis. According to the fiber-type composition, IS is classified as a red muscle (rich in type I fiber), while LD as white muscle (rich in type IIB fiber). Ouali *et al.* [9] showed higher CAPN2 content in red muscle, Muroya *et al.* [10], considering both red and white muscles, had not find differences in CAPN1 and CAPN2 mRNA level. Nowak [11] reviewed that CAPN1 plays a role in the beginning of meat tenderization, while CAPN2 contribute later, and are regulated by calpastatin.

Caspases favors death cell process. CASP9 is activated in response of hypoxia and ischemia related to physiological post-slaughter condition and actives CASP3, which is considered an effector caspase. CASP3 and CASP9 mRNA abundance was similar between muscles (P>0.05; Table 2). From αII spectrin cleavage, 120kDa degradation product (SBDP120) is an indirect indicator of CASP3 activity. Differences in SBDP120 level between muscles were not found (P>0.05; Figure 1). Conversely to Kemp *et al.* [12], correlation between WBSF and SBDP120 was not found in LD neither in IS (P>0.05). Taking into account our results, CASP3 *post-mortem* activity seems not influence the WBSF variability between and within muscles. Underwood *et al.* [13] claimed that CASP3 is not involved in the *post-mortem* tenderization of beef. Positive correlations were found between CAPN1 and CASP9 mRNA abundance both in IS (r=0.680; P=0.03) and LD (r=0.507; P=0.04), confirming the hypothesis that these enzymes are involved in the early *post-mortem* proteolysis [12]. From western blotting analysis, SBDP145 level was higher than SBDP120 both in LD (P<0.01) and IS (P<0.01; Figure 1), it means that the activity of CAPN1 was higher than that of CASP3 in both muscles. In our study significant correlations between SBDP120 level and CASP3 mRNA abundance was not found, however a slightly positive correlation between SBDP145 level and CAPN1 mRNA abundance was highlighted (r=0.355; P=0.08).

Heat Shock Proteins (HSPs) are released by cell in response to hypoxia and ischemia conditions with the aim to preserve the cell functionality inhibiting directly apoptosis. LD showed lower level of HSP27 (P<0.01) and HSP70 (P<0.05), but similar (P>0.05) mRNA level of HSP40, a co-chaperone of HSP70, than IS. Our results are not in agreement with a previous study carried out in pig [14], where white muscles showed higher expression of HSP27 than red ones, but

without statistical significance. However, considering the anti-apoptotic activity of HSPs, the lower HSP27 and HSP70 abundances found in LD than IS, seem consistent with the WBSF results. In literature controversial results concerning relationship between HSPs, HSP27 in particular, and shear force were reported [5,15]. A recent study [5] reported that HSP27 could be positively or negatively correlated to shear force depending on breed.

Table 2 Fold change ratio in relative RNA expression of genes of *Longissimus Dorsi* (LD) and *Infraspinatus* muscle (IS).

Gene	IS	LD	SEM
CAPN1	1.00	1.33	0.190
CAPN2	1.00	1.05	0.138
CASP3	1.00	1.21	0.161
CASP9	1.00	0.94	0.086
HSP70	1.00^{a}	0.68^{b}	0.129
HSP27	1.00^{A}	0.53^{B}	0.074
HSP40	1.00	1.02	0.097

$^{A,B:}$ $P<0.01$; $^{a,b:}$ $P<0.05$

IV. CONCLUSION

Tenderization process of beef is complex. Taking into account the results of the present study, the differences in WBSF between muscles seem to be mainly linked to CAPN1 activity and to HSPs early *post-mortem* expression even if some interesting tendencies were highlighted concerning CAPN1 and CASP9 expression at slaughter.

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Annex II:

Manuscript submitted to ANIMAL Journal

Activity of enzymes and expression of genes of calpain and caspase systems in two bovine muscles

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Running head: Calpain and caspase systems in bovine muscles

The objective of this trial was to determine the early post mortem activity of enzymes and the expression of some genes, belonging to calpain and caspase systems, in two muscle types in Italian Simmental young bulls. Samples of Longissimus dorsi (LD) and Infraspinatus (IS) muscles were collected within 20 min from slaughtering of 17 Italian Simmental young bulls. By SDS-PAGE and Western Blotting, the degradation products of αII spectrin and poly (ADP-ribose) polymerase-1 (PARP-1) were analyzed to assess μ-calpain (CAPN1) and caspase 3 (CASP3) activities. The mRNA abundances of CAPN1, m-calpain (CAPN2), CASP3, and CASP9 were detected by RT-PCR. Also pH after 48h, and WBSF after 7 days of ageing were measured. LD showed lower pH_{48}, WBSF, higher CAPN1 activity, and tended to have

higher CAPN1 mRNA abundance than IS. Within each muscle, CAPN1 activity was higher than that of CASP3. Differences between CASP3 activity, CASP3, CASP9 and CAPN2 mRNA abundance were not found between muscles. Considering calpain and caspase proteolytic systems in the early post mortem period, a different proteolysis between LD and IS muscles in Italian Simmental young bulls was found, this difference is mainly due to CAPN1 in respect to CAPN2, CASP3 and CASP9.

Keywords: cattle, tenderness, calpain, caspase, skeletal muscle

Implications

The tenderness of beef is one of the most important factors that can influence consumer choices. Among the factors that affect tenderness, *post mortem* proteolysis has a key role. The proteolysis process, which involves many enzymes including the calpains and caspases systems, is still not completely clear. The results of this study contribute to a deeper understanding of the role of caspases and calpaine during the early *post mortem* period on meat tenderness in two different muscles of Italian Simmental young bulls.

Introduction

From the consumer point of view, tenderness is one of most important factors able to modify the buying decision process for beef (Miller *et al.*, 2001). Tenderness is affected by many factors such as: breed, amount and solubility of collagen, intramuscular fat content, pH decline, muscle fiber type characteristics. Koohmaraie (1995) explained that 70% of tenderness was accounted for environmental and 30% for genetic factors.

In general, the tenderization process of beef has an enzymatic nature (Ouali *et al.*, 2006). Many endogenous proteolytic systems directly contribute to beef tenderness being involved in muscle myofibrillar degradation. The role of calpain on meat tenderness is well documented, conversely less and conflicting information concerning the role of caspase on meat tenderization process are available (Cao *et al.*, 2010; Underwood *et al.*, 2008). However, in general, the *post mortem* proteolysis process are not completely clear (Mohrhouser *et al.*, 2011).

Guillemin *et al.* (2011) hypothesized different biological pathways for *post mortem* proteolysis in relation to muscle types. Indeed, in relation to their function, muscles are made of different proportion of fiber type, which are able to influence tenderness of beef having different histochemistry and metabolism characteristics. Fibers type I are slow oxidative, type IIA are fast oxido-glycolytic, type IIX and IIB are fast glycolytic.

Aim of this study is to determine different activities of enzymes and expression of some genes belonging to the calpain and caspase proteolytic systems in two muscle types in Italian Simmental young bulls.

Materials and methods

Experimental design and sample collection

Seventeen Italian Simmental young bulls were randomly chosen from 1 farm, fed corn silage-based diets and slaughtered at 694 ± 11.6 kg (mean ± s.e.) of weight. At slaughter, samples of *Longissimus dorsi* muscle (LD) and *Infraspinatus* muscle (IS) were obtained within 20 min of exsanguination, frozen in N_2, and stored at -80 ºC until transcriptomic and SDS-PAGE and Western Blotting analysis. After chilling at 4 oC for 48 h, pH of LD (6th-7th ribs) and IS were measured, and samples of the same muscles were collected; after 7 days of ageing at 4 °C, Warner-Bratzler shear force (WBSF) analysis was performed.

Analysis of beef pH and shear force

pH of LD and IS were measured by a pH-meter (Crison 52-32 electrode). Shear force was measured on raw sample using a Warner-Bratzler device with a triangular hole in the shear blade, mounted on a Lloyd TAPlus texture analyser (ELIS, IT). Measurement was recorded as the peak yield force (N), required to shear, at a 100 mm/min crosshead speed, perpendicular to the direction of fibres, 7 cylindrical cross-section replicates, (15 mm diameter × 30 mm length).

Analysis of mRNA levels

RNA was extracted from 40 mg muscle using RNeasy Fibrous Tissue Mini Kit (Qiagen, DE). Concentration and purity of RNA were assessed using spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific, MA). To obtain cDNA, the iScript cDNA Synthesis kit (BioRad, CA) was used. Each 20 µl of reaction contained 4 µl of 5X iScript reaction mix, 1µl of RNase H+ MMLV-derived reverse transcriptase, 10 µL of RNA, 5 µL of nuclease-free water. The mixture was held 5 min at 25 °C, 1 h at 42 °C, and 5 min at 85 °C before being cooled at 16 °C. The cDNA was quantified using NanoDrop 2000c. A qualitative PCR was carried out to verify primers (Table 1) using the BioRad CFX96 system (BioRad, CA). In particular, 1 µl of cDNA was added to a mixture containing 0.3 µl of each forward and reverse primer (0.3 µM), 8.4 µl of sterile water, and 10 µl of iQ SYBR Green Supermix [100 mM KCl, 40 mM Tris-HCl (pH 8.4), 0.4 mM of dNTPs, 50 U/ml of iTaq DNA polymerase, 6 mM MgCl2, SYBR Green I dye, and 20 nM fluorescein; BioRad]. Amplification conditions were 1 cycle of 3 min at 95 °C, followed by 40 PCR cycles of 15 s at 95 °C, 30 s at the annealing temperature of the primers (60 °C), 30 s at 72 °C; then there was 1 min at 95 °C followed to a melt curve of 55-95 °C with 0.5 °C increments every 5 s. The amplified PCR products were examined by electrophoresis in 1.5 % (wt/vol) agarose gel. The quantitative PCR was performed using the Bio-Rad

CFX96 system (BioRad, CA), on a reaction volume of 20 µL, containing 0.3 µL of each forward and reverse primer (0.3 µM), 10 µL of iQ SYBR Green Supermix (BioRad, CA), 8.4 µL of sterile water, 1 µL of cDNA. Amplification conditions included 1 cycle of 3 min at 95 °C, 40 PCR cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C, then 1 min at 95 °C followed by a melt curve of 55-95 °C with 0.5 °C increments every 5 s. Each sample was analysed in triplicate and relative gene expression was calculated according to the efficiency-corrected method (Pfaffl *et al.*, 2001). β-actin, cyclophilin, glyceraldehyde-3-P dehydrogenas, and ribosomial protein large P0, were treated as reference genes and used for normalization of RT-qPCR data (gene expression stability value, M < 0.10). Data were presented in fold-change ratio having as reference IS; primer efficiency was calculated and verified using the standard curve obtained by serial dilution of the pooled cDNA (Pfaffl *et al.*, 2001).

SDS-PAGE and Western Blotting analysis

CAPN1 and CASP3 activities were assessed through αII spectrin and Poly (ADP-ribose) polymerase-1 (PARP-1) cleavage products. Total protein was extracted from 100 mg of each muscle. Sample were homogenized 3 times for 30 s with Ultra-Turrax® T25 in extraction buffer (0,05 M DTT, 2 % SDS, 60 mM tris-HCl, 0,2 % antiproteases), boiling for 2 min at 98 °C, mixed 30 min at room temperature and centrifuged 10 min at 11000 rpm. Protein was determinate by the Near UV Absorbance (280nm) method (Walker, 2002). Equivalent quantity of protein of each sample were subject to 8 % SDS-PAGE (Laemmli, 1970). Proteins separated via SDS-PAGE were transferred to 0.2 µm pore size nitrocellulose membranes using a semi-dry blotting apparatus (TE 22 transfer unit, Amersham Biosciences) in a transfer buffer for 1 h and 45 min for αII spectrin and PARP-1 cleavage products respectively. After staining with Ponceau S. solution (ATX Poceaus S red staining solution Fluka), to verify transfer efficiency, the nitrocellulose sheets were saturated with 3 % (w/v) non-fat dry milk in PBS buffer plus 0.1 %, Tween 20 for 1 h at room temperature and then incubated overnight with the

spectrin α II antibodies (C-3; Santa Cruz Biotechnology, inc.) or PARP-1 antibody (C-2-10; Novus Biologicals). Blots were then rinsed 3 times with PBS buffer plus Tween 20 and incubated for 1.30 h with anti-mouse-IgG (1/8000) at room temperature. Protein bands were acquired using the instrument SF Launcher and processed using ImageQuant TL. Data were expressed in percentage on total protein detected.

Statistical analysis

The statistical analysis was performed using SPSS version 17 software (SPSS Inc., Illinois) and the free software R version 2.15.2 (R core team, 2012). Normality of data distribution was tested by Kolmogorov-Smirnov test. Differences between muscles were carry out using paired samples t-test procedure. Pearson coefficients were used to determine associations between variables. A probability level of $P \leq 0.05$ was established for statistical significance.

Results and Discussion

IS showed higher pH_{48} (5.72 *vs.* 5.52; $P < 0.01$) and WBSF (30.27 *vs.* 20.20 N; $P < 0.01$; data not tabulated) than LD. According to the fibre type composition, IS is classified as a red muscle (rich in type I fiber) , while LD as white muscle (rich in type IIB fiber). The higher proportion of type IIB fiber could have induced a reduction of pH_{48} (Lee *et al.*, 2010).

αII Spectrin is an actin binding protein that is degraded during proteolysis, its full length is 250 kDa (SBDP250). SBDP250 level was higher in IS than LD ($P < 0.01$; Figure 1), it means that, at slaughter, αII spectrin was less degraded in IS than LD, in agreement with Joo *et al.* (2013) who reviewed, in the early *post mortem* period, a lower proteolysis in type I fibers respect to type IIB and consistent with WBSF results. Indeed a positive correlation between

SBDP250 and WBSF was found (r = 0.810; P < 0.01), indicating that a different early proteolysis between muscles may contribute to explain their different tenderness.

μ-Calpain (CAPN1) and m-calpain (CAPN2) are responsible for *post mortem* tenderization being involved in muscle proteolysis in presence of calcium. CAPN1 mRNA abundance was slightly higher in LD than IS (P = 0.08), while CAPN2 expression was similar between muscles (P > 0.05; Table 2). 150 kDa peptide (SBDP150) and 145 kDa peptide (SBDP145) derived from αII spectrin cleavage. However SBDP145 is an indirect indicator of CAPN1 activity, while SBDP150 can derive both by CAPN1 and caspase activity. As reported in Figure 1, SBDP150 level was similar between muscles (P > 0.05), while SBDP145 was higher in LD than IS (P < 0.01), suggesting that CAPN1 was more active in LD than in IS, that is consistent with the mRNA and WBSF analysis. Ouali and Talmant (1990) showed higher CAPN2 content in red muscle, Muroya *et al.* (2012), considering both red and white muscles, had not find differences in CAPN1 and CAPN2 mRNA level. Negative correlations between WBSF and SBDP145 (r = 0.736; P < 0.01) and between WBSF and CAPN1 mRNA abundance (r = 0.311; P = 0.04) were highlighted. Conversely correlations between WBSF and CAPN2 mRNA abundance was not found (P > 0.05), confirming that CAPN1 plays an important role in the meat tenderization process (Guillemin *et al*., 2011). However, Nowak (2011) reviewed that CAPN1 plays a role in the beginning of meat tenderization, while CAPN2 contribute later. A slightly positive correlation between SBDP145 level and CAPN1 mRNA abundance was highlighted (r = 0.355; P = 0.08).

Caspases favors death cell process being involved in apoptosis process. CASP9 is activated in response of hypoxia and ischemia related to physiological post slaughter condition and actives CASP3, which is considered an effector caspase. CASP3 and CASP9 mRNA abundance was similar between muscles (P > 0.05; Table 2). From αII spectrin cleavage, 120kDa degradation product (SBDP120) is an indirect indicator of CASP3 activity. Differences in SBDP120 level between muscles was not found (P > 0.05; Figure 1). Also Cao *et al.* (2010),

considering three skeletal muscles of bulls, *Longissimus*, *Semitendinosus*, and *Psoas minor*, have not shown differences in the CASP3 activity in the early *post mortem* period. In our study significant correlations between SBDP120 level and CASP3 mRNA abundance was not found ($P > 0.05$). Yang *et al.* (2012), reported that, in cattle, caspases are regulated at post translational level. From western blotting analysis, SBDP145 level was higher than SBDP120 both in LD ($P < 0.01$) and IS ($P < 0.01$), it means that the activity of CAPN1 was higher than that of CASP3 in both muscles. In the present study the activity of CASP3 was also studied through the assessment of PARP-1 cleavage product. PARP-1 is a nuclear protein which has a key role for repairing the DNA damage. After activation, caspase 3 is able to cleave PARP-1 and produced two fragments of 89 kDa and 24 kDa length. In the present study, the protein level of 89 kDa length fragment was detected, and it was similar between LD and IS muscles (49.9 *vs.* 50.9 %; $P > 0.05$; data not reported in Tables). This result means that the CASP3 activity was similar between muscles, moreover the presence of 89 kDa length fragment found (50.4 % of the total protein detected) indicated that CASP3 was active in both muscles in the early *post mortem* period, confirming the αII spectrin cleveage products analysis results, and in agreement with the results of Kemp *et al.* (2006) and Cao *et al.* (2010) obtained in pig and bulls respectively. However, conversely to Kemp *et al.* (2010), correlation between WBSF and SBDP120 was not found either in LD nor in IS ($P > 0.05$). Also Underwood *et al.* (2008) have not shown a correlation between CASP3 activity and WBSF in *Longissimus thoracis* muscle of steers. Taking into account our results, CASP3 seems active in the early *post mortem* period, but it does not play a primary role in the meat tenderization process in the two bovine skeletal muscles considered.

In summary, the results of the present study indicated a different early *post mortem* proteolysis between LD and IS muscles in Italian Simmental young bulls; considering the calpain and caspase proteolitic systems, this difference is mainly due to CAPN1 in respect to CAPN2, CASP3 and CASP9.

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Table 1 *Primer sequences (5' to 3') and real-time amplification products*

Gene	Primers, Forward-Reverse	Amplicon (bp)	Accession No.	R2	Efficiency
CAPN11	F:AACCGGATCCGGAATTACCTGTCCATCTTC R:GTAACCACTTAAACAAGTCAAAGGTCACCA	282	NM_174259	0.992	0.98
CAPN22	F:CGAGGACATGCACACCATTGGCTTCG R:TCCTCGCTGATGTCAATCTCGTCAATGTTG	314	NM_001103086.1	0.995	1.00
CASP33	F:AGAACTGGACTGTGGTATTGAGA R:CACAAAGAGCCTGGATGAAC	167	NM_001077840.1	0.995	1.00
CASP94	F:CCTGTGGTGGAGAGCAGAAAG R:CATCTGGCTCGTCAATGGA*A	134	NM_001205504.1	0.999	0.99
Cycloph.5	F: GGATTTATGTGCCAGGGTGGTGA R: CAAGATGCCAGGACCTGTATG	119	NM _00178320	0.993	1.00
β-actin	F: CTCTTCCAGCCTTCCTTCCT R: GGGCAGTGATCTCTTTCTGC	117	NM_00173979	0.997	0.96
GAPDH6	F: TCATCCCTGCTTCTACTGGC R: CCTGCTTCACCACCTTCTTG	177	NM_001034034	0.998	0.93
RPLP07	F: CAACCCTGAAGTGCTTGACAT R: AGGCAGATGGATCAGCCA	226	NM_001012682	0.998	0.93

1CAPN1 = μ-calpain

2CAPN2 = m-calpain

3CASP3 = caspase 3

4CASP9 = caspase 9

5Cycloph. = cyclophilin

6GAPDH = glyceraldehyde-3-phosphate dehydrogenase

7RPLP0 = ribosomial protein large P0

Table 2 *Fold change ratio in relative RNA expression of genes of Longissimus dorsi (LD) and Infraspinatus muscle (IS), groups were compared to IS group (n=34)*

Gene	IS	LD	SEM
CAPN11	1.00α	1.33β	0.190
CAPN22	1.00	1.05	0.138
CASP33	1.00	1.21	0.161
CASP94	1.00	0.94	0.086

α,β: $P < 0.10$

1CAPN1 = μ-calpain

2CAPN2 = m-calpain

3CASP3 = caspase 3

4CASP9 = caspase 9

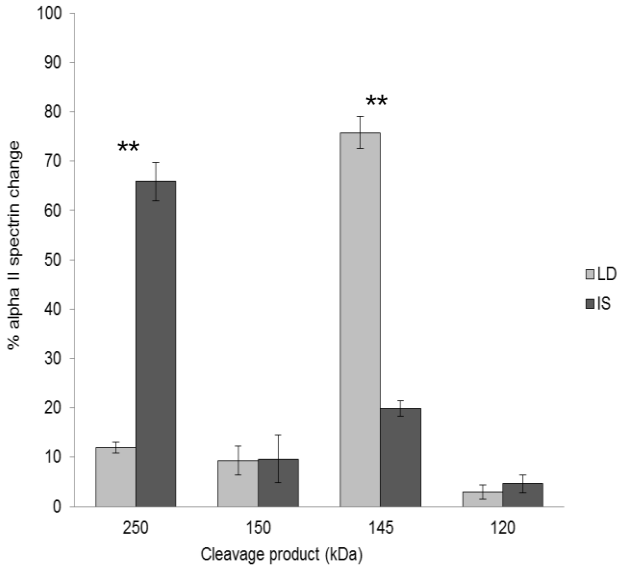

Figure 7 Cleavage products of αII spectrin in Longissimus dorsi (LD), Infraspinatus (IS) muscle at slaughter. Data expressed in percentage ± se on the total protein detected.**: P < 0.01

Annex III:

Oral presentation at the XX ASPA NATIONALCONGRESS - Associazione Per La Scienza E Le Produzioni Animali-Bologna, 11-13 June 2013

The caspase and calpain proteolytic systems in *Longissimus dorsi* and *Infraspinatus* muscles of Italian Simmental young bulls

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Calpains and capsases are two families of cytosolic proteases essential for a proper skeletal muscle function, which recognize specific target proteins for degradation. Moreover, μ-calpain is believed to play a major role in *post mortem* proteolysis and meat tenderization, which in turn depends on fibre type composition. Recent data also support the involvement of caspases in the development of tender meat. In fact, it is plausible that the hypoxic conditions occurring in *post mortem* may activate these proteases, which function as vital executioners of apoptosis in hypoxia/ischemia and degrade structural components of the cytosckeleton. The goal of this work was to evaluate the activities of μ-calpain and caspases in two different muscle types from bulls, immediately after slaughtering by immunodetection of their specific proteolytic products and to compare such activities to their related mRNA expression level. In particular, caspases 9 and 3 have been analysed, which are an initiating and an executer caspase, respectively, to follow the entire cascade of events occurring during a hypoxic stress.
Samples of *Longissimus dorsi* (white/type IIB muscle) and *Infraspinatus* (red/type I muscle) muscles were collected within 20 minutes from slaughtering of 16 Italian Simmental young bulls, and stored at -80° until the analysis. By SDS-PAGE the alpha II spectrin and its degradation products 145kDa (from μ-calpain degradation) and 150kDda and 120kDda (from caspases degradation) were separated and then quantified in relative terms. Total RNA was extracted from the two muscles and cDNA was produced; the DNA was amplified by q-PCR technic. The genes analyzed were CAPN1 (coding for μ-calpain), CASP3 (caspase 3), CASP9 (caspase 9). GAPDH, β-Actin, RPLP0 and Cyclophilin was used as reference genes.
Both caspases and μ-calpain were found active, having been recognized their target degradation products. More specifically, in each animal no difference in the level of alpha II spectrin fragment, derived from the caspase-3, activity was found between the *Longissimus dorsi* and *Infraspinatus* muscles. On the other hand, the μ-calpain activity appeared to be influenced by the type of muscles. These functional data were confirmed by the gene expression assays.