Biomarkers of meat tenderness: Present knowledge and perspectives in regards to our current understanding of the mechanisms involved

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Abstract

Biomarkers of the meat quality are of prime importance for meat industry, which has to satisfy consumers' expectations and, for them, meat tenderness is and will remain the primary and most important quality attribute. The tenderization of meat starts immediately after animal death with the onset of apoptosis followed by a cooperative action of endogenous proteolytic systems. Before consideration of the biomarkers identified so far, we present here some new features on the apoptotic process. Amongst them, the most important is the recent discovery of a complex family of serpins capable to inhibit, in a pseudo-irreversible manner, caspases, the major enzymes responsible of cell dismantling during apoptosis. The biomarkers so far identified have been then sorted and grouped according to their common biological functions. All of them refer to a series of biological pathways including glycolytic and oxidative energy production, cell detoxification, protease inhibition and production of Heat Shock Proteins. Some unusual biomarkers are also presented**:** annexins, galectins and peroxiredoxin. On this basis, a detailed analysis of these metabolic pathways allowed us to identify some domains of interest for future investigations. It was thus emphasized that mitochondria, an important organelle in the production of energy from carbohydrates, lipids and proteins are a central element in the initiation and development of apoptosis. It was therefore stressed forward that, in fact, very little is known about the *postmortem* fate of these organelles and their multiple associated activities. Other topics discussed here would provide avenues for the future in the context of identifying reliable predictors of the ultimate meat tenderness.

Key words: biomarkers, tenderness, metabolism, muscle cell, meat quality, apoptosis

Highlights: Potential Biological markers of meat tenderness

Contents

1. Introduction

Among the different qualities of meat, tenderness is and will always be the first quality sought by the consumer. Since decades, the mechanisms of meat tenderization have focused much interest from meat scientists. Despite these extensive efforts, these mechanisms are still unclear and this probably explains the fact that we have not yet identified a good marker of this quality (Kemp, Sensky, Bardsley, Buttery, & Parr, 2010).

Nevertheless, there is now a general agreement on two major questions: (1) the meat tenderizing mechanisms are enzymatic in nature and involve several intracellular proteolytic systems; (2) the first step of the conversion of muscle into meat is the onset of apoptosis, a finely regulated and complex energy dependent cell death process (Ouali, et al., 2006). In this context, the first proteolytic system likely to be involved is the group of initiator caspases followed by executor caspases, responsible for the degradation of proteinaceous cell constituents. Other proteolytic systems including calpains, proteasome, cathepsins, matrix metalloproteases, thrombin, plasmin etc … will then participate to the cell dismantling process but we still do not know in what order and to which extent, even if some of them are suspected to be able to activate some caspases at least *in vitro*. In this respect, we must keep in mind that *in vitro* successful protease substrates are not necessarily *in vivo* target substrates of the protease considered.

On the other hand, quality markers would be a reflect of the different metabolic pathways contributing to the *postmortem* development of meat tenderness. Hence, a better understanding of these pathways and their interactions is a prerequisite for a successful identification of accurate biological/biochemial markers of this primary quality attribute of meat. In this respect, the advent of modern proteomic technologies has undoubtedly contributed to a better understanding of these processes (D'Alessandro & Zolla, 2013).

In this review, we will first try to update of the mechanisms responsible for the *postmortem* improvement of meat tenderness with some new features according to research carried out during last years. Then we will overview the potential markers of meat tenderness identified

so far and the biological structure(s)/pathway(s) to which they are related. Finally, we will conclude this report by a series of perspectives to improve our knowledge about these mechanisms and the most interesting points to be investigated in the near future for a more efficient search of the best biomarkers of meat tenderness.

2. Meat tenderization mechanisms: some new features

As commented above, meat tenderization is a complex process still not clearly understood. With refinement of the techniques, a large set of new features have been reported during last years, thus making the actual concept of meat tenderization somewhat confusing and much more complex than expected. Nevertheless, we think that there is a consensus on the two features commented above: (1) the multi-enzymatic nature of meat tenderization, and (2) the onset of apoptosis as the first step in the conversion of muscle into meat.

2.1. Major Events following animal bleeding

After animal bleeding, tissues come into an ischemic anoxic state which will affect all metabolic pathways and will lead to an adaptation of most, if not all, metabolic processes (Ouali, et al., 2006; Ouali, et al., 2007). In other words, the cell will develop contradictory tools for cell death or cell survival pathways (Figure 1).

The first objective for the cell after entering into the anoxia state will be to improve its capacities to provide the energy needed for increased metabolic activities. As observed by quantitative analysis of 2D gel spots, this led to an increase in the enzyme associated to glycolytic and tricarboxylic acid (TCA) cycles. A major consequence of this intense metabolic activity will be the accumulation of diverse harmful byproducts including CO_2, HCO_3^-, NH_4 and lactic acid, which are normally transported to the liver where they can be recycled. Later in the context we will consider the energy aspects in the last part of this review as it could be a good source for finding new biomarkers of meat tenderness.

The second step after animal bleeding will be the preservation of cell functions by an increase in the concentration of several Heat Shock Proteins (HSP) including HSP 70, 40, 27, 20, αβ-crystallin, and probably others.

The battle between cell death and cell survival will finally turn to the advantage of the cell death process with the well-known characteristic changes associated to this status, especially cell shrinkage, and phosphatidylserine externalization, together with mitochondria alteration (Becila, et al., 2010; D'Alessandro & Zolla, 2013; Ouali, et al., 2007). A set of pro- and anti-apoptotic proteins will be released from mitochondria and their ratio will define the rate and extent of apoptosis development. For the same reason than for the energy aspects,

mitochondria will be reconsidered later in this review. So it could be interesting to develop studies on mitochondria, in order to establish whether this could be a way to better explain tenderness variability of carcasses.

2.2. Are mono-nucleated cells the first to die in postmortem *muscle?*

2.2.1. Characteristic features of apoptosis in muscle cells

A specific feature of skeletal muscle fibres is being multinucleated in the form of successive muscle-fibre segments controlled by separate nuclei residing in the segments. It has been shown in experimental denervation as well as in infantile spinal muscular atrophy that a minority of muscle fibres reveal apoptotic DNA-fragmentation at the same time (Tews, et al., 1997).

On this basis, we might expect a very different image of muscle cells as compared to dying mononucleated cells generally found in the extracellular matrix (fibroblasts, epithelial cells of blood vessels, adipocytes,…) which might be easier to identify in muscle tissue. The nature of the structural alterations characterizing the death of muscle fibres in *postmortem* muscle is therefore an opened question. To verify this assumption, we looked at muscle fibres in *postmortem* muscle hoping to detect DNA alteration using an antibody labeling specifically regular single-stranded fragments characterizing cell apoptosis but not double stranded fragments observed in necrosis (Ouali, et al., 2007).

2.2.2. Particular structural apoptotic changes in postmortem muscle

The following results were obtained using rat as a model. The animal was anesthetized by etheroxyde and a sample of the *Longissimus* muscle was cut off and immediately frozen for subsequent immunohistochemical examination. The animal was then bled by decapitation, dressed and the carcass stored at +10°C up to 24 h *postmortem* and then transferred to 4°C. Samples were taken at different times after animal death, i.e. just after bleeding, at 15, 30, 60 min *postmortem* and then at 2, 4, 7, 24 and 48 h *postmortem*. Thin cuts were stained using the traditional Hematoxylin/ Erythrosin method and finally labeled with a monoclonal antibody (apostain) labeling specifically single-stranded DNA fragments of regular length characterizing apoptotic cells (Prochazkova, Kylarova, Vranka, & Lichnovsky, 2003).

Examination of the thin cuts was done with the aim to identify structural changes characteristic of the programmed cell death or apoptosis. As compared to Figure 2a where muscle cells are in close proximity to each other, few minutes after bleeding an important shrinkage of muscle cells can be observed (Figure 2b, small arrows). Over the past decades,

much work was devoted to the *postmortem* evolution of intra- and extracellular spaces in relation with intracellular water movements in the muscle (Offer & Knight, 1988a, 1988b). It was generally recognized that the main cause of these changes was the distribution between the two compartments of water, which accounts in weight for approximately 75% of muscle tissue. Acidification of muscle decreases protein electrical charge and induces an increase in their hydrophobicity, thereby reducing water retention. This is confirmed by the very high correlation observed between the increase in extracellular space and muscle pH (Guignot, Vignon, & Monin, 1993). As pointed out by these last authors, the only still unexplained point was the early increase in extracellular space, starting immediately after slaughter, whereas pH was still very close to neutrality. We have recently reported that cell death could provide a realistic explanation of the early volume change in the extracellular compartment in *postmortem* muscle (Becila, et al., 2010). This finding constitutes the first evidence supporting the early increase in the extracellular space resulting from the expulse of intracellular water towards the extracellular compartment whereas the pH is still high. Hence, cell death would provide a more realistic explanation of the early volume change in the extracellular compartment in *postmortem* muscle.

In Figure 2c, DNA fragmentation was detected in mononucleated cells located within the extracellular matrix. Note the important accumulation in the close proximity of other cells which could correspond to resident macrophages, also called ED2 and ED3 macrophages, known to be unable to undertake degenerative cells phagocytosis (McLennan, 1993). Interestingly, such cells can be observed above and below where they seemed to delineate empty cells or blood vessels (Becila, et al., 2010). The alignment of these suspected resident macrophages around the dyed cells might contribute to the isolation of these cells from their neighbors as soon as they are engaged in the "suicide" program. Macrophages are attracted by the cells engaged in apoptosis through the translocation of phosphatidylserine to the external leaflet of the membrane, the major signal of its suicide status (Martin, et al., 1995). By contrast, no DNA fragmentation seemed to take place in muscle cells nucleus.

Figure 2d, also taken from a 15 min sample, emphasized the DNA fragmentation occurring in an apoptotic mononucleated cell and further confirmed the accumulation of resident macrophages in the close proximity of the dying cell and also, above, aligned close to the muscle cell membranes (open arrow). Interestingly, in cuts used for extracellular space measurement on rat samples fixed but no stained (Becila, et al., 2010), we sometimes observed the presence of muscle cell membrane invaginations suggesting that they are ingesting extracellular material or dying small cells by phagocytosis for further degradation

within muscle cells [insert Figure 2D at a low (left) and a slightly higher (right) magnification] supporting the occurrence of phagocytotic activities in *postmortem* muscle. From these findings, it must be therefore stressed that the first cells to die *postmortem* are mononucleated cells from the extracellular matrix, which might be degraded by muscle cells after ingestion by phagocytosis.

2.3. New inhibitors of both caspases and apoptosis

In the 80's, the presence of serine proteases within muscle cells was doubtful and a large set of investigations suggested that some of them if not all originated from mast cells (Ouali, 1990). As a result this protease family was ignored by meat scientists. Later on, serine protease inhibitors have been suggested to be a good marker of meat tenderness (Zamora, et al., 2005; Zamora, et al., 1996), a result strengthening the early proposal that inhibitors are better predictors of tenderness than their target enzymes (Ouali, 1990). As shown in Figure 3a, detailed investigations carried out on these serine protease inhibitors revealed a complex protein family belonging to the serpin superfamily (Boudida, Gagaoua, et al., 2013; Gagaoua, et al., 2012; Pelissier, et al., 2008; Sentandreu, Coulis, & Ouali, 2002).

Serpins, an acronym of **SER**ine **P**rotease **In**hibitors, were discovered in the beginning of the 80's. This superfamily comprise the largest family of protease inhibitors identified to date, now having over 3000 members in all the three kingdoms of life, the archea, the bacteria, and the eukaryotes, as well as in some viruses (Olson & Gettins, 2011).

Besides elestase and trypsin, the serpins identified by our group inhibit pseudo-irreversibly initiator and effector human caspases (caspases 8 and 3) but neither papain nor cathepsins, forming stable complexes with all inhibited proteases including these multimeric proteases containing two active sites per molecule (Figure 3b) (Gagaoua, et al., 2012; Herrera-Mendez, et al., 2009). In addition, we purified an inhibitor of thrombin, an enzyme located at the level of muscle synapses, identified as antithrombin III by mass spectrometry peptide map (Herrera-Mendez, et al., 2010). In this tissue, thrombin is synthesized by muscle cells (Citron, Smirnova, Zoubine, & Festoff, 1997) and acts locally by contributing to synapse remodeling and elimination at the neuromuscular junction (Citron, et al., 1997; Liu, Fields, Festoff, & Nelson, 1994; Zoubine, Ma, Smirnova, Citron, & Festoff, 1996).

Using different approaches, several studies support a degradation of a large set of myofibrillar and cytoskeletal proteins (titin, nebulin, desmin, spectrin, desmin, troponin T, …) by caspases in *postmortem* muscle, as well as other proteins specifically targeted by these proteases during apoptosis (spectrin and poly (ADP-ribose) polymerase or PARP). Note that

most of these proteins are either cytoskeletal proteins or other proteins with proapoptotic activities (Chen, Shun, Zhang, & Gao, 2003; Huang, Huang, Xu, & Zhou, 2009; Kemp, et al., 2010). SERPINA3 like, a group of serpin inhibiting caspases, were assumed to be associated with meat tenderness (Gagaoua, et al., 2012; Zamora, et al., 2005; Zamora, et al., 1996) but also to other quality attributes including drip loss and extent of pH drop (te Pas, et al., 2013). Further investigations should be done to understand the real relationship of these inhibitors and meat quality attributes.

3. Potential biomarkers of meat tenderness identified up to date

For many decades, meat scientists are looking for accurate biological markers of meat tenderness that would make possible: (1) the classification of meat cuts soon after slaughter on the basis of their potential ultimate tenderness and (2) the optimization of the genetic selection of meat animals on the basis of this quality. These challenges have aroused a great interest in this research field by meat scientists. Unfortunately, we still have not identified such markers probably because of a too much limited understanding of the biological mechanisms responsible for the *postmortem* improvement of meat texture.

Overview of the literature has allowed us to identify a series of potential markers of meat tenderness which have been then screened according to the metabolic or biological process they are involved in. This list is far from being exhaustive and numerous other proteins remain to be considered. Such screening has allowed the identification of 8 subgroups, the three most important being glycolytic and oxidative energy supplying pathways together with Heat Shock Proteins (HSPs).

3.1. Markers from the glycolytic energy metabolism pathway

All members of this selected group are enzymes of the glycolytic pathway (Table 1). Glycolysis is a two-phase process. The first phase consuming 2 ATP, corresponds to the conversion of glucose-1-phosphate, generated from either free glucose or from glycogen by phosphorylase, to two trioses phosphate. The second one ensures the conversion of 2 trioses-phosphate (from 1 glucose) to pyruvate and then lactate, producing 2 ATP and one $NADH,H^+$ molecules.

Two enzymes, out of 5, of this first phase were identified as potential markers of tenderness. These are the first and the last of this phase, i.e. Phosphoglucomutase (Bjarnadottir, Hollung, Faergestad, & Veiseth-Kent, 2010; Bouley, Chambon, & Picard, 2004; Chaze, et al., 2013; Laville, et al., 2009) and Triosephosphate isomerase (N. K. Kim, et al.,

2008). In muscle cells, the predominant triose is dihydroxyacetone phosphate (DHAP), an important metabolite at a cross-road between several metabolic pathways (Figure 4).

Besides the normal continuity of the glycolytic pathway through isomerization to glyceraldehyde-3-P (second phase of glycolysis), DHAP can also enter the glycerol–phosphate shuttle (shuttle between cytosolic and mitochondrial glycerol-3-phosphate dehydrogenase), which plays an important role in skeletal muscle (Kalapos, 1999). This shuttle is indeed used to rapidly regenerate NAD necessary for glycolysis and, hence, speed up this pathway. Increased levels of DHAP can also activate methylglyoxal synthase and DHAP then enters the glyoxal pathway which produces methylglyoxal, a toxic component for the cell (Thornalley, 2008). This toxic metabolite can further follow a two-step conversion to lactate, a chain of reactions catalyzed by either lactoylglutathione lyase (identifed as a potential marker of tenderness: see hereafter cell detoxification markers) and Hydroxyacylglutathione hydrolase or by Methylglyoxal reductase and aldehyde dehydrogenase.

Contrary to the first phase, a majority of the enzymes involved in the second phase of glycolysis (5 out of 6) were identified as good markers of tenderness. These were glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (Jia, Hildrum, et al., 2006), enolase or phosphopyruvate hydratase (Choi, et al., 2010; Laville, et al., 2009), pyruvate kinase (Laville, et al., 2009; Polati, et al., 2012) and lactate dehydrogenase (Choi, et al., 2010; Jia, Hollung, Therkildsen, Hildrum, & Bendixen, 2006; Laville, et al., 2009; Polati, et al., 2012). A surprising feature of this second phase is the multifunctional role of GAPDH which is present in different compartments of the cell and can contribute to diverse adverse cellular functions. It could thus have a pro-apoptotic (Tarze, et al., 2007) or a pro-survival function (Colell, et al., 2007).

Aldehyde dehydrogenase, another potential marker increasing in *postmortem* muscle, contributes to the glycolytic pathway through the direct conversion of glyceraldehyde to 2-phosphoglycerate (Jia, Hildrum, et al., 2006). This enzyme is a member of the aldehyde dehydrogenase family, which is known to be implicated in multiple other processes including amino acids and fatty acid metabolism (Vasiliou, Pappa, & Estey, 2004). Some members of this family of aldehyde oxidase protect against aldehydes generated by lipid peroxidation and overall aldehydes cytotoxicity and against both oxidative and osmotic stress (Brocker, Cantore, Failli, & Vasiliou, 2011; Brocker, et al., 2010; Pappa, et al., 2005).

3.2. Markers from the oxidative energy metabolism pathway

Schematically, oxidative energy metabolism ends up within the mitochondrion matrix where ultimate degradation products of lipids, amino acids and polysaccharides are oxidized, providing substrates to the electron transport chain and to the ATP synthase responsible for ATP production. In other words, this pathway uses all ultimate products provided by degradation of sugars, especially glucose (glycolysis), proteins (and more accurately amino-acids coming from either the pool of free amino acids or from protein hydrolysis) and fatty acids generated from triglycerides. Seven markers of tenderness coming from these energy supplying pathways have been so far identified (table 2).

- 3-hydroxyisobutyrate dehydrogenase is an enzyme involved in the degradation of the branched amino acid valine to succinyl-CoA, a member of the tricarboxylic acid cycle (TCA) cycle (Jia, et al., 2007; Jia, Hildrum, et al., 2006; Lokanath, et al., 2005).
- β-hydroxyacyl CoA-dehydrogenase (HADH) is a member of the β-oxidation of lipids, also known as the Lynen cycle (or Lynen helix) which produces acetyl-CoA, a metabolite entering the TCA cycle (Hamill, et al., 2012; Polati, et al., 2012).
- Cytochrome c is a member of the electron transport chain located outside the inner membrane of mitochondria (Ding, Shen, & Ong, 2002).
- The three other markers identified from this pathway, i.e. Succinate dehydrogenase, Succinyl Co-A synthase and Isocitrate dehydrogenase, are all members of the TCA cycle (Hamill, et al., 2012; Hollung, Veiseth, Jia, Faergestad, & Hildrum, 2007; Jia, Hildrum, et al., 2006; G. D. Kim, Jeong, Moon, Hwang, & Joo, 2009; Zapata, Zerby, & Wick, 2009).

From these results, it can be stressed forward that all cellular components including sugars, amino acids (from the free pool or from protein degradation) and lipids could concomitantly be used for energy production in *postmortem* muscle. Regarding mitochondrial enzymes, we might wonder on how the enzymes concentration increases *postmortem*. Whether this concentrations change result from an up regulation of the proteins' expression or from a multiplication of the number of mitochondria by fission as suggested for apoptotic cells remains an opened question. Supporting this last hypothesis, several studies indeed showed that mitochondrial morphology changes during apoptosis, resulting in small, round and more numerous organelles. They further pointed out that mitochondria fission occurs early in the cell death process (Suen, Norris, & Youle, 2008; Youle & Karbowski, 2005).

3.3. Markers involved in cell detoxification

In living cells, several waste metabolites are produced by cell metabolism. Some of them are normally eliminated directly or after blood transport to liver. The most important are:

- NH_4 generated during amino acids degradation for energy production, which is recycled, *in vivo*, through the urea cycle in the liver,
- Methylglyoxal, a byproduct of DHAP,
- Carbon dioxide (CO_2) released from the TCA cycle and during the conversion of glycerate to acetyl-CoA;
- Carbonic acid (H_2CO_3) formed from CO_2 conversion by Carbonic anhydrase;
- Toxic Aldehydes.

In *postmortem* muscle, some enzymes are able to metabolize more or less these metabolites. Carbonic anhydrase can catalyze the conversion of CO_2 to bicarbonate which could explain, at least in part, the discontinuity observed in the pH profiles. Lactoylglutathione lyase, or Glyoxylase 1, is able to catalyze the first step of the conversion of methyglyoxal to lactate. Finally, besides its function in glycolysis (Jia, Hildrum, et al., 2006), some members of the complex Aldehyde dehydrogenase family (Vasiliou, Thompson, Smith, Fujita, & Chen, 2012), could also protect cells against cytotoxic effects of various aldehydes accumulating in the cytosol (O'Brien, Siraki, & Shangari, 2005). It is worth noting that these three enzymes have been identified as potential markers of tenderness (Table 3).

Regarding ammonium ions (NH_4^+), these are normally recycled in liver (glucose-alanine cycle) where it enters the urea cycle. *Postmortem*, this transportation is no more available and thus ammonium ions will accumulate in muscle tissue. These ions can be then used in different ways:

- For alanine synthesis :

  pyruvate (limiting substrate) + NH4 → alanine

- Or for synthesizing glutamate from alpha-ketoglutarate

  NH4 + alpha-ketoglutarate (limiting substrate) → glutamate

- Or used by glutamine synthetase to convert glutamate to glutamine (Adeva, Souto, Blanco, & Donapetry, 2012).

3.4. Markers from the Heat Shock Protein family

Whatever its nature, stress induces synthesis of protective proteins called Heat Shock Proteins (HSPs) which preserve cellular proteins against denaturation and possible loss of function (Kultz, 2003). The many known Heat Shock Proteins are generally classified in subfamilies on the basis of their size (molecular weight in kDa): Hsp 90, Hsp 70, Hsp 40, Hsp

27, etc. Most of them play an important role as molecular chaperones during protein assembly (Haslbeck, Miess, Stromer, Walter, & Buchner, 2005), protein folding and unfolding (Zietkiewicz, Krzewska, & Liberek, 2004), and in the refolding of damaged proteins (Marques, et al., 2006).

One can therefore expect that HSPs, also called stress proteins, will have, at the time of animal death, a pro-survival function and, hence, an antiapoptotic role (Arrigo, 2005; Arrigo, et al., 2002; Beere, 2001, 2004, 2005; Flower, Chesnokova, Froelich, Dixon, & Witt, 2005). Upon apoptotic stimuli, HSPs may therefore have diverse anti-apoptotic actions which can be summarized as follows:

– Formation of a complex with active caspases (initiators or effectors) thus hindering their function.

– Protection of target proteins (substrates) of effector caspases, preventing or delaying their degradation by these enzymes.

– Restoration of the initial and active structure of proteins having undergone structural damage following either the stress itself or the initiation of apoptosis.

As summarized in table 4, a large set of HSPs have been associated with meat tenderness. According to most studies, the increase in HSPs levels results in meat toughening. However, it is difficult to globally analyze the contribution of HSPs to meat tenderization, and each of them must be considered separately, a task impossible here. Furthermore, the role of HSPs in *postmortem* muscle remains unclear and additional investigations on the underlying mechanisms will be needed. Modulation, by HSPs, of apoptosis and cell survival *in vivo* have been reviewed recently and we suggest to refer to these papers for more details on their adverse contribution to cell death and cell survival processes (Arya, Mallik, & Lakhotia, 2007; Lanneau, et al., 2008).

3.5. Annexins A1 and A6 as potential markers of meat tenderness

3.5.1. Annexins family: presentation and functions

Annexins are members of large structurally-related and calcium sensitive protein family. Expressed in all eukaryotic cells, they participate in a variety of cellular processes including apoptosis and intracellular signaling. Annexins are a class of Ca^{2+} regulated proteins, characterized by the unique architecture of their Ca^{2+} binding sites, which enables them to peripherally localize onto negatively charged membrane surfaces in their Ca^{2+} bound conformation (Gerke & Moss, 2002; Monastyrskaya, Babiychuk, & Draeger, 2009).

All are multifunctional proteins, contributing to numerous cellular and physiological processes (Gerke, Creutz, & Moss, 2005; Kenis, et al., 2010; van Genderen, Kenis, Hofstra, Narula, & Reutelingsperger, 2008) including:

- They provide a membrane scaffold, which is relevant to changes in the cell shape.
- They are involved in trafficking and organization of vesicles, exocytosis, endocytosis.
- They contribute to calcium ion channel formation.
- They can be transported to the extracellular space where their activity is linked to fibrinolysis, coagulation, inflammation and apoptosis.

Changes in the concentration of two annexin isoforms, Annexin A1 (also known as lipocortin-1) and Annexin A6, have been recently reported in *postmortem* muscle, a change probably related to apoptosis development and meat tenderization (Bjarnadottir, et al., 2012; Zhao, Basu, Dodson, Basarb, & Guan le, 2010) (Table 5) .

3.5.2. Annexin A1

Besides its complex and not clearly understood role in obesity and hence in fat deposition (Kosicka, et al., 2013; van Erk, et al., 2010) and other proposed functions (Gerke & Moss, 2002; Monastyrskaya, et al., 2009), it is worth noting that Annexin A1 has been also implicated in the apoptotic process where it is rapidly translocated to the cell surface as an "eat me" message to promote the removal of cells that have undergone apoptosis. Similarly to phosphatidylserine, Annexin A1 is therefore a marker of apoptosis onset, but its exportation to the cell surface is dependent on caspase activation. Caspase activation thus induced a recruitment of Annexin A1 from the cytosol, a translocation to the outer plasma membrane leaflet where it colocalizes with phosphatidylserine and is also required for efficient clearance of apoptotic cells (Arur, et al., 2003). This feature supports previous findings proving that caspases are activated in *postmortem* muscle and suggests an over expression of these proteases in tender meat (Ouali, et al., 2006).

3.5.3. Annexin A6

As reviewed by (Cornely, Rentero, Enrich, Grewal, & Gaus, 2011), Annexin A6 is involved in a large set of biological processes and promotes apoptosis. In cells lacking Annexin A6, mitochondrial morphology is indeed abnormal, Ca^{2+} signaling and respiration are impaired and cells have increased resistance to Ca^{2+} mediated apoptosis. Mitochondrial fission is an early event during apoptosis, occurring before caspase activation. This process is

mediated by binding of the fission GTPase Drp 1 (Dynamin-related protein 1) to the outer mitochondrial membrane leading to a preliminary release of small amounts of cytochrome c (cyt c) (Chlystun, et al., 2013; Suen, et al., 2008). Released cyt c translocates to the endoplasmic reticulum where it selectively binds InsP3R (inositol (1,4,5) triosphosphate receptor) resulting in sustained cytosolic calcium increases. Mitochondrial fission can be inhibited by binding of Annexin A6 to Drp1, an inhibition relieved by high cytosolic calcium levels, which dissociates the Drp1-Annexin A6 complex and targets Annexin A6 to the plasma membrane (Boehning, et al., 2003; Boehning, van Rossum, Patterson, & Snyder, 2005). This event causes an amplification of Cytochrome c release and fission of mitochondria, generating a higher number of smaller organelles.

In *postmortem* muscle, Annexin A6 may therefor act as a brake to apoptosis through inhibition of Drp 1 and subsequent intensification of Cytochrome c release occurring soon after animal death (see Figure 6 and related comments). With regards to the present function of Annexin A6 in apoptosis, a lower abundance of this protein in tender meat would be expected as compared to tough meat, a proposal in good agreement with the conclusions of (Bjarnadottir, et al., 2012) who observed lower Annexin A6 levels in tender meat.

3.6. Other less known markers of tenderness: Galectin-1 and Peroxiredoxin 6

Two other potential markers of tenderness have been reported. These are galectin 1, a member of the Galectin family (Bjarnadottir, et al., 2012; Zapata, et al., 2009) and Peroxiredoxin-6, an antioxidant contributing to hydrogen peroxide degradation and exhibiting phospholipase activity (Jia, et al., 2009).

3.6.1. Galectins

Galectins constitute an evolutionary conserved family of β-galactoside binding proteins that are ubiquitous in mammals and other vertebrate taxa, invertebrates, and fungi. Since their discovery in the 1970's, their biological roles, initially understood as limited to recognition of carbohydrate ligands in embryogenesis and development, have expanded in recent years by the identification of multiple other functions including apoptose induction and/or regulation (Vasta, 2012). Most of them including Galectin 1 are believed to exhibit proapoptotic activities whereas Galectin 3 is the only one considered to have an anti-apoptotic activity (Hernandez & Baum, 2002). The role of Galectin 1 in apoptosis is however highly controversial and contradictory findings are reported in the literature (R. Y. Yang, Rabinovich, & Liu, 2008). Therefore, it appears difficult to analyze the conclusion of (Zapata,

et al., 2009) and (Bjarnadottir, et al., 2012) suggesting that a lower expression of Galectin 1 is associated with greater tenderness.

3.6.2. Peroxiredoxin 6

Peroxiredoxin 6 (Prdx6) was the sixth (and final) mammalian member of the Prdx family to be described, shares structural and functional properties with other members of this family but has important characteristics that makes it unique among the Prdxs. The first one is structural in nature since it has only one conserved cysteine residue instead of two for other members. Second, Thioredoxin, the natural co-enzyme of most Prdxs, does not participate in the catalytic cycle as compared to the other members of this family. Glutathione appears indeed to be the physiological reductant for Prdx6. Third, Prdx6 is able to bind and reduce phospholipid hydroperoxides, an essential enzymatic activity in antioxidant defense. Finally, structural studies revealed that Prdx6 is a bifunctional enzyme with phospholipase A2 activity in addition to its peroxidase function (Fisher, 2011). As already postulated by (Manevich & Fisher, 2005), Prdx6 functions in antioxidant defense mainly by hydrolysis of hydrogen peroxides and by facilitating repair of damaged cell membranes via reduction of peroxidized phospholipids (S. Y. Kim, Chun, & Lee, 2011; Pak, et al., 2011; Tulsawani, et al., 2010). (Jia, et al., 2009) reported an over expression of Prdx6 in tender meat, a finding in contradiction with the antiapoptotic and/or cell survival activities of this protein. However, it is presently difficult to get conclusions about the functions of Prdx6 in *postmortem* muscle and thus more detailed investigations will be needed in order to clarify the exact nature of the relationship between Prdx6 increased activities and ultimate meat tenderness.

3.7. Proteases and protease inhibitors as markers of meat tenderness

Since decades, the meat tenderizing process is unanimously recognized to be enzymatic in nature and the most studied proteolytic systems were cathepsins, calpains, the 20S proteasome and, although more recently, members of the caspases family (Kemp & Parr, 2012; Ouali, et al., 2006; Sentandreu, et al., 2002). However, the major peptidases of concern are not identified yet in an unquestionable way and this question is still strongly debated.

In the 80's the search for biological predictors of ultimate meat tenderness focused our attention on the improvement of the enzymatic activity measurement especially with the use of fluorescent substrates, which are much more sensitive than colorimetric ones. Proteolytic enzyme levels were indeed expected to be good predictors of meat tenderness. Unfortunately, this was not the case. In this respect, the at-death enzyme/inhibitor ratio or the inhibitor

concentrations alone are both the best predictors of meat tenderness reported up to date (Ouali & Talmant, 1990).

Since then, all assays attempting to associate enzyme concentrations to meat tenderness have been unsuccessful. Assays with calpastatin, the common specific inhibitor of calpains 1 and 2, have not been convincing and not reproducible. Cystatins, a group of cysteine protease inhibitors, have been identified as potential markers in only one study carried out by (Shackelford, Koohmaraie, Miller, Crouse, & Reagan, 1991). These authors thus stressed that at-death cystatin levels showed a much higher correlation with Warner-Bratzler shear force at 7 days *postmortem* than calpastatin levels alone (r = +0.62 *vs* -0.23 for calpastatin), a feature confirming the unreliability of calpastatin as predictor of meat tenderness as suggested before.

Later on, serine protease inhibitors levels have been shown to be best predictor of meat tenderness amongst about thirty quantitative variables including calpains and calpastatine (Zamora, et al., 2005; Zamora, et al., 1996). This unexpected feature was extremely surprising since no serine protease susceptible to contribute to myofibrillar softening was reported (Gagaoua, et al., 2012; Ouali, 1990). Investigations then performed on bovine muscle aiming at identifying these intracellular serine protease inhibitors revealed that most of them belong to the serpin superfamily (an acronym of **SER**ine **P**roteases **IN**hibitors), the largest serine protease inhibitor subfamily. Serpins are pseudo-irreversible inhibitors of serine proteases, cysteine proteases and possibly other proteases groups. Some of them exhibited no inhibitory activity and serves other functions (Olson & Gettins, 2011). Bovine serpins encompass at least 8 different isoforms (Pelissier, et al., 2008) most of them (6 out of 8) being strong inhibitor of initiator and effector caspases, which are very likely their natural target proteases *in situ* (Herrera-Mendez, et al., 2009). As they bind tightly to caspases, they must be considered as efficient inhibitors of the caspase-dependent apoptotic process which seems to be the case in *postmortem* muscle.

3.8. Structural muscle proteins as marker of tenderness

Numerous myofibrillar proteins are degraded in *postmortem* muscle and the first to be identified was probably troponin T, a regulator and nonstructural protein which does not contribute to meat texture, together with the suspected 30-32 kDa troponin proteolytic fragment which is in fact an electrophoretic band containing principally a proteolytic product of actin (Becila, et al., 2010). Because of the large number of proteins concerned, this point will not be developed here. One of the earliest structural changes resulting from hydrolysis of cytoskeletal structures is the rapid *postmortem* detachment of the basal lamina from the

plasma membrane following the degradation of all transversal connection between muscle cells and the extracellular matrix (Nishimura, Hattori, & Takahashi, 1996a; R. G. Taylor, et al., 1997).

We all know today that the meat tenderization process involves all intracellular proteases and probably also extracellular ones alike plasmin, thrombin …etc. It is therefore essential to emphasize here that the identification of the proteases suspected to be responsible for the degradation of a particular protein is of secondary importance. This is especially true as the degradation of a protein *in vitro* by a particular protease does not necessarily imply that it is the target protein *in situ*. Moreover, a given protein can be often degraded by several different proteases at least *in vitro*.

Most importantly, we have to identify the structural proteins whose hydrolysis is likely to weaken enough the muscle structures. We will come back to this point in the next chapter by reconsidering the transversal cytoskeletal network and the interesting proteins to be considered in relation to this topic.

4. Future perspectives in the search for meat tenderness predictors

Two dimensional gel electrophoresis coupled to mass spectrometry analysis of muscle proteome provides a lot of information about changes occurring during meat ageing. If we want to progress in this area, we must now take a step back with the aim to look and synthetically analyze the mass of available data sets in order to target key points within the biochemical processes implemented during the conversion of muscle into meat and therefore clarify the major and limiting steps of the metabolic pathways of concern. This paper is probably the first attempt to synthetically analyze the data available about meat tenderness predictors identified up to date, mostly by two dimensional gel electrophoresis coupled to mass spectrometry analysis of muscle proteome.

The last part of this review will be an overview of different metabolic pathways and of proteolytic events including structural aspects of muscle cells and protease inhibitors, which are known to be better predictors of tenderness than their target enzymes. For the different reasons previously mentioned, HSPs will not be considered hereafter. Indeed, HSPs and their potential functions in *postmortem* muscle would need a specific review.

4.1. Energy metabolism in postmortem *muscle with reference to living cells*

Postmortem, the first objective of muscle cells will be to mobilize all its resources to produce enough energy in the form of ATP to keep their normal metabolism. In this context,

and as previously suggested, it's not surprising to see that all cellular components rich in energy (carbohydrates, lipids, amino acids …) are degraded for optimizing ATP production (Figure 5a).

4.1.1. Amino acids as the source of energy

Regarding amino acids as a source of energy; it is worth emphasizing that skeletal muscle contains 75% of the entire free amino acid pool of the body and 25% of total body proteins. Glutamate, which comprises 20% of all amino acids in natural proteins, is one of the amino acids in highest concentration in the free amino acid pool in human skeletal muscle and yet has a low concentration in plasma (Graham & MacLean, 1998; Graham, Turcotte, Kiens, & Richter, 1997; Rennie, 2010). Although it is not an essential amino acid, it takes part in numerous important metabolic processes. Glutamate plays a central role in transamination and deamination reactions. These include the formation of aspartate, alanine, and glutamine. Skeletal muscle, in its resting state, normally releases glutamine and alanine in large quantities; they can represent from 50 to 100% of the amino acid efflux in the fasted and fed states, respectively, whereas glutamate is the dominant amino acid that is taken up by skeletal muscle (Graham & MacLean, 1998; Graham, et al., 1997). Amino acids are therefore an important potential source of energy in *postmortem* muscle because the free pool is important and all of them can be degraded to a component of the Krebs cycle or an intermediate compound able to be converted to a component of this cycle (Figure 5b). The carbon skeletons of amino acids are brought back to only seven molecules: pyruvate, acetyl CoA, acetoacetyl CoA, α-ketoglutarate, succinyl CoA, fumarate and oxaloacetate. Those that are degraded to acetyl CoA or acetoacetyl CoA are called ketogenic amino acids because they can be converted in ketone bodies (Figure 5b green frame) whereas those that are converted in the remaining of the seven molecules are called glucogenic amino acids (Figure 5b yellow frame) because they can be converted to phosphoenol pyruvate and from there to glucose. Few of them (Ile, Trp, Phe & Tyr) are both ketogenic and glucogenic.

The major byproduct of amino acid degradation are ammonium ions, which are normally recycled, *in vivo*, after blood transportation as alanine to the liver (glucose-alanine cycle) where the transamination is reversed, releasing this metabolite that would then enter the urea cycle (Figure 5a). This will not be possible *postmortem*, so that muscle cells would have to activate unusual pathways for ammonium ions elimination, an adaptation which may take some time. In the meantime, ammonium ions together with bicarbonate, generated from CO_2 by carbonic anhydrase may slow down the rate of pH drop or may cause transient increase of

the pH like sometimes observed (Boudjellal, et al., 2008; Ouali, et al., 2006). As shown in Figure 7, pH profile showed a transient slowdown of the pH drop during postmortem storage of rat muscle and, in the present case, a slight increase in the pH value. This was observed about 3h *postmortem* when the pH value is close to 6.2. Similar findings have also been observed sometimes in both *postmortem* beef and sheep muscles. The significance of this slight pH increase is still unclear.

The assimilation of ammonium ions can be done through three major pathways:

1) Synthesis of alanine from 2 pyruvate molecules (glucose-alanine cycle)

$$2\ \text{Pyruvates} + NH_4^+ \rightarrow \text{Alanine} \qquad (\textit{alanine transaminase})$$

2) Synthesis of glutamate from α-ketoglutarate

$$NH_4^+ + \alpha\text{-Ketoglutarate} + NADPH + H^+ \leftarrow (\textit{Glutamate dehydrogenase}) \rightarrow \text{Glutamate} + NADP^+ + H_2O$$

3) Synthesis of glutamine from glutamate

$$\text{Glutamate} + NH_4^+ + ATP \rightarrow \text{Glutamine} + ADP + Pi + H^+ \qquad (\textit{glutamine synthetase})$$

The two first pathways consume two important metabolites involved in energy production (pyruvate and α-ketoglutarate) and their availability will be the main limiting step. The last one is therefore the most probable to occur *postmortem* especially because glutamate is abundant and displaces the reaction towards the formation of glutamine. The only one problem is that this reaction is ATP-dependent. What solution muscle cells will choose is an open question and further investigations on the origin of the over expression of the enzymes of concern will be necessary.

Regarding amino acid metabolism, the major questions we will have to answer in future are:

- Amino acid degradation occurs mainly in mitochondria: how long these pathways will be active in postmortem muscle?
- Which amino acids are mainly used for energy production?
- How ammonium ions are assimilated *in situ* and at what rate this could be done?
- What could be the consequence of ammonium and bicarbonate production on the pH drop?

4.1.2. Carbohydrates as the source of energy

In the anoxic conditions of *postmortem* muscle, glycolysis will be the first pathway mobilized for ATP production. The rate of this pathway is dependent on the availability of NAD^+, which is essentially regenerated from NADH (unable to enter mitochondria) by two different shuttles between the cytosol and the mitochondrial matrix:

- ***The malate-aspartate shuttle*** which is the most efficient: cytosolic NADH is oxidized to NAD^+ by reducing oxaloacetate (OAA) to malate, which travels through the inner mitochondrial membrane, then being exchange by α-ketoglutarate that is exported to the cytosol where it will be converted back to OAA.
- *The **glycerol–phosphate shuttle***: cytosolic NADH is oxidized to NAD^+ by reducing DHAP to glycerol-3-P (Gly-3-P). Gly-3-P donates electrons to glycerol-3-P dehydrogenase in the inner mitochondrial membrane, regenerating DHAP and converting FAD^+ to $FADH_2$. Then $FADH_2$ enters the respiratory chain to produce 3 ATP.

As a marker indicative of these exchanges and of the efficiency of the glycolytic pathway we must follow the time dependent changes in the cytosolic and mitochondrial NAD^+/NADH ratio. The second point of interest is the possible accumulation of methylglyoxal in the cytosol. We previously observed that one enzyme (Lactoylglutathione lyase) implicated in the conversion of this metabolite to lactate is upregulated. However we have no information about the quantity and the future of the Methylglyoxal accumulated in meat. Accumulation of this toxic metabolite also means that the concentration of DHAP (Dihydroxyacetone Phosphate**)** increases *postmortem* at the expense of glyceraldehyde 3-P, which is the starting point of the conversion of trioses phosphate to pyruvate and ultimately to either acetyl-CoA or lactate. In summary, we might wonder about the real importance of the methylglyoxal cycle in *postmortem* muscle and the amount of DHAP produced through different pathways. These questions call for further detailed investigations on the different pathways implicated in the synthesis of DHAP (see Figure 4).

4.1.3. Free fatty acids as the source of energy

For long time, lipid contribution to meat tenderness was ascribed to a dilution of the collagen network by deposition within the extracellular matrix (Jeremiah, Dugan, Aalhus, & Gibson, 2003; Nishimura, 2010). On this basis we would expect a proportional increase in meat tenderness with the level of intramuscular fat. However this is not the case and, as reviewed by (Hocquette, et al., 2010), increase in ultimate tenderness of meat was only observed for intramuscular fat content ranging from 0 to about 3-4%. Above this value no effect on sensory qualities could be detected whereas consumer acceptability can be affected.

Another possibility would be to consider a more active participation of lipids to the tenderizing process through a significant contribution to energy production in the first hours after slaughter. This was supported by the reported increase of β-hydroxyacyl CoA-dehydrogenase, a member of the Lynen helix (Hamill, et al., 2012; Polati, et al., 2012). This

implies the degradation of triglycerides by lipases, releasing free fatty acid and glycerol. Glycerol can enter the glycolytic pathway after conversion to DHAP (see Figure 4). Fatty acids are then transported to the mitochondria matrix through binding to carnitine, where they are oxidized to acetyl-CoA within the Lynen helix.

The limiting step for oxidation of lipids is the status of mitochondria. We do not know yet the survival time of these organelles and the time course fate of their major activities. Oxygen pressure in *postmortem* muscle is known to decrease progressively and not sharply, suggesting that mitochondria will maintain their activities until oxygen pressure reach very low levels.

4.2. Mitochondria status and apoptosis regulation

Mitochondria are the center of many events that will contribute to the development of meat qualities (Sierra & Olivan, 2013). They thus play a central role in the initiation and development of apoptosis as well as in the production of energy that is absolutely vital to this process. They are implicated in the final oxidation of all end-products of amino acids, carbohydates and lipid metabolism. Because of the role of mitochondria in energy metabolism and in the initiation/regulation of apoptosis, these organelles could provide new predictors of meat tenderness.

4.2.1. Mitochondria as initiator and regulator of apoptosis

Mitochondria are central to many forms of cell death, usually via the release of pro-apoptotic proteins from the mitochondrial inter-membrane space. Some inter-membrane space proteins, including Cytochrome c, Smac/DIABLO (second mitochondria-derived activator of caspases encoded by the Diablo gene), and Omi/HtrA2 (mitochondrially-located serine protease) can induce or enhance caspase activation whereas others, such as AIF (Apoptosis Inducing Factor) and endonuclease G, might act in a caspase-independent manner (Figure 6). Release of inter-membrane space proteins is often regulated by the Bcl-2 protein family (Brunelle & Letai, 2009; Chipuk & Green, 2008). Recent evidence suggests that proapoptotic members of this family, by themselves, can permeabilize the outer mitochondrial membrane without otherwise damaging mitochondria. Mitochondria can contribute to cell death in other ways. For example, they can respond to calcium release from the endoplasmic reticulum by undergoing the mitochondrial permeability transition, which in turn causes outer membrane rupture and release of inter-membrane space proteins. Bcl-2-family proteins such as Bik can

influence the levels of releasable Ca^{2+} in the endoplasmic reticulum, and thus determine whether the released Ca^{2+} is sufficient to overload mitochondria and induce cell death.

4.2.2. Calcium trafficking

As reported by (Vignon, Beaulaton, & Ouali, 1989), calcium is already very abundant in the sarcoplasm and the inter-myofibrillar space of beef muscle at 4 h *postmortem*. This suggests a rapid *postmortem* translocation of calcium ions from the sarcoplasmic reticulum to the cytosol. A large set of biochemical processes triggers this translocation. This could result from different but cooperative processes affecting the permeability of the sarcoplasmic reticulum membrane including binding of proapototic Bcl2 members such as Bik, binding of Cytochrome c to a membrane glycoprotein complex acting as Ca^{2+} channel and known as inositol (1,4,5) trisphosphate receptor (InsP3R), and probably other calcium release processes (Figure 6). Large amounts of this calcium are transferred to mitochondria and overcharge them and triggering apoptosis (Mattson & Chan, 2003; Orrenius, Zhivotovsky, & Nicotera, 2003).

4.2.3. Mitochondria fission

Mitochondrial fission is an early event during apoptosis, occurring before caspase activation. It is well recognized that mitochondrial morphology changes during apoptosis, resulting in small, round and more numerous organelles. This process is associated with earlier binding of several proteins including proapoptotic Bcl2 family members to outer mitochondrial membrane, a translocation altering the membrane permeability to calcium and proteins of the inter-membrane space (Youle & Karbowski, 2005).

The large GTPase Dynamin-related protein 1 (Drp1) mediates mitochondrial fission in mammalian cells. This cytosolic protein moves to mitochondria and assembles into spirals at division sites around the outer mitochondrial membrane to drive the fission process (insert Figure 6). Oligomerisation of Drp1 is inhibited by Annexin 6 at low levels of calcium. By contrast, increase in the cytosolic calcium levels relieves this inhibition, mediating the translocation of Annexin 6 to the plasma membrane (Suen, et al., 2008). Bik, a BH3 protein of the Bcl-2 family, contributes very likely to the translocation of Drp1 to the mitochondrial membrane through a calcium release from the sarcoplasmic reticulum, which prevents it from Annexin 6's inhibition.

Fission of mitochondria has never been demonstrated so far in *postmortem* muscle although all needed conditions are gathered together. Because it is an essential event in apoptosis initiation and development, this point needs to be addressed with appropriate

techniques and research on specific markers such as the expression of Drp 1 and the existence of Cytochrome c-InsP3R complexes, which amplify the release of calcium and translocation of Annexin 6 to the cell membrane, cancelling the inhibition of the fission process.

We need indeed to explain how levels of the different enzymes of the tricarboxylic acid cycle are increased *postmortem* and, in this context, we might also consider the mitochondria fusion process (fusion also causes an increase in mitochondrial metabolic enzymes) assuming that this event is possible in the particular *postmortem* conditions. Markers and mechanisms of mitochondria fusion have been reviewed by (Westermann, 2008) and (Suen, et al., 2008).

For more details, the reader would have to refer to different reviews covering these early events of the apoptotic process initiation (J. Li, Li, Qin, von Harsdorf, & Li, 2010; Otera & Mihara, 2012; Suen, et al., 2008; Youle & Karbowski, 2005) and some others focused on the role of Annexins in apoptosis (Arur, et al., 2003; Chlystun, et al., 2013; Cornely, et al., 2011; Monastyrskaya, et al., 2009; Suen, et al., 2008).

4.2.4. Pro- and anti-apoptotic proteins from mitochondria inter-membrane space

Reports from Horvitz's group summarized in their latest review (Ellis, Yuan, & Horvitz, 1991) clearly indicated that apoptosis is a fundamental property of animal cells and that proteins that mediate and regulate this process have been largely conserved along the evolution, from worms to humans. In *postmortem* muscle, the apoptotic process will be qualitatively identical to what we know *in vivo* and regulatory pathways will be the same. The main limiting factors will be the absence of blood flow, the pH drop and the lower level of energy available. These settings will slow down, but not prevent, the development of the cell death process.

Following apoptotic signals and outer mitochondrial membrane alteration, several pro-apoptotic [Cytochrome c, Endonuclease G (Endo G), Apoptosis Inducing Factor (AIF)] and anti-apoptotic proteins (**HtrA2**: high-temperature-requirement protein A2; **Omi**: Omi stress-regulated endopeptidase; **Smac**: second mitochondria-derived activator of caspase) are released from mitochondria in a caspase independent manner (Figure 6).

Upon release from mitochondria, Cytochrome c will trigger apoptosome complex formation through association with Apaf 1 (apoptotic protease activating factor). Once formed, the apoptosome can then recruit and activate the inactive pro-caspase 9. We recently followed the release of Cytochrome c in rat *Longissimus* muscle from at death to 72h of storage at low temperature (for experimental details see (Becila, et al., 2010)). As assessed by western blot and densitometry on the supernatant obtained after mitochondria sedimentation

at 20000 g, release of this protein into the cytosol starts immediately after death and reach its maximum concentration about 6h postmortem at a pH value of 6.0 (**Figure 7**). Quantification of Cytochrome c soon after death using more accurate methods might provide interesting information on apoptosis development and, hence, on meat tenderness.

Apoptosis Inducing Factor (AIF) is a protein that triggers chromatin condensation and DNA degradation in the cell. The mitochondrial AIF protein was found to be a caspase-independent death effector that can allow independent nuclei to undergo apoptotic changes (Hangen, Blomgren, Benit, Kroemer, & Modjtahedi, 2010).

Endonuclease G is a proapoptotic DNase synthetized in cell nucleus and further stored within mitochondria. After release from mitochondria, Endonuclease G is translocated to cell nucleus during apoptosis where it cleaves chromatin DNA into nucleosomal fragments (Burhans & Weinberger, 2007; L. Y. Li, Luo, & Wang, 2001; Yoshida, Pommier, & Ueda, 2006).

SMAC (second mitochondria-derived activator of caspases) is a mitochondrial protein encoded in human by the DIABLO gene. This explains why it is often also designed DIABLO. SMAC is an IAP-binding protein. This mitochondrial protein enters the cytosol when cells undergo apoptosis, and it moderates the caspase inhibition by IAPs (Adrain, Creagh, & Martin, 2001).

The mitochondrial Serine protease HtrA2 is an enzyme that in humans is encoded by the *HTRA2* gene. HtrA2, also known as Omi, can be released from the mitochondria during apoptosis, using its first four N-terminal amino acids to mimic a caspase substrate with the objective to be recruited by IAP caspase inhibitors (Verhagen, et al., 2002). They also are able to cleave IAPs (Q.-H. Yang, Church-Hajduk, Ren, Newton, & Du, 2003).

4.2.5. Conclusion about the central role of mitochondria in postmortem muscle

It is worth noting that most of the mentioned mitochondrion associated proteins that contribute in different ways to the regulation of apoptosis have never been identified by 2D gel proteome analysis. Therefore, specifically targeted investigations will be needed in future research to clarify their implication in *postmortem* apoptosis and, hence, in meat tenderness development, together with their ability to be good predictors of meat tenderness or not.

4.3. Inhibitors of caspases and apoptosis

Naturally occurring caspase inhibitors include several members of the mammalian IAP (inhibitors of apoptosis proteins) family. As shown in **Figure 6**, some are efficient inhibitors

of effector caspases and caspase 9, one of the initiator caspase family. Inhibition of caspase 8 by IAPs is still uncertain. The mode of inhibition of caspases by IAPs greatly differs from the traditional mechanisms known for cystatins, serpins and other peptidase inhibitor families, mostly interacting with their target peptidase in a substrate-enzyme manner. By contrast, binding with relatively low affinity (*Kd* in the μM range) of IAPs in the vicinity of the active site creates a sufficient steric obstruction to prevent access of the active site to protein substrates, small peptide substrates being hydrolysed after IAP binding (Chiou, Jones, & Tarnawski, 2003; Fuentes-Prior & Salvesen, 2004; Fulda, 2009; Philchenkov, 2004; R. C. Taylor, Cullen, & Martin, 2008; Wei, Fan, & Yu, 2008). The anti-apoptotic function of the IAP proteins family can be cancelled by specific inhibitors of this interaction, *i.e.* Smac/DIABLO and Omi/HtrA2 proteins (Saelens, et al., 2004).

Besides IAPs, we have recently identified new caspase inhibitors, firstly described as elastase and trypsin inhibitors and further characterized as initiator and effectors caspases inhibitors. This highly polymorphic group of protease inhibitors, which has already been presented in chapter 1, belongs to the serpin superfamily and forms, with their target enzymes including caspases, a covalent complex detectable upon SDS-PAGE analysis (Gagaoua, et al., 2012). Preliminary assays using prostate cancer cells in culture showed that *in situ* inactivation of some of these serpins is able to decrease cell proliferation probably through an increase of cell death at the expense of cell proliferation.

(Zamora, et al., 2005) determined the levels of serine protease inhibitor in bovine muscle extracts, obtained immediately after animal death, by titration with trypsin, and found that at-death serine proteinase inhibitor concentration is a better predictor of meat tenderness than all other variables considered including calpains 1 and 2, cysteine protease inhibitors, calpastatin, …etc. These serine protease inhibitors are integrated by members of the identified caspase inhibitors of the serpin superfamily since these are by far the most predominant in muscle tissue. Characterization of these serpins is currently going on and will be further used to confirm their efficiency as predictors of meat tenderness using more specific quantification methods such as ELISA.

4.4. Proteases and Proteolysis

We already stressed forward that levels of proteases in muscle tissue are not between the best options to look for potential adequate predictors of meat tenderness. We must emphasize that most, if not all, protein substrates can be hydrolyzed *in vitro* by a series of endogenous proteases and it is often difficult or impossible to identify the target *in situ* substrates for each proteolytic system, with the possible exception of living or cultured cells models. In addition,

we are not in a position today to certify that a given protein is degraded by a particular protease. The answer to this question is, in our view, beyond our present capabilities, so that we have to overcome this difficulty by defining other new priorities if we want to progress in this area. One possibility will be to improve our knowledge about inhibitors of each proteolytic system, which would provide much more information about the role of their target enzymes in *postmortem* muscle, including proteasome, papain-like cysteine proteases, serine proteases, caspases …etc.

Identification of potential protein substrates as good predictor of meat tenderness therefore needs to select potentially interesting proteins according to their function in the myofibrillar and cytoskeletal protein network. It is clear that the cytoskeletal network is of primary interest for our objective, as this architecture plays the same role in muscle than bones in our body.

With regard to the apoptotic underlying mechanisms, caspases are probably the first proteolytic enzymes to be implicated in protein disruption and the transversal cytoskeletal network linking myofibrils to the extracellular matrix network should be between the primary substrate targets.

In postmortem muscle, the basal membrane is quickly detached from the plasma membrane (Nishimura, Hattori, & Takahashi, 1996b; R. G. Taylor, et al., 1997) suggesting an early *postmortem* degradation of the connections between these two membranes. This results very likely from a proteolytic hydrolysis of integrins, laminin and/or both sarco- and dystroglycans, three major protein structures needing further refined investigations. Other proteins involved in these transversal connecting structures are αβ-crystallin, dystrophin, spectrin, syntrophin and dystrobrevin (Capetanaki, Bloch, Kouloumenta, Mavroidis, & Psarras, 2007).

Another protein of great interest is actin. During apoptosis, actin is the first protein targeted by effector caspases and some actin fragments have been considered to be accurate markers of apoptosis (F. Yang, et al., 1998). Generated actin peptides by themselves can induce morphological apoptotic changes comparable to those observed in apoptotic cells (Mashima, Naito, & Tsuruo, 1999). Within muscle cells actin is present in thin filaments, in association with nebulin, and also in intermediate transversal cytoskeletal filaments where it serves to connect myofibrils to the sarcolemme, this link extending further to the extracellular matrix. Degradation of transversal cytoskeletal actin filaments causes a detachment of sarcolemma from the basal lamina and the extracellular matrix network, a finding supported by the proteolytic hydrolysis of laminin, the major component of the basal lamina (Becila, et al., 2010).

One of the major fragments generated in postmortem muscle coming from actin showed à Mr of about 32 kDa. The appearance of this fragment was followed in rat *Longissimus* muscle by western blot and densitometry. The relative concentration of the soluble 32 kDa fragment was plotted versus the storage time (Figure 7). The concentration reached a maximum value about 3-4h postmortem and then decreased sharply, suggesting a degradation of this fragment to lower Mr ones (Becila, et al., 2010); see also hereafter comments of figure 8).

Interestingly, some situations of muscle atrophy such as uremia, aging or myocardial infarct, are associated to an accelerated degradation of muscle proteins. According to results obtained by (Du, et al., 2004), in such catabolic conditions the initial step in myofibrilar proteolysis would be the breakdown of the actomyosin complex by the direct action of caspase 3. These authors pointed out that caspase 3 action would be the first step in dissociating the actomyosin complex, giving rise to both intact monomeric actin and fragments of actin. In a second step, these products will be then further degraded by the ATP-dependent ubiquitin-proteasome system. As characteristic product fragments, they found the generation of an actin fragment of around 14 kDa when actomyosin complexes were incubated with caspase 3. It is worth highlighting that this characteristic 14 kDa actin fragment has been detected in atrophying muscles of diabetic or uremic rats, where the activity of caspase 3 is increased (Du, et al., 2004). In relation to this, we have carried out some experiments with the aim to characterize the ability of caspase 3 to hydrolyze bovine muscle actin. As it can be seen in **Figure 8**, the action of caspase 3 on bovine actin is remarkable from 24 hours of incubation and later on. As main products of this action, we have also observed the generation of a 14 kDa fragment, in addition to the 32 kDa fragment that has been already observed in postmortem muscle (Becila, et al., 2010). A third and less intense actin fragment at around 25 kDa, not previously described, was also observed after 24 hours of incubation (**Figure 8**). Future research would need to be done in order to establish the utility of these fragments as potential useful markers of meat tenderness and of caspase action.

4.5. Protease inhibitors

As demonstrated for calpains (Ouali & Talmant, 1990), cysteine proteases (Shackelford, et al., 1991) and caspases (Gagaoua, et al., 2012; Zamora, et al., 2005), inhibitor levels and or Inhibitor/enzyme ratios are better predictors than the concentration of their target enzymes alone. Hence, a prerequisite for any investigation on the role of a proteolytic system in the meat tenderizing process is to know the nature of their specific inhibitors. If inhibitors have

been identified for proteases of the papain family, the calpain and the caspase families, nothing is known about proteasome inhibitors which are undoubtedly present in muscle cells.

Furthermore, we previously showed the presence, in muscle tissue, of papain-like cysteine protease inhibitors with Mr ranging from 30 to 70 kDa which were inactive against any of the serine proteases tested. However, their identity and their *in situ* target protease are still not known. The major question about these last inhibitors will be to clarify whether they are able to inactivate caspases or not, a group of enzymes discovered in the middle of the 90's and thus not tested at that time (Berri, Rouchon, Zabari, & Ouali, 1998; Berri, Venien, Levieux, & Ouali, 1996; Ouali, 1995; Ouali, et al., 1995).

We must stress forward that much remain to be done about muscle proteases inhibitors, concerning the characterization of their interaction with potential target proteases and their identification at the protein level.

5. Conclusions

In recent years, we have accumulated a lot of data using two-dimensional gel electrophoresis and mass spectrometry to identify spots of interest in relation to meat tenderness. As reviewed here, we think that we are arriving at the limits of these approaches and that is because the synthetic analysis of these data has been one of the main objectives of this paper. From this analysis we have then proposed some perspectives to improve our understanding of the biological mechanisms responsible for meat tenderization. The best way to do this now would be to more specifically target stakeholders of metabolic pathways that seem most conducive to better understand the mechanisms of meat tenderization.

Apoptosis is generally believed to begin after caspase activation. In fact a large set of events precedes this step, strongly contributing to the definition of the extent and intensity of the cell's dismantling process, first by the action of caspases, then followed with the help of the other proteolytic systems.

This paper reviews a large set of proteins of interest as potential predictors of meat tenderness. Most of them are enzymes of the energy metabolic pathways and/or direct or indirect regulators of these pathways. Whatever the source of energy used by muscle cells, the limiting step will always be the mitochondrion where all end products of these pathways are finally oxidized to produce a maximum of ATP. In near future, we will have to analyze the *postmortem* fate of these organelles in muscle tissue.

On the other hand, proteins involved in the regulation of the apoptotic process whether released from mitochondria (AIF, Endo G, Cytochrome c, …) or expressed in muscle cells (IAPs, Caspase inhibiting Serpin, …) have never been revealed by 2D gel electrophoresis

proteome analysis and thus they would merit to be selectively targeted using alternative more specific approaches.

Finally, muscle cells were shown to express strong inhibitors of initiator and effector caspases belonging to the serpin family. Screening and characterization of protease inhibitors in muscle tissue are far from being completed and much work remains to be done in this context. As inhibitors are better predictors of meat tenderness than their target enzymes, such investigations would help to clarify the role of the different endogenous proteolytic systems in *postmortem* proteolysis.

To conclude, it is therefore obvious that early apoptotic signaling processes converge on the mitochondria and that response of mitochondria to upstream proapoptotic signals is a critical control point for the regulation of cell death. Another important feature to be considered is the role of phospholipids in phospholipid/protein interactions, which are essential for the maintenance of mitochondrial dynamics and hoemostasis (Lehmann & Shatrov, 2002).

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References

Adeva, M. M., Souto, G., Blanco, N., & Donapetry, C. (2012). Ammonium metabolism in humans. *Metabolism, 61*(11), 1495-1511.

Adrain, C., Creagh, E. M., & Martin, S. J. (2001). Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. *Embo J, 20*(23), 6627-6636.

Arrigo, A. P. (2005). Heat shock proteins as molecular chaperones. *M S-Medecine Sciences, 21*(6-7), 619-625.

Arrigo, A. P., Paul, C., Ducasse, C., Manero, F., Kretz-Remy, C., Virot, S., Javouhey, E., Mounier, N., & Diaz-Latoud, C. (2002). Small Stress Proteins: Novel Negative Modulators of Apoptosis Induced Independently of Reactive Oxygen Species. In A.-P. Arrigo & W. E. G. Müller (Eds.), *Small Stress Proteins* (Vol. 28, pp. 185-204): Springer Berlin Heidelberg.

Arur, S., Uche, U. E., Rezaul, K., Fong, M., Scranton, V., Cowan, A. E., Mohler, W., & Han, D. K. (2003). Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. *Dev Cell, 4*(4), 587-598.

Arya, R., Mallik, M., & Lakhotia, S. C. (2007). Heat shock genes - integrating cell survival and death. *J Biosci, 32*(3), 595-610.

Becila, S., Herrera-Mendez, C. H., Coulis, G., Labas, R., Astruc, T., Picard, B., Boudjellal, A., Pelissier, P., Bremaud, L., & Ouali, A. (2010). Postmortem muscle cells die through apoptosis. *European Food Research and Technology, 231*(3), 485-493.

Beere, H. M. (2001). Stressed to death: regulation of apoptotic signaling pathways by the heat shock proteins. *Sci STKE, 2001*(93), re1.

Beere, H. M. (2004). "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. *J Cell Sci, 117*(Pt 13), 2641-2651.

Beere, H. M. (2005). Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *Journal of Clinical Investigation, 115*(10), 2633-2639.

Bernard, C., Cassar-Malek, I., Le Cunff, M., Dubroeucq, H., Renand, G., & Hocquette, J. F. (2007). New indicators of beef sensory quality revealed by expression of specific genes. *J Agric Food Chem, 55*(13), 5229-5237.

Berri, M., Rouchon, P., Zabari, M., & Ouali, A. (1998). Purification and characterization of a new potential in vivo inhibitor of cathepsin L from bovine skeletal muscle. *Comp Biochem Physiol B Biochem Mol Biol, 119*(2), 283-288.

Berri, M., Venien, A., Levieux, D., & Ouali, A. (1996). Tissue distribution and characterization of a 30-kDa cysteine proteinase inhibitor from bovine skeletal muscle. *Comp Biochem Physiol B Biochem Mol Biol, 113*(2), 275-279.

Bjarnadottir, S. G., Hollung, K., Faergestad, E. M., & Veiseth-Kent, E. (2010). Proteome changes in bovine longissimus thoracis muscle during the first 48 h postmortem: shifts in energy status and myofibrillar stability. *J Agric Food Chem, 58*(12), 7408-7414.

Bjarnadottir, S. G., Hollung, K., Hoy, M., Bendixen, E., Codrea, M. C., & Veiseth-Kent, E. (2012). Changes in protein abundance between tender and tough meat from bovine longissimus thoracis muscle assessed by isobaric Tag for Relative and Absolute Quantitation (iTRAQ) and 2-dimensional gel electrophoresis analysis. *J Anim Sci, 90*(6), 2035-2043.

Boehning, D., Patterson, R. L., Sedaghat, L., Glebova, N. O., Kurosaki, T., & Snyder, S. H. (2003). Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. *Nat Cell Biol, 5*(12), 1051-1061.

Boehning, D., van Rossum, D. B., Patterson, R. L., & Snyder, S. H. (2005). A peptide inhibitor of cytochrome c/inositol 1,4,5-trisphosphate receptor binding blocks intrinsic and extrinsic cell death pathways. *Proc Natl Acad Sci U S A, 102*(5), 1466-1471.

Boudida, Y., Gagaoua, M., Becila, S., Picard, B., Boudjellal, A., Herrera-Mendez, C. H., Sentandreu, M. A., & Ouali, A. (2013). Serine protease inhibitors as good predictors of meat tenderness: which are they and what are their functions? *Critical Reviews in Food Science and Nutrition, In press*.

Boudjellal, A., Becila, S., Coulis, G., Herrera-Mendez, C. H., Aubry, L., Lepetit, J., Harhoura, K., Sentandreu, M. A., Ait-Amar, H., & Ouali, A. (2008). Is the pH drop profile curvilinear and either monophasic or polyphasic? Consequences on the ultimate bovine meat texture. *African Journal of Agricultural Research, 3*(3), 195-204.

Bouley, J., Chambon, C., & Picard, B. (2004). Mapping of bovine skeletal muscle proteins using two-dimensional gel electrophoresis and mass spectrometry. *Proteomics, 4*(6), 1811-1824.

Brocker, C., Cantore, M., Failli, P., & Vasiliou, V. (2011). Aldehyde dehydrogenase 7A1 (ALDH7A1) attenuates reactive aldehyde and oxidative stress induced cytotoxicity. *Chem Biol Interact, 191*(1-3), 269-277.

Brocker, C., Lassen, N., Estey, T., Pappa, A., Cantore, M., Orlova, V. V., Chavakis, T., Kavanagh, K. L., Oppermann, U., & Vasiliou, V. (2010). Aldehyde dehydrogenase 7A1 (ALDH7A1) is a novel enzyme involved in cellular defense against hyperosmotic stress. *J Biol Chem, 285*(24), 18452-18463.

Brunelle, J. K., & Letai, A. (2009). Control of mitochondrial apoptosis by the Bcl-2 family. *J Cell Sci, 122*(Pt 4), 437-441.

Burhans, W. C., & Weinberger, M. (2007). Yeast endonuclease G: complex matters of death, and of life. *Mol Cell, 25*(3), 323-325.

Capetanaki, Y., Bloch, R. J., Kouloumenta, A., Mavroidis, M., & Psarras, S. (2007). Muscle intermediate filaments and their links to membranes and membranous organelles. *Exp Cell Res, 313*(10), 2063-2076.

Cassar-Malek, I., Guillemin, N., Hocquette, J. F., Micol, D., Bauchart, D., Picard, B., & Jurie, C. (2011). Expression of DNAJA1 in bovine muscles according to developmental age and management factors. *Animal, 5*(6), 867-874.

Chaze, T., Hocquette, J.-F., Meunier, B., Renand, G., Jurie, C., Chambon, C., Journaux, L., Rousset, S., Denoyelle, C., Lepetit, J., & Picard, B. (2013). Biological Markers for Meat Tenderness of the Three Main French Beef Breeds Using 2-DE and MS Approach. In F. Toldrá & L. M. L. Nollet (Eds.), *Proteomics in Foods* (Vol. 2, pp. 127-146): Springer US.

Chen, X. L., Shun, C. Y., Zhang, Y. Z., & Gao, P. J. (2003). Rapid monitoring of autolysis process of proteases by capillary electrophoresis. *Biotechnol Lett, 25*(20), 1763-1767.

Chiou, S. K., Jones, M. K., & Tarnawski, A. S. (2003). Survivin - an anti-apoptosis protein: its biological roles and implications for cancer and beyond. *Med Sci Monit, 9*(4), PI25-29.

Chipuk, J. E., & Green, D. R. (2008). How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends in cell biology, 18*(4), 157-164.

Chlystun, M., Campanella, M., Law, A. L., Duchen, M. R., Fatimathas, L., Levine, T. P., Gerke, V., & Moss, S. E. (2013). Regulation of mitochondrial morphogenesis by annexin a6. *PLoS One, 8*(1), e53774.

Choi, Y. M., Lee, S. H., Choe, J. H., Rhee, M. S., Lee, S. K., Joo, S. T., & Kim, B. C. (2010). Protein solubility is related to myosin isoforms, muscle fiber types, meat quality traits, and postmortem protein changes in porcine longissimus dorsi muscle. *Livestock Science, 127*(2), 183-191.

Citron, B. A., Smirnova, I. V., Zoubine, M. N., & Festoff, B. W. (1997). Quantitative PCR analysis reveals novel expression of prothrombin mRNA and regulation of its levels in developing mouse muscle. *Thromb Res, 87*(3), 303-313.

Colell, A., Green, D. R., & Ricci, J. E. (2009). Novel roles for GAPDH in cell death and carcinogenesis. *Cell Death Differ, 16*(12), 1573-1581.

Colell, A., Ricci, J.-E., Tait, S., Milasta, S., Maurer, U., Bouchier-Hayes, L., Fitzgerald, P., Guio-Carrion, A., Waterhouse, N. J., Li, C. W., Mari, B., Barbry, P., Newmeyer, D. D., Beere, H. M., & Green, D. R. (2007). GAPDH and Autophagy Preserve Survival after Apoptotic Cytochrome c Release in the Absence of Caspase Activation. *Cell, 129*(5), 983-997.

Cornely, R., Rentero, C., Enrich, C., Grewal, T., & Gaus, K. (2011). Annexin A6 is an organizer of membrane microdomains to regulate receptor localization and signalling. *IUBMB Life, 63*(11), 1009-1017.

D'Alessandro, A., & Zolla, L. (2013). Meat science: From proteomics to integrated omics towards system biology. *J Proteomics, 78*(0), 558-577.

Ding, W. X., Shen, H. M., & Ong, C. N. (2002). Calpain activation after mitochondrial permeability transition in microcystin-induced cell death in rat hepatocytes. *Biochem Biophys Res Commun, 291*(2), 321-331.

Du, J., Wang, X., Miereles, C., Bailey, J. L., Debigare, R., Zheng, B., Price, S. R., & Mitch, W. E. (2004). Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions. *J Clin Invest, 113*(1), 115-123.

Ellis, R. E., Yuan, J. Y., & Horvitz, H. R. (1991). Mechanisms and functions of cell death. *Annu Rev Cell Biol, 7*, 663-698.

Fisher, A. B. (2011). Peroxiredoxin 6: a bifunctional enzyme with glutathione peroxidase and phospholipase A(2) activities. *Antioxid Redox Signal, 15*(3), 831-844.

Flower, T. R., Chesnokova, L. S., Froelich, C. A., Dixon, C., & Witt, S. N. (2005). Heat shock prevents alpha-synuclein-induced apoptosis in a yeast model of Parkinson's disease. *J Mol Biol, 351*(5), 1081-1100.

Fuentes-Prior, P., & Salvesen, G. S. (2004). The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J, 384*(Pt 2), 201-232.

Fulda, S. (2009). Inhibitor of apoptosis proteins in hematological malignancies. *Leukemia, 23*(3), 467-476.

Gagaoua, M., Boudida, Y., Becila, S., Picard, B., Boudjellal, A., Sentandreu, M. A., & Ouali, A. (2012). New Caspases' inhibitors belonging to the serpin superfamily: A novel key control point of apoptosis in mammalian tissues. *Advances in Bioscience and Biotechnology, 3*, 740-750.

Gerke, V., Creutz, C. E., & Moss, S. E. (2005). Annexins: linking Ca2+ signalling to membrane dynamics. *Nat Rev Mol Cell Biol, 6*(6), 449-461.

Gerke, V., & Moss, S. E. (2002). Annexins: from structure to function. *Physiol Rev, 82*(2), 331-371.

Graham, T. E., & MacLean, D. A. (1998). Ammonia and amino acid metabolism in skeletal muscle: human, rodent and canine models. *Med Sci Sports Exerc, 30*(1), 34-46.

Graham, T. E., Turcotte, L. P., Kiens, B., & Richter, E. A. (1997). Effect of endurance training on ammonia and amino acid metabolism in humans. *Med Sci Sports Exerc, 29*(5), 646-653.

Guignot, F., Vignon, X., & Monin, G. (1993). Post mortem evolution of myofilament spacing and extracellular space in veal muscle. *Meat Sci, 33*(3), 333-347.

Guillemin, N., Bonnet, M., Jurie, C., & Picard, B. (2011). Functional analysis of beef tenderness. *J Proteomics, 75*(2), 352-365.

Guillemin, N., Jurie, C., Cassar-Malek, I., Hocquette, J. F., Renand, G., & Picard, B. (2011). Variations in the abundance of 24 protein biomarkers of beef tenderness according to muscle and animal type. *Animal, 5*(6), 885-894.

Hamill, R. M., McBryan, J., McGee, C., Mullen, A. M., Sweeney, T., Talbot, A., Cairns, M. T., & Davey, G. C. (2012). Functional analysis of muscle gene expression profiles associated with tenderness and intramuscular fat content in pork. *Meat Science, 92*(4), 440-450.

Hangen, E., Blomgren, K., Benit, P., Kroemer, G., & Modjtahedi, N. (2010). Life with or without AIF. *Trends Biochem Sci, 35*(5), 278-287.

Haslbeck, M., Miess, A., Stromer, T., Walter, S., & Buchner, J. (2005). Disassembling protein aggregates in the yeast cytosol. The cooperation of Hsp26 with Ssa1 and Hsp104. *J Biol Chem, 280*(25), 23861-23868.

Hernandez, J. D., & Baum, L. G. (2002). Ah, sweet mystery of death! Galectins and control of cell fate. *Glycobiology, 12*(10), 127R-136R.

Herrera-Mendez, C. H., Becila, S., Blanchet, X., Pelissier, P., Delourme, D., Coulis, G., Sentandreu, M. A., Boudjellal, A., Bremaud, L., & Ouali, A. (2009). Inhibition of human initiator caspase 8 and effector caspase 3 by cross-class inhibitory bovSERPINA3-1 and A3-3. *FEBS Lett, 583*(17), 2743-2748.

Herrera-Mendez, C. H., Becila, S., Coulis, G., Sentandreu, M. A., Aubry, L., & Ouali, A. (2010). Purification and partial characterization of antithrombin III from bovine skeletal muscle and possible role of thrombin in postmortem apoptosis development and in efficiency of low voltage electrical stimulation. *Food Research International, 43*(1), 356-363.

Hocquette, J. F., Gondret, F., Baeza, E., Medale, F., Jurie, C., & Pethick, D. W. (2010). Intramuscular fat content in meat-producing animals: development, genetic and nutritional control, and identification of putative markers. *Animal, 4*(2), 303-319.

Hollung, K., Veiseth, E., Jia, X., Faergestad, E. M., & Hildrum, K. I. (2007). Application of proteomics to understand the molecular mechanisms behind meat quality. *Meat Sci, 77*(1), 97-104.

Huang, M., Huang, F., Xu, X., & Zhou, G. (2009). Influence of caspase3 selective inhibitor on proteolysis of chicken skeletal muscle proteins during post mortem aging. *Food Chemistry, 115*(1), 181-186.

Jeremiah, L. E., Dugan, M. E., Aalhus, J. L., & Gibson, L. L. (2003). Assessment of the relationship between chemical components and palatability of major beef muscles and muscle groups. *Meat Sci, 65*(3), 1013-1019.

Jia, X., Ekman, M., Grove, H., Faergestad, E. M., Aass, L., Hildrum, K. I., & Hollung, K. (2007). Proteome changes in bovine longissimus thoracis muscle during the early postmortem storage period. *J Proteome Res, 6*(7), 2720-2731.

Jia, X., Hildrum, K. I., Westad, F., Kummen, E., Aass, L., & Hollung, K. (2006). Changes in enzymes associated with energy metabolism during the early post mortem period in longissimus thoracis bovine muscle analyzed by proteomics. *J Proteome Res, 5*(7), 1763-1769.

Jia, X., Hollung, K., Therkildsen, M., Hildrum, K. I., & Bendixen, E. (2006). Proteome analysis of early post-mortem changes in two bovine muscle types: M. longissimus dorsi and M. semitendinosis. *Proteomics, 6*(3), 936-944.

Jia, X., Veiseth-Kent, E., Grove, H., Kuziora, P., Aass, L., Hildrum, K. I., & Hollung, K. (2009). Peroxiredoxin-6--a potential protein marker for meat tenderness in bovine longissimus thoracis muscle. *J Anim Sci, 87*(7), 2391-2399.

Kalapos, M. P. (1999). Methylglyoxal in living organisms: chemistry, biochemistry, toxicology and biological implications. *Toxicol Lett, 110*(3), 145-175.

Kemp, C. M., & Parr, T. (2012). Advances in apoptotic mediated proteolysis in meat tenderisation. *Meat Sci, 92*(3), 252-259.

Kemp, C. M., Sensky, P. L., Bardsley, R. G., Buttery, P. J., & Parr, T. (2010). Tenderness--an enzymatic view. *Meat Sci, 84*(2), 248-256.

Kenis, H., Zandbergen, H. R., Hofstra, L., Petrov, A. D., Dumont, E. A., Blankenberg, F. D., Haider, N., Bitsch, N., Gijbels, M., Verjans, J. W., Narula, N., Narula, J., & Reutelingsperger, C. P. (2010). Annexin A5 uptake in ischemic myocardium: demonstration of reversible phosphatidylserine externalization and feasibility of radionuclide imaging. *J Nucl Med, 51*(2), 259-267.

Kim, G. D., Jeong, J. Y., Moon, S. H., Hwang, Y. H., & Joo, S. T. (2009). Influences of carcass weight on histochemical characteristics and meat quality of crossbred (Korean native black pig X Landrace) pigs. In *Proceedings of 55th International Congress of Meat Science and Technology* (Vol. PS1.05a.). Denmark: Copenhagen.

Kim, N. K., Cho, S., Lee, S. H., Park, H. R., Lee, C. S., Cho, Y. M., Choy, Y. H., Yoon, D., Im, S. K., & Park, E. W. (2008). Proteins in longissimus muscle of Korean native cattle and their relationship to meat quality. *Meat Sci, 80*(4), 1068-1073.

Kim, S. Y., Chun, E., & Lee, K. Y. (2011). Phospholipase A(2) of peroxiredoxin 6 has a critical role in tumor necrosis factor-induced apoptosis. *Cell Death Differ, 18*(10), 1573-1583.

Kosicka, A., Cunliffe, A. D., Mackenzie, R., Zariwala, M. G., Perretti, M., Flower, R. J., & Renshaw, D. (2013). Attenuation of plasma annexin A1 in human obesity. *Faseb J, 27*(1), 368-378.

Kultz, D. (2003). Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. *J Exp Biol, 206*(Pt 18), 3119-3124.

Lanneau, D., Brunet, M., Frisan, E., Solary, E., Fontenay, M., & Garrido, C. (2008). Heat shock proteins: essential proteins for apoptosis regulation. *J Cell Mol Med, 12*(3), 743-761.

Laville, E., Sayd, T., Morzel, M., Blinet, S., Chambon, C., Lepetit, J., Renand, G., & Hocquette, J. F. o. (2009). Proteome Changes during Meat Aging in Tough and Tender Beef Suggest the Importance of Apoptosis and Protein Solubility for Beef Aging and Tenderization. *Journal of Agricultural and Food Chemistry, 57*(22), 10755-10764.

Lehmann, V., & Shatrov, V. (2002). Lipid metabolism and release of cytochrome c from mitochondria. *Subcell Biochem, 36*, 1-17.

Li, J., Li, Y., Qin, D., von Harsdorf, R., & Li, P. (2010). Mitochondrial fission leads to Smac/DIABLO release quenched by ARC. *Apoptosis, 15*(10), 1187-1196.

Li, L. Y., Luo, X., & Wang, X. (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature, 412*(6842), 95-99.

Liu, Y., Fields, R. D., Festoff, B. W., & Nelson, P. G. (1994). Proteolytic Action of Thrombin Is Required for Electrical Activity-Dependent Synapse Reduction. *Proc Natl Acad Sci U S A, 91*(22), 10300-10304.

Lokanath, N. K., Ohshima, N., Takio, K., Shiromizu, I., Kuroishi, C., Okazaki, N., Kuramitsu, S., Yokoyama, S., Miyano, M., & Kunishima, N. (2005). Crystal structure of novel NADP-dependent 3-hydroxyisobutyrate dehydrogenase from Thermus thermophilus HB8. *J Mol Biol, 352*(4), 905-917.

Manevich, Y., & Fisher, A. B. (2005). Peroxiredoxin 6, a 1-Cys peroxiredoxin, functions in antioxidant defense and lung phospholipid metabolism. *Free Radic Biol Med, 38*(11), 1422-1432.

Marques, C., Guo, W., Pereira, P., Taylor, A., Patterson, C., Evans, P. C., & Shang, F. (2006). The triage of damaged proteins: degradation by the ubiquitin-proteasome pathway or repair by molecular chaperones. *Faseb J, 20*(6), 741-743.

Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., & Green, D. R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med, 182*(5), 1545-1556.

Mashima, T., Naito, M., & Tsuruo, T. (1999). Caspase-mediated cleavage of cytoskeletal actin plays a positive role in the process of morphological apoptosis. *Oncogene, 18*(15), 2423-2430.

Mattson, M. P., & Chan, S. L. (2003). *Calcium orchestrates apoptosis*: Nat Cell Biol. 2003 Dec;5(12):1041-3.

McLennan, I. S. (1993). Resident macrophages (ED2- and ED3-positive) do not phagocytose degenerating rat skeletal muscle fibres. *Cell & Tissue Research, 272*(1), 193-196.

Monastyrskaya, K., Babiychuk, E. B., & Draeger, A. (2009). The annexins: spatial and temporal coordination of signaling events during cellular stress. *Cell Mol Life Sci, 66*(16), 2623-2642.

Morzel, M., Terlouw, C., Chambon, C., Micol, D., & Picard, B. (2008). Muscle proteome and meat eating qualities of Longissimus thoracis of "Blonde d'Aquitaine" young bulls: A central role of HSP27 isoforms. *Meat Science, 78*(3), 297-304.

Nishimura, T. (2010). The role of intramuscular connective tissue in meat texture. *Anim Sci J, 81*(1), 21-27.

Nishimura, T., Hattori, A., & Takahashi, K. (1996a). Relationship between degradation of proteoglycans and weakening of the intramuscular connective tissue during post-mortem ageing of beef. *Meat Sci, 42*(3), 251-260.

Nishimura, T., Hattori, A., & Takahashi, K. (1996b). Relationship between degradation of proteoglycans and weakening of the intramuscular connective tissue during post-mortem ageing of beef. *Meat Science, 42*(3), 251-260.

O'Brien, P. J., Siraki, A. G., & Shangari, N. (2005). Aldehyde sources, metabolism, molecular toxicity mechanisms, and possible effects on human health. *Crit Rev Toxicol, 35*(7), 609-662.

Offer, G., & Knight, P. (1988a). The structural basis of water-holding in meat. I. General principles and water uptake in meat processing. *Developments in Meat Science, 4*, 63-171.

Offer, G., & Knight, P. (1988b). The structural basis of water-holding in meat. II. Drip losses. *Developments in Meat Science, 4*, 173-243.

Olson, S. T., & Gettins, P. G. (2011). Regulation of proteases by protein inhibitors of the serpin superfamily. *Prog Mol Biol Transl Sci, 99*, 185-240.

Orrenius, S., Zhivotovsky, B., & Nicotera, P. (2003). Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol, 4*(7), 552-565.

Otera, H., & Mihara, K. (2012). Mitochondrial dynamics: functional link with apoptosis. *Int J Cell Biol, 821676*(10), 22.

Ouali, A. (1990). Meat Tenderization: Possible Causes and Mechanisms. A Review. *Journal of Muscle Foods, 1*(2), 129-165.

Ouali, A. (1995). Proteolytic enzymes and tenderness of meat. *Viandes et Produits Carnes, 16*(3), 81-88.

Ouali, A., Herrera-Mendez, C. H., Coulis, G., Becila, S., Boudjellal, A., Aubry, L., & Sentandreu, M. A. (2006). Revisiting the conversion of muscle into meat and the underlying mechanisms. *Meat Science, 74*(1), 44-58.

Ouali, A., Herrera-Mendez, C. H., Coulis, G., Samira, B., Boudjellal, A., Harhoura, K., Aubry, L., & Sentandreu, M. A. (2007). Meat tenderisation and muscle cell death, two highly related events. *Tehnologija Mesa, 48*(1/2), 1-15.

Ouali, A., Rouchon, P., Zabari, M., Berri, M., Zamora, F., & Tassy, C. (1995). Natural serine and cysteine proteinase inhibitors in bovine skeletal muscle. *In Expression of tissue proteinases and regulation of protein degradation as related to meat quality Edited by: Ouali A, Demeyer D, Smulders FJM. Utrecht (NL): ECCEAMST,*, 173 - 198.

Ouali, A., & Talmant, A. (1990). Calpains and calpastatin distribution in bovine, porcine and ovine skeletal muscles. *Meat Sci, 28*(4), 331-348.

Pak, J. H., Choi, W. H., Lee, H. M., Joo, W. D., Kim, J. H., Kim, Y. T., Kim, Y. M., & Nam, J. H. (2011). Peroxiredoxin 6 overexpression attenuates cisplatin-induced apoptosis in human ovarian cancer cells. *Cancer Invest, 29*(1), 21-28.

Pappa, A., Brown, D., Koutalos, Y., DeGregori, J., White, C., & Vasiliou, V. (2005). Human aldehyde dehydrogenase 3A1 inhibits proliferation and promotes survival of human corneal epithelial cells. *J Biol Chem, 280*(30), 27998-28006.

Pelissier, P., Delourme, D., Germot, A., Blanchet, X., Becila, S., Maftah, A., Leveziel, H., Ouali, A., & Bremaud, L. (2008). An original SERPINA3 gene cluster: elucidation of genomic organization and gene expression in the Bos taurus 21q24 region. *BMC Genomics, 9*, 151.

Philchenkov, A. (2004). Caspases: potential targets for regulating cell death. *J Cell Mol Med, 8*(4), 432-444.

Picard, B., Berri, C., Lefaucheur, L., Molette, C., Sayd, T., & Terlouw, C. (2010). Skeletal muscle proteomics in livestock production. *Brief Funct Genomics, 9*(3), 259-278.

Polati, R., Menini, M., Robotti, E., Millioni, R., Marengo, E., Novelli, E., Balzan, S., & Cecconi, D. (2012). Proteomic changes involved in tenderization of bovine Longissimus dorsi muscle during prolonged ageing. *Food Chemistry, 135*(3), 2052-2069.

Prochazkova, J., Kylarova, D., Vranka, P., & Lichnovsky, V. (2003). *Comparative study of apoptosis-detecting techniques: TUNEL, apostain, and lamin B*: Biotechniques. 2003 Sep;35(3):528-34.

Rennie, M. J. (2010). Influence of Exercise on Protein and Amino Acid Metabolism. In *Comprehensive Physiology*: John Wiley & Sons, Inc.

Saelens, X., Festjens, N., Vande Walle, L., van Gurp, M., van Loo, G., & Vandenabeele, P. (2004). Toxic proteins released from mitochondria in cell death. *Oncogene, 23*(16), 2861-2874.

Sentandreu, M. A., Coulis, G., & Ouali, A. (2002). Role of muscle endopeptidases and their inhibitors in meat tenderness. *Trends in Food Science & Technology, 13*(12), 400-421.

Shackelford, S. D., Koohmaraie, M., Miller, M. F., Crouse, J. D., & Reagan, J. O. (1991). An evaluation of tenderness of the longissimus muscle of Angus by Hereford versus Brahman crossbred heifers. *J Anim Sci, 69*(1), 171-177.

Sierra, V., & Olivan, M. (2013). Role of Mitochondriaon Muscle Cell Death and Meat Tenderization. *Recent Pat Endocr Metab Immune Drug Discov, 13*, 13.

Suen, D. F., Norris, K. L., & Youle, R. J. (2008). Mitochondrial dynamics and apoptosis. *Genes Dev, 22*(12), 1577-1590.

Tarze, A., Deniaud, A., Le Bras, M., Maillier, E., Molle, D., Larochette, N., Zamzami, N., Jan, G., Kroemer, G., & Brenner, C. (2007). GAPDH, a novel regulator of the pro-apoptotic mitochondrial membrane permeabilization. *Oncogene, 26*(18), 2606-2620.

Tassy, C., Herrera-Mendez, C. H., Sentandreu, M. A., Aubry, L., Bremaud, L., Pelissier, P., Delourme, D., Brillard, M., Gauthier, F., Leveziel, H., & Ouali, A. (2005). Muscle endopin 1, a muscle intracellular serpin which strongly inhibits elastase: purification, characterization, cellular localization and tissue distribution. *Biochem J, 388*(Pt 1), 273-280.

Taylor, R. C., Cullen, S. P., & Martin, S. J. (2008). Apoptosis: controlled demolition at the cellular level. *Nature Reviews Molecular Cell Biology, 9*(3), 231-241.

Taylor, R. G., Papa, I., Astier, C., Ventre, F., Benyamin, Y., & Ouali, A. (1997). Fish muscle cytoskeleton integrity is not dependent on intact thin filaments. *J Muscle Res Cell Motil, 18*(3), 285-294.

te Pas, M. F. W., Kruijt, L., Pierzchala, M., Crump, R. E., Boeren, S., Keuning, E., Hoving-Bolink, R., Hortós, M., Gispert, M., Arnau, J., Diestre, A., & Mulder, H. A. (2013). Identification of proteomic biomarkers in M. Longissimus dorsi as potential predictors of pork quality. *Meat Science*(0).

Tews, D. S., Goebel, H. H., Schneider, I., Gunkel, A., Stennert, E., & Neiss, W. F. (1997). DNA-fragmentation and expression of apoptosis-related proteins in experimentally denervated and reinnervated rat facial muscle. *Neuropathol Appl Neurobiol, 23*(2), 141-149.

Thornalley, P. J. (2008). Protein and nucleotide damage by glyoxal and methylglyoxal in physiological systems--role in ageing and disease. *Drug Metabol Drug Interact, 23*(1-2), 125-150.

Tulsawani, R., Kelly, L. S., Fatma, N., Chhunchha, B., Kubo, E., Kumar, A., & Singh, D. P. (2010). Neuroprotective effect of peroxiredoxin 6 against hypoxia-induced retinal ganglion cell damage. *BMC Neurosci, 11*(125), 1471-2202.

van Erk, M. J., Wopereis, S., Rubingh, C., van Vliet, T., Verheij, E., Cnubben, N. H., Pedersen, T. L., Newman, J. W., Smilde, A. K., van der Greef, J., Hendriks, H. F., &

van Ommen, B. (2010). Insight in modulation of inflammation in response to diclofenac intervention: a human intervention study. *BMC Med Genomics, 3*, 5.

van Genderen, H. O., Kenis, H., Hofstra, L., Narula, J., & Reutelingsperger, C. P. (2008). Extracellular annexin A5: functions of phosphatidylserine-binding and two-dimensional crystallization. *Biochim Biophys Acta, 1783*(6), 953-963.

Vasiliou, V., Pappa, A., & Estey, T. (2004). Role of human aldehyde dehydrogenases in endobiotic and xenobiotic metabolism. *Drug Metab Rev, 36*(2), 279-299.

Vasiliou, V., Thompson, D. C., Smith, C., Fujita, M., & Chen, Y. (2012). Aldehyde dehydrogenases: From eye crystallins to metabolic disease and cancer stem cells. *Chem Biol Interact, 16*(12), 00233-00235.

Vasta, G. R. (2012). Galectins as pattern recognition receptors: structure, function, and evolution. *Adv Exp Med Biol, 946*, 21-36.

Verhagen, A. M., Silke, J., Ekert, P. G., Pakusch, M., Kaufmann, H., Connolly, L. M., Day, C. L., Tikoo, A., Burke, R., Wrobel, C., Moritz, R. L., Simpson, R. J., & Vaux, D. L. (2002). HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J Biol Chem, 277*(1), 445-454.

Vignon, X., Beaulaton, J., & Ouali, A. (1989). Ultrastructural localization of calcium in post-mortem bovine muscle: a cytochemical and X-ray microanalytical study. *Histochem J, 21*(7), 403-411.

Wei, Y., Fan, T., & Yu, M. (2008). Inhibitor of apoptosis proteins and apoptosis. *Acta Biochimica et Biophysica Sinica, 40*(4), 278-288.

Westermann, B. (2008). Molecular machinery of mitochondrial fusion and fission. *J Biol Chem, 283*(20), 13501-13505.

Yang, F., Sun, X., Beech, W., Teter, B., Wu, S., Sigel, J., Vinters, H. V., Frautschy, S. A., & Cole, G. M. (1998). Antibody to caspase-cleaved actin detects apoptosis in differentiated neuroblastoma and plaque-associated neurons and microglia in Alzheimer's disease. *Am J Pathol, 152*(2), 379-389.

Yang, Q.-H., Church-Hajduk, R., Ren, J., Newton, M. L., & Du, C. (2003). Omi/HtrA2 catalytic cleavage of inhibitor of apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis. *Genes Dev, 17*(12), 1487-1496.

Yang, R. Y., Rabinovich, G. A., & Liu, F. T. (2008). Galectins: structure, function and therapeutic potential. *Expert Rev Mol Med, 13*(10).

Yoshida, A., Pommier, Y., & Ueda, T. (2006). Endonuclease activation and chromosomal DNA fragmentation during apoptosis in leukemia cells. *Int J Hematol, 84*(1), 31-37.

Youle, R. J., & Karbowski, M. (2005). Mitochondrial fission in apoptosis. *Nat Rev Mol Cell Biol, 6*(8), 657-663.

Zamora, F., Aubry, L., Sayd, T., Lepetit, J., Lebert, A., Sentandreu, M. A., & Ouali, A. (2005). Serine peptidase inhibitors, the best predictor of beef ageing amongst a large set of quantitative variables. *Meat Sci, 71*(4), 730-742.

Zamora, F., Debiton, E., Lepetit, J., Lebert, A., Dransfield, E., & Ouali, A. (1996). Predicting variability of ageing and toughness in beef M. Longissimus lumborum et thoracis. *Meat Sci, 43*(3-4), 321-333.

Zapata, I., Zerby, H. N., & Wick, M. (2009). Functional proteomic analysis predicts beef tenderness and the tenderness differential. *J Agric Food Chem, 57*(11), 4956-4963.

Zhao, Y. M., Basu, U., Dodson, M. V., Basarb, J. A., & Guan le, L. (2010). Proteome differences associated with fat accumulation in bovine subcutaneous adipose tissues. *Proteome Sci, 8*(14), 14.

Zietkiewicz, S., Krzewska, J., & Liberek, K. (2004). Successive and synergistic action of the Hsp70 and Hsp100 chaperones in protein disaggregation. *J Biol Chem, 279*(43), 44376-44383.

Zoubine, M. N., Ma, J. Y., Smirnova, I. V., Citron, B. A., & Festoff, B. W. (1996). A molecular mechanism for synapse elimination: novel inhibition of locally generated thrombin delays synapse loss in neonatal mouse muscle. *Dev Biol*, *179*(2), 447-457.

Tables and legends

Table 1: Potential markers of tenderness belonging to the glycolytic pathway.

Abbreviations used: *[M], marker concentration increases (+) or decreases (-); *Tend: meat tenderness increases (+) or decreases (-) as a consequence of the marker level modification. (+/-), marker induced change in meat tenderness is variable.

Marker Name	Localization	Functions	*[M]	*Tend	References
Phosphoglucomutase	Cytoplasm	Gluc-1P <==> Gluc 6P	+	+	(Bjarnadottir, et al., 2010; Bouley, et al., 2004 ; Laville, et al., 2009)
Triosephosphate isomerase	Cytoplasm	Dihydroxyacetone phosphate (DHAP) <===> glyceraldehyde 3-phosphate	+	+	(G. D. Kim, et al., 2009)
Glyceraldehyde-3 phosphate dehydrogenase (GAPDH)	Cytoplasm, Nucleus	glycéraldéhyde.3.phosphate <=> 1,3.diphosphoglycerase **Cell death & cell survival**	+	+	(Colell, Green, & Ricci, 2009; Laville, et al., 2009)
3-Phosphoglycerate kinase	Cytoplasm	3-phosphoglycerate + ADP <==> 1,3-bisphosphoglycerate + ADP	+	+	(Jia, Hildrum, et al., 2006)
Aldehyde dehydrogenase (ALDH)	Cytosol and organelles	Aldehyde oxidation to carboxylic acids (glycolysis, amino acids and lipids degradation)	+	+/-	(Hollung, et al., 2007; Jia, Hildrum, et al., 2006)
Enolase 3 or Phospho-pyruvate hydratase	Cytoplasm **Muscle specific**	2-phosphoglycerate <===> phosphoenolpyruvate	+	+	(Choi, et al., 2010; Laville, et al., 2009)
Pyruvate kinase	Cytoplasm	phosphoenolpyruvate <===> pyruvate	+	+	(Laville, et al., 2009 ; Polati, et al., 2012)
Lactate dehydrogenase	Cytoplasm	pyruvate <===> lactate.	+	+	(Laville, et al., 2009 ; Polati, et al., 2012)

Table 2: Potential markers of tenderness belonging to the aerobic Pathway.

Abbreviations used: Mito.: mitochondria. Mb: membrane. TCA: Tri-Carboxylic Acid. *[M]: marker concentration increase (+) or decrease (-). *Tend: meat tenderness increases (+) or decreases (-) as a consequence of the marker level modification. (+/-): marker induced change in meat tenderness is variable.

Marker Name	Localization	Functions	*[M]	*Tend	References
3-hydroxyisobutyrate dehydrogenase (1)	Mito.	1 of the 9 steps of valine degrad. to succinyl-CoA	+	+	(Jia, et al., 2007; Jia, Hollung, et al., 2006)
β-hydroxyacyl CoA-dehydrogenase	Matrix Mito.	Fatty acid degradation (Lynen helix)	+	+	(Hamill, et al., 2012 ; Polati, et al., 2012)
Cytochrome c	Mito.	Electron transport chain; external side of inner Mito mb. Apoptosis	+	+	(Ding, et al., 2002)
Succinate dehydrogenase	Matrix Mito	TCA cycle	+	+	(G. D. Kim, et al., 2009)
Succinyl Co-A synthase	Matrix Mito.	TCA cycle	+	+	(Hollung, et al., 2007; Jia, Hildrum, et al., 2006)
Isocitrate dehydrogenase	Matrix Mito.	TCA cycle	+	+/-	(Hamill, et al., 2012 ; Zapata, et al., 2009)

ATP synthase	inner Mito. Mb	ATP synthesis	+	+	(Hamill, et al., 2012 ; Zapata, et al., 2009)

Table 3: Potential markers of tenderness involved in cell detoxification.

Abbreviations used: Mito.: mitochondria. *[M], marker concentration increases (+) or decreases (-); *Tend: meat tenderness increases (+) or decreases (-) as a consequence of the marker level modification. (+/-), marker induced change in meat tenderness is variable.

Marker Name	Localization	Functions	*[M]	*Tend	References
Carbonic anhydrase	Cytosol, Mito.	Elimination CO2; H2O+CO2=>HCO3 + H+	+	+	(D'Alessandro & Zolla, 2013; Polati, et al., 2012 ; Zapata, et al., 2009)
Lactoylglutathione lyase or Glyoxylase 1	Cytosol	Detoxification methylglyoxal => lactate	+	+	(Jia, Hildrum, et al., 2006)
Aldehyde dehy-drogenases (ALDHs)	Cytosol and all organelles	Protect cell from Cytotoxic aldehydes	+	+/-	(Hollung, et al., 2007; Jia, Hildrum, et al., 2006)

Table 4: Candidate Heat Shock Proteins as potential markers of tenderness.

Abbreviations used: *[M], marker concentration increases (+) or decreases (-); *Tend: meat tenderness increases (+) or decreases (-) as a consequence of the marker level modification.

Marker Name	Localization	Functions	*[M]	*Tend	References
HSP 70	Cytoplasm	Slow down the process of cellular death. Protection of tissues against oxidative stress. Fat deposition	+	-	(Cassar-Malek, et al., 2011; Guillemin, Jurie, et al., 2011)
DNAJA1 (HSP 40)	Nucleus / Cytoplasm	DNAJA1 gene encodes HSP40, a chaperone involved in protein import into mitochondria and a co-chaperone of HSP70	+	-	(Bernard, et al., 2007 ; Picard, et al., 2010)
HSPB1 (HSP 27)	Cell surface / Cytoplasm / Nucleus	Response to heat and stress. Regulation and stabilization of myofibrillar proteins, and protects actin filaments and desmin	+	-	(Bernard, et al., 2007 ; Guillemin, Bonnet, Jurie, & Picard, 2011; Morzel, Terlouw, Chambon, Micol, & Picard, 2008)
α-crystallin (CRYAB)	Nucleus	Protein homodimerization activity. Protection of structural proteins	+	-	(Bernard, et al., 2007 ; Guillemin, Bonnet, et al., 2011; Morzel, et al., 2008)
HSP 60	Cell surface / Cytoplasm	Prevent degradation & structure damage of proteins from apoptotic processes in muscle cells.	+	-	(Beere, 2005; Jia, Hollung, et al., 2006)

Figures Captions

Figure 1: Major events occurring in post mortem muscle after cessation of blood flow. Abbreviations: TCA, Tricarboxylic acids; HSP, Heat Shock Proteins; PS, Phosphatidyserine; IAP, Inhibitors of Apoptosis Proteins.

Figure 2: *Postmortem* structural changes in fibres from rat Longissimus muscle. (a) transversal cuts of an ante-mortem sample showing cells in close proximity and very few extracellular space (Open arrows); (b)transversal cut showing a shrinkage of muscle fibres and an increase in the extracellular space (Open arrows). The sample was obtained just after bleeding and prepared for subsequent examination after hematoxylin/Erythrosin staining. Nucleus were also immunostained according to (Prochazkova, et al., 2003) with a monoclonal antibody labelling specifically single strand DNA fragments characteristic of apoptotic cells. Apoptotic nucleus are stained in dark brown. **(c)** Similar muscle sample excised 15 min after bleeding showing 1DNA fragment staining in a mononucleated cells (open arrow head). In this view of the sample we noticed a high concentration of small cells, probably resident macrophages, delinating empty cells (open arrows). Muscle cells nucleus showed no DNA degradation (close arrow heads). **(d)** Similar sample than in c showing a mononucleated cell with fragmented DNA (open arrow head), a high concentration of small cells corresponding very likely to resident macrophages and aligned along fibre membranes(open arrow). Inserts showed muscle cell membrane invaginations (short black arrows) indicative of phagocytosis activities for elimination of extracellular material/cells within muscle cells. Scale bars: 100 µm

Figure 3: (a) Polymorphism of the Bov-SERPINA3 family as assessed by 2D gel electrophoresis of the high Mr fraction eluted from a crude muscle extract by chromatography on a sephadex G100 column as assessed by 2D Gel analysis using a specific rabbit polyclonal antibody raised against purified bovSERPINA3-1. In this figure, the line with a down arrowhead indicates aligned spots corresponding to various degree of phosphorylation. The black arrow indicates a comma shape alignment of spots corresponding to different degree of glycosylation (Boudida, Gagaoua, et al., 2013; Gagaoua, et al., 2012). (b) Covalent complexes upon SDS-PAGE between Bov-SerpinA3-1 and proteases as revealed by western blot, using the rabbit polyclonal antibody raised against this serpin (Herrera-Mendez, et al., 2009). Similar findings were obtained with Bov-SerpinA3-3 (not shown). Note that the 140 kDa band is a dimer of the 70 kDa serpin (Tassy, et al., 2005). Abbreviations used: C, band

corresponding to the complex formed with the different target enzymes: trypsin, Human Leukocyte Elastase (HLE) and human recombinant caspases 3 and 8. (-) inhibitor alone; (+) inhibitor incubated with the target enzyme (Enz).

Figure 4: Metabolic pathways generating or using Dihydroxyacetone phosphate. Enzyme names: (1) Fructokinase, (2) Fructose-1-P aldolase, (3) 1st steps Glycolysis, (4) Aldolase, (5) Triose phosphate isomerase, (6) Lipases, (7) Glycerol kinase, (8) Glycerol-3-phosphate dehydrogenase (G3PDH), (9)Aldehyde dehydrogenase, (10) Glycerate kinase, (11) Methylglyoxal synthase. c/m-8: cytosolic and mitochondrial G3PDH.

Figure 5: Overview of the energy supplying pathways: (a) Major energy production pathways from amino-acids, glucose and free fatty acids (adapted from http://www.takween.com/metabolisme/metabolisme-sucres.html. (b) Major end products of amino acids degradation at the Krebs cycle level (adapted from http://www.natuurlijkerwijs.com/english/Amino_acid_metabolism.htm#aminozuurafbraak.

Figure 6: Calcium trafficking between endoplasmic reticulum (ER) and mitochondria which are close to each other (left part of the figure): Cytochrome c released from Mitochondria binds to InsP3R resulting in sustained calcium efflux. Bik, a BH3 member of the Bcl-2 family also mediates calcium release from ER. Increase in cytosolic calcium induces Drp1 recruitment to the mitochondria which initiates the fission process (see insert). Mitochondria take up calcium into the matrix via calcium channel as MICU. The massive calcium influx into the matrix leads to mitochondria fission, accelerates cytochrome c release and amplify apoptosis. **Apoptose regulation** (right part of the figure): Proteins (Prot) released from mitochondria comprise cytochrome c, AIF, Endo G, Smac/Diablo and Omi/HtrA2. Cytochrome c binds to Apaf1 to form the apoptosome, a complex activating procaspase 9. Endo G and AIF translocate to the nucleus where they contribute to chromatine condensation and DNA fragmentation. IAPs (Inhibitors of Apoptosis Proteins) are inhibitors of caspases 3, 9 and 7 and the interaction with their target enzymes can be reversed by SMAC/Diablo and Omi/HtrA2. SERPINA3 like are pseudo-irreversible inhibitors of initiator and effector caspases and hence will be essential regulators of the caspase-dependent apoptotic process. Abbreviations used: AIF, Apoptosis Inducing Factor ; Apaf 1, apoptotic protease activating factor); Bcl2, Bax, Bik: members of the Bcl2 (B-cell lymphoma 2) family; Drp1, dynamin related protein 1; Endo G, endonuclease G; InsP3D, inositol (1,4,5) trisphosphate receptor; MICU, mitochondrial calcium uptake; Omi/HtrA2, Omi stress-regulated endopeptidase /high-

temperature-requirement protein A2; SERCA, sarco/endoplasmic reticulum Ca2+-ATPase; SMAC/Diablo, second mitochondria-derived activator of caspases encoded by the Diablo gene. Adapted from (Otera & Mihara, 2012).

Figure 7: Postmortem changes in muscle pH, in the soluble 32 kDa actin fragment levels and in the extent of cytochrome c release in *Longissimus* rat muscle maintained on the carcass which was stored at 10°C for 24h and then transferred at 4°C. More details can be found in (Becila, et al., 2010).

Figure 8: 12% SDS-PAGE of the protein degradation profile obtained when bovine skeletal muscle actin was incubated in the presence of recombinant human caspase 3 at different time intervals (data not shown previously). (A): protein band corresponding to intact monomeric actin; (B), (C) and (D): actin fragments generated after incubation with caspase 3, having molecular mass values around 32, 25 and 14 kDa, respectively.

Figure 1:

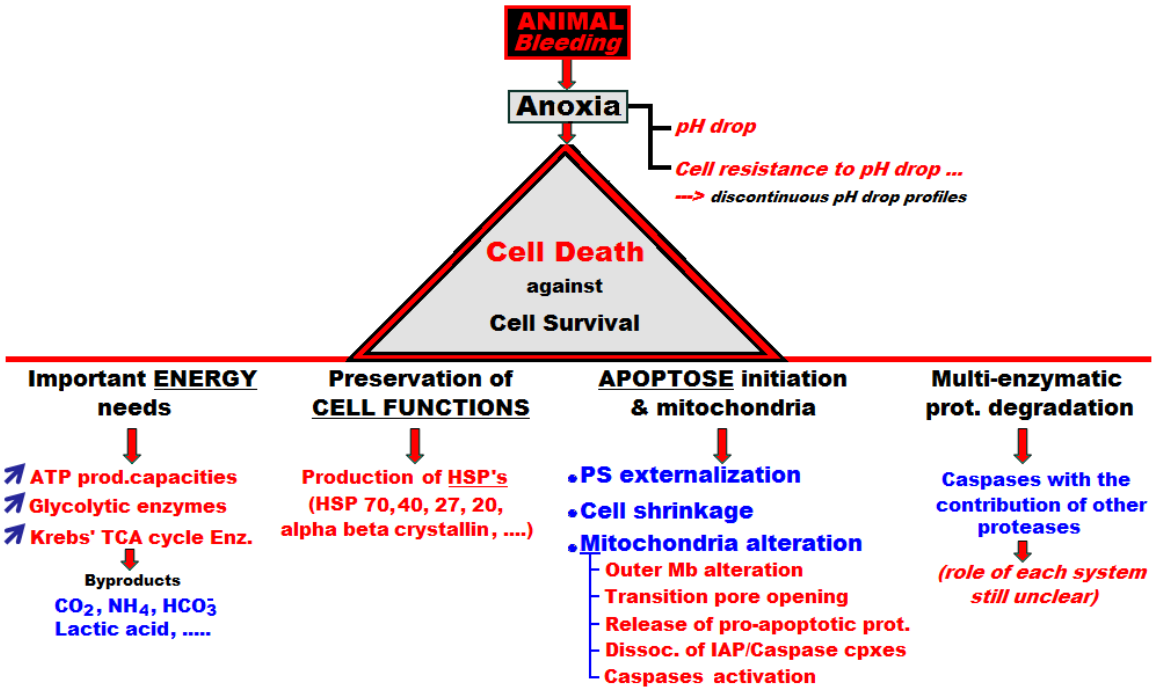


Figure 2:

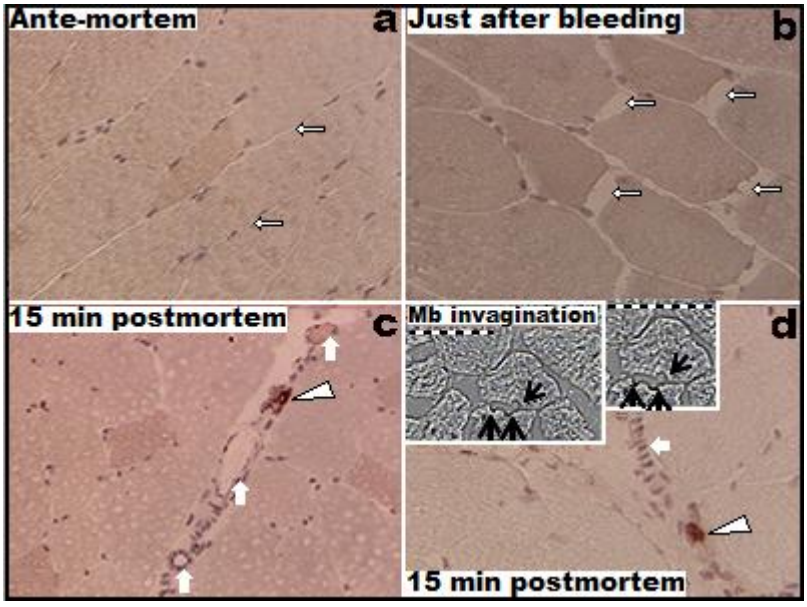


Figure 3:

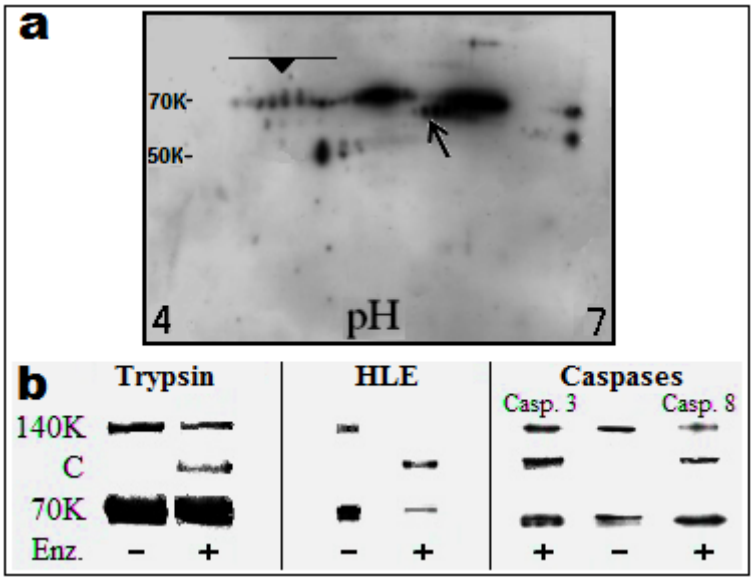
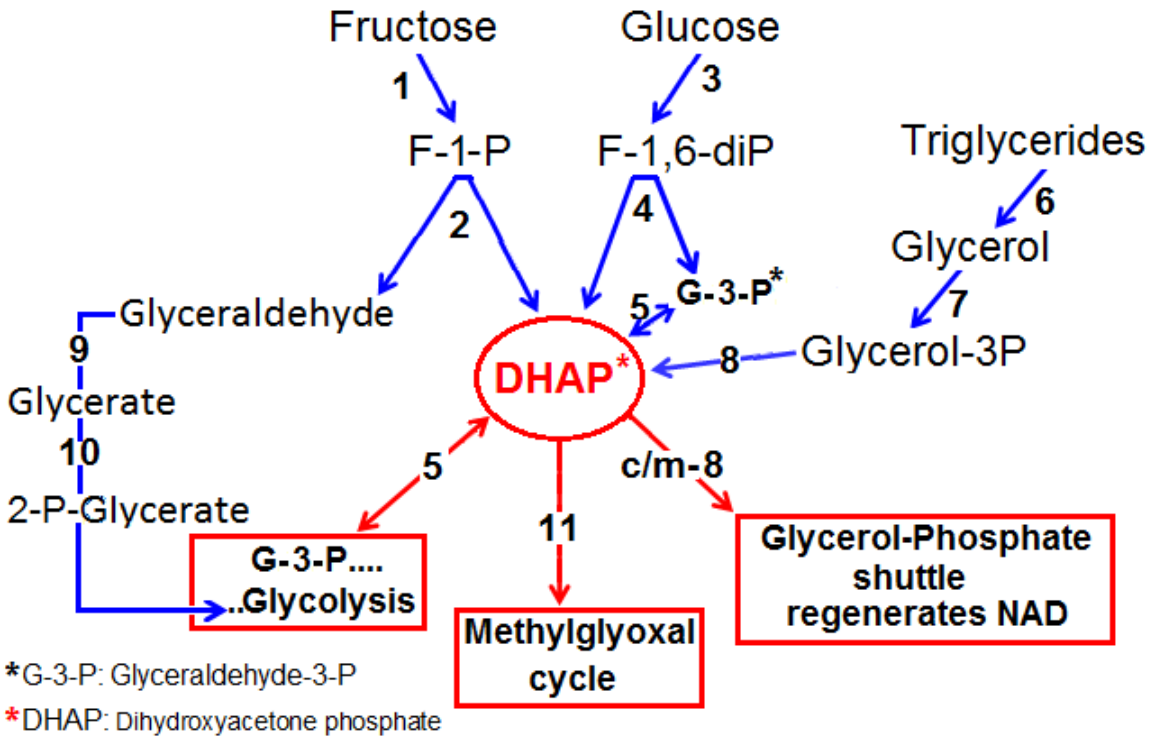


Figure 4:



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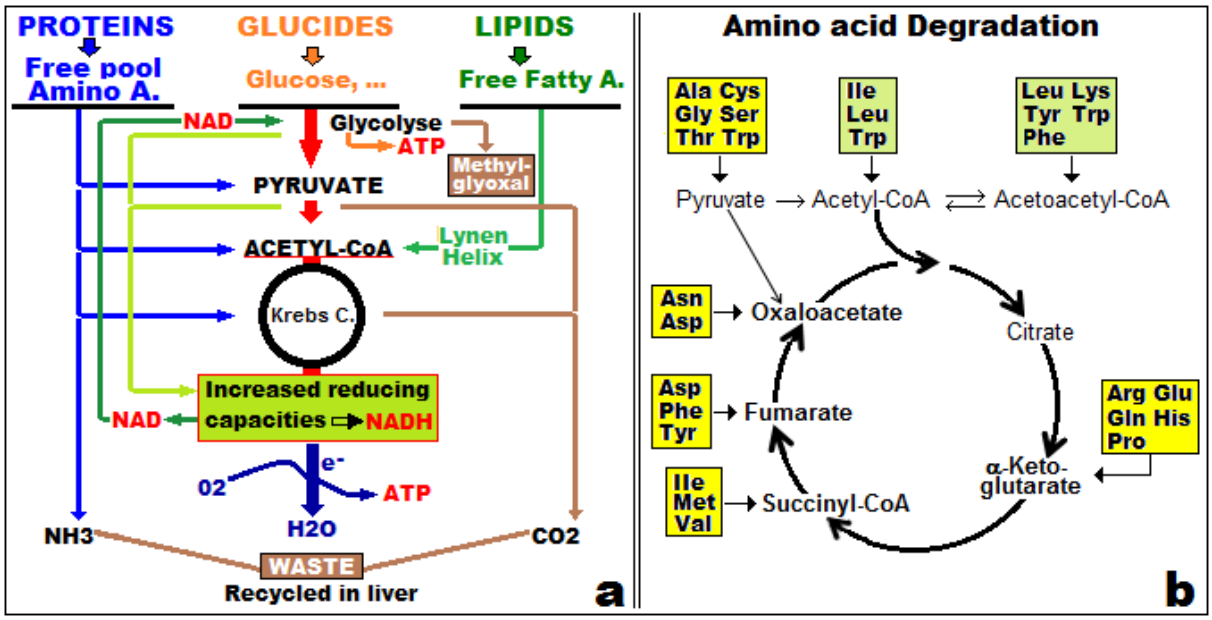

Figure 6:

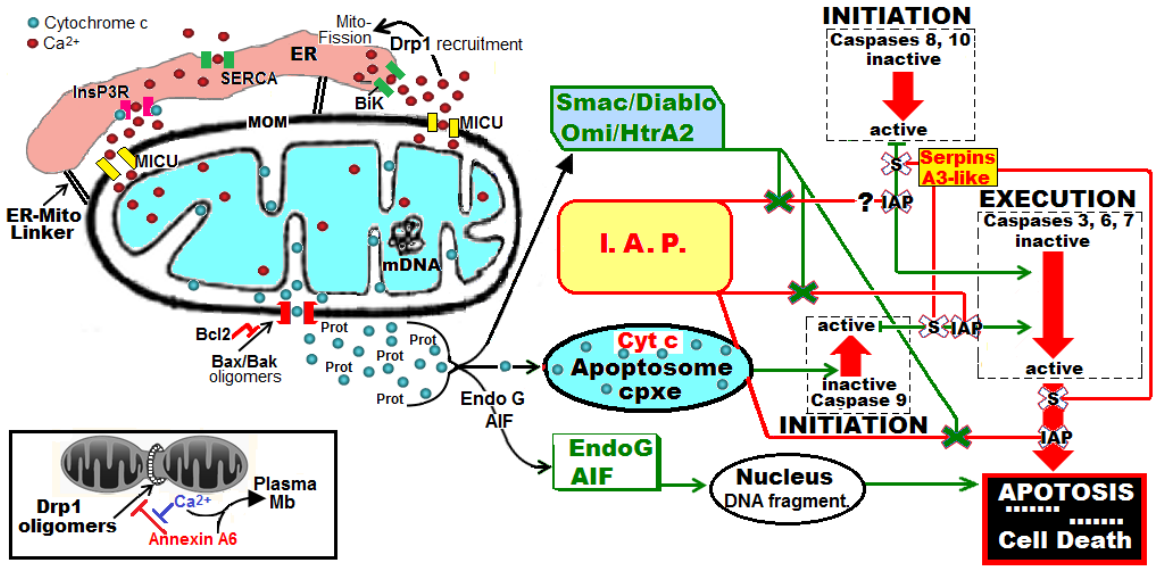

Figure 7:

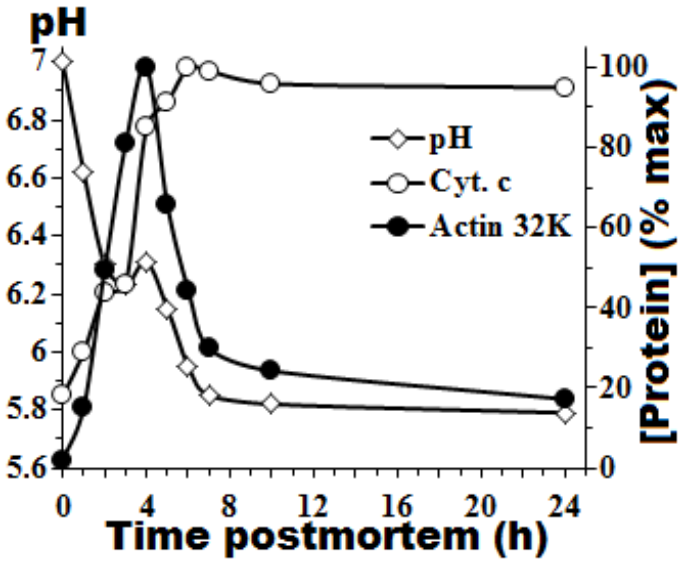

Figure 8: