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

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► To cite this version:

Ahmed Ouali, Mohammed Gagaoua, Yasmine Boudida, Samira Becila, Abdelghani Boudjellal, et al. Biomarkers of meat tenderness: Present knowledge and perspectives in regards to our current understanding of the mechanisms involved. *Meat Science*, 2013, 95 (4), pp.854 - 870. 10.1016/j.meatsci.2013.05.010 . hal-02652573

HAL Id: hal-02652573

<https://hal.inrae.fr/hal-02652573>

Submitted on 21 Sep 2023

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

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Abstract

1
2 Biomarkers of the meat quality are of prime importance for meat industry, which has to
3 satisfy consumers' expectations and, for them, meat tenderness is and will remain the primary
4 and most important quality attribute. The tenderization of meat starts immediately after
5 animal death with the onset of apoptosis followed by a cooperative action of endogenous
6 proteolytic systems. Before consideration of the biomarkers identified so far, we present here
7 some new features on the apoptotic process. Amongst them, the most important is the recent
8 discovery of a complex family of serpins capable to inhibit, in a pseudo-irreversible manner,
9 caspases, the major enzymes responsible of cell dismantling during apoptosis. The biomarkers
10 so far identified have been then sorted and grouped according to their common biological
11 functions. All of them refer to a series of biological pathways including glycolytic and
12 oxidative energy production, cell detoxification, protease inhibition and production of Heat
13 Shock Proteins. Some unusual biomarkers are also presented: annexins, galectins and
14 peroxiredoxin. On this basis, a detailed analysis of these metabolic pathways allowed us to
15 identify some domains of interest for future investigations. It was thus emphasized that
16 mitochondria, an important organelle in the production of energy from carbohydrates, lipids
17 and proteins are a central element in the initiation and development of apoptosis. It was
18 therefore stressed forward that, in fact, very little is known about the *postmortem* fate of these
19 organelles and their multiple associated activities. Other topics discussed here would provide
20 avenues for the future in the context of identifying reliable predictors of the ultimate meat
21 tenderness.
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Key words: biomarkers, tenderness, metabolism, muscle cell, meat quality, apoptosis

Highlights: Potential Biological markers of meat tenderness

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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 ([Quali, 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/biochemical 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

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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 CO₂, HCO₃⁻, NH₄ 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,

1 mitochondria will be reconsidered later in this review. So it could be interesting to develop
2 studies on mitochondria, in order to establish whether this could be a way to better explain
3 tenderness variability of carcasses.
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5 6 2.2. Are mono-nucleated cells the first to die in postmortem muscle?

7 2.2.1. Characteristic features of apoptosis in muscle cells 8

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10 A specific feature of skeletal muscle fibres is being multinucleated in the form of
11 successive muscle-fibre segments controlled by separate nuclei residing in the segments. It
12 has been shown in experimental denervation as well as in infantile spinal muscular atrophy
13 that a minority of muscle fibres reveal apoptotic DNA-fragmentation at the same time ([Tews,
14 et al., 1997](#)).
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18 On this basis, we might expect a very different image of muscle cells as compared to dying
19 mononucleated cells generally found in the extracellular matrix (fibroblasts, epithelial cells of
20 blood vessels, adipocytes,...) which might be easier to identify in muscle tissue. The nature of
21 the structural alterations characterizing the death of muscle fibres in *postmortem* muscle is
22 therefore an opened question. To verify this assumption, we looked at muscle fibres in
23 *postmortem* muscle hoping to detect DNA alteration using an antibody labeling specifically
24 regular single-stranded fragments characterizing cell apoptosis but not double stranded
25 fragments observed in necrosis ([Ouali, et al., 2007](#)).
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28 2.2.2. Particular structural apoptotic changes in postmortem muscle 29

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31 The following results were obtained using rat as a model. The animal was anesthetized by
32 etheroxyde and a sample of the *Longissimus* muscle was cut off and immediately frozen for
33 subsequent immunohistochemical examination. The animal was then bled by decapitation,
34 dressed and the carcass stored at +10°C up to 24 h *postmortem* and then transferred to 4°C.
35 Samples were taken at different times after animal death, i.e. just after bleeding, at 15, 30, 60
36 min *postmortem* and then at 2, 4, 7, 24 and 48 h *postmortem*. Thin cuts were stained using the
37 traditional Hematoxylin/ Erythrosin method and finally labeled with a monoclonal antibody
38 (apostain) labeling specifically single-stranded DNA fragments of regular length
39 characterizing apoptotic cells ([Prochazkova, Kylarova, Vranka, & Lichnovsky, 2003](#)).
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43 Examination of the thin cuts was done with the aim to identify structural changes
44 characteristic of the programmed cell death or apoptosis. As compared to Figure 2a where
45 muscle cells are in close proximity to each other, few minutes after bleeding an important
46 shrinkage of muscle cells can be observed (Figure 2b, small arrows). Over the past decades,
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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

1 within muscle cells [insert Figure 2D at a low (left) and a slightly higher (right)
2 magnification] supporting the occurrence of phagocytotic activities in *postmortem* muscle.
3 From these findings, it must be therefore stressed that the first cells to die *postmortem* are
4 mononucleated cells from the extracellular matrix, which might be degraded by muscle cells
5 after ingestion by phagocytosis.
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10 2.3. *New inhibitors of both caspases and apoptosis*

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12 In the 80's, the presence of serine proteases within muscle cells was doubtful and a large
13 set of investigations suggested that some of them if not all originated from mast cells (Ouali,
14 1990). As a result this protease family was ignored by meat scientists. Later on, serine
15 protease inhibitors have been suggested to be a good marker of meat tenderness ([Zamora, et](#)
16 [al., 2005](#); [Zamora, et al., 1996](#)), a result strengthening the early proposal that inhibitors are
17 better predictors of tenderness than their target enzymes ([Ouali, 1990](#)). As shown in Figure
18 3a, detailed investigations carried out on these serine protease inhibitors revealed a complex
19 protein family belonging to the serpin superfamily ([Boudida, Gagaoua, et al., 2013](#); [Gagaoua,](#)
20 [et al., 2012](#); [Pelissier, et al., 2008](#); [Sentandreu, Coulis, & Ouali, 2002](#)).

21
22 Serpins, an acronym of **SER**ine **P**rotease **I**nhibitors, were discovered in the beginning of
23 the 80's. This superfamily comprise the largest family of protease inhibitors identified to date,
24 now having over 3000 members in all the three kingdoms of life, the archea, the bacteria, and
25 the eukaryotes, as well as in some viruses ([Olson & Gettins, 2011](#)).

26
27 Besides elastase and trypsin, the serpins identified by our group inhibit pseudo-irreversibly
28 initiator and effector human caspases (caspases 8 and 3) but neither papain nor cathepsins,
29 forming stable complexes with all inhibited proteases including these multimeric proteases
30 containing two active sites per molecule (Figure 3b) ([Gagaoua, et al., 2012](#); [Herrera-Mendez,](#)
31 [et al., 2009](#)). In addition, we purified an inhibitor of thrombin, an enzyme located at the level
32 of muscle synapses, identified as antithrombin III by mass spectrometry peptide map
33 ([Herrera-Mendez, et al., 2010](#)). In this tissue, thrombin is synthesized by muscle cells ([Citron,](#)
34 [Smirnova, Zoubine, & Festoff, 1997](#)) and acts locally by contributing to synapse remodeling
35 and elimination at the neuromuscular junction ([Citron, et al., 1997](#); [Liu, Fields, Festoff, &](#)
36 [Nelson, 1994](#); [Zoubine, Ma, Smirnova, Citron, & Festoff, 1996](#)).

37
38 Using different approaches, several studies support a degradation of a large set of
39 myofibrillar and cytoskeletal proteins (titin, nebulin, desmin, spectrin, desmin, troponin T, ...)
40 by caspases in *postmortem* muscle, as well as other proteins specifically targeted by these
41 proteases during apoptosis (spectrin and poly (ADP-ribose) polymerase or PARP). Note that
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1 most of these proteins are either cytoskeletal proteins or other proteins with proapoptotic
2 activities ([Chen, Shun, Zhang, & Gao, 2003](#); [Huang, Huang, Xu, & Zhou, 2009](#); [Kemp, et al.,
3 2010](#)). SERPINA3 like, a group of serpin inhibiting caspases, were assumed to be associated
4 with meat tenderness ([Gagaoua, et al., 2012](#); [Zamora, et al., 2005](#); [Zamora, et al., 1996](#)) but
5 also to other quality attributes including drip loss and extent of pH drop ([te Pas, et al., 2013](#)).
6 Further investigations should be done to understand the real relationship of these inhibitors
7 and meat quality attributes.
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10 **3. Potential biomarkers of meat tenderness identified up to date**

11 For many decades, meat scientists are looking for accurate biological markers of meat
12 tenderness that would make possible: (1) the classification of meat cuts soon after slaughter
13 on the basis of their potential ultimate tenderness and (2) the optimization of the genetic
14 selection of meat animals on the basis of this quality. These challenges have aroused a great
15 interest in this research field by meat scientists. Unfortunately, we still have not identified
16 such markers probably because of a too much limited understanding of the biological
17 mechanisms responsible for the *postmortem* improvement of meat texture.
18
19

20 Overview of the literature has allowed us to identify a series of potential markers of meat
21 tenderness which have been then screened according to the metabolic or biological process
22 they are involved in. This list is far from being exhaustive and numerous other proteins
23 remain to be considered. Such screening has allowed the identification of 8 subgroups, the
24 three most important being glycolytic and oxidative energy supplying pathways together with
25 Heat Shock Proteins (HSPs).
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27

28 *3.1. Markers from the glycolytic energy metabolism pathway*

29 All members of this selected group are enzymes of the glycolytic pathway (Table 1).
30 Glycolysis is a two-phase process. The first phase consuming 2 ATP, corresponds to the
31 conversion of glucose-1-phosphate, generated from either free glucose or from glycogen by
32 phosphorylase, to two trioses phosphate. The second one ensures the conversion of 2 trioses-
33 phosphate (from 1 glucose) to pyruvate and then lactate, producing 2 ATP and one NADH,H⁺
34 molecules.
35
36

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

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

- 14 - 3-hydroxyisobutyrate dehydrogenase is an enzyme involved in the degradation of the
15 branched amino acid valine to succinyl-CoA, a member of the tricarboxylic acid cycle
16 (TCA) cycle ([Jia, et al., 2007](#); [Jia, Hildrum, et al., 2006](#); [Lokanath, et al., 2005](#)).
- 17 - β -hydroxyacyl CoA-dehydrogenase (HADH) is a member of the β -oxidation of lipids,
18 also known as the Lynen cycle (or Lynen helix) which produces acetyl-CoA, a
19 metabolite entering the TCA cycle ([Hamill, et al., 2012](#); [Polati, et al., 2012](#)).
- 20 - Cytochrome c is a member of the electron transport chain located outside the inner
21 membrane of mitochondria ([Ding, Shen, & Ong, 2002](#)).
- 22 - The three other markers identified from this pathway, i.e. Succinate dehydrogenase,
23 Succinyl Co-A synthase and Isocitrate dehydrogenase, are all members of the TCA
24 cycle ([Hamill, et al., 2012](#); [Hollung, Veiseth, Jia, Faergestad, & Hildrum, 2007](#); [Jia,
25 Hildrum, et al., 2006](#); [G. D. Kim, Jeong, Moon, Hwang, & Joo, 2009](#); [Zapata, Zerby, &
26 Wick, 2009](#)).

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

3.3. Markers involved in cell detoxification

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

- 3 - NH_4 generated during amino acids degradation for energy production, which is
4 recycled, *in vivo*, through the urea cycle in the liver,
5
6
- 7 - Methylglyoxal, a byproduct of DHAP,
8
- 9 - Carbon dioxide (CO_2) released from the TCA cycle and during the conversion of
10 glycerate to acetyl-CoA;
11
- 12 - Carbonic acid (H_2CO_3) formed from CO_2 conversion by Carbonic anhydrase;
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- 14 - Toxic Aldehydes.
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16 In *postmortem* muscle, some enzymes are able to metabolize more or less these
17 metabolites. Carbonic anhydrase can catalyze the conversion of CO_2 to bicarbonate which
18 could explain, at least in part, the discontinuity observed in the pH profiles.
19 Lactoylglutathione lyase, or Glyoxylase 1, is able to catalyze the first step of the conversion
20 of methylglyoxal to lactate. Finally, besides its function in glycolysis ([Jia, Hildrum, et al.,
21 2006](#)), some members of the complex Aldehyde dehydrogenase family ([Vasiliou, Thompson,
22 Smith, Fujita, & Chen, 2012](#)), could also protect cells against cytotoxic effects of various
23 aldehydes accumulating in the cytosol ([O'Brien, Siraki, & Shangari, 2005](#)). It is worth noting
24 that these three enzymes have been identified as potential markers of tenderness (Table 3).
25

26 Regarding ammonium ions (NH_4^+), these are normally recycled in liver (glucose-alanine
27 cycle) where it enters the urea cycle. *Postmortem*, this transportation is no more available and
28 thus ammonium ions will accumulate in muscle tissue. These ions can be then used in
29 different ways:
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- 31 - For alanine synthesis :
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33 pyruvate (limiting substrate) + NH_4 \rightarrow alanine
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- 35 - Or for synthesizing glutamate from alpha-ketoglutarate
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37 NH_4 + alpha-ketoglutarate (limiting substrate) \rightarrow glutamate
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- 39 - Or used by glutamine synthetase to convert glutamate to glutamine ([Adeva, Souto,
40 Blanco, & Donapetry, 2012](#)).
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51 3.4. Markers from the Heat Shock Protein family

52 Whatever its nature, stress induces synthesis of protective proteins called Heat Shock
53 Proteins (HSPs) which preserve cellular proteins against denaturation and possible loss of
54 function ([Kultz, 2003](#)). The many known Heat Shock Proteins are generally classified in
55 subfamilies on the basis of their size (molecular weight in kDa): Hsp 90, Hsp 70, Hsp 40, Hsp
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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 Ca²⁺ regulated proteins, characterized by the unique architecture of their Ca²⁺ binding sites, which enables them to peripherally localize onto negatively charged membrane surfaces in their Ca²⁺ bound conformation ([Gerke & Moss, 2002](#); [Monastyrskaya, Babiychuk, & Draeger, 2009](#)).

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

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

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

22 23 3.5.2. Annexin A1

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26 Besides its complex and not clearly understood role in obesity and hence in fat deposition
27 ([Kosicka, et al., 2013](#); [van Erk, et al., 2010](#)) and other proposed functions ([Gerke & Moss,](#)
28 [2002](#); [Monastyrskaya, et al., 2009](#)), it is worth noting that Annexin A1 has been also
29 implicated in the apoptotic process where it is rapidly translocated to the cell surface as an
30 “eat me” message to promote the removal of cells that have undergone apoptosis. Similarly to
31 phosphatidylserine, Annexin A1 is therefore a marker of apoptosis onset, but its exportation to
32 the cell surface is dependent on caspase activation. Caspase activation thus induced a
33 recruitment of Annexin A1 from the cytosol, a translocation to the outer plasma membrane
34 leaflet where it colocalizes with phosphatidylserine and is also required for efficient clearance
35 of apoptotic cells ([Arur, et al., 2003](#)). This feature supports previous findings proving that
36 caspases are activated in *postmortem* muscle and suggests an over expression of these
37 proteases in tender meat ([Ouali, et al., 2006](#)).

38 39 40 41 42 43 44 45 46 47 48 3.5.3. Annexin A6

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51 As reviewed by ([Cornely, Rentero, Enrich, Grewal, & Gaus, 2011](#)), Annexin A6 is
52 involved in a large set of biological processes and promotes apoptosis. In cells lacking
53 Annexin A6, mitochondrial morphology is indeed abnormal, Ca²⁺ signaling and respiration
54 are impaired and cells have increased resistance to Ca²⁺ mediated apoptosis. Mitochondrial
55 fission is an early event during apoptosis, occurring before caspase activation. This process is
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1 mediated by binding of the fission GTPase Drp 1 (Dynamin-related protein 1) to the outer
2 mitochondrial membrane leading to a preliminary release of small amounts of cytochrome c
3 (cyt c) ([Chlystun, et al., 2013](#); [Suen, et al., 2008](#)). Released cyt c translocates to the
4 endoplasmic reticulum where it selectively binds InsP3R (inositol (1,4,5) triphosphate
5 receptor) resulting in sustained cytosolic calcium increases. Mitochondrial fission can be
6 inhibited by binding of Annexin A6 to Drp1, an inhibition relieved by high cytosolic calcium
7 levels, which dissociates the Drp1-Annexin A6 complex and targets Annexin A6 to the
8 plasma membrane ([Boehning, et al., 2003](#); [Boehning, van Rossum, Patterson, & Snyder,
9 2005](#)). This event causes an amplification of Cytochrome c release and fission of
10 mitochondria, generating a higher number of smaller organelles.
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19 In *postmortem* muscle, Annexin A6 may therefore act as a brake to apoptosis through
20 inhibition of Drp 1 and subsequent intensification of Cytochrome c release occurring soon
21 after animal death (see Figure 6 and related comments). With regards to the present function
22 of Annexin A6 in apoptosis, a lower abundance of this protein in tender meat would be
23 expected as compared to tough meat, a proposal in good agreement with the conclusions of
24 ([Bjarnadottir, et al., 2012](#)) who observed lower Annexin A6 levels in tender meat.
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31 3.6. Other less known markers of tenderness: Galectin-1 and Peroxiredoxin 6

32 Two other potential markers of tenderness have been reported. These are galectin 1, a
33 member of the Galectin family ([Bjarnadottir, et al., 2012](#); [Zapata, et al., 2009](#)) and
34 Peroxiredoxin-6, an antioxidant contributing to hydrogen peroxide degradation and exhibiting
35 phospholipase activity ([Jia, et al., 2009](#)).
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41 3.6.1. Galectins

42 Galectins constitute an evolutionary conserved family of β -galactoside binding proteins
43 that are ubiquitous in mammals and other vertebrate taxa, invertebrates, and fungi. Since their
44 discovery in the 1970's, their biological roles, initially understood as limited to recognition of
45 carbohydrate ligands in embryogenesis and development, have expanded in recent years by
46 the identification of multiple other functions including apoptose induction and/or regulation
47 ([Vasta, 2012](#)). Most of them including Galectin 1 are believed to exhibit proapoptotic
48 activities whereas Galectin 3 is the only one considered to have an anti-apoptotic activity
49 ([Hernandez & Baum, 2002](#)). The role of Galectin 1 in apoptosis is however highly
50 controversial and contradictory findings are reported in the literature ([R. Y. Yang,
51 Rabinovich, & Liu, 2008](#)). Therefore, it appears difficult to analyze the conclusion of ([Zapata,
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2 [et al., 2009](#)) and ([Bjarnadottir, et al., 2012](#)) suggesting that a lower expression of Galectin 1 is
3 associated with greater tenderness.

4 5 3.6.2. *Peroxiredoxin 6* 6

7 Peroxiredoxin 6 (Prdx6) was the sixth (and final) mammalian member of the Prdx family
8 to be described, shares structural and functional properties with other members of this family
9 but has important characteristics that makes it unique among the Prdxs. The first one is
10 structural in nature since it has only one conserved cysteine residue instead of two for other
11 members. Second, Thioredoxin, the natural co-enzyme of most Prdxs, does not participate in
12 the catalytic cycle as compared to the other members of this family. Glutathione appears
13 indeed to be the physiological reductant for Prdx6. Third, Prdx6 is able to bind and reduce
14 phospholipid hydroperoxides, an essential enzymatic activity in antioxidant defense. Finally,
15 structural studies revealed that Prdx6 is a bifunctional enzyme with phospholipase A2 activity
16 in addition to its peroxidase function ([Fisher, 2011](#)). As already postulated by ([Manevich &](#)
17 [Fisher, 2005](#)), Prdx6 functions in antioxidant defense mainly by hydrolysis of hydrogen
18 peroxides and by facilitating repair of damaged cell membranes via reduction of peroxidized
19 phospholipids ([S. Y. Kim, Chun, & Lee, 2011](#); [Pak, et al., 2011](#); [Tulsawani, et al., 2010](#)). ([Jia,](#)
20 [et al., 2009](#)) reported an over expression of Prdx6 in tender meat, a finding in contradiction
21 with the antiapoptotic and/or cell survival activities of this protein. However, it is presently
22 difficult to get conclusions about the functions of Prdx6 in *postmortem* muscle and thus more
23 detailed investigations will be needed in order to clarify the exact nature of the relationship
24 between Prdx6 increased activities and ultimate meat tenderness.
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41 3.7. *Proteases and protease inhibitors as markers of meat tenderness* 42

43 Since decades, the meat tenderizing process is unanimously recognized to be enzymatic in
44 nature and the most studied proteolytic systems were cathepsins, calpains, the 20S proteasome
45 and, although more recently, members of the caspases family ([Kemp & Parr, 2012](#); [Ouali, et](#)
46 [al., 2006](#); [Sentandreu, et al., 2002](#)). However, the major peptidases of concern are not
47 identified yet in an unquestionable way and this question is still strongly debated.
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53 In the 80's the search for biological predictors of ultimate meat tenderness focused our
54 attention on the improvement of the enzymatic activity measurement especially with the use
55 of fluorescent substrates, which are much more sensitive than colorimetric ones. Proteolytic
56 enzyme levels were indeed expected to be good predictors of meat tenderness. Unfortunately,
57 this was not the case. In this respect, the at-death enzyme/inhibitor ratio or the inhibitor
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1 concentrations alone are both the best predictors of meat tenderness reported up to date ([Ouali](#)
2 [& Talmant, 1990](#)).

3
4 Since then, all assays attempting to associate enzyme concentrations to meat tenderness
5 have been unsuccessful. Assays with calpastatin, the common specific inhibitor of calpains 1
6 and 2, have not been convincing and not reproducible. Cystatins, a group of cysteine protease
7 inhibitors, have been identified as potential markers in only one study carried out by
8 ([Shackelford, Koochmaraie, Miller, Crouse, & Reagan, 1991](#)). These authors thus stressed that
9 at-death cystatin levels showed a much higher correlation with Warner-Bratzler shear force at
10 7 days *postmortem* than calpastatin levels alone ($r = +0.62$ vs -0.23 for calpastatin), a feature
11 confirming the unreliability of calpastatin as predictor of meat tenderness as suggested before.
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18 Later on, serine protease inhibitors levels have been shown to be best predictor of meat
19 tenderness amongst about thirty quantitative variables including calpains and calpastatin
20 ([Zamora, et al., 2005](#); [Zamora, et al., 1996](#)). This unexpected feature was extremely surprising
21 since no serine protease susceptible to contribute to myofibrillar softening was reported
22 ([Gagaoua, et al., 2012](#); [Ouali, 1990](#)). Investigations then performed on bovine muscle aiming
23 at identifying these intracellular serine protease inhibitors revealed that most of them belong
24 to the serpin superfamily (an acronym of **SER**ine **P**roteases **I**Nhibitors), the largest serine
25 protease inhibitor subfamily. Serpins are pseudo-irreversible inhibitors of serine proteases,
26 cysteine proteases and possibly other proteases groups. Some of them exhibited no inhibitory
27 activity and serves other functions ([Olson & Gettins, 2011](#)). Bovine serpins encompass at
28 least 8 different isoforms ([Pelissier, et al., 2008](#)) most of them (6 out of 8) being strong
29 inhibitor of initiator and effector caspases, which are very likely their natural target proteases
30 *in situ* ([Herrera-Mendez, et al., 2009](#)). As they bind tightly to caspases, they must be
31 considered as efficient inhibitors of the caspase-dependent apoptotic process which seems to
32 be the case in *postmortem* muscle.
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46 3.8. Structural muscle proteins as marker of tenderness

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49 Numerous myofibrillar proteins are degraded in *postmortem* muscle and the first to be
50 identified was probably troponin T, a regulator and nonstructural protein which does not
51 contribute to meat texture, together with the suspected 30-32 kDa troponin proteolytic
52 fragment which is in fact an electrophoretic band containing principally a proteolytic product
53 of actin ([Becila, et al., 2010](#)). Because of the large number of proteins concerned, this point
54 will not be developed here. One of the earliest structural changes resulting from hydrolysis of
55 cytoskeletal structures is the rapid *postmortem* detachment of the basal lamina from the
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1 plasma membrane following the degradation of all transversal connection between muscle
2 cells and the extracellular matrix ([Nishimura, Hattori, & Takahashi, 1996a](#); [R. G. Taylor, et](#)
3 [al., 1997](#)).

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

12
13 Most importantly, we have to identify the structural proteins whose hydrolysis is likely to
14 weaken enough the muscle structures. We will come back to this point in the next chapter by
15 reconsidering the transversal cytoskeletal network and the interesting proteins to be
16 considered in relation to this topic.

17 18 **4. Future perspectives in the search for meat tenderness predictors**

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

29
30 The last part of this review will be an overview of different metabolic pathways and of
31 proteolytic events including structural aspects of muscle cells and protease inhibitors, which
32 are known to be better predictors of tenderness than their target enzymes. For the different
33 reasons previously mentioned, HSPs will not be considered hereafter. Indeed, HSPs and their
34 potential functions in *postmortem* muscle would need a specific review.

35 36 *4.1. Energy metabolism in postmortem muscle with reference to living cells*

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38 *Postmortem*, the first objective of muscle cells will be to mobilize all its resources to
39 produce enough energy in the form of ATP to keep their normal metabolism. In this context,
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1 and as previously suggested, it's not surprising to see that all cellular components rich in
2 energy (carbohydrates, lipids, amino acids ...) are degraded for optimizing ATP production
3 (Figure 5a).
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5 6 4.1.1. Amino acids as the source of energy

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8 Regarding amino acids as a source of energy; it is worth emphasizing that skeletal muscle
9 contains 75% of the entire free amino acid pool of the body and 25% of total body proteins.
10 Glutamate, which comprises 20% of all amino acids in natural proteins, is one of the amino
11 acids in highest concentration in the free amino acid pool in human skeletal muscle and yet
12 has a low concentration in plasma ([Graham & MacLean, 1998](#); [Graham, Turcotte, Kiens, &
13 Richter, 1997](#); [Rennie, 2010](#)). Although it is not an essential amino acid, it takes part in
14 numerous important metabolic processes. Glutamate plays a central role in transamination and
15 deamination reactions. These include the formation of aspartate, alanine, and glutamine.
16 Skeletal muscle, in its resting state, normally releases glutamine and alanine in large
17 quantities; they can represent from 50 to 100% of the amino acid efflux in the fasted and fed
18 states, respectively, whereas glutamate is the dominant amino acid that is taken up by skeletal
19 muscle ([Graham & MacLean, 1998](#); [Graham, et al., 1997](#)). Amino acids are therefore an
20 important potential source of energy in *postmortem* muscle because the free pool is important
21 and all of them can be degraded to a component of the Krebs cycle or an intermediate
22 compound able to be converted to a component of this cycle (Figure 5b). The carbon
23 skeletons of amino acids are brought back to only seven molecules: pyruvate, acetyl CoA,
24 acetoacetyl CoA, α -ketoglutarate, succinyl CoA, fumarate and oxaloacetate. Those that are
25 degraded to acetyl CoA or acetoacetyl CoA are called ketogenic amino acids because they can
26 be converted in ketone bodies (Figure 5b green frame) whereas those that are converted in the
27 remaining of the seven molecules are called glucogenic amino acids (Figure 5b yellow frame)
28 because they can be converted to phosphoenol pyruvate and from there to glucose. Few of
29 them (Ile, Trp, Phe & Tyr) are both ketogenic and glucogenic.
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49 The major byproduct of amino acid degradation are ammonium ions, which are normally
50 recycled, *in vivo*, after blood transportation as alanine to the liver (glucose-alanine cycle)
51 where the transamination is reversed, releasing this metabolite that would then enter the urea
52 cycle (Figure 5a). This will not be possible *postmortem*, so that muscle cells would have to
53 activate unusual pathways for ammonium ions elimination, an adaptation which may take
54 some time. In the meantime, ammonium ions together with bicarbonate, generated from CO₂
55 by carbonic anhydrase may slow down the rate of pH drop or may cause transient increase of
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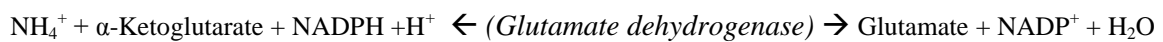
1 the pH like sometimes observed ([Boudjellal, et al., 2008](#); [Ouali, et al., 2006](#)). As shown in
2 Figure 7, pH profile showed a transient slowdown of the pH drop during postmortem storage
3 of rat muscle and, in the present case, a slight increase in the pH value. This was observed
4 about 3h *postmortem* when the pH value is close to 6.2. Similar findings have also been
5 observed sometimes in both *postmortem* beef and sheep muscles. The significance of this
6 slight pH increase is still unclear.
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10 The assimilation of ammonium ions can be done through three major pathways:

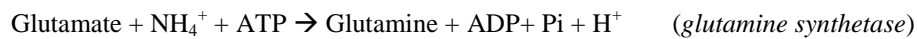
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12 1) Synthesis of alanine from 2 pyruvate molecules (glucose-alanine cycle)



- 15
16 2) Synthesis of glutamate from α -ketoglutarate



- 19
20 3) Synthesis of glutamine from glutamate



23 The two first pathways consume two important metabolites involved in energy production
24 (pyruvate and α -ketoglutarate) and their availability will be the main limiting step. The last
25 one is therefore the most probable to occur *postmortem* especially because glutamate is
26 abundant and displaces the reaction towards the formation of glutamine. The only one
27 problem is that this reaction is ATP-dependent. What solution muscle cells will choose is an
28 open question and further investigations on the origin of the over expression of the enzymes
29 of concern will be necessary.
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35 Regarding amino acid metabolism, the major questions we will have to answer in future
36 are:
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39 - Amino acid degradation occurs mainly in mitochondria: how long these pathways will
40 be active in postmortem muscle?
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42 - Which amino acids are mainly used for energy production?
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44 - How ammonium ions are assimilated *in situ* and at what rate this could be done?
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46 - What could be the consequence of ammonium and bicarbonate production on the pH
47 drop?
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51 4.1.2. Carbohydrates as the source of energy

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53 In the anoxic conditions of *postmortem* muscle, glycolysis will be the first pathway
54 mobilized for ATP production. The rate of this pathway is dependent on the availability of
55 NAD^+ , which is essentially regenerated from NADH (unable to enter mitochondria) by two
56 different shuttles between the cytosol and the mitochondrial matrix:
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- ***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 FADH₂. Then FADH₂ enters the respiratory chain to produce 3 ATP.

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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).

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4.1.3. *Free fatty acids as the source of energy*

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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.

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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

1 implies the degradation of triglycerides by lipases, releasing free fatty acid and glycerol.
2 Glycerol can enter the glycolytic pathway after conversion to DHAP (see Figure 4). Fatty
3 acids are then transported to the mitochondria matrix through binding to carnitine, where they
4 are oxidized to acetyl-CoA within the Lynen helix.
5
6

7 The limiting step for oxidation of lipids is the status of mitochondria. We do not know yet
8 the survival time of these organelles and the time course fate of their major activities. Oxygen
9 pressure in *postmortem* muscle is known to decrease progressively and not sharply,
10 suggesting that mitochondria will maintain their activities until oxygen pressure reach very
11 low levels.
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14 4.2. Mitochondria status and apoptosis regulation 15

16 Mitochondria are the center of many events that will contribute to the development of meat
17 qualities ([Sierra & Olivan, 2013](#)). They thus play a central role in the initiation and
18 development of apoptosis as well as in the production of energy that is absolutely vital to this
19 process. They are implicated in the final oxidation of all end-products of amino acids,
20 carbohydrates and lipid metabolism. Because of the role of mitochondria in energy metabolism
21 and in the initiation/regulation of apoptosis, these organelles could provide new predictors of
22 meat tenderness.
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31 4.2.1. Mitochondria as initiator and regulator of apoptosis 32 33

34 Mitochondria are central to many forms of cell death, usually via the release of pro-
35 apoptotic proteins from the mitochondrial inter-membrane space. Some inter-membrane space
36 proteins, including Cytochrome c, Smac/DIABLO (second mitochondria-derived activator of
37 caspases encoded by the Diablo gene), and Omi/HtrA2 (mitochondrially-located serine
38 protease) can induce or enhance caspase activation whereas others, such as AIF (Apoptosis
39 Inducing Factor) and endonuclease G, might act in a caspase-independent manner (Figure 6).
40 Release of inter-membrane space proteins is often regulated by the Bcl-2 protein family
41 ([Brunelle & Letai, 2009](#); [Chipuk & Green, 2008](#)). Recent evidence suggests that proapoptotic
42 members of this family, by themselves, can permeabilize the outer mitochondrial membrane
43 without otherwise damaging mitochondria. Mitochondria can contribute to cell death in other
44 ways. For example, they can respond to calcium release from the endoplasmic reticulum by
45 undergoing the mitochondrial permeability transition, which in turn causes outer membrane
46 rupture and release of inter-membrane space proteins. Bcl-2-family proteins such as Bik can
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1 influence the levels of releasable Ca^{2+} in the endoplasmic reticulum, and thus determine
2 whether the released Ca^{2+} is sufficient to overload mitochondria and induce cell death.
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4 4.2.2. *Calcium trafficking* 5 6

7 As reported by ([Vignon, Beaulaton, & Ouali, 1989](#)), calcium is already very abundant in
8 the sarcoplasm and the inter-myofibrillar space of beef muscle at 4 h *postmortem*. This
9 suggests a rapid *postmortem* translocation of calcium ions from the sarcoplasmic reticulum to
10 the cytosol. A large set of biochemical processes triggers this translocation. This could result
11 from different but cooperative processes affecting the permeability of the sarcoplasmic
12 reticulum membrane including binding of proapoptotic Bcl2 members such as Bik, binding of
13 Cytochrome c to a membrane glycoprotein complex acting as Ca^{2+} channel and known as
14 inositol (1,4,5) trisphosphate receptor (InsP3R), and probably other calcium release processes
15 (Figure 6). Large amounts of this calcium are transferred to mitochondria and overcharge
16 them and triggering apoptosis ([Mattson & Chan, 2003](#); [Orrenius, Zhivotovsky, & Nicotera,
17 2003](#)).
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27 4.2.3. *Mitochondria fission* 28

29 Mitochondrial fission is an early event during apoptosis, occurring before caspase
30 activation. It is well recognized that mitochondrial morphology changes during apoptosis,
31 resulting in small, round and more numerous organelles. This process is associated with
32 earlier binding of several proteins including proapoptotic Bcl2 family members to outer
33 mitochondrial membrane, a translocation altering the membrane permeability to calcium and
34 proteins of the inter-membrane space ([Youle & Karbowski, 2005](#)).
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40 The large GTPase Dynamin-related protein 1 (Drp1) mediates mitochondrial fission in
41 mammalian cells. This cytosolic protein moves to mitochondria and assembles into spirals at
42 division sites around the outer mitochondrial membrane to drive the fission process (insert
43 Figure 6). Oligomerisation of Drp1 is inhibited by Annexin 6 at low levels of calcium. By
44 contrast, increase in the cytosolic calcium levels relieves this inhibition, mediating the
45 translocation of Annexin 6 to the plasma membrane ([Suen, et al., 2008](#)). Bik, a BH3 protein of
46 the Bcl-2 family, contributes very likely to the translocation of Drp1 to the mitochondrial
47 membrane through a calcium release from the sarcoplasmic reticulum, which prevents it from
48 Annexin 6's inhibition.
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56 Fission of mitochondria has never been demonstrated so far in *postmortem* muscle
57 although all needed conditions are gathered together. Because it is an essential event in
58 apoptosis initiation and development, this point needs to be addressed with appropriate
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1 techniques and research on specific markers such as the expression of Drp 1 and the existence
2 of Cytochrome c-InsP3R complexes, which amplify the release of calcium and translocation
3 of Annexin 6 to the cell membrane, cancelling the inhibition of the fission process.
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5 We need indeed to explain how levels of the different enzymes of the tricarboxylic acid
6 cycle are increased *postmortem* and, in this context, we might also consider the mitochondria
7 fusion process (fusion also causes an increase in mitochondrial metabolic enzymes) assuming
8 that this event is possible in the particular *postmortem* conditions. Markers and mechanisms
9 of mitochondria fusion have been reviewed by ([Westermann, 2008](#)) and ([Suen, et al., 2008](#)).
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11 For more details, the reader would have to refer to different reviews covering these early
12 events of the apoptotic process initiation ([J. Li, Li, Qin, von Harsdorf, & Li, 2010](#); [Otera &](#)
13 [Mihara, 2012](#); [Suen, et al., 2008](#); [Youle & Karbowski, 2005](#)) and some others focused on the
14 role of Annexins in apoptosis ([Arur, et al., 2003](#); [Chlystun, et al., 2013](#); [Cornely, et al., 2011](#);
15 [Monastyrskaya, et al., 2009](#); [Suen, et al., 2008](#)).
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17 4.2.4. Pro- and anti-apoptotic proteins from mitochondria inter-membrane space

18 Reports from Horvitz's group summarized in their latest review ([Ellis, Yuan, & Horvitz,](#)
19 [1991](#)) clearly indicated that apoptosis is a fundamental property of animal cells and that
20 proteins that mediate and regulate this process have been largely conserved along the
21 evolution, from worms to humans. In *postmortem* muscle, the apoptotic process will be
22 qualitatively identical to what we know *in vivo* and regulatory pathways will be the same. The
23 main limiting factors will be the absence of blood flow, the pH drop and the lower level of
24 energy available. These settings will slow down, but not prevent, the development of the cell
25 death process.
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27 Following apoptotic signals and outer mitochondrial membrane alteration, several pro-
28 apoptotic [Cytochrome c, Endonuclease G (Endo G), Apoptosis Inducing Factor (AIF)] and
29 anti-apoptotic proteins (**HtrA2**: high-temperature-requirement protein A2; **Omi**: Omi stress-
30 regulated endopeptidase; **Smac**: second mitochondria-derived activator of caspase) are
31 released from mitochondria in a caspase independent manner (Figure 6).
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33 Upon release from mitochondria, Cytochrome c will trigger apoptosome complex
34 formation through association with Apaf 1 (apoptotic protease activating factor). Once
35 formed, the apoptosome can then recruit and activate the inactive pro-caspase 9. We recently
36 followed the release of Cytochrome c in rat *Longissimus* muscle from at death to 72h of
37 storage at low temperature (for experimental details see ([Becila, et al., 2010](#))). As assessed by
38 western blot and densitometry on the supernatant obtained after mitochondria sedimentation
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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.

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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](#)).

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Endonuclease G is a proapoptotic DNase synthesized 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](#)).

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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](#)).

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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](#)).

42 43 44 4.2.5. *Conclusion about the central role of mitochondria in postmortem muscle*

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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.

55 56 57 4.3. *Inhibitors of caspases and apoptosis*

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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

1 of effector caspases and caspase 9, one of the initiator caspase family. Inhibition of caspase 8
2 by IAPs is still uncertain. The mode of inhibition of caspases by IAPs greatly differs from the
3 traditional mechanisms known for cystatins, serpins and other peptidase inhibitor families,
4 mostly interacting with their target peptidase in a substrate-enzyme manner. By contrast,
5 binding with relatively low affinity (K_d in the μM range) of IAPs in the vicinity of the active
6 site creates a sufficient steric obstruction to prevent access of the active site to protein
7 substrates, small peptide substrates being hydrolysed after IAP binding ([Chiou, Jones, &](#)
8 [Tarnawski, 2003](#); [Fuentes-Prior & Salvesen, 2004](#); [Fulda, 2009](#); [Philchenkov, 2004](#); [R. C.](#)
9 [Taylor, Cullen, & Martin, 2008](#); [Wei, Fan, & Yu, 2008](#)). The anti-apoptotic function of the
10 IAP proteins family can be cancelled by specific inhibitors of this interaction, *i.e.*
11 Smac/DIABLO and Omi/HtrA2 proteins ([Saelens, et al., 2004](#)).
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20 Besides IAPs, we have recently identified new caspase inhibitors, firstly described as
21 elastase and trypsin inhibitors and further characterized as initiator and effectors caspases
22 inhibitors. This highly polymorphic group of protease inhibitors, which has already been
23 presented in chapter 1, belongs to the serpin superfamily and forms, with their target enzymes
24 including caspases, a covalent complex detectable upon SDS-PAGE analysis ([Gagaoua, et al.,](#)
25 [2012](#)). Preliminary assays using prostate cancer cells in culture showed that *in situ*
26 inactivation of some of these serpins is able to decrease cell proliferation probably through an
27 increase of cell death at the expense of cell proliferation.
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34 ([Zamora, et al., 2005](#)) determined the levels of serine protease inhibitor in bovine muscle
35 extracts, obtained immediately after animal death, by titration with trypsin, and found that at-
36 death serine proteinase inhibitor concentration is a better predictor of meat tenderness than all
37 other variables considered including calpains 1 and 2, cysteine protease inhibitors, calpastatin,
38 ...etc. These serine protease inhibitors are integrated by members of the identified caspase
39 inhibitors of the serpin superfamily since these are by far the most predominant in muscle
40 tissue. Characterization of these serpins is currently going on and will be further used to
41 confirm their efficiency as predictors of meat tenderness using more specific quantification
42 methods such as ELISA.
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51 4.4. *Proteases and Proteolysis*

52 We already stressed forward that levels of proteases in muscle tissue are not between the
53 best options to look for potential adequate predictors of meat tenderness. We must emphasize
54 that most, if not all, protein substrates can be hydrolyzed *in vitro* by a series of endogenous
55 proteases and it is often difficult or impossible to identify the target *in situ* substrates for each
56 proteolytic system, with the possible exception of living or cultured cells models. In addition,
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1 we are not in a position today to certify that a given protein is degraded by a particular
2 protease. The answer to this question is, in our view, beyond our present capabilities, so that
3 we have to overcome this difficulty by defining other new priorities if we want to progress in
4 this area. One possibility will be to improve our knowledge about inhibitors of each
5 proteolytic system, which would provide much more information about the role of their target
6 enzymes in *postmortem* muscle, including proteasome, papain-like cysteine proteases, serine
7 proteases, caspases ...etc.

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

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

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

41 Another protein of great interest is actin. During apoptosis, actin is the first protein targeted
42 by effector caspases and some actin fragments have been considered to be accurate markers of
43 apoptosis ([F. Yang, et al., 1998](#)). Generated actin peptides by themselves can induce
44 morphological apoptotic changes comparable to those observed in apoptotic cells ([Mashima,](#)
45 [Naito, & Tsuruo, 1999](#)). Within muscle cells actin is present in thin filaments, in association
46 with nebulin, and also in intermediate transversal cytoskeletal filaments where it serves to
47 connect myofibrils to the sarcolemme, this link extending further to the extracellular matrix.
48 Degradation of transversal cytoskeletal actin filaments causes a detachment of sarcolemma
49 from the basal lamina and the extracellular matrix network, a finding supported by the
50 proteolytic hydrolysis of laminin, the major component of the basal lamina ([Becila, et al.,](#)
51 [2010](#)).

1 One of the major fragments generated in postmortem muscle coming from actin showed à
2 Mr of about 32 kDa. The appearance of this fragment was followed in rat *Longissimus* muscle
3 by western blot and densitometry. The relative concentration of the soluble 32 kDa fragment
4 was plotted versus the storage time (Figure 7). The concentration reached a maximum value
5 about 3-4h postmortem and then decreased sharply, suggesting a degradation of this fragment
6 to lower Mr ones ([Becila, et al., 2010](#)); see also hereafter comments of figure 8).
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10 Interestingly, some situations of muscle atrophy such as uremia, aging or myocardial
11 infarct, are associated to an accelerated degradation of muscle proteins. According to results
12 obtained by ([Du, et al., 2004](#)), in such catabolic conditions the initial step in myofibrillar
13 proteolysis would be the breakdown of the actomyosin complex by the direct action of
14 caspase 3. These authors pointed out that caspase 3 action would be the first step in
15 dissociating the actomyosin complex, giving rise to both intact monomeric actin and
16 fragments of actin. In a second step, these products will be then further degraded by the ATP-
17 dependent ubiquitin-proteasome system. As characteristic product fragments, they found the
18 generation of an actin fragment of around 14 kDa when actomyosin complexes were
19 incubated with caspase 3. It is worth highlighting that this characteristic 14 kDa actin
20 fragment has been detected in atrophying muscles of diabetic or uremic rats, where the
21 activity of caspase 3 is increased ([Du, et al., 2004](#)). In relation to this, we have carried out
22 some experiments with the aim to characterize the ability of caspase 3 to hydrolyze bovine
23 muscle actin. As it can be seen in **Figure 8**, the action of caspase 3 on bovine actin is
24 remarkable from 24 hours of incubation and later on. As main products of this action, we have
25 also observed the generation of a 14 kDa fragment, in addition to the 32 kDa fragment that
26 has been already observed in postmortem muscle ([Becila, et al., 2010](#)). A third and less
27 intense actin fragment at around 25 kDa, not previously described, was also observed after 24
28 hours of incubation (**Figure 8**). Future research would need to be done in order to establish
29 the utility of these fragments as potential useful markers of meat tenderness and of caspase
30 action.
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49 *4.5. Protease inhibitors*

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

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

3 Finally, muscle cells were shown to express strong inhibitors of initiator and effector
4 caspases belonging to the serpin family. Screening and characterization of protease inhibitors
5 in muscle tissue are far from being completed and much work remains to be done in this
6 context. As inhibitors are better predictors of meat tenderness than their target enzymes, such
7 investigations would help to clarify the role of the different endogenous proteolytic systems in
8 *postmortem* proteolysis.
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10 To conclude, it is therefore obvious that early apoptotic signaling processes converge on
11 the mitochondria and that response of mitochondria to upstream proapoptotic signals is a
12 critical control point for the regulation of cell death. Another important feature to be
13 considered is the role of phospholipids in phospholipid/protein interactions, which are
14 essential for the maintenance of mitochondrial dynamics and hoemostasis ([Lehmann &
15 Shatrov, 2002](#)).

16 **Acknowledgements**

17 We gratefully acknowledge funding and support from INRA and EGIDE (Tassili program).
18 Financial support of project AGL2009-12992 from the Spanish Ministry of Economy and
19 Competiveness is also fully acknowledged.
20

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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 \rightleftharpoons Gluc 6P	+	+	(Bjarnadottir, et al., 2010 ; Bouley, et al., 2004 ; Laville, et al., 2009)
Triosephosphate isomerase	Cytoplasm	Dihydroxyacetone phosphate (DHAP) \rightleftharpoons glyceraldehyde 3-phosphate	+	+	(G. D. Kim, et al., 2009)
Glyceraldehyde-3 phosphate dehydrogenase (GAPDH)	Cytoplasm, Nucleus	glycéraldéhyde.3.phosphate \rightleftharpoons 1,3.diphosphoglycerase Cell death & cell survival	+	+	(Colell, Green, & Ricci, 2009 ; Laville, et al., 2009)
3-Phosphoglycerate kinase	Cytoplasm	3-phosphoglycerate + ADP \rightleftharpoons 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 Phosphopyruvate hydratase	Cytoplasm Muscle specific	2-phosphoglycerate \rightleftharpoons phosphoenolpyruvate	+	+	(Choi, et al., 2010 ; Laville, et al., 2009)
Pyruvate kinase	Cytoplasm	phosphoenolpyruvate \rightleftharpoons pyruvate	+	+	(Laville, et al., 2009 ; Polati, et al., 2012)
Lactate dehydrogenase	Cytoplasm	pyruvate \rightleftharpoons 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)
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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 CO ₂ ; H ₂ O+CO ₂ =>HCO ₃ + 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 dehydrogenases (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)
<i>DNAJA1</i> (HSP 40)	Nucleus / Cytoplasm	<i>DNAJA1</i> 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, Phosphatidylserine; 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

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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 chromatin 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-

1 temperature-requirement protein A2; SERCA, sarco/endoplasmic reticulum Ca²⁺-ATPase;
2 SMAC/Diablo, second mitochondria-derived activator of caspases encoded by the Diablo
3 gene. Adapted from ([Otera & Mihara, 2012](#)).
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5 **Figure 7:** Postmortem changes in muscle pH, in the soluble 32 kDa actin fragment levels and
6 in the extent of cytochrome c release in *Longissimus* rat muscle maintained on the carcass
7 which was stored at 10°C for 24h and then transferred at 4°C. More details can be found in
8 ([Becila, et al., 2010](#)).
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12 **Figure 8:** 12% SDS-PAGE of the protein degradation profile obtained when bovine skeletal
13 muscle actin was incubated in the presence of recombinant human caspase 3 at different time
14 intervals (data not shown previously). (A): protein band corresponding to intact monomeric
15 actin; (B), (C) and (D): actin fragments generated after incubation with caspase 3, having
16 molecular mass values around 32, 25 and 14 kDa, respectively.
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Figure 1:

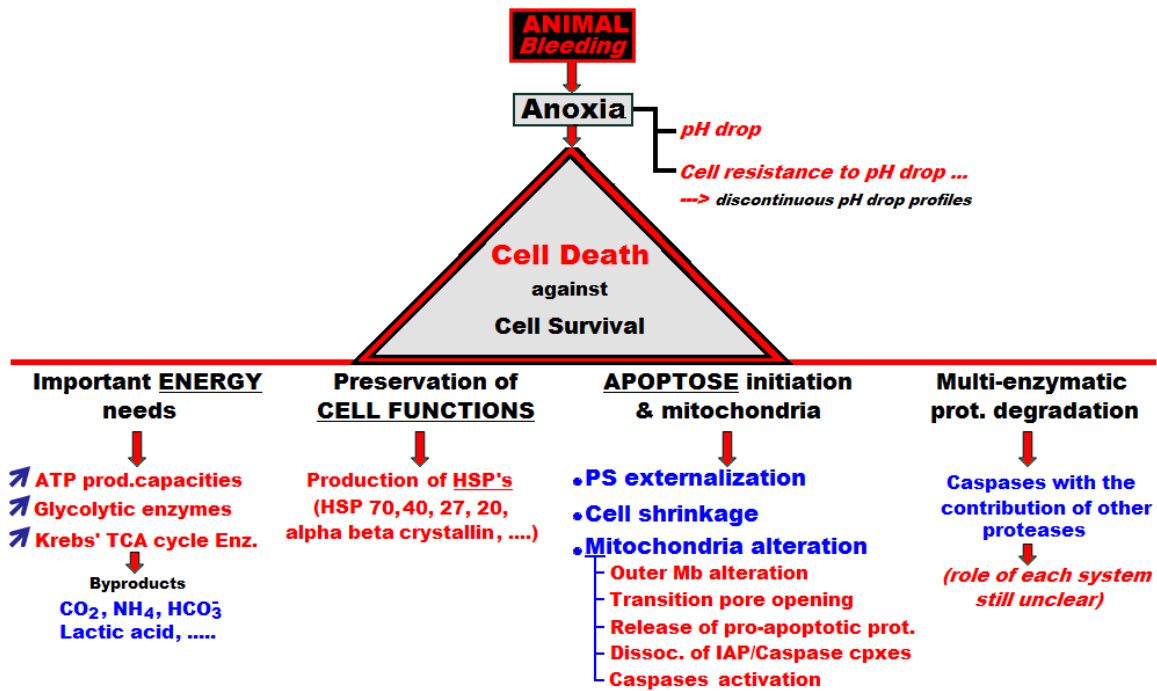


Figure 2:

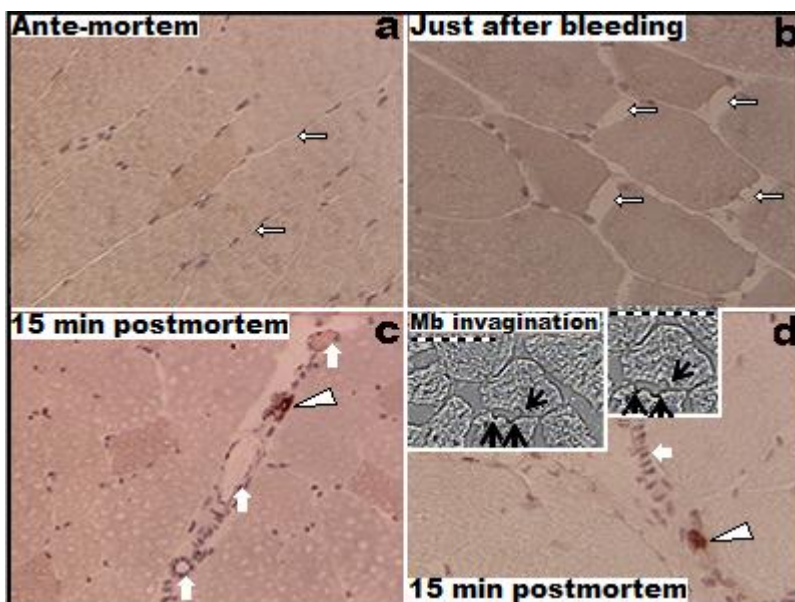


Figure 3:

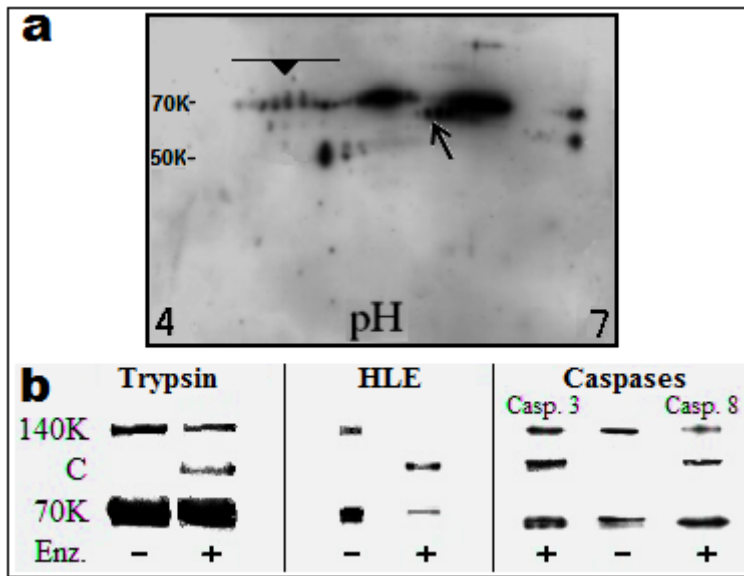


Figure 4:

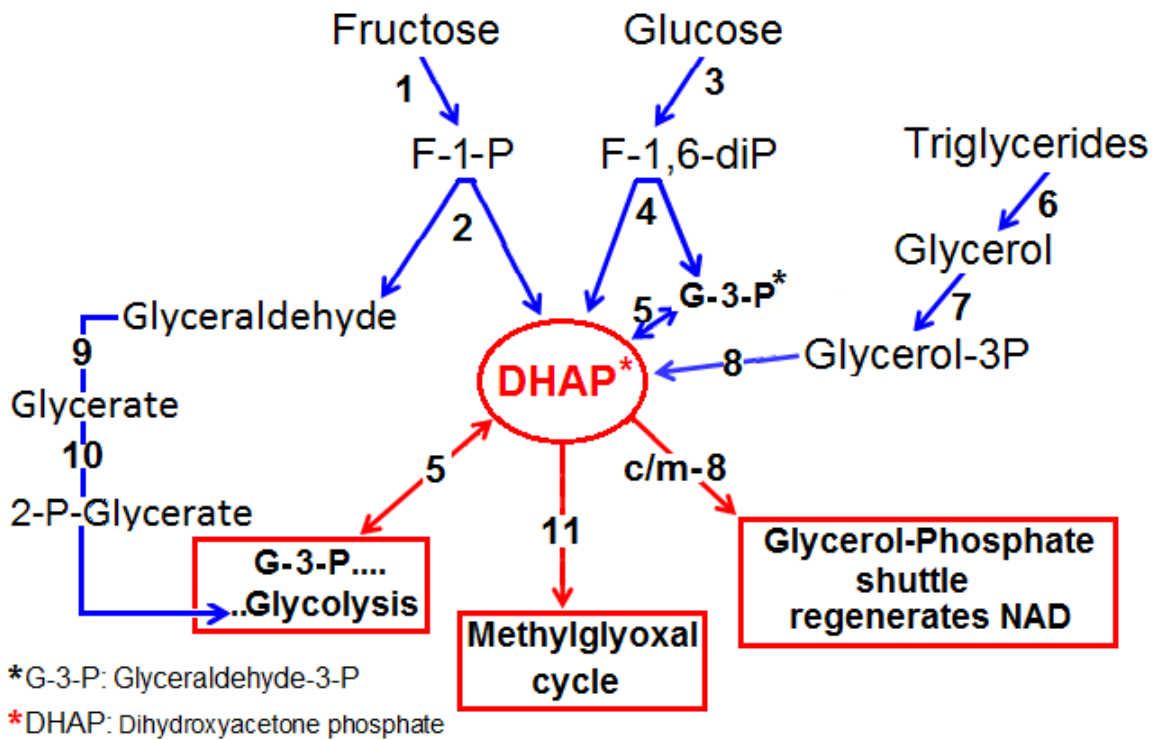


Figure 5:

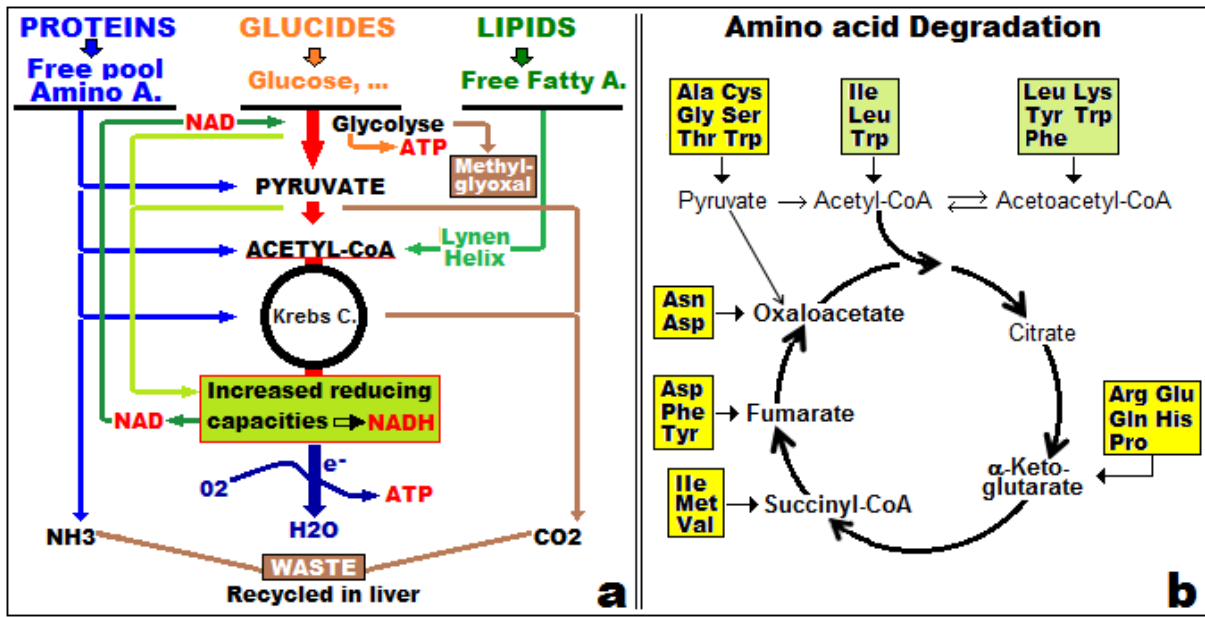


Figure 6:

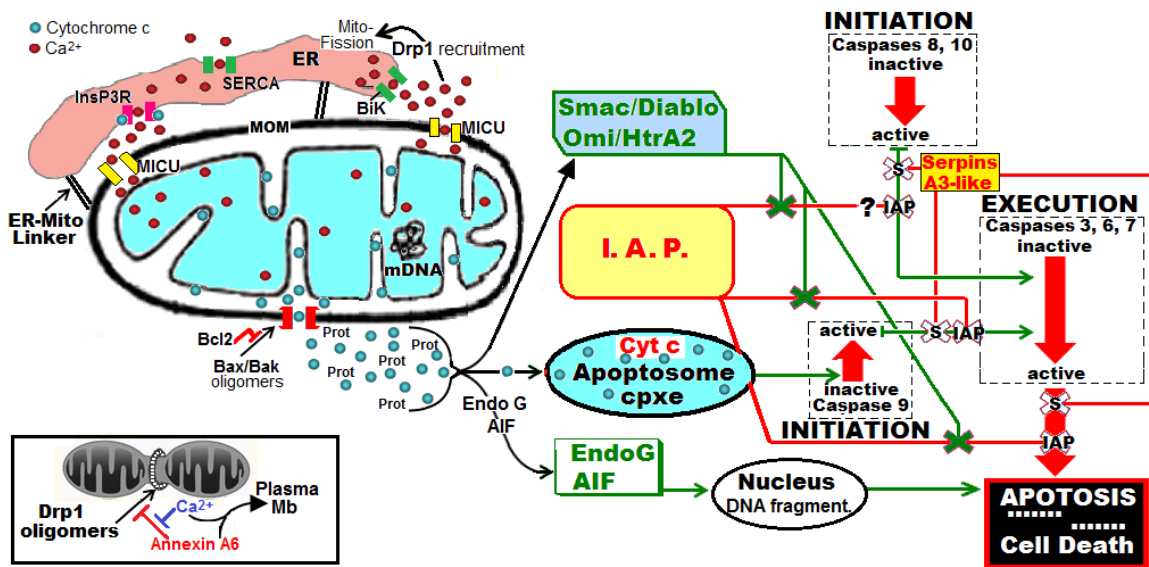


Figure 7:

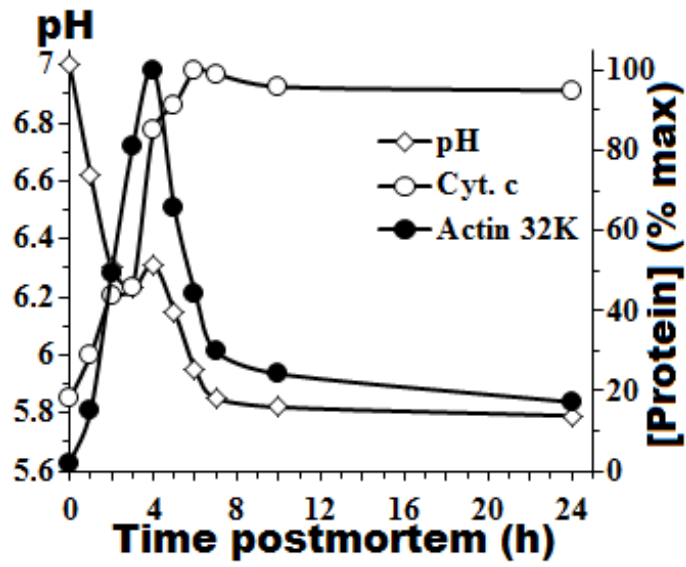


Figure 8:

