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Tesi di Laurea

CHARACTERIZATION OF CALPAIN PROTEOLYTIC ACTIVITIES IN LIVERS ISSUED FROM FORCE-FED DUCKS

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ABSTRACT

Technological yield of a food product is one of the most valuable parameters for manufacturers. In the case of *foie gras*, it is mainly affected by cooking losses, which are represented by lipidic exudations during pasteurization or sterilization. Being *foie* gras appreciated for its richness in fat, lipid exudation degrades the image of the final product at the eyes of the consumer. Moreover, if cooking losses exceed 30% the fatty liver can't be sold in the most valuable commercial forms, thus representing an economic loss for the producer. During the last decade, manipulation and cooking practices have been studied to guarantee the uniformity of the processed flock. Despite that the inter-individual technological yield variability remains high and cooking losses still represent the major quality issue that faces the industry of *foie gras*. The present study focuses on the role of calpains neutral cystein-proteases on the determinism of *foie gras* technological yield. The objective was establishing whether the extent of their lytic activity in the liver could be counted among the biological causes influencing *foie gras* cooking losses. To achieve it, two groups of 8 fatty liver samples each (CL+ and CL-) with equal weight and biochemical characteristics (lipids and dry matter content) but with different cooking losses values were analysed through calpain casein zymography. No statistically significant difference was revealed for the calpain lytic activity toward the casein substrate between the CL+ and the CL- samples.

In addition to this first study, three other studies were done. Their aim was to better characterize the calpain casein zymography and the calpain 2 Western Blot (WB) techniques. Moreover, one of these studies was perform in order to investigate Mule duck (*C. moschata* × *A. platyrhynchos*) calpain 1 and 2 autolytic rate, with the objective to gain insight in the calpain system. A better characterization of calpain casein zymography consisted on determining calpains stability in the Extraction Buffer and calpain lytic activity at different Ca^{2+} concentrations. Mainly qualitative data were obtained. Yet we could conclude that calpains are stable in the Extraction Buffer for long times (at least until 17 hours after defrosting) and we could identify two isoforms of calpain 2 which were previously undistinguishable. Calpain casein zymography was also adapted to study calpains autolytic rate. Finally, some time was devoted to investigate calpain 2 WB repeatability and anti calpain 2 antibody specificity.

Key words: *foie gras*, technological yield, calpains, calpain casein zymography, autolysis.

RIASSUNTO

La resa tecnologica di un prodotto alimentare rappresenta uno dei parametri più influenti agli occhi del produttore. Nel caso del *foie gras*, questo parametro è per lo più determinato dalla perdita in cottura, che interviene sotto forma di essudazioni lipidiche durante la pastorizzazione o la sterilizzazione. Essendo il *foie gras* apprezzato per la sua ricchezza in grasso, questo fenomeno è causa di degrado dell'immagine del prodotto per il consumatore. Inoltre, se la perdita in cottura supera il 30%, il *foie gras* non può per legge essere venduto nelle forme più costose, e ciò rappresenta una considerevole perdita economica per il produttore. Durante l'ultimo decennio, una standardizzazione delle pratiche di manipolazione e di cottura è stata perseguita in modo da garantire l'uniformità dei lotti di prodotto. Nonostante ciò, la variabilità inter-individuale rimane elevata e le perdite in cottura rappresentano ancora la maggiore preoccupazione in termini di qualità per l'industria del *foie gras.* Il presente studio sperimentale ha considerato il ruolo delle calpaine, cisteina-proteasi neutre, nel determinismo della resa tecnologica del *foie gras,* con l'obiettivo di stabilire se l'entità della loro attività litica possa essere annoverata tra le cause biologiche delle perdite in cottura del *foie gras.* A tale scopo l'attività calpainica è stata misurata tramite zimografia in due gruppi (CL+ e CL-) composti ciascuno da 8 campioni di *foie gras* con peso e caratteristiche biochimiche (contenuto in lipidi e sostanza secca) simili, ma diverse rese tecnologiche. Nessuna differenza statisticamente significativa è stata rilevata per l'attività litica nei confronti del substrato caseinico tra campioni appartenenti rispettivamente ai gruppi CL+ e CL-. Oltre a questo primo studio, sono state eseguite ulteriori tre indagini, allo scopo di meglio caratterizzare le tecniche di zimografia per le calpaine e di Western Blot (WB) per la calpaina 2. Inoltre, uno di questi studi ha avuto l'obiettivo di quantificare la velocità autolitica delle calpaine 1 e 2 in anatra Mulard (*C. moschata × A. platyrhynchos*), utilizzando una versione appositamente modificata del protocollo di zimografia. La prima esperienza ha previsto lo studio della stabilità delle calpaine nel tampone di estrazione e della loro attività litica a differenti concentrazioni di Ca^{2+}. I dati ottenuti sono stati principalmente qualitativi. Si è potuto concludere che le calpaine sono stabili nel tampone di estrazione per un relativamente lungo periodo di tempo (almeno fino a 17 ore dopo lo scongelamento) e si sono potute identificare due isoforme della calpaina 2 precedentemente indistinguibili. Infine, del tempo è stato dedicato per meglio caratterizzare i profili risultanti dal WB per la calpaina 2 ed indagarne la ripetibilità.

1. INTRODUCTION

1.1. *Foie gras*

Foie gras or fatty liver is a typical, traditional and popular delicacy in French gastronomy. According to the French law it is "the liver of a duck or a goose that has been obtained by force feeding" (French *Code rural* law L654-27-1). French Regulation (CE) N° 543/2008 dated 16th June 2008 defines the main parameters of *foie gras* production as:

- the exclusive derivation from goose (*Anser anser*) or duck (*Caïrina moschata* or *Caïrina moschata* x *Anas platyrhynchos*), fed so that to induce a lipid hypertrophy in the hepatocytes;
- the withdrawal from completely bled animals, slaughtered not before 95 days for geese, 70 days for the females of *Caïrina moschata* (Muskovy duck), 84 days for the Muscovy duck males and 92 days for the Mule ducks (*Caïrina moschata* x *Anas platyrhynchos*) individuals;
- the sanitary safety;
- a net weight of at least 300 g if derived from duck, and at least 400 g if issued from geese.

The physiological basis for the fatty liver production is the natural capacity of migrating birds to over consume food in order to store energy before long travels (Bairlein, 1987). In France, about 97% of *foie gras* production is obtained from ducks (*Comité Interprofessionnel des palmipèdes à Foie Gras*, 2011). Two genotypes are mainly used: the Muscovy duck (*Caïrina moschata*) and the Mule duck, which results from the mating of a Muscovy male duck and a Pekin female (*Anas platyrhynchos*).

Since ancient times, Egyptians noticed that geese and ducks used to ingest large quantities of food in order to be able to survive during winter migrations, so they started to force feed them to obtain energy food for human consumption (Odum, 1960). Afterwards, Romans discovered and adopted the Egyptians' technique and the fatty liver became soon a popular and appreciated product. In the middle age Jewish people spread this method of production, and the force feeding of birds got expanded in a lot of European countries like Poland, Hungary and France. Nowadays, the *foie gras*, which is seen in France as a food for special occasion, has acquired a new production target: the point is now to produce a high quality product that could satisfy the demands of the

consumer.

Although the production of fatty liver is common in some European countries, such as Spain, Hungary and Bulgaria, France is the first consumer and producer with 19,000 tons in 2010 which represents more than 70% of the world production (*Comité Interprofessionnel des palmipèdes à Foie Gras*, 2011). This was probably caused by the fact that for the European Union to be recognized as *foie gras*, the fatty liver has to be produced according to traditional farming method in the south west of France and with commercial policies based on a geographical indication of provenance (*Indication Géographique Protégée, IGP)*. But it can be caused also by the fact that many countries see the force feeding as harmful to the health of the involved animals. For example, some Europeans countries, like Denmark (1991), Germany (1993) and Italy (2004), have enacted explicit bans against overfeeding. The Council of Europe, with the "COUNCIL DIRECTIVE 98/58/EC, 20 July 1998 concerning the protection of animals kept for farming purposes", introduced prohibition regulations that ban force feeding in all countries where it isn't a common method, and demands research on alternative methods which do not include force feeding (Official Journal of the European Communities, L221/23). On the other hand, France defends its traditional product, and tries to get the approval of the European Union and society on animal's welfare by introducing changes in the farming conditions (i.e. abolition of the individual cage and decrease of the duration of the force feeding period).

1.2. Physiological and metabolic bases of hepatic steatosis in palmipeds

Foie gras in palmipeds is the result of a nutritional, hypertrophic and reversible hepatic steatosis. In breeds adapted to fatty liver production, this phenomenon is intense; for example, in goose at the end of the force-feeding period the liver weight can reach 10 times the initial weight, passing in 15 days from 100 g to 1 kg, that is a 10% of goose body weight (Hermier *et al*., 1999). Force-feeding caused hepatic steatosis is the result of a *de novo* lipogenesis more than food lipids storage. In fact, the feed is highly energetic (3,250 kcal/kg) but disequilibrated, being rich in carbohydrates (70%) but poor in lipids (4%) and proteins (8-11%). The lipidic content is mainly represented by essentials polyunsaturated fatty acids, especially linoleic acid (C18:2n-6) which accounts for a 53% of the total. Polyunsaturated fatty acids are assembled in the enterocytes as portomicrons (Fraser *et*

al., 1986), which are liberated in the portal venous system and collected by the liver before entering the general circulation. Therefore, a part of them can be directly stocked in the liver. However, Saez *et al.* (2008) showed that a 90% of the triglycerides stocked in the liver is derived from the carbohydrate component of the feed. In birds, the *de novo* lipogenesis (Léveille *et al*., 1975) is principally performed in the liver and depends from the carbohydrate precursors availability. Therefore, it is stimulated during the force-feeding period as the corn is rich in starch, and because the quantity of feed distributed at each meal is high and increased from 300 g to 600-700 g between the beginning and the end of the feeding period. Moreover, bird liver has a better capacity of converting carbohydrates than phopholipids in neutral lipids.

After the ingestion, starch is dismantled in dextrin and maltose by the pancreatic amylases. Maltose is then quickly degraded in glucose by the intestinal maltases and isomaltases. During the *de novo* lipogenesis (Figure 1), the exceeding glucose is collected from blood circulation by the hepatocytes and converted in pyruvate, which enters the Krebs cycle in the mitochondria. The citrate produced by the Krebs cycle is transported in the cytosol, where it is transformed in acetyl-CoA by the ATP Citrate Lyase (ACLY). The Acetyl-CoA Carboxilase 1 (ACC1) converts the acetyl-CoA in malonyl-CoA, which is used by the Fatty Acid Synthase (FAS) in order to produce palmitic acid (C16:0). Palmitic acid is desaturated by the Stearoyl-CoA desaturase (SCD) in palmitoleic acid (C16:1n-7), or converted in stearic acid (C18:0) by the Long-Chain Fatty Acid Elongase. The stearic acid can then be desaturated to form the oleic acid (18:1n-9). These different fatty acids are all used to synthesize triglycerides. The whole process is positively regulated by insulin and glucose, through the Sterol Regulatory Element Binding Protein-1c (SREBP-1c) and Carbohydrate Responsive Element Binding Protein (ChREBP) transcription factors, respectively. On the contrary, it is negatively regulated by glucagon. In particular, insulin activates ACC1, while glucagon inhibits it. Therefore, high levels of insulin and glucose promotes the lipid storage, while high levels of glucagon the energetic stock demolition and exportation.

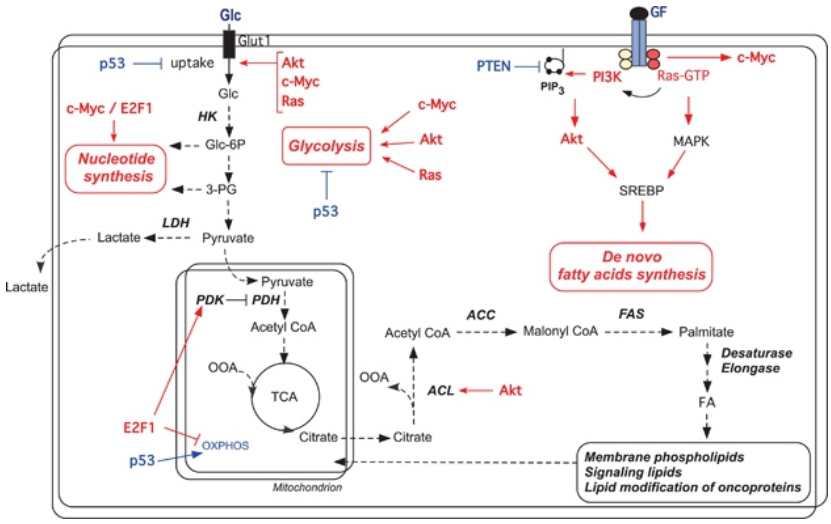


Figure 1. Schematic pathway for the *de novo* lipogenesis (Fritz & Frjas, 2010).

The development of hepatic steatosis depends on various factors, first of all, the ratio between the lipid storage and the exportation rate. Triglycerides, phospholipids and cholesterol assembled in the liver are exported as lipoproteins. Triglycerides associate mainly apolipoprotein B (apoB) in order to form Very Low Density Lipoproteins (VLDL), while cholesterol and phospholipids mainly constitute High Density Lipoproteins (HDL) together with apolipoprotein A-I. VLDLs are then hydrolysed by the lipoprotein lipases of the peripheric tissues in order to absorb the fatty acids, esterificate and store them. The fraction which is not taken returns to the liver and contributes to the development of the hepatic steatosis. So, the extent of the lipid storage depends on insulin and glucose levels and on the ability of the peripheric tissues to collect lipids, while the exportation rate is influenced by the glucagon blood level. In force-fed ducks and geese, the onset of the hepatic steatosis is favoured by a permanent postprandial insulin stimulation. Also feed composition seems to predispose the liver to lipid storage. Feed is mainly composed by corn which is poor in choline, folic acid and methionin and lacking in vitamin B12. Mc Neil *et al*. (2008) and Kumar *et al*. (2013) proved that rats subjected to diets poor in these elements had a tendency to develop hepatic steatosis. In fact, a deficiency in methyl group donors causes an increase in homocystein levels in the hepatocytes, which leads to the SREBP-1 over-expression. Finally, hepatic steatosis onset is favoured also by enzymatic and not only hormonal mechanisms. For example, Hérault *et al*. (2010) showed that in ducks at the end of the force-feeding period, genes involved in the lipogenesis are over-expressed, while the apoB and the CPT-1 genes are under-expressed, suggesting an

increase in the production of the fatty acids and a diminution of VLDL formation and fatty acids oxidation. Also, a proteomic analysis performed on male Mule ducks force-fed for a different number of times (0, 14 and 23 meals of overfeeding) highlighted 5 main categories of protein expression modifications: enzymes, transcription factors, cellular structure, antioxidants and calcium binding (Bax *et al*., 2012) level changes. It is interesting to remark that an increase in calpain activation was detected at the end of the force-feeding period. So, as a general consideration, predisposition to develop hepatic steatosis is primarily determined by the animal genetics, as demonstrated also by the choice of particular species and breeds in *foie gras* production.

1.3. *Foie Gras* Quality Features

As defined by the International Organization for Standardization, food quality is "the totality of characteristics of an entity that bears on its ability to satisfy stated and implied needs" (ISO 8402: 1994). In other words, it is all the characteristics of food that make it acceptable by consumers. So, depending upon the level of satisfaction, a product may be termed as excellent, good or poor quality product. Characteristics influencing quality can be classified as following:

- External:
  - Appearance (size, shape, colour, glossy, and consistency)
  - Sensory qualities (texture, flavour and odour)
- Internal:
  - Nutritional value
  - Adulterants
  - Contaminants (physical, chemical and microbiological)
  - Hygiene

The quality of a food product can also be more precisely defined by:

- its nutritional quality: levels of different nutrients (proteins, lipids, sugars, vitamins, etc.);
- its sensory quality: how the food is appreciated by consumers (taste, colour, aspect, flavour, etc.);
- its technological quality: how the food is appreciated by processing managers

(storage ability, water holding capacity, value of yield after a given transformation);

- safety quality: lack of any danger (physical, chemical or biological) for consumers;
- psychosocial quality: how the product is, subjectively, appreciated or not by consumers.

All those characteristics can be influence by intrinsic parameters linked to the animal (genetics, age, nutrition, conditions of rearing, etc.) or by extrinsic factors (temperature, duration of storage, environment, etc.).

A good quality *foie gras* is supposed to have the following characteristics:

- its colour should be uniformly light with no visible lesion or blood. The colour depends on the feed composition, in particular on corn characteristics, and it varies from light ochre to white-pink. In general, goose *foie gras* has a light pink tone, while duck's one has a yellowish tone.
- its texture must be firm but not hard, smooth but not malleable, homogenous and with no grittiness.
- its taste is pronounced with a rustic consonance in duck, while it is light, buttery and delicate in goose (Rousset-Akrim *et al*., 1994).

Two other properties are typical of *foie* gras, but not strictly related to the definition of quality, as they are requirements for a liver to be classified as *foie gras*. In fact they are regulated at a legislative level. First, the liver must have a net weight of at least 300 g if derived from duck, and at least 400 g if issued from goose. Second, the fat cooking losses should not exceed 30% after processing. In the case this second property is not respected, the liver can't be sold as *foie gras* and must be redirected to other and usually cheaper preparations. This represents an important economic loss for producers.

Three commercial forms of *foie gras* exist: *foie gras entier*, *foie gras* and *bloc de foie gras* (Figure 2). *Foie gras entier* consists on one or two lobes of fatty liver with the addition of stuffing. *Foie gras* is made of parts of liver agglomerated and added with stuffing. These are the two most valuable commercial forms. *Bloc de foie gras* is the result of the mechanical processing of the fatty liver, so that the chucks of liver are not visible. Less valuable preparations are represented by:

- *Parfait de foie*, which includes at least 75% of mechanically processed fatty liver;

- *Medallion de foie* and *Paté de foie*, produced with at least 50% of chunks or mechanically processed fatty liver;
- *Gelatin de foie*, a preparation containing at least 50% of fatty liver mixed with stuffing;
- *Mousse de foie,* a soft spongy preparation with a minimum of 50% of fatty liver
- A general category comprehensive of products which contain at least 20% of fatty liver named *Produits au foie gras.*

All these products are less expensive and represent both an alternative to the classic preparation and a way to use the fatty livers discarded because of their cooking losses.

a. b. c.

Figure 2. Main *foie gras* commercial forms. *Foie gras entier* (a), *foie gras* (b) and *foie gras en bloc* (c).

1.4. *Foie Gras* Technological Yield

Technological yield is the yield of a product after processing, and can refer to physical parameters (weight, nutritional content, etc.) or to quality aspects. In the case of *foie gras*, the parameter that affects technological yield the most is represented by cooking losses.

Even if *foie gras* can be sold as a raw product, in this form it has a short shell-life. Therefore, it is commercialized cooked or half-cooked. Half-cooked (*mi-cuit*) *foie gras* is pasteurized, so it keeps in the fridge and preserves the aromas of the fresh product. Cooked (*cuit*) *foie gras* is sterilized in autoclave and keeps many years in a fresh and dry environment. During the last decade, manipulation and cooking practices have been studied to guarantee the uniformity of the processed flock. Despite that, cooking losses still represent the major quality issue that faces the industry of fatty liver. In fact, standardization of the practices -from slaughtering to transformation- still doesn't prevent the inter-individual variability to remain high. The coefficient of variation within a flock

can even exceed 50% under commercial conditions (Theron *et al*., 2011). Cooking losses are caused by the lysis of the hepatocytes cellular membrane, with the consequent release of the lipids in the extracellular environment (exudation), which in that case is represented by the glass can. As a consequence, this phenomenon degrades the image of the final product, that is appreciated for its particular richness in fat, at the extent that this aspect is legislated. This becomes of great importance for processors as it affects their output and thus their profitability. Moreover, cooking losses cause alteration of the organoleptic properties of *foie gras*.

1.4.1. Parameters Influencing the Technological Yield

Various parameters are at the basis of the cooking losses determinism, and therefore influence the technological yield. Biological and technological factors can be recognized, and among the last *peri-* and *post- mortem* parameters are distinguished.

Biological factors are:

- The liver weight, which is directly proportional to cooking losses extent. This is the reason why acceptable liver weights range between 300 and 400 g depending on the considered species, and lower or higher weights give a lower ratio between profitability and production investment.
- The liver fattening extent, that is negatively correlated with the technological yield (Rousselot-Pailley, 1992). In fact, the higher the lipidic content, the lesser the ratio between proteins and lipids is. As a consequence, the capacity of the proteic structures to maintain the lipidic inclusions in the cell diminishes, the plasma membrane weakens and the probability to release the cellular content during the heat treatment increases (Blum *et al*., 1990).
- Breed. It is well-known that Muscovy ducks have a higher technological yield than Pekin ducks (Blum et al., 1992) and that mule duck fatty livers show lower cooking losses than both the parental livers, because of an effect of heterosis.
- Sex. In duck, just livers issued from male individuals are used to produce *foie gras*, because of a better technological yield and better quality features, while in goose the sex has no influence on these parameters.
- Zootechnical factors, such as the age of the animal, feed additions and force-feeding rhythm.

Technological factors are:

- Among the *peri-mortem* parameters:
  - Fasting period duration. A 12-24 hours fasting period is necessary to make sure the digestive system has emptied and avoid the risk of contamination during evisceration. Longer fasting periods are well-known to diminish the animal body and the liver weights, and to compromise the technological yield.
  - Transport conditions, whose duration was recently demonstrated to influence the technological yield (Fernandez *et al*., 2011).
- Among the *post-mortem* parameters, the most important are the evisceration conditions, which are comprehensive of :
  - Evisceration methods. Fatty liver processors have noticed that hot eviscerated livers (i.e. livers withdrawn immediately after slaughter) rapidly chilled presented a higher technological yield than cold eviscerated ones (i.e. after cooling of the entire carcass at +4 °C) (Babile & Auvergne, 1986; Rousselot-Pailley *et al*., 1992). That seems to be related to the time necessary for the liver to cool, which is longer while it is in the carcass. Bouillier-Oudot *et al*. (2004), showed that during this period carbohydrates and glycogenolytic activities reduced glycogen reserves during refrigeration, but it is also possible to hypothesize that longer time extents for the proteases activity favour the demolition of the plasma membrane and thus the lipids release.
  - Fatty livers chilling rates. Sahar *et al.* (2013) demonstrated that faster chilling rates of the fatty livers after hot evisceration ameliorate the final quality of *foie gras.* Hardness of the liver is related to its lipidic content and thus constitute a parameter to evaluate fatty livers predisposition to cooking losses before transformation. Soft livers have higher content in water and lesser cooking losses, but are not desired by producers because of the issues posed by their manipulation. On the contrary, hard livers have higher lipidic contents and lower technological yield. Therefore, the desired consistence is a firm intermediate one. The study showed that hardness and cooking losses are directly proportional to the chilling time, and that in fast cooled samples the proteolytic activity is less modified. This seems to confirm the hypothesis that the more rapidly the liver is chilled, the least proteases are active and thus the

least proteins are degraded *post-mortem*.

1.4.2. Relationship between Proteolytic Activities and Cooking Losses Variability

In addition to the previously described parameters, Sahar *et al.* (2014), recently showed the implication of proteases in *foie gras* technological yield determinism.

Proteases represent an ubiquitous category of hydrolitic enzymes whose activity is directed toward the peptide bond (proteolysis). They are responsible for the protein turnover in the cell and for incorrectly folded protein removal, as well as for cellular processes regulation and protein digestion. On the basis of the site of the cleavage along the substrate polypeptide chain, proteases can be distinguished in endoproteases, if they break peptide bonds within the molecule, and exoproteases, if they cleave at the N- or C-terminal of the polypeptide. Exoproteases are so divided in aminopeptidases and carboxypeptidases, respectively. On the contrary, among endoproteases, a further classification can be made on the basis of the catalytic residue involved in proteolysis. As far, six broad groups of proteases have been so identified: serine proteases, threonine proteases, cysteine proteases, aspartate proteases, glutamic acid proteases and metalloproteases. Both intra- and extra- cellular proteases can be identified for their localization toward the plasma membrane.

It is well-known that specific families of proteases play an important role in the onset of *post-mortem* phenomenons, in human as well as in animals. Many studies have been focusing on their activity in *post-mortem* animal tissues, as it affects the quality of food products. In particular, cellular structure alterations due to proteases activity during *post-mortem* storage of meat has been extensively investigated (Nowak, 2011). On the basis of the existing knowledges, Awde *et al.* (2014a), hypothesized a role for proteases in *foie gras* technological yield determinism. In meat during *post-mortem* storage proteases are known to be involved in a tenderness increase, a feature which is regarded as the most important factor in determining the acceptability of meat both by the consumer and the producer. On the contrary, a possible implication of proteases in the weakening of the cellular structures in fatty liver would be negatively correlated with *foie gras* quality, as it enhances its tendency to release lipidic exudates during cooking. In order to demonstrate this assumption, two groups of fatty livers were investigated: a high cooking losses (CL+) and a low cooking losses (CL-) group. Fatty livers samples were used to perform both

biochemical (dry matter content and lipids content) and proteomic analyses. Three family of proteases were considered for their previously demonstrated implication in *post-mortem* phenomenons: metalloproteases (MMPs), cathepsins and calpains. It was shown that the two groups of fatty livers had significantly different proteolytic activities. However, as a PCA (Principal Component Analysis) performed with all the data obtained from the three investigated parameters demonstrated a higher correlation between cooking losses and biochemical parameters, no conclusion could be done on the exact role of the proteases. Therefore, further studies are necessary to clarify whether the improved proteases activity represents a cause of the cooking losses or the consequence of other predisposing factors. In fact, it could be possible that high proteolytic activities correspond to very active hepatic metabolisms (high level of lipid accumulation), as well as they are linked to the animal genetics and thus represent a factor influencing the technological yield.

1.5. The Calpain System

1.5.1. Calpains

Calpains are a family of complex multi-domain intracellular enzymes that share a Ca^{2+}-dependent cysteine protease core. These cytoplasmic enzymes respond to calcium signalling by carrying out limited cleavage of target proteins, rather than serving as degradative enzymes.

The first evidence of calpains came from two different groups in the 1960s who reported the presence of a Ca^{2+}-activated proteolytic activity in soluble extracts from rat brain (Guroff, 1964) and skeletal muscle (Huston *et al.*, 1968). In the 1970s, the enzyme was purified from skeletal muscle (Dayton *et al.*, 1976) and was called CANP (Calcium-Activated Neutral Protease), where 'neutral' referred to its optimal pH for activity, which is compatible with a cytoplasmic location. Murachi *et al.* (1980) then introduced the current used name 'calpain', referring to a Calcium-dependent Papain Like enzyme.

The first mammalian calpain isoforms to be resolved were the micromolar (μ-) and the millimolar (m-) calpains, which were named on the basis of different Ca^{2+} requirements for their activation (Wheelock, 1982). μ- and m- calpain were shown to be heterodimers of an isoform specific 80 kDa subunit and a common small 28 kDa subunit. It was not until the human genome sequencing that the extent of the mammalian calpain family was

revealed. At present, 15 human calpain genes (CAPN1–15) are known, one of which (CAPN4) codes for the small subunit (Table 1). The criterion for designation as a calpain is generally agreed to be the possession of a cysteine protease core sequence, even if some of the recognized calpain family members (such as the CAPN6 gene product) are not enzymatically functional because of the absence of one or more catalytic triad residues. Calpains 1 and 2 respectively correspond to μ- and m- calpains. Recently Campbell *et al.* (2012), suggested the use of numbers instead of letters to designate the two isoforms, in the attempt to render the nomenclature less misleading. In fact, the difference in the activating Ca^{2+} concentration is only one order of magnitude, being 3–50 μM for μ-calpain and 400–800 μM for m-calpain isolated from bovine skeletal muscle (Goll *et al.*, 2003).

As calpains 1 and 2 are the two most abundant and extensively studied members of the calpain family, the next sections will focus on these two isoforms properties. Where nucleotide or amino acids are numbered, the human calpain gene or protein sequence is considered. In the other investigated species, here included a bird species (chicken), calpain 1 and 2 characteristics are maintained, but positions or nucleotides/amino acids identity slightly change.

Table 1 (Ravulapalli, 2009). Calpain nomenclature. T-S: tissue-specific; U: ubiquitous; X: no; Y: yes.

Calpain	Gene products	Aliases	PEF	Tissue distribution pattern	Expression (highest)
Calpain-1	CAPN1 and CAPNS1	μ-Calpain, calpain I, μCANP	Y	U	Placenta, oesophagus, trachea
Calpain-2	CAPN2 and CAPNS1	m-Calpain, calpain II, mCANP	Y	U	Kidney, lung, trachea
Calpain-3	CAPN3 dimer?	p94, nCL-1 (Lp82, Lp85, Rt88)	Y	T-S	Skeletal muscle (lens, retina)
Calpain-5	CAPN5	nCL-3, htra-3, CAPN5	X	U	Brain, kidney, liver
Calpain-6	CAPN6	CAPN6	X	T-S	Placenta
Calpain-7	CAPN7	CAPN7, palBH	X	U	
Calpain-8	CAPN8	CAPN8, nCL-2	Y	T-S	Stomach, digestive tract
Calpain-9	CAPN9 plus CAPNS1	CAPN9, nCL-4	Y	T-S	Digestive tract, heart
Calpain-10	CAPN10	CAPN10	X	U	Heart
Calpain-11	CAPN11 plus ?	CAPN11	Y	T-S	Testis
Calpain-12	CAPN12 plus ?	CAPN12	Y	T-S	Hair follicle
Calpain-13	CAPN13 plus ?	CAPN13	Y	U	Lung, testis
Calpain-14	CAPN14 plus ?	CAPN14	Y	U	
Calpain-15	CAPN15	CAPN15, SolH	X	U	Brain
Calpain small subunit 1	CAPNS1	CAPN4, CPNS1*	Y	U	Heart, pancreas, kidney

Calpain 1 and 2 Genes Structure and Coding Products.

Calpain 1 is a heterodimer of the *CAPN1* gene product, CAPN1, and the *CAPNS1* (formerly *CAPN4*) gene product, CAPNS1. Calpain 2 is a heterodimer of the *CAPN2* gene product, CAPN2, and *CAPNS1.* Calpain 1 and 2 can thus be shortly referred as CAPN1/S1 and CAPN2/S1. *CAPN1* and *CAPN2* encode for a 80 kDa catalytic subunit, while *CAPNS1* a small

28 kDa subunit. A second small subunit homologue, *CAPNS2*, has been found in both human and mice (Schà *et al.*, 2002). It encodes for a 248 aa polypeptide having a predicted molecular mass of 27,659 Da and 63% amino acid sequence identity with CAPNS1. Although CAPNS2 could potentially form heterodimers with penta-EF-hand (PEF) containing CAPN gene products, these variants have not been identified *in vivo*.

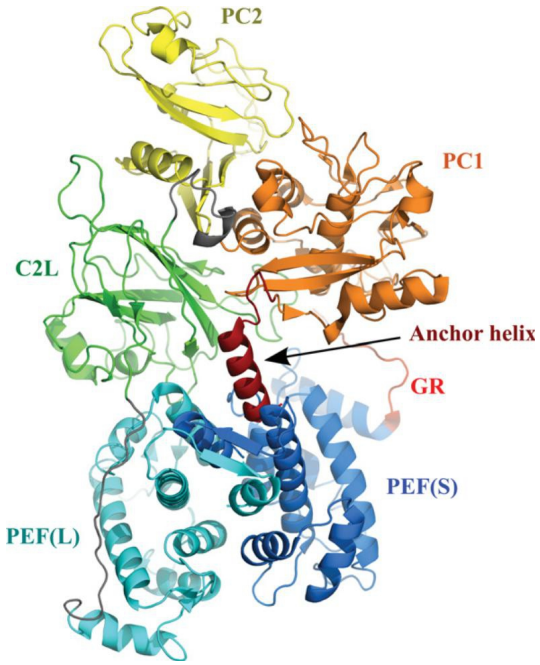
Detailed gene structure informations are available for human, monkey, mouse, rat, bovine, porcine, rabbit, sheep, fruit fly and chicken (UniProt consulted on August 2014). On the contrary, informations concerning duck calpain gene and protein sequences are still at a prediction level, deriving from the genome sequencing analysis of a female Pekin duck (*Anas platyrhynchos*) (Huang *et al.*, 2013). On the basis of sequence homology, two ORFs have been annotated as the catalytic subunit gene of duck calpain 1, both of unknown chromosome position and genetic structure: one is a 1,977 nt ORF (ID# KB744603.1, ENA, EMBL-EBI) whose translation give a predicted protein of 659 aa length (ID# R0KNG7_ANAPL, UniProt) and 74,414 Da molecular mass, while the other one is represented by a 2,058 nt polynucleotide (ID# KB742809.1, ENA, EMBL-EBI) coding for a 686 aa polypeptide (ID# R0LQS4_ANAPL, UniProt) with a predicted 77,934 Da molecular mass. Duck calpain 2 catalytic subunit predicted sequence (ID# KB742799.1, ENA, EMBL-EBI) is 1,830 nt long and its translational product is a 610 aa protein with a molecular mass of 69,734 Da (R0LRI5_ANAPL , UniProt). No informations are available for duck calpain small subunit.

Calpain 1 and 2 Crystallographic Protein Structure.

In the last ten years calpain crystallographic images have been helpful to elucidate their tertiary structure and their mode of action. In 2000 Strobl *at al.* first solved the 2.3-Å crystal structure of full length heterodimeric human calpain 2 crystallized in the absence of Ca^{2+} (Figure 3). Additional informations came from the work of Hanna *et al.* (2008), who managed to crystallize a Ca^{2+}-bound recombinant rat calpain 2 in complex with calpastatin. Attempts to obtain the complete crystallographic structure of calpain 1 have not yet been successful, although the similarity in amino acid sequence suggests that the two structures will be similar. This was partly confirmed by the crystallization of the protease core of calpain 1 by Moldoveanu *et al.* (2002). For these reasons the three-dimensional representation of calpain 2 will be here used to discuss both the isoforms

properties.

Figure 3. Crystal structure of human Ca^{2+} -free calpain-2 (Campbell *et al.*, 2012).



Domain structure of human calpain 2 (PDB code 1KFU) produced in baculovirus-infected insect cells with the GR domain present (Strobl *et al.*, 2000). Calpain 2 has an overall oval shape. The N-terminus of the large (80 kDa) subunits begins with the anchor helix (red) and leads into the protease core domains PC1 (orange) and PC2 (yellow), followed by the C2L domain (green) and then into the C-terminal PEF domain (light blue). This PEF domain is paired with the C-terminal PEF domain of the small (28 kDa) subunit (dark blue). The N-terminal GR domain is unstructured and electron density is lost in 11 residues from the C-terminal end of this domain.

Till 2011 calpain domain nomenclature and boundaries identification were designate in different ways, not necessarily consistent with one another. In a recent review Sorimachi *et al.* (2011) proposed the currently used nomenclature. According to them, the domain boundaries were established on the basis of the available crystallographic structures and of evolutional conservation studies, while the domain names were decided to be the acronyms representing their function or their structure (Figure 3). Five structural elements were recognized as part of the 80 kDa catalytic subunit:

- A N-terminal anchor helix, once identified as Domain I.
- Protease Core domains 1 (PC1) and 2 (PC2), representing the two halves part of the catalytic (CysPc: Cys-protease core) domain, which in the previous nomenclature were named as Domain I and II or Domain IIa and IIb. The active site is represented by the catalytic triad (Cys-His-Asn), characteristic of cysteine proteases such as papain or cathepsins B, L and S. The CysPc was shown to have elements that bind Ca^{2+} ions and are directly responsible for the main activation event.

- C2-like domain (C2L), once Domain III. The name is due to the presence of a β-sandwich structure that at the level of tertiary conformation resembles the C2 domain found in various enzymes, such as protein kinase C, where it seems to be implicated in their translocation to the plasma and/or other subcellular compartments membranes (Corbalán-García *et al.*, 2010). Therefore, domain C2L may have a phospholipid-binding function (Gil-Parrado *et al.*, 2003). Analysis of the amino acid sequence indicates that this domain also contains two clusters of acidic residues (Glu392, Glu393, Glu394, Asp395, Glu396, Glu481 and Asp508; and Asp351, Asp398, Glu397, Asp399, Glu402 and Glu504) situated at the N-terminal and at the C-terminal, which were assumed to bind Ca^{2+}-ions through coordination and charge neutralization. However, the N-terminal cluster conformation in the crystallographic structure was shown to be incompatible with the Ca^{2+}-binding, as later confirmed by the crystal structure of a Ca^{2+}-bound calpain 2 when in complex with calpastatin (Hanna *et al.*, 2008). C2L has also been assigned a role in the transmission of the conformational changing from the PEF Ca^{2+}-bounded domain to the CysPc domain, until the moment this was demonstrated not to be the main event in calpain activation.
- A penta-EF domain (PEF), or Domain IV. As from the name, this domain contains five EF hands sub domains, four of which are able to bind Ca^{2+}-ions, while one is involved in the dimerization of the two calpain subunits.

A similar PEF domain was identified also in the 28 kDa small subunit (domain VI), together with a N-terminal Gly-rich domain (GR), once domain V. GR domain is made up of 95 amino acids, 40 of which are Gly organised in two contiguous stretches of 11 and 20 residues. A 5 Pro stretch is also present. Hence, the amino acid sequence of GR domain suggests an unordered hydrophobic structure that could serve as a tether to other molecules or structures (possibly the target proteins).

Domains involved in the dimerization of the large catalytic and the small subunits are the anchor helix and the PEF domains. The main association is done by the pairing of the C-terminal EF-hand domains of each subunit through extensive intimate hydrophobic contacts. A lesser contact is made between the N-terminal anchor helix of the large subunit and the PEF domain of the small subunit.

Activation Mechanism: The Role of Calcium.

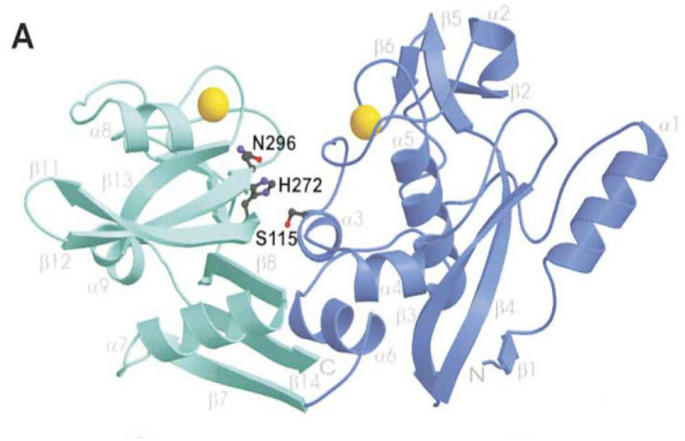
The Ca^{2+} free structure of human calpain (Strobl *et al.*, 2000) revealed that the inactivity of the enzyme was due to the misalignment of the catalytic triad. In the inactive state the histidine side chain is 8.5 Å apart from the cysteine, a distance which is far from the ~3.5 Å optimum for the histidine to deprotonate the cysteine, thus activating it for nucleophilic attack on the substrate. The alignment of the catalytic triad is enabled by a 25° rigid-body rotation of PC1 and PC2 relative to each other, with the consequent formation of an active site cleft (Figure 4) which accommodate the substrate. Various events have been identified as possible responsible of calpain activation:

- the release (Strobl *et al.*, 2000) and the possible autolysis (Chou *et al.*, 2011) of the anchor helix , a conformational change which would relieve the tension between PC1 and CAPNS1 PEF domain, thus facilitating the approach of the two CysPc domains. Dissociation of the anchor helix was hypothesized to be led by electrostatic repulsion following subtle conformational changes and charge re-organisation in the two Ca^{2+}-bound PEF domains.
- Ca^{2+}-binding by a negatively charged loop of 10 residues belonging to C2L domain (Strobl *et al.*, 2000). In the Ca^{2+} free conformation, this loop directly contacts the amphipathic α_7-helix and the open loop of PC2 domain (Figure 5). Even if it wasn't demonstrated through crystallography, it is plausible that positively charged particles such as Ca^{2+}-ions bind the acidic loop under partial charge compensation. This event would reduce the acidic loop strongly negative potential and lower the electrostatic interaction that it forms with PC2, thus allowing this latter to turn over to PC1 and form the active-site cleft. Such an "electrostatic switch mechanism" could also account for the different Ca^{2+} requirements of calpain 1 and 2, the acidic loop of the first one having eight negative charges distributed over a longer segment.
- cooperative Ca^{2+}-binding by two non-EF-Hand Ca^{2+}-binding sites in the CysPc, which was showed through crystal structure of the protease core of calpain 1 (μI-II or mini-calpain 1) obtained by Moldoveanu *et al.* (2002). After Ca^{2+}-binding, peptide loops rearrangement at the level of individual PC1 and PC2 causes the alignment of these two domains to form the active-site cleft. This would seem to be the leading event of CysPc conformational change into the active form, as μI-II

is inactive in the absence of Ca^{2+} even if free of restraining interactions from the neighbouring domains. It is worth to notice, however, that *in vivo* the presence of the accessory domains would enhance the activation rate, as demonstrated by the slower proteolytic rate of µI-II *in vitro*.

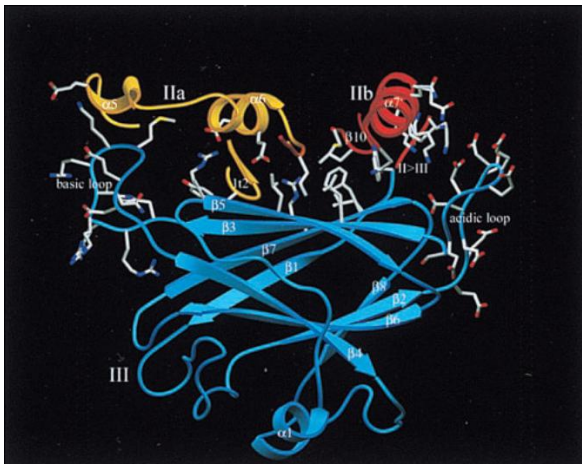
Therefore, a two-stage model for Ca^{2+}-triggered calpain activation can be hypothesized (Moldoveanu *et al.*, 2002). The first stage consists on the release of constraints imposed by the non-core domains, while the second is represented by the alignment of the active-site cleft caused by the cooperative binding of Ca^{2+} to PC1 and PC2.

Figure 4. X-Ray Structure of Ca2 Bound µI-II (Moldoveanu *et al.*, 2002).



The front view of µI-II with PC1 colored in blue and PC2 in cyan. β-strands and α-helices are numbered sequentially from the N-terminus (N) to the C-terminus (C). Gold-colored spheres indicate the positions of the two Ca^{2+}. The side chain atoms of the catalytic triad residues are colored in red (oxygen), dark blue (nitrogen), and gray (carbon), and the bonds are colored in gray.

Fig. 5. Ribbon plot of domain C2L (III, in blue) **and contacting segments from subdomains PC1** (IIa, in gold) **and PC2** (IIb, in red) **of human Ca^{2+} free calpain 2** (Strobl *et al.*, 2000).



The side chains of the right-side acidic loop and of the left-side basic loop, and some polar and hydrophobic residues forming the polar (center, left) and hydrophobic (center, right) interface between both catalytic subdomains and domain C2L are given.

Autoproteolysis and the Proenzyme Question.

It is well known that both calpains 1 and 2 autolyze when incubated with Ca^{2+} (Goll *et al.*, 2001). Although it is not uncommon for proteolytic enzymes to autolyze, autolysis (or autoproteolysis) of calpains has several unique features. Autolysis reduces the Ca^{2+} concentration required for half-maximal proteolytic activity of calpain 1 from 3–50 to 0.5–2.0 μM, and that of calpain 2 from 400–800 to 50–150 μM. For this reason and for the fact that Ca^{2+} concentrations otherwise necessary for calpain activation are higher than those typical of normal cells, autolysis has for long being thought as a mechanism for calpain activation. Thus, the 80 kDa forms of the catalytic subunit were considered proenzymes, while the 76 kDa and the 78 kDa autolysed forms of calpain 1 and 2 catalytic subunit respectively were considered the active forms in the cell. This same autolysis reduced the mass of the 28-kDa subunit to 18 kDa. The autolytic process was further characterized when the 80 kDa subunit of calpain 1 was found out to autolyze more rapidly than the calpain 2 80 kDa subunit, if both compared to the 28 kDa subunit autolysis rate. In 1992, Saido *et al.*, demonstrated that the autolytic event consists on the removal of a N-terminal sequence, which has now been identified in the anchor helix. However, Hanna *et al.* (2008), showed that for calpain 1 and 2 autolysis must be an intermolecular event, as the anchor helix is far from the active-site cleft in both the crystallographic structures of the apo- and the Ca^{2+}-bound forms of calpain 2.

Even if the biochemical changes that accompany autolysis have been well characterized, its physiological significance *in vivo* remains controversial. Various arguments can be made against autoproteolysis being a physiological activation mechanism. First of all, as autolysis is performed by calpain in its proenzyme form, Ca^{2+} concentrations required are out of the range of physiological Ca^{2+} levels in normal cells. Furthermore, autolysis being an intermolecular event, activation of a calpain molecule would need an already active calpain. Also, it appears clear (Hannah *et al.*, 2008) that in the inactive state the anchor peptide cleavage sites would be protected from hydrolysis by being part of an *α*-helix, and only after activation would assume a disordered conformation, hence becoming susceptible to hydrolysis. The fact that autolysis does not precede activation was demonstrated by Chou *et al.*, 2011, too. In fact, anchor helix removal occurs in the same frame-time as several potentially inactivating cleavages in C2L domain. Hence, calpain autolysis would be the result of activation, and not the cause. Moreover, the observation

that *in vivo* calpain would be surrounded by other proteins, including its substrates, and would not naturally be concentrated to the same extent as it is *in vitro*, arises the question whether autolysis takes place *in vivo* or is an artificial situation promoted *in vitro* when calpains are concentrated through purification.

The Small Subunit Dissociation Question.

Another incompletely substantiated claim that is deeply entrenched in the calpain literature is that the small 'regulatory' subunit dissociates from the large 'catalytic' subunit during activation or even as a calpain activation mechanism (Yoshizawa, 1995; Goll *et al.*, 2003). One reason why this 'subunit dissociation mechanism' is hard to accept from the perspective of structural biology is that the interface between the PEF domains of the small and large subunits is extensive and hydrophobic. It is difficult to imagine this interaction being broken and reformed without the involvement of a chaperone or denaturing conditions. Moreover, crystal structures of the apo- and Ca^{2+}-bound forms of calpain 2 only show small differences in conformation or area of contact between the PEF domain. In 2001 Nakagawa *et al.* showed that the small subunit dissociation was a consequence of the autolysis and was not required for activation. Recently, Chou *et al.* (2011) demonstrated that intermolecular proteolysis of calpain produces a stable heterodimer of the two PEF domains, while there is no evidence that small subunit itself is released. As this heterodimer has a molecular weight of ≈40 kDa, it could have been difficult to distinguish it from the small subunit homodimer and it might have been mistaken for this last in some of the reports of subunit dissociation.

Activation of the Protease Core in the Absence of the Other Domains Supports a Simplified Hypothesis.

Having found fault with autoproteolysis and subunit dissociation as activation mechanisms, there is an obligation to explain how calpain can be activated in the cell when resting calcium levels are three orders of magnitude lower than those needed to achieve half-maximal activation. Chou *et al.*, 2011, proposed a simplified model where the extremely localized calcium concentration near the inflow from channels and pumps should be high enough to transiently activate calpain where needed – for example at a focal adhesion complex. By the time this calcium influx has diffused, calpain will already

have returned to its inactive state. Hence, physiological activation of calpain would occur in an extremely localized fashion, with a tiny fraction of calpains activated in extremely localized areas for very brief moments. Indeed, the argument has been made that the requirement for high calcium levels acts as a biological safety device that helps prevent calpain overactivation under physiological conditions. In this model, calpastatin might only be needed as a stop gap to prevent prolonged calpain activity under conditions of cell stress.

1.5.2. Calpastatin

The second member of the Calpain system is calpastatin, the endogenous inhibitor of calpain. Calpastatin is an intrinsically unstructured protein capable of reversibly binding and inhibiting four molecules of calpain in the presence of calcium (Goll *et al.*, 2003). Human calpastatin is encoded by the CAST gene. CAST contains multiple promoters, thus generating several different transcripts, which are also alternatively spliced into different mRNAs: as a result multiple protein isoforms are derived from a single gene (Meyers & Beever, 2008). Recently Hanna *et al.* (2008) have identified the nature of the interaction of calpastatin with calpain, which has unique aspects for a protein inhibitor interacting with a proteolytic enzyme. In fact, calpastatin is an unstructured protein, but when it binds calpain it adopts a structure which allows inhibition to take place. The interaction is favoured by a conformational change of calpain after binding of calcium. Calpastatin contains 4 inhibitory domains, each of which can inhibit calpain activity. Within these domains three regions (A, B and C) are predicted to interact with calpain. The peptide chain helices found in regions A and C interact with calpain at two separate sites causing the inhibitory domain to wrap around calpain. The region between A and C, region B, then blocks the active site of calpain. The crystallography studies suggest that region B does not directly bind to the active site thereby preventing it becoming a substrate for the enzyme.

1.5.3. Roles of Calpain System

Physiological roles of calpains. The target proteins and processes in which calpains are implicated are extensive (Goll *et al.*, 2003). Perhaps the main process they have been reported to be involved is cell motility, where they act in the cutting of the focal adhesion

complexes (Chan *et al.*, 2010) and in the reorganization of the cytoskeleton (Cortesio *et al.*, 2010).

Calpains in diseases. Calpain 1 and 2 high calcium requirement ensures that activation is temporally and spatially limited, occurring only near the epicentre of a Ca^{2+} influx, which is then quickly dissipated by diffusion and Ca^{2+} removal by various pumps (Campbell *et al.*, 2012). Pathological conditions where a role has been attributed to calpains are situations in which Ca^{2+} levels in the cell rises out of control and causes prolonged widespread overactivation of calpain, with off-target proteolysis leading to necrotic cell death (McCall, 2010). This is the case of reperfusion injury, neurodegenerative diseases, muscular dystrophies, retinopathy and cataract, where calcium homoeostasis can be compromised (Campbell *et al.*, 2012). In various animal models, the administration of low molecular mass calpain inhibitors can bring about some amelioration of the damage and has prompted the search and development of more specific and efficacious calpain inhibitors (Donkor, 2011).

Role of Calpain System in Post-Mortem Proteolysis. It is widely accepted that proteolytic calpain activity does contribute to meat tenderization, with considerable evidence in beef, lamb and pork. Correlations have shown that the different tenderization rates between species (beef < lamb < pork) relate inversely to the ratio of calpastatin:calpain (beef > lamb > pork) (Koohmaraie *et al.*, 1991).

The final tenderness of meat depends on the degree of alteration of the muscle structural and associated proteins, in particular myofibrillar, myofibril cytoskeleton and costamere proteins. Calpain 1 was demonstrated to be responsible for the cleavage of key myofibrillar proteins including nebulin, dystrophin, metavinculin, vinculin, troponin-T and desmin (Geesink *et al.*, 2006). On the contrary, muscle specific calpain 3 and calpain 2 did not appear to have a major role in *post-mortem* proteolysis and development of tenderness (Geesink *et al.*, 2005; Geesink *et al.*, 2006), at least not in the short-term, as suggested for calpain 2 by the fact that it persists longer than the less stable calpain 1 isoform in ageing muscle from all species studied.

Although the experimental evidence available suggests that calpain 1 is the central enzyme component of the calpain system in *post-mortem* meat tenderization, calpastatin role too has been turned out to be significant. High levels of calpastatin in the few hours after slaughter are associated with poor quality meat (Kemp *et al.*, 2010), the model being

that high levels of calpastatin reduce the activity of calpain thereby reducing the proteolysis required for tender meat. Experimental observations have therefore highlighted the possibility to monitor calpastatin during this period of time, making calpastatin a marker for meat quality, useful to predict whether or not any given carcass will tenderize to an acceptable degree. Recent studies showed also that calpain system might represent the link between pre-slaughter animal stress and meat toughness; for example, Parr *et al.* (2000) demonstrated that elevated plasma adrenaline increases calpastatin activity and expression in pigs.

Recently, interactions between the calpain system and other protease systems have been investigated, such has caspases, whose role in meat tenderization is well-established. Nakagawa & Yuan (2000) demonstrated that a disruption in Ca^{2+} homeostasis in the ER as a result of ischemic injury induced calpain-mediated activation of caspase 12. Calpain inhibition through over-expression of calpastatin has been shown to increase caspase 3 activity and apoptosis (Neumar *et al.*, 2003). Additionally the endogenous calpain inhibitor calpastatin is also cleaved by caspases 1, 3 and 7, generating distinct degradation patterns (Wang *et al.*, 1998). Therefore if caspases are active in the muscle *post-mortem* they may influence meat quality by proteolysis of calpastatin. This in turn could result in the activation of calpains which are known to be involved in meat tenderness and thus reducing toughness. Therefore, examining the interactions of the calpain system and other molecular components of the cell represents the future direction in the investigation of meat tenderness development.

2. HYPOTHESES AND OBJECTIVES OF THE PROJECT

This Master Thesis work was accomplished during my six month Erasmus exchange mobility at the Department GenPhySE (*Génétique, Physiologie et Systèmes d'Elevage*, UMR 1388 INRA/INPT) at the ENSAT National Institute of Agronomy (É*cole Nationale Superieure Agronomique deToulouse*, France).

A part of this project (study 1) was supposed to be the following of a PhD project accomplished by Sahar Awde (Awde, 2014b) and aimed at investigating the relationship between the hepatic proteolysis and the technological and sensory qualities of duck *foie gras*. Awde *et al*. (2014a) found that differences in the proteolytic activities of three main classes of proteases (calpains, cathepsins and MMPs) partly accounts for the technological yield of duck fatty livers. However, lipids and dry matter contents were shown to have a stronger influence on the technological yield value than proteases activities *per se*. Therefore, it remained unclear whether differences in the proteases activities were due to differences in hepatic metabolism (the higher the lipids anabolism, the more active the hepatic metabolism and the higher the proteases content) or whether they were at the basis of the inter-individual technological yield variability, thus constituting one of its biological deterministic factors. In order to validate one of these two hypotheses, I was supposed to investigate the proteolytic activity in two groups of fatty liver samples (CL+ and CL-) with equal dry matter and lipids contents. In fact, without the influence of the biochemical parameters just the role of proteases on *foie gras* technological yield was expected to be seen. For practical reasons, just the calpain proteolytic activity toward the casein substrate was measured through calpain casein zymographic approach.

A second part of my Master Thesis work focused on the better characterization of the calpain casein zymography technique (study 2). Limited information is available on duck calpain system, partly because it has only recently become an object of study (Awde *et* al., 2014a) and partly because duck fatty liver production represents a niche in the international market. In particular, at present nothing is known on the relationship between calpains and their activator (Ca^{2+} ions) and inhibitors (calpastatin) in duck hepatic cell, and whether a possible interaction continues to take place also during calpain casein zymography performance. As *in vitro* and in the presence of their activator calpains are endowed with autolytic activity, calpains stability in the Extraction Buffer was investigated

in order to prove that differences in the zymograms are only determined by differences in calpain content at the moment of the extraction. On the contrary, no investigation was done on calpastatin content after extraction. A second part of study 2 focused on calpain lytic activity at different Ca^{2+} concentrations. This was done to prove that zymograms resulting from the lysis of the casein substrate by duck calpains showed the same profile as those in rat, which were recently investigated by Kanzaki *et al*. (2014).

Finally, duck calpain autolysis rate (study 3) and calpain 2 Western Blot profiles (study 4) were investigated. The first was done to gain insight in duck calpain system, according to that part of the scientific community who supports the hypothesis that autolysis represents an activation mechanism for calpain *in vivo*. On the other side, calpain 2 Western Blot profiles were studied to establish the identity of the displayed bands and possibly make a correlation between the Western Blot profile and the zymogram.

3. MATERIAL AND METHODS

All the experiments described here fully comply with the legislation on research involving animal subjects, according to the European Communities Council Directive of Nov 24, 1986 (86/609/EC). Furthermore, investigators were qualified by the French governmental authority.

Both biochemical and proteomic analyses were performed with liver powder samples obtained through a grinding process. Fatty liver samples were taken from the freezer (-80 °C) and crushed in liquid nitrogen with a ball-grinder. The procedure was aimed at producing a fine, homogeneous and representative powder for each sample, that was then stored in specific and enumerated plastic cups. All the chemical products used to perform the analyses have been bought from Sigma- Aldrich, if no other specification is present.

3.1. STUDY 1: Relationship between proteolytic activities and cooking losses values in livers issued from force-fed ducks

3.1.1. Sample Processing

Fatty livers used in this experiment were issued from two commercial flocks of male Mule ducks (*C. moschata* × *A. platyrhynchos*) reared and force-fed according to standard practices. At the end of the force-feeding period, the slaughtering procedure was conventionally conducted in a commercial plant. About 20 minutes *post-mortem* the fatty livers were removed from the carcasses and weighed. Fifty fatty livers were selected on the basis of similar weights. Samples of 60 g were withdrawn from the middle part of the livers and directly used to accomplish physical analysis. At the same time samples of 15 and 10 g were taken and frozen in liquid nitrogen, to be assigned to biochemical and proteolytic analyses respectively. Those samples were stored separately at -80 °C until grinding and further processing.

Dry matter and total lipid contents were determined for all the ground samples. Using the technological yield values, they were divided in two numerically equal groups: a high cooking losses (CL+) and a low cooking losses (CL-) group. Eight CL+ and eight CL- samples were then selected on the basis of similar values for dry matter and lipid contents and used for the proteolytic analysis.

3.1.2. Physical Analysis

a) **Technological Yield or Cooking Losses**

Technological yield or cooking losses was calculated by performing the melting test. Each one of the 60 g samples was placed into a glass can. Salt (13 g/kg) and pepper (2 g/kg) were added and the glass cans were placed in an autoclave at 90 °C for 75 min for cooking. The cans were subsequently chilled using tap water and stored for two months at 4 °C. After this storage period, livers were removed from glass cans and all visible exterior fat was trimmed by a gentle scrapping using a knife. The technological yield was then evaluated by the expression of fat losses during cooking and storage, and expressed as a percentage of the initial weight.

3.1.3. Biochemical Analyses

A) **Dry matter content**

The dry matter content of the fatty livers was determined by drying a mass of ground liver (Wdm_{raw}) in an oven at 105 °C for 24 h (Molee *et al*., 2005). Sand was added to previously weighed cups (Wdm_{cup}) to increase the surface of exchange. Once dried in the oven, the samples were cooled to room temperature in desiccators and then weighed (Wdm_{dry}). The percentage of dry matter (DM) was then determined by the formula:

$$\text{DM content} = ((Wdm_{dry} - Wdm_{cup})/Wdm_{raw}) \times 100.$$

B) **Lipids extraction and quantification**

The total lipid extraction was performed according to the method of Folch *et al*. (1957).

The ground samples were homogenized with an Ultra-Turrax T25 homogenizer (JK- IKA Labortechnik, Germany) with 2:1 v/v chloroform-methanol mixture (Folch Mix) to a final dilution of 100-fold the volume of the sample. The volume of the sample was computed on the assumption that it has the specific gravity of water, that is the volume of 1 g of tissue sample is 1 ml. The homogenate was incubated at 4 °C overnight, in order to maximize the extraction yield, and then filtered through a type Fisher paper filter (grade 12). The crude extract obtained was mixed thoroughly with 0.2-fold its volume of a salt solution (0.73% NaCl), to allow the formation of a biphasic system: a lower chloroform phase containing the lipids and an upper aqueous phase (methanol and water) containing other compounds. A known quantity of chloroform phase was withdrawn, filtered on Whatman 1PS filter papers (Sigma- Aldrich) and collected in a glass Wheaton tube. Finally, the chloroform was subtracted by evaporation thanks to a concentrator (RC10-10, Jouan S.A., France). The extracted lipid weight was calculated by subtracting the weight of the empty tube from the final total weight. The total lipid content was calculated as:

$$\text{total lipids (\%)} = \frac{2 * V * R}{3 * Vlip * S} * 100$$

V= Folch Mix Volume (ml);

R= Extracted Lipid Weight (g);

Vlip= Withdrawn Chlorophorm Phase Volume (ml);

S= Sample Weight (g).

3.1.4. Proteolytic Analysis

I. **Protein extraction**

The extraction was carried out in reducing and non-denaturing conditions, thus maximizing the extraction yield for calpains, among other proteins. Approximately 0.3 g of ground fatty liver were weighted in a 2 ml eppendorf tube and added with 1.5 ml of Calpains Extraction Buffer (50 mM Tris Base, 20 mM EDTA, 10 mM EGTA, 0.1% β-mercaptoethanol, pH= 8.8) and a glass ball to enhance the cell wall lysis. The samples were ground for 20 seconds with FastPrep 24 Instrument (Biomedical MP) and centrifuged (1,200 g for 15 minutes at 4 °C). After centrifugation, three phases could be distinguished: a lipid phase at the top, a soluble phase containing the protein components and a precipitate representing the cellular debris. The lipid phase (fat-cake) was removed. The centrifugation and the removal of the fat-cake were repeated twice. Depending on the sample, it was possible to regain 800-1000 µl of the soluble phase. This quantity was used to make some aliquots and to compose a pool containing all samples in equal parts.

II. **Extracted proteins quantification**

The extracted proteins quantification was performed according to Bradford's method (Bradford, 1976), using the Bradford Reagent (Sigma-Aldrich). The Bradford Reagent contains the Brilliant Blue G dye which complexes with proteins in solution. The protein-dye complex causes a shift in the maximum absorption of the dye from 465 to 595 nm. The amount of absorption is proportional to the protein content, thus, using the BSA (Bovine Serum Albumin) as standard protein, it is possible to infer the sample total proteins concentration. The protein concentration as a function of the absorption is calculated with BSA solution (2 mg/ml, Thermo Scientific™ Pierce™) dilutions (a-H), and represents the standard calibration curve (Table 2). This curve is supposed to be linear in the range 0.1-1.4 mg/ ml of proteins.

Each sample was diluted to a 1:10 (10 µl sample + 90 µl Calpain Extraction Buffer) and 1:20 (5 µl sample + 95 µl Calpain Extraction Buffer) ratios. Replicates (5 µl) of each sample dilution, each standard point, a positive control (C) and the blank (H) were deposited on a 96-Well Polypropylene Microplates (Greiner Bio-One). 250 µl of Bradford Reagent were added to each well, and the plate was incubated in absence of light at room temperature for 15 minutes. The plate was transferred in the FLUOstar Omega microplate reader (BMG

LABTECH), which was setted to make a 30 seconds agitation followed by the optical density (OD) measurement at 595 nm. The data were analysed by the use of MARS Data Analysis software package provided with the microplate reader. The software calculates automatically the blank corrected OD values, the calibration curve as well as the protein concentration of each dilution. For each sample dilution, the values of the replicates were used to calculate the mean total protein concentration, its standard deviation (SD) and its coefficient of variation (CV). The dilution with a lower CV was chosen to calculate the total protein concentration of the respective sample.

Table 2. BSA standards for the calibration curve.

Standard	Calpain Extraction Buffer	BSA	$[BSA]_{final}$
A	25 µl	75 µl BSA solution	1500 µg/ml
a	15 µl	35 µl BSA solution	1400 µg/ml
B	32.5 µl	32.5 µl BSA solution	1000 µg/ml
C	35 µl	35 µl A	750 µg/ml
D	32.5 µl	32.5 µl B	500 µg/ml
E	32.5 µl	32.5 µl D	250 µg/ml
F	32.5 µl	32.5 µl E	125 µg/ml
G	40 µl	10 µl F	25 µg/ml
H	50 µl	0	0 µg/ml

III. **Calpains casein zymography**

Calpain casein zymography is a semi-quantitative technique based on the possibility to reveal calpain lytic activity toward its casein substrate on casein-containing polyacrylamide gels after mono-dimensional electrophoresis. The essay relies on the fact that the extent of the lytic activity is directly proportional to the sample calpain content, in a range of protein concentrations whose inferior limit is determined by the detection limit of the technique, and upper limit by the casein content.

The calpains casein zymography protocol (Awde *et al*., 2014a) was derived from the method of Raser *et al*. (1995).

The samples were diluted to obtain a final protein concentration of 60 µg/µl. The dilution was carried out with the Calpains Extraction Buffer and the Sample Buffer (150 mM Tris Base/HCl pH 6.8, 30% Glycerol, 0.2% Bromophenol Blue, 0.75% β-Mercaptoethanol) in a concentration of 1:4 with the final volume.

Four mini-gels were prepared for the migration. First the Separation Gel (10% Acrylamide 0.5% Bis-acrylamide, Table 3) was prepared and left polymerize between two glass plates (Mini-PROTEAN® Tetra Handcast Systems, Bio-Rad): a short plate and a spacer plate with 1.0 mm integrated spacers. A layer of ultra-pure water was temporarily added to accelerate the polymerization. Then, the Concentration Gel (3.84% Acrylamide 0.1% Bis-acrylamide, Table 4) was added and the 10-wells combs were placed before its polymerization.

Table 3. Calpains casein zymography Separation Gel composition for 4 mini- gels.

Separation Gel (Vf= 20 ml)		
2	ml	Casein 2%
3	ml	Ultra-pure water
5	ml	Acrylamide 40%
4	ml	Bis-acrylamide 2%
5	ml	Tris Base/HCl 1.5M pH 8.8
1	ml	Glycerol
100	µl	APS 10%
10	µl	TEMED

Table 4. Calpains casein zymography Concentration Gel composition for 4 mini- gels.

Separation Gel (Vf= 5 ml)		
3	ml	Ultra-pure water
480	µl	Acrylamide 40%
250	µl	Bis-acrylamide 2%
1.25	ml	Tris Base/HCl 0.5M pH 6.8
25	µl	APS 10%
5	µl	TEMED

Twenty-five µl of each sample and the pool were deposited into the wells just after the removal of the combs. To obtain a good sampling and a correct statistical analysis four replicates were done for each sample and eight replicates for the pool (two wells loaded per gel).

The migration was carried out by electrophoresis in a Mini-PROTEAN® Tetra Cell (Bio-Rad) at 100 V and 4 °C for 5 hours, in the Running Buffer (192 mM Glycine, 25 mM Tris Base, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, pH 8.3).

The gels were then rinsed twice for 30 minutes in 50 ml of Activation Buffer (20 mM Tris base, 7.6 mM $CaCl_2$, pH 7.4). An overnight incubation at 37 °C in the DTT added Activation Buffer (20 mM Tris base, 7.6 mM $CaCl_2$, 10 mM, pH 7.4) was then performed.

The next day the gels were stained with 10 ml of Calpain Staining Solution (0.5% Coomassie Blue, 45% Ethanol, 10% Acetic Acid) in agitation for 2 hours at room temperature. Gels were then washed three times for 30 minutes in distilled water. Digestion of casein due to calpain activity appeared as white bands on a blue background (Figure 6).

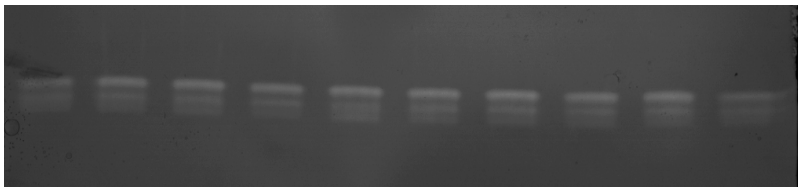


Figure 6. Zymogram detecting the calpain lytic activity toward the casein gel substrate. The band with higher molecular weight represents calpain 1, while the two low molecular weight bands correspond to the two calpain 2 isoforms (full-length and autolysed calpains 2).

IV. **Image analysis**

Gels were scanned with an Image Scanner III using ImageMaster Platinum software (GE Healthcare, Uppsala, Sweden). The resulting images were analysed by the use of ImageJ (Schneider *et al*., 2012). Shortly, every band was circled with the freehand selector tool (Figure 7), and for each selection the area and the pixel average intensity were automatically displayed. The band volume was the result of the area multiplied by the pixel average intensity. For each gel image, the area, the pixel average intensity and the volume values of the samples were divided by the correspondent mean values of the two pool bands in order to standardize them. For the same sample, eight standardized values for each one of the three considered parameters were obtained, as a result of the image analysis of the four gels. Among them, four values were produced for calpain 1 band and four for calpain 2 band. Calpain 2 band consists of two bands: the high molecular weight one corresponding to the full-length isoform, and the low molecular weight one to the autolysed isoform. These values were used to calculate the average area, the average pixel

intensity and the average volume for calpain 1 and calpain 2 of each sample.

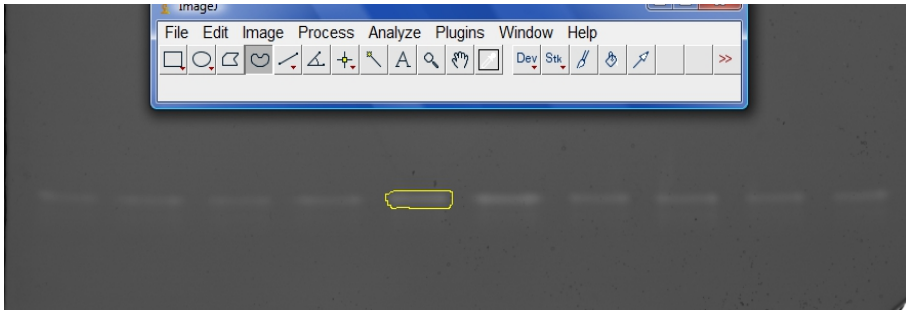


Figure 7. Image analysis by the use of the free hand selector tool of ImageJ.

V. **Statistical analysis**

A one tailed student test (T-test) for two groups of samples with unequal variance (performed with Excel software) was run with the values of average area, average pixel intensity and average volume for the two groups of samples (CL^+ and CL^-), both for calpain 1 and calpain 2. The analysis was aimed at establishing whether the differences in the proteolytic activity between the two groups were statistically significant ($p< 0.05$). The same was done with the liver weight, dry matter and lipids contents, technological yield and cooking losses values of the two groups of samples.

3.2. STUDY 2: Better characterization and comprehension of the calpains casein zymography protocol

Fatty livers samples used for this series of research were issued from commercial flocks of male Mule ducks (*C. moschata* × *A. platyrhynchos*) reared until the age of 14 weeks according to standard practices. The birds were then force-fed by the distribution of a soak corn mixture (25% grain, 35% flour, and 40% water, 3330 kcal/kg, 8.28% crude proteins and 3.38% lipids) twice daily for 12 days. At the end of the force-feeding period, the slaughtering procedure was conventionally conducted in a commercial plant.

3.2.1. Calpains stability in the Extraction Buffer

Calpains stability was tested in the course of time and with different Ca^{2+} concentrations. Proteins were extracted from three fatty liver powder samples (samples no. 1, 10 and 4) and quantified as described before (I. Protein extraction, II. Extracted protein quantification). A pool (pool 1) was made up of equal aliquots of the three samples. For each sample and after defrosting, three aliquots were incubated at room temperature for 20 minutes, three aliquots for 2 hours and three aliquots overnight. Furthermore, for each incubation time each one of the three aliquots was added with a $CaCl_2$ solution to obtain different final concentrations (0 µM, 5 µM and 5 mM) (Figure 8).

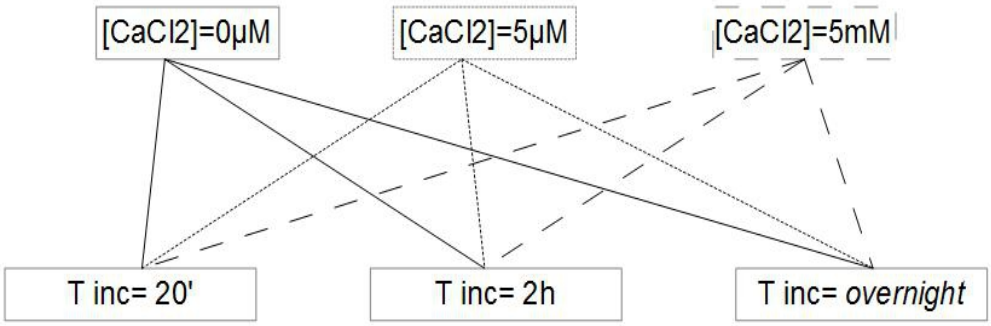


Figure 8. Graphic representation of the experimental design for the incubations.

All the nine samples were processed with calpains casein zymography as described in section III. Three replicates were done for each sample and the pool. The electrophoretic run was carried out at 125 V and 4 °C for 2 hours, as done by Awde *et al*. (2014a). Finally, a visual and qualitative analysis of calpains activities was conducted as described before.

In a second time, calpains stability in the Extraction Buffer was investigated just in the course of time and for more time points. Six fatty liver powder samples (samples no. 4, 8, 9, 10, 19, 20) were subjected to protein extraction and quantification. A pool (pool 2) was constituted with equal volumes of the obtained extracts. Two extracts were incubated at 0, 20, 40, 60, 80, 100 and 120 minutes, before being analysed by calpains casein zymography respecting the same conditions described in section III. The image analysis was conducted as described in section IV. A further investigation was led for 8 time points between 2 and 17 hours after defrosting of one of the two samples (2, 3, 4, 5, 6, 7, 8 and 17 hours). Data were used to produce dispersion graphs, where the points were interpolated with a linear curve. The coefficient of determination (R^2) was calculated to understand how well the data fitted the model.

3.2.2. Calpain lytic activity at different calcium concentrations

The calpain lytic activity was investigated at the following concentrations of $CaCl_2$: 0, 5 µM, 200 µM, 800 µM, 1 mM and 5 mM. Proteins were extracted from one fatty liver sample and quantified as described in section I and II. Pool 1 mentioned in the previous section was used to monitor the correct course of the casein calpain zymography. Casein calpain zymography was then performed as in section III. The pool was deposited in the 4th, 5th, 9th and 10th well of each of the three mini-gels, while the other wells were loaded with the sample. Just after the electrophoretic run, the mini-gels were cut vertically between the 5th and the 6th well, so that six gels containing three replicates of the sample and two replicates of the pool were obtained. Each half gel was rinsed twice and incubated with previously prepared Activation Buffer solutions at the selected concentrations of $CaCl_2$. The visual and qualitative analysis was then performed.

3.3. STUDY 3: Total Ca^{2+}-activated proteases activity in the Extraction Buffer at saturated EDTA/EGTA system

The present study aimed at investigating the total proteolytic activity of the Ca^{2+}-activated proteases toward calpains 1 and 2 in the Extraction Buffer, by using a modified version of the protocol for the casein calpain zymography.

As a first step, eight aliquots of each of the two selected samples (samples no. 4 and 9, section 3.2.1.) were defrosted at eight different time points before loading on the mini-gels (0, 30, 60, 90, 120, 150, 180, 210 minutes). $CaCl_2$ was added to a final concentration of forty mM in order to saturate the EDTA/EGTA system (30 mM) and to activate the Ca^{2+}-dependent proteases (10 mM). After the incubation, the casein calpains zymography was performed as described in section III. Four replicates were done for each of the investigated time points for sample no. 4, while two were done for those of sample 9. Pool 2 was used to standardize the results and monitor the correct course of the procedure. Image analysis was then performed as described in section IV. Data were used to produce dispersion graphs where points were interpolated with a linear curve. The coefficient of determination (R^2) was calculated to understand how well the data fitted the model.

Afterwards, eight aliquots of sample no. 9 were treated in the same way, with the difference that the $CaCl_2$ was added to a final concentration of thirty-five mM (30 to saturate the EDTA/EGTA system, and 5 mM to activate the Ca^{2+}-dependent proteases) instead of forty before incubation. That was done to understand if any change in the Ca^{2+} concentration could affect the level of activation of Ca^{2+}-dependent proteases and thus the rate of calpains degradation.

3.4. STUDY 4: Calpain 2 Western Blot repeatability and WB proteolytic profiles after differential incubations

Western blot. The Western blot is a semi-quantitative technique aimed at detecting a target protein through the use of a specific antibody. In a first step, proteins are separated by SDS-PAGE electrophoresis on a polyacrylamide gel, then they are transferred into a nitrocellulose paper (Transfer), where the protein of interest is recognized by its antibody (Immunoblot).

SDS-PAGE. The SDS-PAGE (Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis) allows the separation of proteins on the base of their dimensions. In non denaturing conditions, proteins electrophoretic mobility is described by the formula

$$a = (q/m) \times E$$

and thus it is directly proportional to the protein total charge (q) and the electric field (E), and inversely proportional to the protein mass (m). Proteins are run in a polyacrylamide gel, which works at the same time as a semi- solid support and a molecular sieve. Therefore proteins electrophoretic mobility depends on their dimensions, too. In SDS-PAGE the ratio q/ m is constant thanks to the addition of the SDS, an amphiphilic and negatively charged molecule. In fact, the SDS bounds to proteins with a constant stoichiometry (1 SDS molecule the 3- 4 amino acids), giving them a negative charge which is proportional to their length. The result is that proteins electrophoretic mobility depends just on the gel porosity and proteins dimensions.

A better characterization of the calpain 2 Western Blot (calpain 2 WB) was performed following the protocol developed by Mrs Nathalie Marty-Gasset (engineer of the GenPhySE lab).

Samples no. 1, 4 and 10 (see section 3.2.1) were used. Three aliquots of sample no. 1 were defrosted and incubated at room temperature for 20 minutes, 2 hours and 17 hours respectively. Furthermore, for all the three samples two aliquots were defrosted just before the electrophoretic run. All the samples were mixed with a 1:1 v/v of Laemmli Buffer (4% w/ v SDS, 1% β-mercaptoethanol, 40.32% Tris-HCl 0.5 M pH 6.8, 10% Glycerol, 0.2% Bromophenol Blue) referring to the final total volume. They were then diluted with the Calpain Extraction Buffer in order to have 100 µg of proteins in 20 µl, which represents the loading volume.

Three mini-gels were prepared as described for the casein calpains zymography (section

III). The composition of the separation gel (10% Acrylamide 0.1% Bis-acrylamide) and concentration gels are shown in table 5 and 6.

Table 5. Anti calpain 2 WB Separation Gel composition for 3 mini-gels.

Separation Gel (Vf= 14 ml)		
5.5	ml	Ultra- pure water
3.5	ml	Acrylamide 40%
705	µl	Bis- acrylamide 2%
3.45	ml	Tris Base/ HCl 1.5M pH 8.8
705	µl	Glycerol
141	µl	SDS
70.5	µl	APS 10%
7.05	µl	TEMED

Table 6. Anti calpain 2 WB Concentration Gel composition for 3 mini-gels.

Separation Gel (Vf= 5 ml)		
3	ml	Ultra- pure water
480	µl	Acrylamide 40%
250	µl	Bis- acrylamide 2%
1.25	ml	Tris Base/ HCl 0.5M pH 6.8
25	µl	APS 10%
5	µl	TEMED

Samples were heated at 95 °C for 5 minutes, to allow the complete denaturation of proteins and bounding to the SDS. The mini-gels were soon after loaded twice with 20 µl of each sample and 5 µl of a molecular weight markers mix (Bio-Rad). The electrophoretic run was carried out in a Mini-PROTEAN® Tetra Cell (Bio-Rad) at 52 mA in the Running Buffer (7.2% w/v Glycine, 1.51% w/v Trizma-Base, 0.5% w/v SDS). The run was considered to be achieved once the front dye migrated out of the gel. The gels were then rinsed in 10 ml of Transfer Buffer (25 mM Tris-base, 192 mM Glycine, 20% v/v Methanol, pH 8.3).

Transfer. The transfer was performed in a Criterion Blotter (Bio-Rad) according to the instructions given in the Criterion Blotter Instruction Manual (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4006190b.pdf).

The Criterion Gel/Blot Assembly Tray (Bio-Rad) was filled with some Transfer Buffer (25 mM Tris-base, 192 mM Glycine, 20% v/v Methanol). A gel holder cassette was opened and

the cathode (black) side submerged in the Transfer Buffer. A gel/membrane sandwich (Figure 9) was then prepared as following. A foam pad was soaked of Transfer Buffer and positioned on the submerged side of the cassette. A piece of filter paper previously cut to the required dimensions was wetted in the Transfer Buffer and placed on the top of the foam pad: trapped air was then removed by the use of a blot roller. The rinsed gel was then placed on the top of the filter paper, and the trapped air gently removed. The gel was then covered with a second piece of wet filter paper that was rolled if needed. Finally, a soaked foam pad was placed on the top to complete the sandwich and the cassette was locked. After this operation was repeated for each one of the three mini-gels, the blotting machinery could be assembled. The transfer tank was placed on a magnetic stir plate and half filled with the Transfer Buffer. A stir bar was added. The gel holder cassettes were inserted into the blotting module latch side up, with the black side of the cassette facing the black side of the blotting module. Each blotting module could contain 2 gel holder cassettes. An ice block was positioned in the tank to limit possible protein damages due to the heat generated during the electrophoretic transfer which entails large power loads. Buffer Transfer was added until its level reached the fill line, and the stirring was begun to help maintaining uniform the conductivity and the temperature during the transfer. Finally, the lid was placed on the top of the cell and its cables were connected to the power supply making sure to match the colours of the cable to those on the power supply inputs. The transfer was run for 30 minutes at 100V, then the cassettes were removed and the gel/membrane sandwich disassembled. The gels were stained with the Western Blot Staining Solution (0.5% Coomassie Blue, 45% Ethanol, 5% Acetic Acid), to prove the correct proteins transfer.

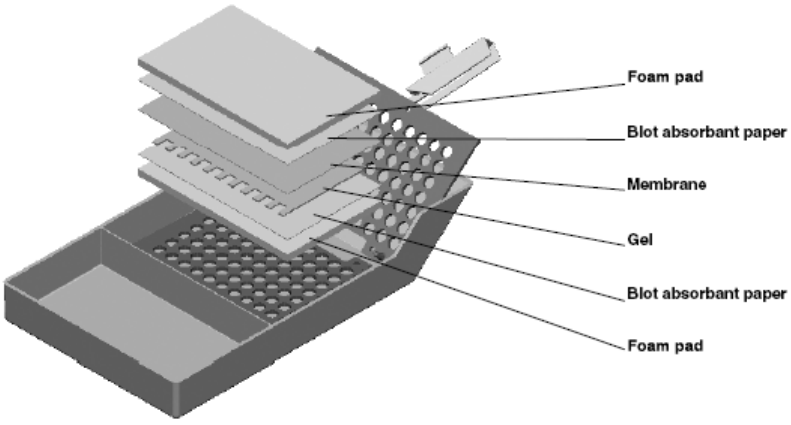


Figure 9. Gel/membrane sandwich for the Western Blot Transfer.

Immunoblot. This phase relies on the ability of the anti calpain 2 rabbit polyclonal primary antibody (GTX102499, GeneTex Inc) to specifically recognize a region within amino acids 162 and 383 of the large subunit of the calpain 2. The bound primary antibody is detectable thanks to a goat anti rabbit IgG secondary antibody (sc-2004, Santa Cruz) HRP conjugated (Horseradish Peroxidase). The HRP enzyme promotes the oxidation of its substrates in the presence of an oxidant agent as the peroxide. In particular, the oxidation of the luminol produces light. Thus, the luminol is one of the most common used substrates in the chemiluminescent detection, and, together with the specificity of the primary and secondary antibodies, allows the revelation of the target protein.

Practically, the membranes were rinsed with 20 ml of TBS (T5912-IL, SIGMA) for 5 minutes in agitation. The TBS was thrown away and 10 ml of milk/TBS-T (1:2000 TBS:Tween) were added. The membranes were incubated for 1 hour at room temperature in agitation in order to block the non specific primary antibody bounding sites. The anti m-calpain rabbit polyclonal primary antibody was diluted 1:2000 in the milk/TBS-T, which was used to replace the milk/TBS-T of the previous step (10ml/ membrane). The membranes were incubated overnight at 4 °C in agitation. The next day they were rinsed three times with 20 ml of TBS-T solution for 10 minutes in agitation to make sure that all the primary antibody molecules that had not bound the calpain 2 were eliminated and thus avoiding non specific signals at the detection. The goat anti rabbit IgG- HRP conjugated was diluted 1:5000 in the milk/TBS-T and 10 ml of this solution were used to replace the TBS-T. An incubation step of 1 hour followed. The membranes were rinsed three times with 20 ml of TBS-T for 10 minutes and with 20 ml of TBS for 5 minutes in agitation. They were then incubated with a 1:1 Luminol/Enhancer:Stable Peroxide Buffer (SuperSignal West Pico Chemiluminescent Substrate, Thermo PIERCE) for 5 minutes in agitation and in absence of light (4ml/membrane). The membranes were then drained and placed between two transparent and cleaned plastic papers. In a dark room they were first exposed to an X-ray film in an auto-radiographic cassette for 30 seconds, 5 minutes and 10 minutes. The X-ray films were incubated for 1 minute respectively in Revealing Buffer, distilled water and Fixation Buffer. They were then washed with distilled water and dried. Finally the membranes were stained with the Red Ponceau Staining (6.6 mM Red Ponceau, 1% Glacial Acetic Acid), in order to prove the correct transfer and to identify the band corresponding to the target protein.

Analysis. A visual analysis was conducted by superimposing the X-ray films and the correspondent Red Ponceau stained dried membranes showing the molecular weight markers lane, in order to approximately derive the molecular weight of the bands obtained by radiography. Resulting X-ray films were also submitted to image analysis as described in section IV. Area, pixel intensity and volume values for each sample within the same X-ray film were used to calculate the average area, average pixel intensity and average volume. Standard Deviation (SD) and the Coefficient of Variation (CV) values were established, too. The same was done with each sample regardless of its position on the X-ray films.

4. RESULTS AND DISCUSSION

4.1. STUDY 1: Relationship between proteolytic activities and cooking losses values in livers issued from force-fed ducks

This study aimed at investigating any possible differences in the calpain lytic activity between two groups of fatty liver samples differing in cooking losses values.

It is of common knowledge that the technological yield (or cooking losses) of duck fatty livers is inversely proportional to the liver weight, but it is also affected by other biological and technological factors, mainly the genetic type of ducks, the length of the force-feeding period, the evisceration and the way of cooking. Moreover, Awde *et al*. (2014a) demonstrated that the technological yield is also affected by dry matter and lipids contents, and by proteolytic activities of the liver. Among proteases, only cathepsins, MMPs and calpains were considered, because of their well-known role in the *post-mortem* tissue degradation. However, a principal component analysis (PCA) performed with those measured variables showed that dry matter and lipids contents have a stronger influence on the technological yield value than proteases activities *per se*.

Consequently, the present study was planned to understand whether and at which extent the proteolytic activity could be correlated to the technological yield in duck fatty livers presenting equal weights and dry matter and lipids contents. Selection of fatty livers on the basis of this assumption was done to remove the influence of the biochemical parameters and thus to focus on the role of proteases in determining cooking losses values. All cathepsins MMPs and calpains activities were supposed to be investigated to perform a new PCA with the resulting data. However because of time limits, I only had the opportunity to measure calpains activities.

Registered average values for the liver weight, the dry matter content, the lipids content and the cooking losses are shown in Table 7 for the CL+ and the CL- groups of fatty livers.

Table 7. Biochemical characteristics and cooking losses (CL) of fatty livers from the two studied groups CL+ (High Cooking Losses) and CL- (Low Cooking Losses).

Group	Weight (g)	p	Dry Matter (%)	p	Lipids Content (% DM)	p	CL (%)	p
CL+	576.25±18.66	0.39	69.12±2.13	0.24	57.60±2.66	0.47	12.94±2.81	0.00
CL-	578.75±15.06		68.41±1.72		57.68±1.30		7.19±1.08	

Table 8. Values for average area, average pixel intensity and average volume resulting from the image analysis of the zymograms for the low cooking losses (CL-) and for the high cooking losses (CL+) fatty liver samples, respectively for calpain 1 and calpain 2. A one tailed student test for two groups of samples with unequal variance was performed with the average sample values of each group, for each one of the three parameters.

		CL-	CL+	p-value
calpain 1	Area	0.987 ± 0.086	1.056 ± 0.085	0.063
	Pixel Intensity	1.059 ± 0.019	1.059 ± 0.017	0.480
	Volume	1.045 ± 0.091	1.118 ± 0.101	0.075
calpain 2	Area	1.177 ± 0.088	1.143 ± 0.119	0.264
	Pixel Intensity	1.041 ± 0.006	1.042 ± 0.016	0.432
	Volume	1.228 ± 0.095	1.193 ± 0.129	0.274

Data obtained from the proteolytic analysis are shown in Table 8. The p-values for the three considered parameters were >0.05, thus the differences between the CL- and the CL+ group were not significant. Therefore, calpains lytic activity alone seems to have no influence on the technological yield in fatty liver samples presenting equal weights, and dry matter and lipids contents.

In their experiment, Awde *et al*. (2014a) reported that, with the same liver weight, lipids and dry matter contents have a higher determinism on cooking losses than proteases activities, which are only of secondary roles. In the present study, we confirm that when lipids and dry matter contents are similar, as well as liver weight, calpains activities did not differ and therefore could not explain observed cooking losses values. If this observation is confirmed by next proteases activities measurements (MMPs and cathepsins), we might admit that proteases definitely have a low impact on cooking losses values. Consequently, observed alterations of cooking losses values might not be due to accelerate tissue degradation associated with higher proteases activities. However, it has also to take into account that those observations have been conducted on samples harvested soon after evisceration (20 min *post-mortem*), that is an insufficient time for proteases to really alter tissue structures. Therefore it could be interesting to evaluate proteolytic activities consequences in samples kept longer before cooking. In this conditions we could expect to see the same effects as those observed during *post-mortem* meat tenderization, with an extensive degradation of cellular components and an associated decrease in cooking yields.

4.2. STUDY 2: Calpains stability in the Extraction Buffer

Investigation of calpain stability in the extraction buffer was conducted to better characterize the calpain zymography protocol.

Calpain extracts have always been manipulated with a special care, supposing that calpains are highly unstable at room temperature. As a consequence, extracts were kept in ice all the time of their preparation for the electrophoretic run long, that is during the phases of the dilution and gels loading. This assumption has been posing questions, such as whether or not the calpain activity could partly be compromised between the loading and the beginning of the electrophoresis. In fact, this period of time can nearly double if the first and the last loaded samples are compared. Therefore, the aim of this part of the study was to ascertain that any difference in the calpain activity between the first and the last loaded samples was not related to an increased instability in the extraction buffer. Instability was supposed to be due to a temperature-related proteolytic activation, which could consist either in a calpain autolytic activity or in other proteases activation.

The second investigated point was the capacity of the Extraction Buffer to chelate Ca^{2+} ions at different concentrations. Typical Ca^{2+} ions concentrations of the mammalian cells are 10^{-4} mM in the intracellular space and 1-2 mM in the extracellular space (Alberts *et al*., 2002, Table 11-1). Ca^{2+} intracellular levels favour calpain 1 activation more than calpain 2 activation, which however depends also on the calpastatin levels. When proteins are extracted from the ground fatty liver samples, Ca^{2+} present in the Extraction Buffer derives both from the intracellular and the extracellular space. This so called endogenous Ca^{2+} varies from sample to sample and could pose a problem for calpain stability after extraction, if not completely buffered. In fact, calpains are Ca^{2+}-activated proteases endowed with autolytic activity *in vitro*. For this reason the Extraction Buffer is normally added with 20 mM of EDTA and 10 mM of EGTA, which are ideally able to chelate the Ca^{2+} ions in a bigger concentration than the one supposed for the endogenous Ca^{2+}. The EDTA-EGTA affinity depends on the buffer pH value with a sigmoid shaped proportional relation (McGuigan *et al*., 2006). In our case, the EDTA-EGTA Ca^{2+}-affinity in the Extraction Buffer (pH= 8.8) is high but not optimal. For these reasons, the effect of three different Ca^{2+} concentrations (0 µM, 5 µM, 5 mM) in the Extraction Buffer was tested, in order to mime possible variations of the endogenous Ca^{2+} levels. The aim was to make sure that the Extraction Buffer could well accomplish its function in a wide range of Ca^{2+} concentrations.

The zymograms resulting from the first study were submitted to a visual and qualitative analysis as no change in the calpain lytic activity could be perceived by eye between the differentially treated samples (Figure 10). As a conclusion, we could affirm that variations of the Ca^{2+} concentration are well withstood by the Extraction Buffer, even when they were important (in the order of the mM). That gave us the certainty that variations in the sample endogenous Ca^{2+} concentration are always prevented from activating Ca^{2+}-dependent proteases, including calpains themselves. Consequently, any possible differences revealed between samples through zymography is not related to the calpains degradation extent. On the contrary, as expected, they can be assigned to a differential activation state of the calpain system in the cell.

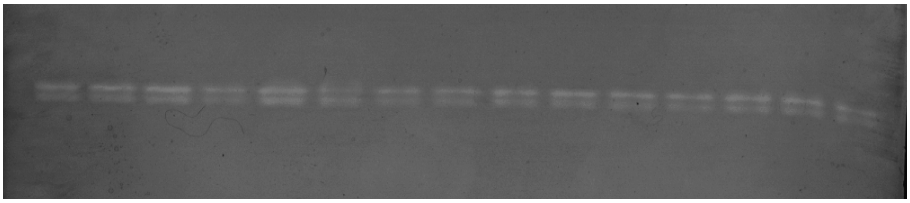


Figure 10. Zymogram of the sample no. 1. From the left to the right, bands correspond to: the pool, sample no 1 incubated for 20 min with 0, 5 µM and 5 mM $CaCl_2$, sample no. 1 incubated for 2 hours with 0, 5 µM and 5 mM $CaCl_2$, and sample no. 1 incubated for 17 hours with 0, 5 µM and 5 mM $CaCl_2$.

No difference between samples incubated for 20 minutes, 2 hours and 17 hours could be either observed (Fig. X.1). That could be related to the fact that I made this trial at the beginning of my training period, following a protocol with an electrophoretic run of two hours instead of five. As a consequence, the two bands representing the full-length and the autolysed isoforms of calpain 2 were both present in a unique band. For the fact no conclusion could be drawn for this two isoforms, the trial was repeated a second time with a longer electrophoretic run, more time points and no variations in the Ca^{2+} content of the Extraction Buffer.

The zymograms obtained for the series of incubations between 0 and 120 min were submitted to a visual analysis, because no evident change could be revealed at the different time points (data not shown). This consideration was later confirmed by the fact that a slight decrease in calpain activity could be detected only after some hours of

incubation.

Zymograms of the second series of incubations (2, 3, 4, 5, 6, 7, 8 and 17 hours) were firstly analysed by eye and then through image analysis. At a visual analysis a decrease in the intensity and a slight increase in the area of the bands was evident for the full-length isoform of calpain 2, along the course of time and in particular between 8 and 17 hours (Figure 11). The linear interpolation of the data produced by image analysis confirmed the observation and highlighted the same trend also for calpain 1 (Figures 12 and 13). Bands corresponding to the full-length and the autolysed form of calpain 2 could not be analysed separately because not all of the gels had the necessary resolution. For both calpains 1 and 2 the area shows a positive variation in an interval of 0.1, while a negative variation is remarkable for the pixel intensity in an interval of 0.02. The volume (Area X Pixel Intensity) follows the trend of the area. However, even if exactly the same trend is maintained for both calpain isoforms, the values of the coefficient of determination (R^2) and of the standard deviation (SD) prevent from making any statistically significant consideration. In fact, values of $R^2 < 0.9$ are representative of the fact the data don't fit well the model, and standard deviations are often bigger than the differences between differential incubations values. This could be assigned to the bias introduced all along the procedure, as it consists of a considerable number of manipulations with a high number of intervention conducted by the operator. To conclude, it would be interesting to prove that the full-length isoform of calpain 2 shows a statistically significant decrease of the band intensity and that this decrease reflects a reduction in calpain quantity due to proteolysis.

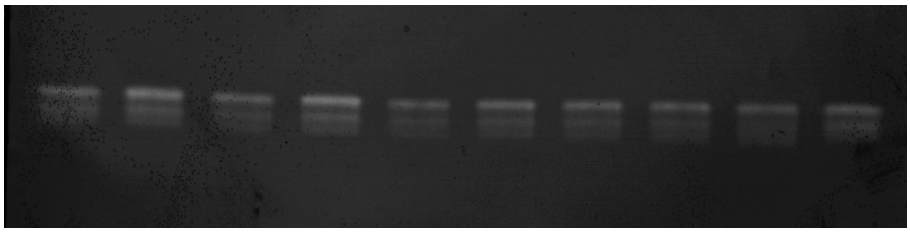
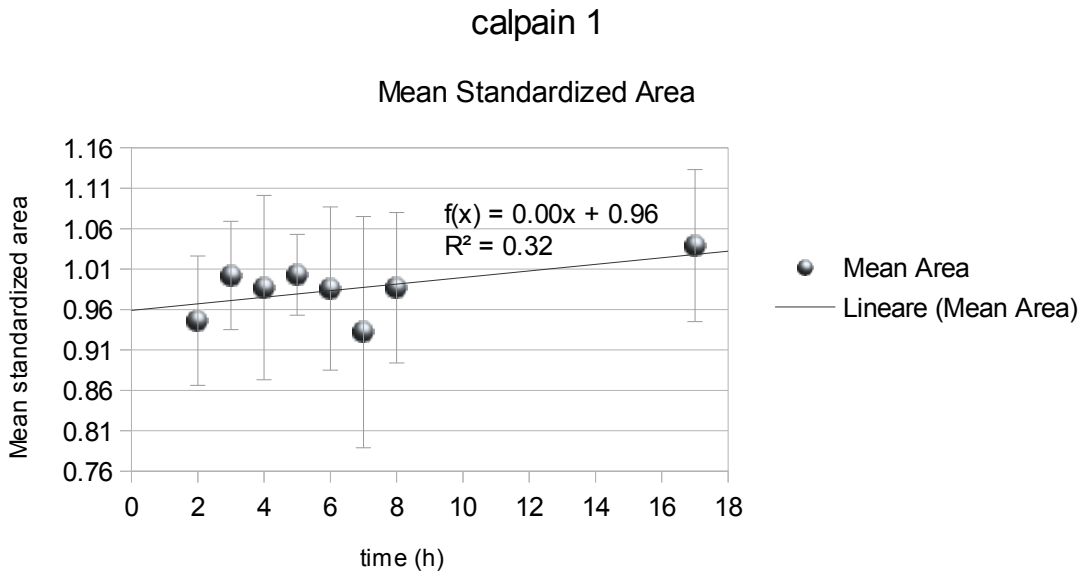


Figure 11. Zymogram of sample no. 4. Pool was loaded in the first and last well of the mini-gel. For the other wells, from the left to the right, bands correspond to increasing time of incubations (2, 3, 4, 5, 6, 7, 8 and 17 hours).

Some general considerations can be made for the whole experimental plan. First, the

Extraction buffer has been proved to well comply its function. Secondly and the most interesting, no proteolytic activity can be revealed unless on the long term, which means that mainly Ca^{2+}-dependent proteases are supposed to be found after the extraction. Ca^{2+}-independent proteases are either inactive in the Extraction Buffer or not well extracted. Reasons for being in the inactive state could be represented by unfavourable conditions, such as pH or the presence of inhibitors at a cellular level or in the Extraction Buffer.

a)



b)

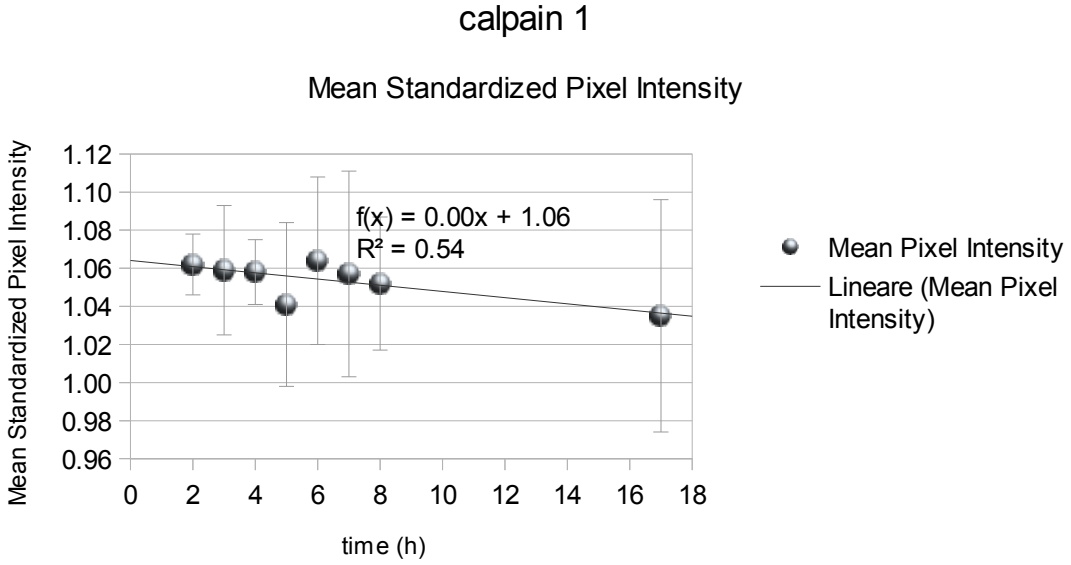
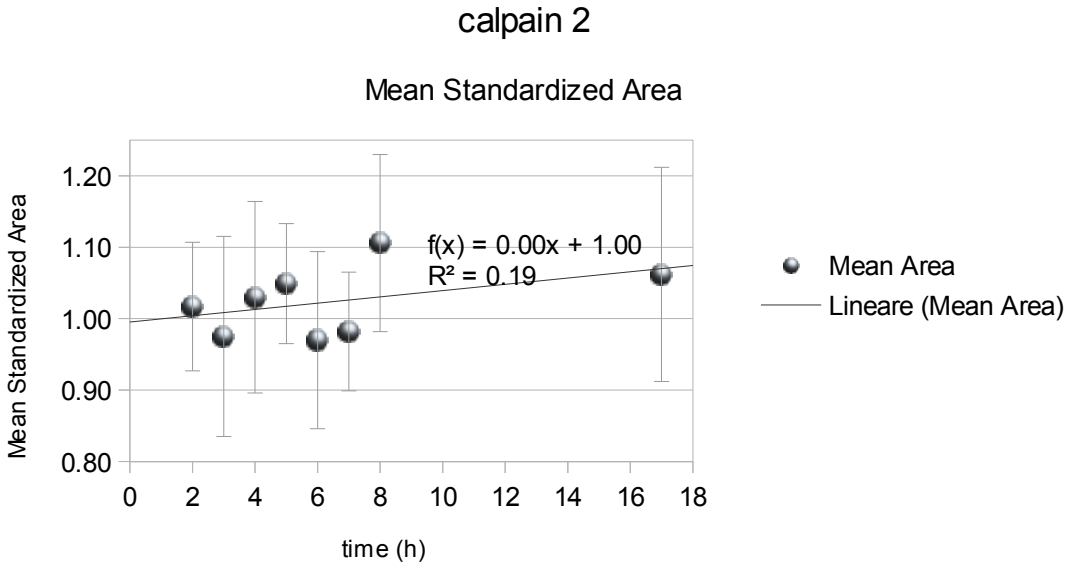


Figure 12. Mean standardized area (a) and pixel intensity (b) for sample no. 4 calpain 1. Bars represent the standard deviation (SD).

a)



b)

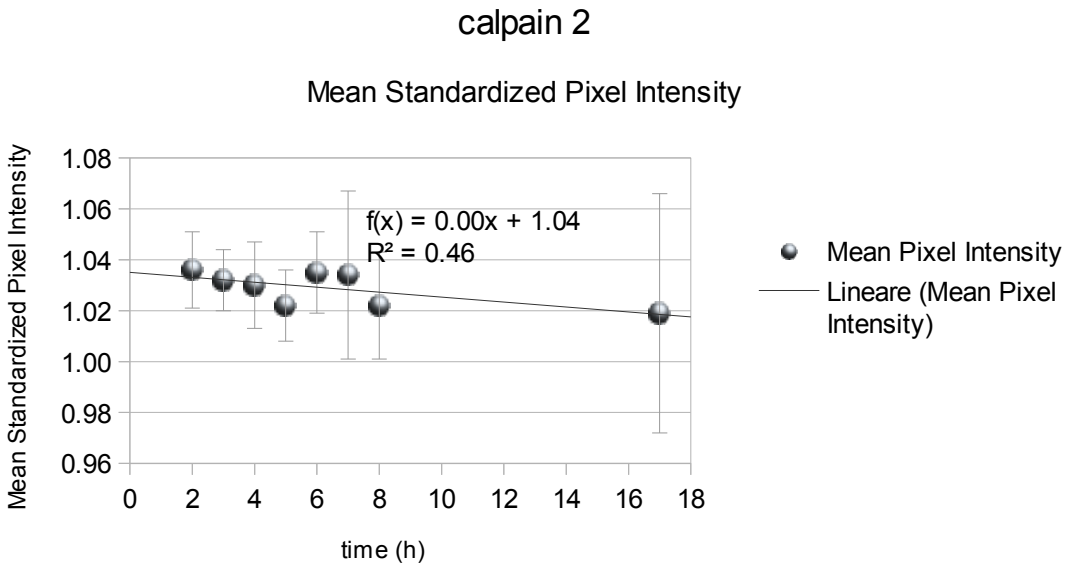


Figure 13. Mean standardized area (a) and pixel intensity (b) for sample no. 4 calpain 2. Bars represent the standard deviation (SD).

4.3. STUDY 2: Calpain lytic activity at different Ca^{2+} concentrations

In a recent study Kanzaki *et al*. (2014) discussed the possibility to identify by zymography two rat calpain 2 isoforms with slightly different molecular weight (≈80 KDa): a full-length isoform and an autolysed isoform with a lower molecular weight. This result was achieved by performing the electrophoretic run for 4 hours at a constant voltage (170 V).

Until the moment we read the publication, the zymography protocol has been performed according to Awde *et al*. (2014a), using a 2 hours electrophoretic run at a constant voltage (125 V). As a result, two bands were obtained in the zymogram: a high molecular weight band which was supposed to correspond to calpain 1 and a low molecular weight one hypothesized to represent calpain 2. However, with this protocol another higher molecular band occasionally appeared (Figure 14). It is common knowledge that calpain 1 is the first to be activated in *post-mortem* beef muscle (Boehm *et al.*, 1998; Cruzen *et al*., 2014). Furthermore, calpains are endowed with autolytic activity but the rate of this phenomenon is unknown in duck fatty liver. For these reasons we could not exclude that calpain 1 autolysis rate could be fast enough to make its content in the sample lower than the detection limit of the calpain casein zymography technique. That posed the question whether the occasionally appearing band was an artefact of the technique or the product of calpain 1 lytic activity in high content of active calpain 1 samples (CL-).

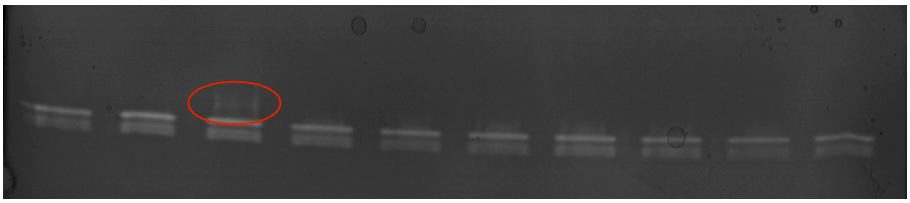


Figure 14. Zymogram obtained with the protocol described by *Awde et al*. (2014). A high molecular band occasionally appearing is circled in red.

In order to investigate all that, two strategies were adopted:

- a longer electrophoresis was run (5 h, 100 V, 4 °C);
- at the same time, Activation Buffers at different Ca^{2+} concentrations were used to activate the calpains during the zymographic procedure.

Concentrations of Ca^{2+} of the order of the micromolar activate calpain 1, while calpain 2 requires Ca^{2+} concentration of the order of the millimolar. Therefore in a millimolar Ca^{2+} Activation Buffer both calpains 1 and 2 are active. With no Ca^{2+} added, no or a very low lytic activity is expected to be revealed. A feeble lytic activity could be due to the binding

of Ca^{2+} ions in the cell before extraction, in the case the strength of the bond was sufficient to prevent the separation of the Ca^{2+} ions during the electrophoretic run.

As shown in Figure 15, at a 0 and 5 µM $CaCl_2$ concentration, no lytic activity could be revealed, probably because it was under the detection limit of the technique. At 200 µM the band corresponding to the higher molecular weight calpain could be observed, highlighting how optimal lysis bands are produced at higher micromolar concentrations through zymography for this duck calpain isoform. Finally, in the range of concentrations from 800 µM to 5 mM the intensity and extension of the higher molecular weight calpain band remained constant, while the ones corresponding to the two lower molecular weight calpain bands increased. In no case the upper occasional appearing band appeared. Therefore, this study confirmed that the isoform with the higher molecular weight corresponded to calpain 1, while the two lower molecular weight matched with calpain 2. Furthermore, between the two calpain 2 isoforms, the one with a higher molecular weight corresponded to the full-length isoform whereas the low molecular weight one represented the autolysed form. The occasionally appearing upper band was probably an artefact related to the technique.

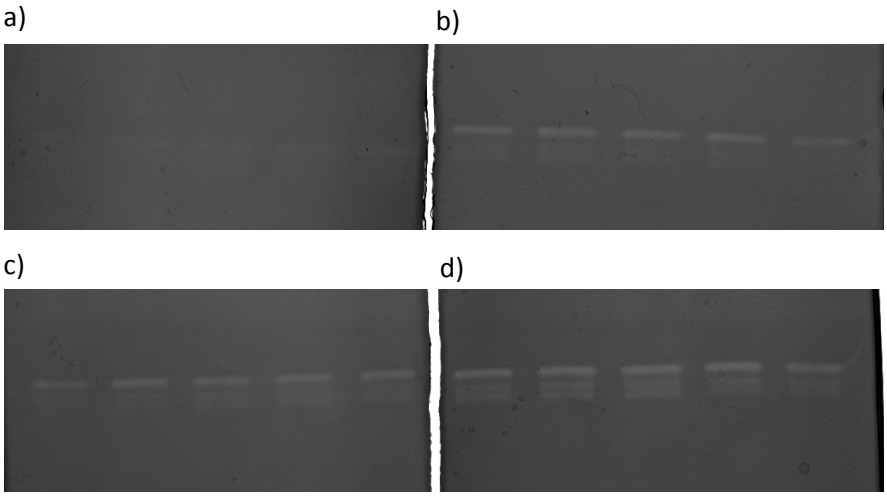


Figure 15. Zymograms obtained from incubation at different $CaCl_2$ concentration. [$CaCl_2$]= 200 µM (a); [$CaCl_2$]= 800 µM (b); [$CaCl_2$]= 1mM (c); [$CaCl_2$]= 5mM (d). Pool was loaded in the first and last well of each mini-gels.

4.4. STUDY 3: Total Ca^{2+}-activated proteases activity in the Extraction Buffer at saturated EDTA/EGTA system

The *in vivo* role of calpain autolysis, considered as an inter-molecular event (Chou *et al*., 2011), has for long been discussed (Goll *et al*., 2003). Although recent studies (Hanna *et al.*, 2008; Chou *et al*., 2011) support the hypothesis that autolysis is favoured by *in vitro* conditions and thus have no physiological meaning, the question isn't considered solved by the scientific community yet. This is proved by the amount of studies that still investigates the dynamics of calpain autolysis (Kanzaki *et al*., 2013) in order to gain insight in the characterization of the calpain system in the cell.

The present study was aimed at investigating the calpain autolysis rate during the course of time, in samples incubated in the Extraction Buffer at saturated EDTA/EGTA system. Calpain lysis was expected to be viewed as a decrease in active calpain quantity and consequently in the intensity of bands deriving from casein degradation in the zymographic gels. At saturated EDTA/EGTA system, it was reasonable to suppose that this decrease was due to the sum of the activity of all the Ca^{2+}-activated proteases present in the sample after extraction. If this assumption was true, a possible decrease of active calpain quantity would have been due to proteolysis. Moreover, considering the amino acid sequence similarity between the two calpain isoforms, the proteolysis rate was expected to be the same for both calpain 1 and 2. However, the fact that calpain 1 and 2 showed different lysis rates supported the hypothesis that mainly autolysis and not proteolysis was being observed. Possible explanations for the limited extent of other Ca^{2+}-activated proteases activity on calpains could be related to their low extractability, as calpains need reducing but non denaturing conditions, which result to be insufficient for the extraction of other proteases, as was demonstrated for cathepsins and MMPs (Kizaki *et al*., 2008). On the other side, Ca^{2+}-activated proteases with a good extractability could not have been finding optimum conditions for their activity in the used Extraction Buffer.

Ideally, at saturated EDTA/EGTA system, Ca^{2+} concentration in the Extraction Buffer corresponds to the endogenous Ca^{2+} level typical of the sample. Typical endogenous Ca^{2+} ions concentrations are inadequate for calpains to perform the lysis of the substrate in the casein calpain zymography (Alberts *et al*., 2002, Table 11-1). Therefore, in order to investigate calpain autolysis rate, Ca^{2+} ions were supplied through $CaCl_2$ addition, for a final concentration of 10 or 5 mM. First, a final $CaCl_2$ concentration of 10 mM was chosen

on the basis of the scientific literature (Kanzaki *et al*., 2011). In this condition, calpain 1 was activated so fast that the correspondent band on the zymogram was barely visible between 0 and 120 minutes, however showing a slightly higher intensity at 0, 30 and 60 minutes, while at the following investigating time points its activity went under the detection limit of the technique (Figure 16). Differences between our results and those obtained by Kanzaki *et al.* (2011), could be explained either with a different Ca^{2+}-dependent activation dynamic between rat and duck, or a resulting final Ca^{2+} concentration higher than 10 mM due to the fact that at the Extraction Buffer pH (pH= 7.4) EDTA/EGTA system does not chelate Ca^{2+} ions in a 1:1 molar ratio (McGuigan *et al*., 2006). In order to investigate calpain 1 autolysis rate, too, a second trial was performed with the same time points but using a final $CaCl_2$ concentration of 5 mM.

At a 10 mM final $CaCl_2$ concentration, calpain 2 full-length isoform activity decreased along the course of time (Figure 18), as confirmed also by the image analysis data (Figure 17). For calpain 2 autolysed isoform, data don't fit well the linear model (R^2<0.9) and only show that in the incubated samples its activity is higher than in non-incubated pool, which stand as a negative control.

Data produced for a 5 mM final $CaCl_2$ concentration (not shown) were not significant because the analysis was performed for one sample and two replicates only. On the other side, the gels show the same profile as that obtained at a 10 mM final $CaCl_2$ concentration, with the difference that calpain 1 band appears between 0 and 90 minutes (Figure 17).

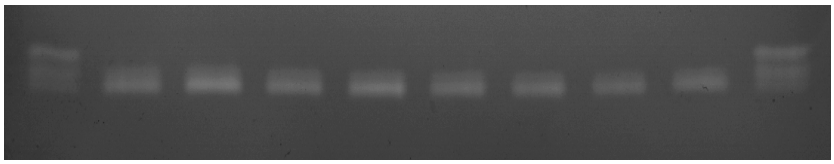
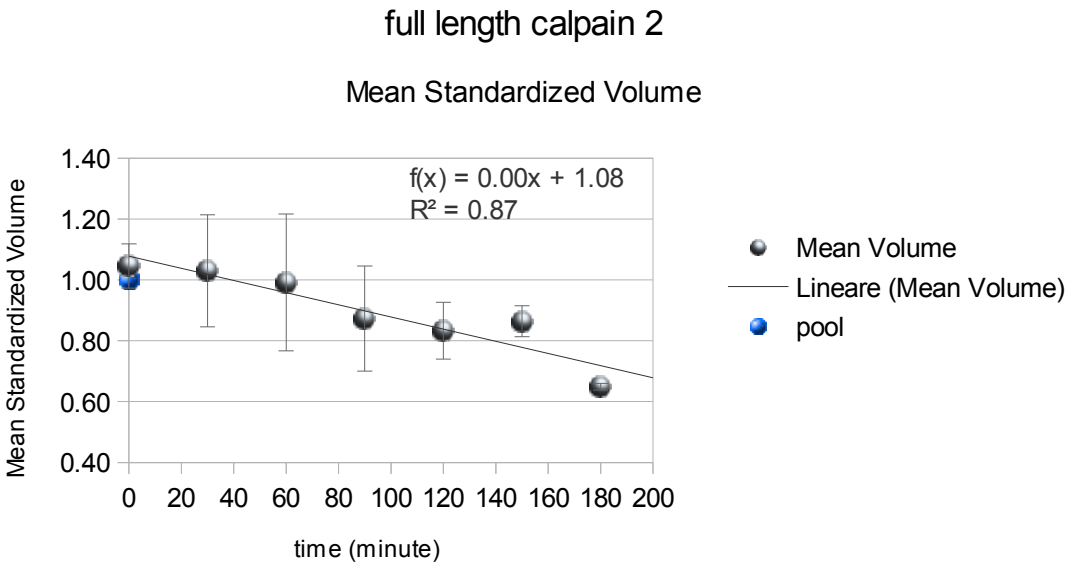


Figure 16. Zymogram for sample no. 4 treated at a final $CaCl_2$ concentration of 10 mM. Pool in the 1st and 2nd well. Treated samples are in an increasing order of incubation (0, 30, 60, 90, 120, 150, 180, 210 min).

Figure 17. Zymogram for sample no. 4 treated at a final $CaCl_2$ concentration of 5 mM. Pool in the 1st and 2nd well. Treated samples are in an increasing order of incubation (0, 30, 60, 90, 120, 150, 180, 210 min).

In the present study we observed a decrease along the course of time of the calpain 2 full-length isoform and an increase of the calpain 2 autolysed isoform if compared to the negative control (pool). Therefore, it is reasonable to hypothesize that under conditions favourable for autolysis to be accomplished (saturated EGTA/EDTA system and free Ca^{2+} ions) calpain 2 full-length isoform autolyzes following a linear course. As a consequence, at the same time calpain 2 autolysed isoform quantity increased and that was accompanied by an increase of its activity toward the casein substrate. Moreover, we could notice that at a 5 mM final $CaCl_2$ concentration also calpain 1 corresponding band appeared, while at a 10 mM final $CaCl_2$ concentration it did not. This will be useful for further studies, as it indicates that lower concentrations have to be used to detect and monitor calpain 1 autolytic activity along the time course.

a)



b)

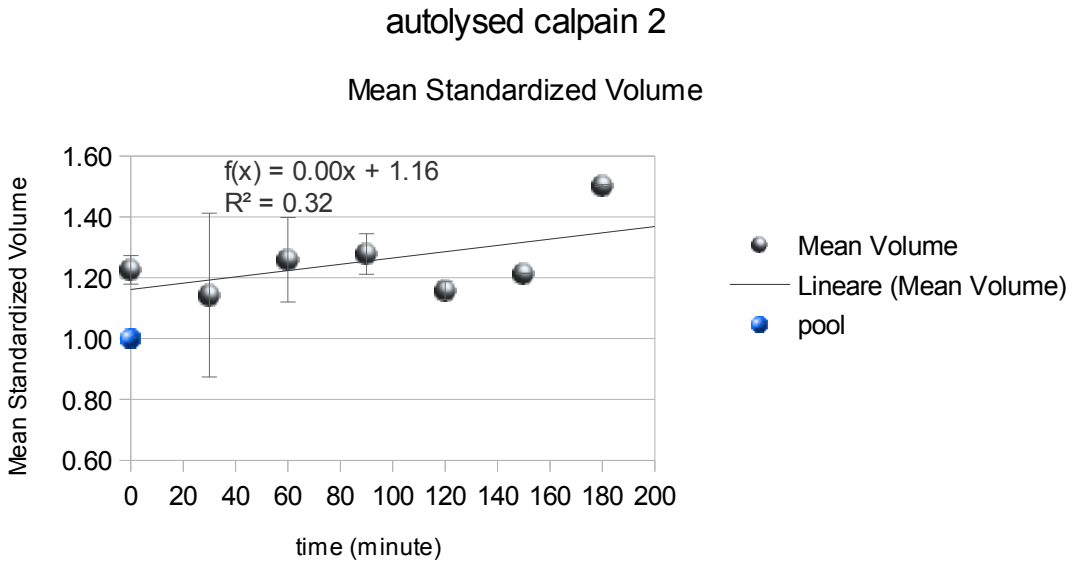


Figure 18. Mean standardized volume for sample no. 4 and 9 full-length (a) and autolysed (b) calpain 2, relative to treatment at a final $CaCl_2$ concentration of 10 mM. Bars represent the standard deviation (SD).

4.5. STUDY 4: Calpain 2 Western blot repeatability and WB proteolytic profiles after differential incubations

The present study was aimed at investigating calpain 2 Western Blot (WB) profiles after different times of incubation. Incubations were performed before the electrophoretic run in the Extraction Buffer, therefore any variations in the profile was due to Ca^{2+}-independent proteases. At the same time, six replicates of three non incubated fatty liver samples were used to establish the calpain 2 WB repeatability.

A previous study on calpain stability in the Extraction Buffer through zymography (see section 4.2) showed the effect of the proteolysis in the course of time towards the active forms of calpain. Here the effect of the proteolysis on both the active and the inactive isoforms of calpain 2 was investigated. The GTX102499 primary antibody recognizes a specific region within amino acids 162 and 383 of human calpain 2 catalytic subunit (UniProt ID#P17655) and its ability to bind duck calpain 2 was previously demonstrated in the laboratory. Therefore it can be assumed that only calpain 2 catalytic subunit isoforms and products of autolysis which possess the GTX102499 primary antibody epitope are recognized and revealed through WB.

The following bands were individuated (Figure 19):

- a ≈95 kDa band;
- a ≈70 kDa band;
- two bands of approximatively 50 kDa;
- a ≈30 kDa band.

An attempt of identification was done on the basis of the GTX102499 primary antibody data sheet (http://www.funakoshi.co.jp/data/datasheet/GNT/GTX102499.pdf) and the information obtained from the calpain stability in the Extraction Buffer study. Human calpain 2 catalytic subunit exists in two gene-splicing products: isoform 1 is a 80 kDa protein, while isoform 2 weights 65 kDa. Data sheet informations for the primary antibody refer to the first isoform, therefore a 80 kDa band is supposed to be revealed. On the basis of the predicted informations available on UniProt (www.uniprot.org), duck calpain 2 catalytic subunit is a 610 amino acids long protein with a molecular weight of approximatively 70 kDa (UniProt ID#R0LRI5). The band corresponding to this molecular weight on the WB profile was therefore supposed to be the active isoform of duck calpain

2 catalytic subunit. This consideration could seem to be in contrast with the fact that two active isoforms were revealed through zymography, but a possible explanation could be found in the different electrophoretic conditions used for zymography and SDS-PAGE. For this reason and for the fact that the two ≈50 kDa bands completely disappeared between the 2 and the 17 hours, the assumption that they correspond to the active isoforms of calpain 2 could be excluded. In fact, at 17 hours the two isoforms still showed to possess lytic activity in the zymographic gels. Two hypotheses can thus be made on the identity of the two ≈50 kDa bands.

The first one consists of associating them to two proteolytic products already present in the fatty liver cell at the moment of the sampling. For the fact they represent clear bands in the non incubated samples whose intensity diminishes during the course of time, it is also plausible to think that they still contain the target site of some Ca^{2+}-independent proteases present in the extract. The second possibility is assigning one or both bands to calpain 2 gene-splicing products, exactly as for the human calpain 2 70 kDa isoform. Both hypotheses could also be made for the ≈30 kDa band; however, as there is no evidence for such a low molecular weight calpain 2 isoform in other organism, it was more reasonable to associate it to a small proteolytic product still containing the epitope and susceptible of proteolysis.

Last but not least the ≈95 kDa band could correspond to a little quantity of non denatured calpain proteic complex, made up of the catalytic subunit and the small subunit. No information on duck small subunit is available in UniProt, but it is known that human small subunit is a 28 kDa protein, common to both calpain 1 and 2 (Goll *et al*., 2003). For this reason a complex between the small and the catalytic subunit could be supposed to weight ≈100 kDa, a molecular weight not so far from the one of the band.

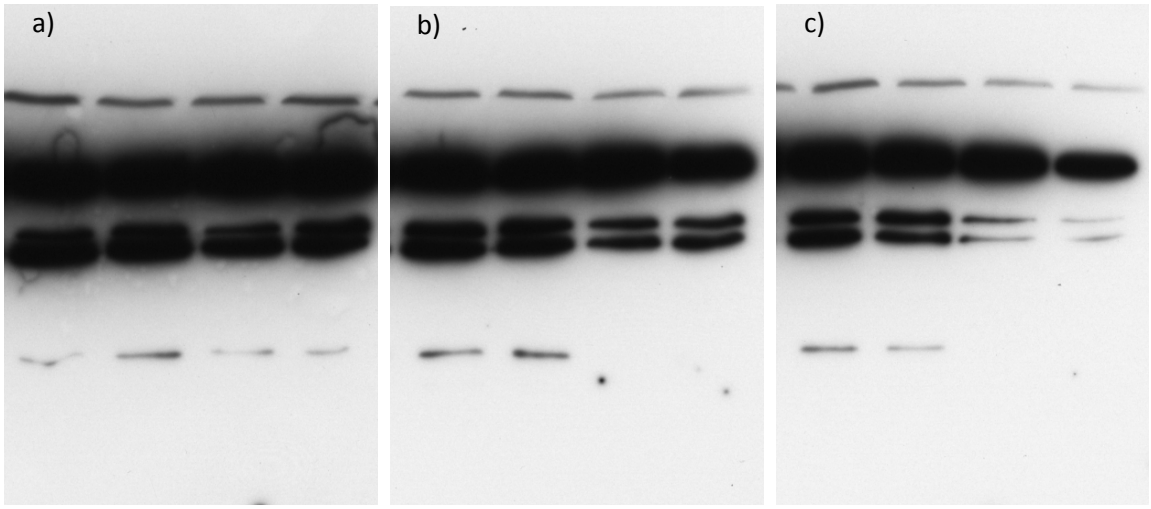


Figure 19. X-ray films obtained by Western Blot using GTX102499 anti-calpain 2 primary antibody. Each film shows two non incubated samples (left side of the box) and two treated samples (right side of the box), which were incubated for 20 minutes (a), 2 hours (b) and 17 hours (c).

A second part of the present study was devoted to the image analysis of six replicates of three non incubated fatty liver samples, aiming at investigating the repeatability of duck calpain 2 Western Blot technique. Results are shown in Table 9 and 10. CV values are high both for the replicates of the same samples (between membranes) and for the samples within the same membrane.

Western Blot is a semi-quantitative technique and data shown here represent a further confirmation for duck calpain 2. Therefore, calpain 2 WB can be used to reveal the presence of calpain 2 and its products, but quantification should be conducted using the reference method (DEAE-Sephacel chromatography and casein assay) or the two-step separation method, as reported by Kent *et al.* (2005).

Table 9. Coefficients of variation (CV) of area, pixel intensity and volume of the three non-incubated samples. Each value refers to six replicates (two replicates per membrane).

Sample	Area CV	Pixel Intensity CV	Volume CV
1	17.1	20.7	28.6
4	14.7	11.4	12.2
10	14.7	13.8	16.3

Table 10. Coefficients of variation (CV) of area, pixel intensity and volume of all the samples in the same membrane, with the respective average values.

Membrane	Area CV	Mean Area CV	Pixel Intensity CV	Mean Pixel Intensity CV	Volume CV	Mean Volume CV
I	26.1		18.4		21.3	
II	16.5	14.8	13.6	17.1	25.0	22.8
III	2.0		19.2		22.2	

5. CONCLUSIONS

The aim of study 1 was to compare calpains proteolytic activity between two groups of duck fatty livers, chosen so that different values of cooking losses were independent from other parameters involved in *foie gras* technological yield determinism. Data obtained from this study showed that calpain activity does not differ between the two groups of samples selected for the same weight, lipids and dry matter contents. If this observation will be confirmed with further studies on cathepsins and MMPs lytic activities, we might admit that proteases do not have a role in *foie gras* technological yield determinism. However, in the specific case of calpains, it is important to take into account that at present nothing is known on the state of the whole calpain system *in vivo*. Therefore, future perspectives on the better understanding of calpains role *post-mortem* could be represented by measurements of calpastatin content in the cell and by better defining calpains Ca^{2+}-triggered activation mechanism.

Study 2 focused on a better characterization of calpain casein zymography. No quantitative statistically significant data were obtained, but qualitative information showed to be useful for a better manipulation and a better interpretation of zymograms. Investigation of calpains stability in the Extraction Buffer allowed us to conclude that Extraction Buffer is effective in a wide range of Ca^{2+} concentrations and thus endogenous Ca^{2+} concentrations do not affect the extent of calpain lytic activity toward the casein gel substrate. Furthermore, it was proved that calpains are stable in the Extraction buffer at room temperature for hours, indicating the possibility to lessen the level of cares during their manipulation. Study of calpain lytic activity at different Ca^{2+} concentrations proved that also in duck two calpain 2 isoforms (a full-length and an autolysed isoform) can be detected through calpain casein zymography. It was also confirmed that calpain 1 is revealable in the zymogram.

Study 3 was performed in order to investigate calpain autolytic rate during the course of time. Quantitative significant data were obtained just for the full-length form of calpain 2 and showed that its activity (expressed as volume of the band), and therefore its content, decreases following a linear trend. We could also conclude that the way we adapted the protocol of calpain casein zymography was adequate to study calpain autolytic activity and that if further studies are meant to be performed on this subject, it will be necessary

to lower $CaCl_2$ final concentration in the Extraction Buffer in order to obtain data on calpain 1.

Finally, with study 4 we meant to do an attempt of identification of the bands in the calpain 2 Western blot profiles, using the knowledge acquired from the scientific literature and the previous experiments. We hypothesized that 5 calpain 2 forms were identified: a ≈95 kDa band corresponding to a little quantity of non denatured CAPN2/S1 proteic complex, a ≈70 kDa band representing only CAPN2 (the full-length isoform of calpain 2), two ≈50 kDa bands supposed to be either gene-splicing products or lysis products, and a ≈30 kDa band most probably identified as a small proteolytic product. Moreover, data for calpain 2 Western blot repeatability were produced: this short analysis showed high CV values for samples within the same membranes and for replicates of the same sample, giving us a numerical idea of calpain 2 WB variability.

BIBLIOGRAPHIC REFERENCES

Alberts B., Johnson A., Lewis J., Raff M., Roberts K., Walter P., 2002. Molecular Biology of the Cell. Garland Science, New York, pp 1463.

Awde S., Marty-Gasset N., Prahkarnkaeo K., Rémignon H., 2014a. Relationship between Proteolytic Activities and Cooking Loss Variability in Liver Issued from Force-Fed Mule Ducks. J. Agri. Food Chem., 62, 3262−3268.

Awde S., 2014b. *Relation entre Proteolyse Hepatique et Qualités Technologiques et Sensorielles du Foie Gras de Canard.* PhD work.

Babilé R., Auvergne A., 1986. *Qualité des foie gras: Comparaison de différentes méthodes d'appréciation de la fonte lipidique. Évolution en fonction du temps.* Recl. Med. Vet., 162(2), 151-156.

Baéza E., Marie-Etancelin C., Davail S., Diot C., 2013. *La stéatose hépatique chez les palmipèdes.* INRA Prod. Anim., 26 (5), 403-414.

Bairlein F., 1987. Nutritional requirements for maintenance of body weight and fat deposition in the long-distance migratory garden warbler Sylvia borin (Boddaert). Comp. Biochem. Physiol. A Comp. Physiol., 86, 337-347.

Bax M. L., Chambon C., Marty-Gasset N., Rémignon H., Fernandez X., Molette C., 2012. Proteomic profile evolution during steatosis development in ducks. Poult. Sci., 91, 112-120.

Blum J. C., Labie C., Raynaud P., 1990. *Influence du poids et de la composition chimique du foie gras d'oie sur la fonte mésurée après stérilisation a 104 °C.* Sci. Aliments, 10, 543-554.

Blum J. C., Salichon M. R., Guy G., Rousselot-Pailley D., 1992. Comparative development, chemical composition and quality of ducks and goose foie gras obtained by cramming. XIX

World's Poultry Congress, W.P.S.A. Amsterdam, The Netherlands, 240-244.

Boehm M. L., Kendall T. L., Thompson V. F., Goll D E., 1998. Changes in the calpains and calpastatin during postmortem storage of bovine muscle. J. Anim. Sci., 76, 2415-2434.

Bouiller-Oudot M., Leprettre S., Dubois J. P., Babilé R., 2004. *Évolution de la composition hépatique lors du refroidissement post mortem de foies gras d'oies dans la carcasse*. 6èmes Journées de la recherche sur les Palmipèdes à Foie Gras. Arcachon, France, 203-206.

Bradford M. M., 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem., 72, 248-254.

Campbell R. L., Davies P., 2012. Structure–function relationships in calpains. Biochem. J., 447, 335–351.

Chan K. T., Bennin D. A., Huttenlocher A., 2010. Regulation of adhesion dynamics by calpain-mediated proteolysis of focal adhesion kinase (FAK). J. Biol. Chem., 285(15), 11418-26.

Chou J. S., Impens F., Gevaert K., Davies P. L., 2011. M-Calpain activation in vitro does not require autolysis or subunit dissociation. Biochem. Biophys. Acta, 1814(7), 864-72.

Comité Interprofessionnel des Palmipèdes à Foie Gras (CIFOG), 2011. *Assemblée generale du 24 juin 2011. Rapport économique de l'année 2010*. 75p.

Corbalán-García S., Gómez-Fernández J.C., 2010. The C2 domains of classical and novel PKCs as versatile decoders of membrane signals. Biofactors, 36(1), 1-7.

Cortesio C. L., Perrin B. J., Bennin D. A., Huttenlocher A., 2010. Actin-binding protein-1 interacts with WASp-interacting protein to regulate growth factor-induced dorsal ruffle formation. Mol. Biol. Cell., 21(1), 186-97.

Criterion Blotter Instruction Manual
http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4006190b.pdf. Web site consulted in August 2014.

Cruzen S. M., Paulino P. V., Lonergan S. M., Huff-Lonergan E., 2014. Postmortem proteolysis in three muscles from growing and mature beef cattle. Meat Sci., 96(2 Pt A), 854-61.

Dayton W. R., Goll D. E., Zeece M. G., Robson R. M., Reville W. J., 1976. A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Purification from porcine muscle. Biochemistry-US, 15, 2150–2158.

Donkor I. O., 2011. Calpain inhibitors: a survey of compounds reported in the patent and scientific literature. Expert Opin. Ther. Pat., 21(5), 601-36.

Fernandez X., Bouillier-Oudot M., Molette C., Bernadet M. D., Manse H., 2011. Duration of transport and holding in lairage at constant postprandial delay to slaughter. Effects on fatty liver and breast muscle quality in mule ducks. Poultry Sci., 90, 2360-2369.

Fraser R., Heslop V. R., Murray F. E., Day W., 1986. Ultrastructural studies of the portal transport of fat in chickens. Brit. J. Exp. Pathol., 67(6), 783-91.

French *Code rural*, Article N° L654-27-1 5 (consulted on 25th August 2014).

Fritz V., Fajas L., 2010. Metabolism and proliferation share common regulatory pathways in cancer cells. Oncogene, 29(31), 4369-77.

Folch J., Lees M., Sloane Stanley G. H., 1957. A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. J. Biol. Chem., 226, 497−509.

Geesink G. H., Taylor R. G., Koohmaraie M., 2005. Calpain 3/p94 is not involved in postmortem proteolysis. J. Anim. Sci., 83, 1646–1652.

Geesink G. H., Kuchay S., Chishti A. H., Koohmaraie M., 2006. μ-Calpain is essential for postmortem proteolysis of muscle proteins. J. Anim. Sci., 84(10), 2834–2840.

Gil-Parrado S., Popp O., Knoch T. A, Zahler S., Bestvater F., Felgenträger M., Holloschi A., Fernández-Montalván A., Auerswald E. A., Fritz H., Fuentes-Prior P., Machleidt W., Spiess E., 2003. Subcellular localization and in vivo subunit interactions of ubiquitous μ-calpain. J. Biol. Chem., 278(18), 16336-46.

Goll D. E., Thompson V. F., Li H., Wei W., Cong J., 2003. The calpain system. Physiol. Rev., 83, 731–801.

Guroff G., 1964. A neutral, calcium-activated proteinase from the soluble fraction of rat brain. J. Biol. Chem., 239, 149–155.

Hanna R. A., Campbell R. L., Davies P. L., 2008. Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. Nature, 456, 409–412.

Hérault F., Saez G., Robert E., Al Mohammad A., Davail S., Chartrin P., Baéza E., Diot C., 2010. Liver gene expression in relation to hepatic steatosis and lipid secretion in two duck species. Anim. Genet., 41, 12-20.

Hermier D., Salichon M. R., Guy G., Peresson R., Mourot J., Lagarrigue S., 1999. *La stéatose hépatique des palmipèdes gavés: bases métaboliques et sensibilité génétique.* In: *Numéro spécial, Lipogenèse et qualité des produits.* Chilliard Y. (Ed). INRA Prod. Anim., 12, 265-271.

Huang Y., Li Y., Burt D. W., Chen H., et al., 2013. The genome sequence and transcriptome of duck provide insight into an avian influenza virus resorvoir species. Nat. Genet., 45, 776–783.

Huston R. B. & Krebs E. G., 1968. Activation of skeletal muscle phosphorylase kinase by Ca^{2+}. Identification of the kinase activating factor as a proteolytic enzyme. Biochemistry-

US, 7, 2116–2122.

Kanzaki K., Kuratani M., Matsunaga S., Yanaka N., Wada M., 2014. Three Calpain Isoforms are Autolyzed in Rat Fast-twitch Muscle after Eccentric Contractions. J. Muscle Res. Cell. Motil., 35(2), 179-89.

Kemp C. M., Sensky P. L., Bardsley R. G., Buttery P. J., Parr T., 2010. Tenderness - an enzymatic view. Meat Sci., 84(2), 248-56.

Kent M. P., Veiseth E., Therkildsen M., Koohmaraie M., 2005. An assessment of extraction and assay techniques for quantification of calpain and calpastatin from small tissue samples. J. Anim. Sci., 83(9), 2182-8.

Kizaki K., Ushizawa K., Takahashi T., Yamada O., Todoroki J., Sato T., Ito A., Hashizume K., 2008. Gelatinase (MMP-2 and -9) expression profiles during gestation in the bovine endometrium. Reprod. Biol. Endocrinol., Dec 31, 6-66.

Koohmaraie M., Whipple G., Kretchmar D. H., Crouse J. D., Mersmann H. J., 1991. Postmortem proteolysis in longissimus muscle from beef, lamb and pork carcasses. J. Anim. Sci., 69(2), 617-24.

Kumar K. A., Lalitha A., Pavithra D., Padmavathi I. J. N., Ganeshan M., Rao K. R., Venu L., Balakrishna N., Shanker N. H., Reddy S. U., Chandak G. R., Sengupta S., Raghunath M., 2013. Maternal dietary folate and/or vitamin B12 restrictions alter body composition (adiposity) and lipid metabolism in Wistar rat offspring. J. Nutr. Biochem., 24, 25-31.

Léveille G. A., Romsos D. R., Yeh Y. Y., O'Hea E. K., 1975. Lipid biosynthesis in the chicken. A consideration of the site of synthesis, influence of diet and possible regulatory mechanisms. Poult. Sci., 54, 1075-1093.

McCall K., 2010. Genetic control of necrosis - another type of programmed cell death. Curr. Opin. Cell. Biol., 22(6), 882-8.

McGuigan J. A., Kay J. W., Elder H. Y., 2006. Critical review of the methods used to measure the apparent dissociation constant and ligand purity in Ca^{2+} and Mg^{2+} buffer solutions. Prog. Biophys. Mol. Bio., 92(3), 333-70.

Mc Neil C. J., Hay S. M., Rucklidge G. J., Reid M. D., Duncan G. J., Malonye C. A., Rees W. D., 2008. Disruption of lipid metabolism in the liver of the pregnant rat fed folate deficient and methyl donor-deficient diets. Brit. J. Nutr., 99, 262-271.

Meyers S. N. & Beever J. E., 2008. Investigating the genetic basis of pork tenderness: Genomic analysis of porcine CAST. Anim. Genet., 39, 531–543.

Moldoveanu T., Hosfield C. M., Lim D., Elce J.S., Jia Z., Davies P. L., 2002. A Ca^{2+} switch aligns the active site of calpain. Cell, 108(5), 649-60.

Molee W., Bouillier-Oudot M., Auvergne A., Babilé R., 2005. Changes in Lipid Composition of Hepatocyte Plasma Membrane Induced by Overfeeding in Duck. Biochem. Mol. Biol., 141, 437–444.

Murachi T., Tanaka K., Hatanaka M., Murakami T., 1980. Intracellular Ca^{2+}-dependent protease (calpain) and its high-molecular-weight endogenous inhibitor (calpastatin). Adv. Enzyme Regul., 19, 407–424.

Nakagawa T. & Yuan J., 2000. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. J. Cell Biol., 150(4), 887-94.

Nakagawa K., Masumoto H., Sorimachi H., Suzuki K., 2001. Dissociation of m-calpain subunits occurs after autolysis of the N-terminus of the catalytic subunit, and is not required for activation. J. Biochem., 130(5), 605-11.

Neumar R. W., Xu Y. A., Gada H., Guttmann R. P., Siman R., 2003. Cross-talk between calpain and caspase proteolytic systems during neuronal apoptosis. J. Biol. Chem., 278(16), 14162-7.

Nowak D., 2011. Enzymes in Tenderization of Meat: The System of Calpains and Other Systems. A Review. Pol. J. Food Nutr. Sci., 61, 231−237.

Odum E. P., 1960. Premigratory hyperphagia in birds. Am. J. Clin. Nutr., 8, 621-629.

Official Journal of the European Communities, L221/23, COUNCIL DIRECTIVE 98/58/EC, 20 July 1998 concerning the protection of animals kept for farming purposes.

Parr T., Sensky P. L., Arnold M. K., Bardsley R. G., Buttery P. J., 2000. Effects of ephinephrine infusion on expression of calpastatin in porcine cardiac and skeletal muscle. Arch. Biochem. Biophys., 374, 299–305.

Raser K. J., Posner A., Wang K. K. W., 1995. Casein Zymography: a Method to Study μ-calpain, m-calpain, and their Inhibitory Agents. Arch. Biochem. Biophys., 319 (1), 211-216.

Ravulapalli R., 2009. Regulatory domains of the human calpain family. Ph.D. Thesis, Department of Biochemistry, Queen's University, Kingston, Canada.

Rousselot-Pailley D., Guy G., Gourichon D., Sellier N., Blum J. C., 1992. *Influence des conditions d'abattage et de refrigération sur la qualité des foies gras d'oie.* INRA Prod. Anim., 5(3), 167-172.

Rousset-Akrim S., Bayle M. C., Martin J. F., Touraille C. 1994. *Comment distinguer par évaluation sensorielle les foie gras d'oie et de canard? Étude préliminaire.* Sci. Aliment., 14, 777-784.

Saez G., Baéza E., Davail S., Durand D., Bauchart D., Gruffat D., 2008. Is the hepatic metabolism of glucose and linoleic acid influenced by species in overfed ducks? Comp. Biochem. Physiol. A, 151, 576-581.

Saido T. C., Nagao S., Shiramine M., Tsukaguchi M., Sorimachi H., Murofushi H., Tsuchiya T., Ito H., Suzuki K., 1992. Autolytic transition of μ-calpain upon activation as resolved by

antibodies distinguishing between the pre- and post-autolysis forms. J. Biochem., 111(1), 81-6.

Schà D. É., Farkas A. J. É., Kely G., Tompa P., Friedrich P., 2002. A novel small subunit of the calpains. Biochem. J., 362, 383–388.

Schneider C. A., Rasband W. S., Eliceiri K. W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods, 9 (7), 671–675.

Strobl S., Fernandez-Catalan C., Braun M., Huber R., Masumoto H., Nakagawa K., Irie A., Sorimachi H., Bourenkow G., Bartunik H., et al., 2000. The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. Proc. Natl. Acad. Sci. U.S.A., 97, 588–592.

Sorimachi H., Hata S., Ono Y., 2011. Impact of genetic insights into calpain biology. J. Biochem., 150, 23–37.

Theron L., 2011. *Déterminisme biologique de la variabilité de la fonte lipidique à la cuisson du foie gras de canard. Thèse de Doctorat, Institut National Polytechnique de Toulouse*, p161.

Yoshizawa T., Sorimachi H., Tomioka S., Ishiura S., Suzuki K., 1995. Calpain dissociates into subunits in the presence of calcium ions. Biochem. Biophys. Res. Co., 208(1), 376-83.

Wang K. K., Posmantur R., Nadimpalli R., Nath R., Mohan P., Nixon R. A., Talanian R. V., Keegan M., Herzog L., Allen H., 1998. Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. Arch. Biochem. Biophys., 356(2), 187-96.

Wheelock M. J., 1982. Evidence for two structurally different forms of skeletal muscle Ca^{2+}-activated protease. J. Biol. Chem., 257, 12471–12474.