DEVELOPMENT OF LABORATORY METHODS TO DETECT MEAT TENDERNESS

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DEVELOPMENT OF LABORATORY METHODS TO DETECT MEAT TENDERNESS

Renata M. Dolazza

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Abstract

Tenderness has been identified as one of the most important attributes for consumers concerning their meat quality desire, so there is a clear need to accurately determine and grade meat accordingly to tenderness. A tenderness grading system has been proposed to be used to segregate carcasses in order to provide a more consistent prediction of eating quality to the consumers and also enable more carcasses to be segregated into the highest USDA quality grade and categorized as guaranteed tender. Known as an endogenous specific inhibitor of calpain, an enzyme responsible for proteolysis of myofibrillar proteins during post-mortem degradation of muscle, calpastatin presence in the muscle indicates that the activity of calpain can potentially be down regulated, resulting in meat toughness. Thus tenderness can be predicted with the assessment of calpastatin activity in the meat. Independent contribution of the sarcomere length to tenderness is also well established as classic scientific works have demonstrated that muscles with longer sarcomere lengths have lower resistance to shear force. Previous research have been done using enzymatic biosensor to predict calpastatin activity, this

being a faster method compared to the traditional method, but the previous biosensor research might have been detecting fragments of inactive calpastatin. Hence more research was necessary in order to determine the rate of calpastatin degradation in samples, comparing quantity versus activity of calpastatin. In order to investigate the degradation of calpastatin mechanism and its activity an experiment was conducted using three different methodologies to measure calpastatin activity or quantity over a period of 180 total days. *Longissimus dorsi* samples from between the 12^{th} and 13^{th} rib of the beef carcass (n = 12) were extracted at 0 hour postmortem. These samples were assayed for calpastatin by the traditional method at day 0, and then kept under refrigeration until day 90 and day 180, when the same traditional assay was performed. While the traditional assay part of the sample was kept under refrigeration (4^{o}C) within reading, a 1 mL aliquot of sample for each other analysis (ELISA and Western Blot) were separated and frozen at day 0, 90, and 180 so the assays for these two other methods could be performed at the same time. Our traditional method results showed that calpastatin activity decreased from a maximum of 3.3 on day 0 to a minimum of 0.1 on day 180, while on day 90 the maximum activity was 1.1 and minimum of 1.4, staying within the results found for day 0 (with maximum activity), and day 180 (minimum activity) as expected. The correlation between day 0 and 90 was 77%, 49% between day 0 and 180 and 70% between day 0 and day 180 for the traditional assay. The ELISA and Western Blot analysis did not show any results for any of the days. One of the possible explanation would be that by pooling out partially active fraction samples and mixing them with their respective active fraction samples made the protein (enzyme) content too diluted for it to be able to be measured. There is also a concern about the NaCl used to wash off the unwanted protein and

impurities out of the columns, which might have hurt or diminished calpastatin present in the samples or have worked against the ionic strength for calpastatin activity. The freezing step conducted in order to stop the decrease of activity in samples until the ELISA and Western Blot reading could be performed is although our most likely explanation for these results. Crystals possibly formed in the samples when freezing could have hurt the enzyme (protein) structure, which might have prevented enzyme-antibody interaction to occur in a scale to be seen in our analysis, thus ELISA and Western Blot results showed a low protein content. Thereby more research is needed in order to be able to compare these methods and answer the question about calpastatin degradation over a long period of time, whereas there could be differences between calpastatin activity and quantity.

Besides postmortem proteolysis, sarcomere length is also well known for its independent contribution to meat tenderness. However, the interaction between these two elements is still not clear and sometimes controversial, it is believed that shortened sarcomeres likely fail to provide substrate for proteolysis to occur. Beyond that, different methodologies have been applied when preparing samples for sarcomere length analysis which makes comparisons between different studies results difficult. Furthermore a lot of time is spent preparing the samples for the readings and it can vary between methods. Some methods can also use more or less chemicals than others as well as taking more or less storage space and being good for less or more time, what makes the choice of the method a monetary, a logistic and a hazard management matter as well, since the homogenization solution contains sodium iodoacetate. Thus, the objective of the second study was to assess the effectiveness of three different sample preparation methods in

determining sarcomere length in three livestock species. In our study, the sarcomere lengths were measured by light diffraction with a Helium-Neon laser. Ten 7.5 g *Longissimus dorsi* samples from beef, pork and lamb were prepared using three different preparation methods: fresh, frozen conventionally (frozen), and frozen in liquid nitrogen and powdered (powdered). Frozen and fresh 7.5 g samples were then homogenized in 50 mL of a 0.25M sucrose, 0.002M potassium chloride, and 0.005M sodium iodoacetate solution at pH 7.0. Powdered samples were given only a drop of the same homogenization solution, applied on them when already in the glass slide. Sarcomere length results for lamb and beef from *Longissimus dorsi* muscle were in accordance with previous research while for pork the sarcomere readings range was a little smaller when comparing to the literature. Statistical analysis showed that there is difference in sarcomere length within species, as expected, but no difference was found ($P > 0.05$) within sample preparation methods other than for fresh and frozen beef samples. These results allow us to assume that these three sample preparation methodologies used can give us the same results in fresh and frozen beef. There is also the logistic matter that can be taken in consideration since powdered samples had the same results as the other two methodologies, it could be used and also have the advantage of needing a lot less homogenization solution, therefore being a much cheaper and safer method.

Chapter I

1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), world meat consumption is increasing. It is estimated that in the year 2030 livestock products consumption will be as high as 100 kg per person per year in the industrialized countries (Bruinsma, 2003). In the human diet, particularly in the developed countries, meat continues to be one of the most important types of food (Delgado, 2003; McAfee et al., 2010; Speedy, 2003). Therefore the meat chain needs to keep improving in production and also in quality in order to meet the demand and deliver high quality and safe meat to the increasing world population. As the most important quality attributes affecting consumers eating experience satisfaction, tenderness of beef has driven the livestock and meat industry to focus on improving consistency. Tenderness is very important, especially in beef, since consumers can distinguish between different tenderness levels (Lusk et al., 2001). Whereas there are several studies showing that consumers are willing to pay for improved beef tenderness (Feuz et al., 2004; Lusk et al., 1999; Platter el al., 2005; Robbins et al., 2003; Shackelford et al., 2001) the beef industry desires to find a tool able to accurately predict this attribute. Being able to identify precisely the tenderness level in the carcass and tenderness of marketed cuts can help to increase their value and balance the cost of sorting carcasses, genetic and nutrition improvements, benefiting the whole meat chain system, from the producers to the final product distributors. Shackelford et al. (2001) mentions a logical use for a tenderness classification system as a tool to add value to undervalued carcasses, such as U.S. Select grade in the current marbling based marketing system. The lack of clearly defined

incentives for the producers to invest in tenderness as the result of an inexistent accurate tool to predict tenderness in a commercial scale has been the primary impediments to tenderness improvements (Weaber and Lusk, 2010). Although as discussed on the last two National Beef Tenderness Surveys (Voges et al., 2007; Guelker et al., 2013) using the Warner-Bratzler shear force values at retail, industry wide improvement in beef tenderness has been slow, the last National Tenderness Survey shows that the range of days that beef is in transit can be from 1 to 358 days, the range has become wider since the first survey in 1991.

There are several methods for measuring tenderness that are accepted scientifically: Warner-Bratzler shear force, slice shear force, sarcomere length and sensory panels, but all of them are invasive, expensive and/or time consuming. A better knowledge about how tenderness develops and the ability to accurately measure tenderness could be used as a tool to market meat as "guaranteed" tender, as well as enabling the meat industry to screen beef carcasses at an early stage. The knowledge of the likely rate of tenderization would make it possible to sort carcasses into those needing minimal or maximal aging time or into those needing the use of some other mechanical mean in order to achieve a satisfactory degree of tenderness. Also, the high cost and time consumed during carcass storage while aging takes place could be reduced, thus optimizing the quality grading system. Although postmortem tenderization mechanism during storage remains unclear, there is a general agreement that the proteolysis of myofibrillar protein is the major contributor to this meat tenderization process (Dutson, 1983). Calpain, a protease located within the skeletal muscle cells, on the myofibril structures (highest on the Z-disks), and calpastatin, its inhibitor, are both calcium

activated and play a big role on postmortem tenderization. Having relatively constant μ-calpain levels leads calpastatin level to be the one which regulates the proteolytic system during postmortem degradation, thus the measurement of calpastatin activity can be used to predict tenderness. A traditional laboratory assay method developed by (Koomaraie, 1990) is used as a 'gold standard' but it is time consuming and laborious. A lot of research has been done in order to develop a faster and more accurate tool to measure calpastatin activity, hence tenderness. The latest research has tried to develop biosensors to measure tenderness of the meat, as methodology technologies evolve, biosensors can achieve greater sensitivity and more accurate results. Previous research (Bratcher et al., 2008) developed a biological sensor has been moderately successful but may have been detecting inactive fragments of calpastatin. A better understanding of the calpastatin degradation overtime and also the rationalization of quantity versus activity calpastatin is necessary. The knowledge of calpastatin's degradation ratio and mechanism postmortem can be really valuable to the tenderization research related work, as well as to a lot of other muscle structure research related studies, even in the medical field. The development of a tool able to provide a rapid method for predicting tenderness to segregate carcasses and to market beef products accordingly to the expected tenderness for a certain aged product will be a great gain to the beef industry. Thus the objective of the first study was to investigate and compare calpastatin activity versus quantity during its degradation overtime on meat extracted calpastatin samples using the traditional assay, ELISA and Western Blot methods in order to access this enzyme mechanism.

Sarcomeres are the smallest unit of contraction in a muscle, which are composed of long, fibrous proteins (actin and myosin) and delimited by two Z-lines (Marieb, 2009),

which appear under the microscope as dark and light bands. The relationship between sarcomere length and tenderness has been proven, as longer sarcomere lengths have lower resistance to shear force, thus being more tender (Herring et al., 1965; Marsh and Case, 1974). Differences in sarcomere length are found within meat species, muscles and are also affected during rigor mortis (DeVol et al., 1988; Weaver, et al., 2008). Sarcomere length role in regulating proteolysis remains unclear and more research has continuously been done. Different methodologies have been used on scientific research when measuring sarcomeres. The preparation of the samples step previous the readings can vary in time consumption, amount of chemicals needed, as well as differing in sample storage longevity and storage space required. The objective of this study was to access the effectiveness of three different sample preparation methods in determining sarcomere length in livestock species.

Chapter II

2. Literature Review

2.1. Tenderness

Tenderness as a quality attribute in meat can be influenced by innumerous different factors. Kerth (2013) when discussing factors affecting tenderness says that we must be aware that tenderness perception can be influenced by indirect factors, which are called "background effects". These effects or factors can indirectly influence a person's ability to judge tenderness. The four more important factors influencing meat tenderness are postmortem proteolysis, intramuscular fat or marbling, connective tissue, and the contractile state of the muscle (Belew et al., 2003). Chemical and physical properties of muscle interact to determine the tenderness of meat products (King et al., 2009). However, meat scientists agree that the main determinant of ultimate tenderness is the extent of endogenous proteolytic enzymes (Koomaraie and Geesink, 2006; Sentandreu, et al., 2002; Taylor et al., 1995; Smith et al., 1978), the rate of tenderization during post-rigor aging is variable. The completion of the tenderization phase varies between species with beef taking approximately (14 to 21 days), pork (4 to10 days) and lamb (7 to 14 days; Warriss, 2000). The weakening of muscle cells post-mortem is the result of collective disruption of structural protein within the muscle and its rate is highly variable, most of the changes are primarily from the weakening of myofibrillar structure as the result of initiation of proteolysis (Huff Lonergan et al., 2010).

2.1.1. Tenderness Importance for Consumers

The most important quality attributes for meat, to achieve consumer satisfaction and what the meat industry is trying to supply are taste, tenderness and leanness

(Steenkamp, 1990). Among them all, tenderness has been pointed to be the most important attribute in regard to meat quality (Savell et al., 1989; Miller et al., 2001). Thus there is a clear need to regulate and determine meat tenderness within the meat industry (Huffman et al., 1996). Studies have shown that consumers are willing to pay for improved beef tenderness (Feuz et al. 2004; Platter et al. 2005). The National Beef Quality Audits (NBQA) from 1991 (Smith et al., 1992; 1995), 2000 (McKenna et al., 2002), and 2005 (Garcia et al., 2008) tried to determine goals and objectives for producers to implement or improve the quality, consistency, value, and competitiveness of beef. The greatest packers concern in the 1991 and 1995 surveys included hide damage and the lack of uniformity in live cattle (Smith et al., 1992, Boleman et al., 1998), while retailers and restaurateurs were concerned about excessive external fat, low overall uniformity, and consistency of beef cuts (Smith et al., 1992; Boleman et al., 1998). NBQA-2000 data revealed improvements in certain producer management practices resulting in an improvement of end-product characteristics (Roeber et al., 2002). Differently, in 2005, NBQA results revealed numerous improvements in beef production at the packer/merchandiser level but also identified several ongoing opportunities for improving cattle production practices to more consistently meet packer, merchandiser and consumers demands (Shook et al., 2008). In 2010 National Beef Tenderness Survey most steaks evaluated were considered tender (Guelker et al. 2013). However, not all WBSF values decreased and all the 2010 Tenderness survey Warner Bratzler Shear Force (WBSF) values were similar numerically to those in the 2006 (Table 1). This may be due to increased attention given to steaks from the round after the last survey and a possible plateau of beef tenderness. Numerous programs focusing on beef tenderness are evident

and will continue to play a role in maximizing beef tenderness and consumer satisfaction. Tenderness values for the 2010 survey Guelker et al. (2013), along with Voges et al. (2007), appear to be improved (having lower WBSF values, greater sensory panel ratings, fewer steaks above certain WBSF threshold values) compared with those reported by Morgan et al. (1991) and Brooks et al. (2000, Table 1), which may indicate a general shift in palatability in the United States over the past decade. Although there has been some improvement by the meat industry on beef tenderness compared to previous surveys, but results still show a lot of variation in tenderness along the retail and foodservice establishments in the US (Voges et al., 2007). In addition to that the lack of clear incentives for any individual producer to invest in tenderness since this attribute cannot yet accurately be predicted on a commercial scale has been one of the primary impediments to tenderness improvements (Weaber and Lusk, 2010).

Platter et al. (2005); Shackelford et al. (2001), Boleman at al. (1997) and Wheeler and Koohmaraie (1994) have conducted studies in order to determine consumers perception of tenderness and their willingness to pay for a product guaranteed tender. Their results suggest that consumers can discern between categories of tenderness and that consumers are willing to pay a premium for improved tender meat. Boleman at al. (1997) thereby asserted that monetary incentives may be used to promote the production, identification and marketing of guaranteed tender beef. Mullen et al. (2000) when talking about eating quality assurance, also states the need of a reliable method which can allow the meat industry to guarantee eating quality and the meat market to grow and be viable economically. Mullen et al. (2000) also discussed variation of eating quality due to

differences between animals and cuts, what makes more difficulty for the meat industry is marketing its product according to quality.

2.1.2. Tenderness Research Importance for Industry

As far as aging and tenderness mechanisms are concerned and as shown on the 2011 National Tenderness Survey showed (Table 2) that the numbers of days in transit of beef products can be really wide (up to 358 days), thus tenderness in those products can be affected. Understanding better how time can affect tenderness in those long transit meat products is necessary and it justifies our present research when comparing tenderness prediction methods overtime. A large effort has been devoted to the development of systems able to classify beef carcasses according to tenderness (Koohmaraie and Geesink, 2006).

Although there are numerous methods to measure tenderness that have been extensively used such as WBSF, slice shear force and sensory panels, they are all invasive, time and expensive due to sample (longissimus dorsi) collection. Once used for one of these methods, longissimus dorsi, a valuable muscle, cannot be sold for profit. Thus, efforts have been devoted in order to develop a new non-invasive instrument grading technique for the evaluation of beef quality in the abattoirs (Brøndum et al., 1998). Also more understanding about the tenderization rate and mechanism over time has become necessary for the scientific tenderness research related methods, in order to be able to better predict and compare laboratory methods. Zór et al. (2011) affirms that if the meat industry could perform the screening of the beef carcasses as soon as possible postmortem, it would be possible to predict the likely rate of tenderization, thus it would enable carcasses to be classified at an early stage. A successful screening program could

Table 1
Warner-Bratzler Shear Force at Retail (kg)

Steak	1990	1998	2006	2011
Ribeye	3.39	2.84	2.37	2.47
Top Loin	3.25	2.77	2.12	2.38
Top Sirloin	3.56	3.04	2.51	2.46
Top Round	5.23	3.74	3.02	3.04
Bottom Round	4.38	4.19	3.67	3.18
Eye of Round	4.67	5.09	3.39	

Adapted from National Beef Tenderness Survey 1991, 1998, 2006 and 2011
Classified as tough at retail with a Warner-Bratzler shear force ≥ 4.6 kg (Shackelford et al.,1991).

reduce time of the high cost carcass storage while aging takes place. Furthermore, control of tenderness during the aging phase could enable producers to optimize and ensure quality provided to the marketplace (Zór et al. 2011) increasing with it the value of the carcass or final products.

2.2. Factors affecting tenderness

Among the factors affecting tenderness perception is marbling, although as Wheeler et al. (1994) stated the tenderness increments are very small as marbling increases, and these little differences may or may not be important to consumers (Save11 et al., 1987). However, a few studies have found stronger relationships where 5 to 10% of the variation in tenderness was accounted for by marbling degree (Blumer, 1963; Jeremiah, 1978). Kerth (2013) describes intramuscular fat as a "lubricant" that improves the tenderness perception when a bite of the meat is taken. Collagen is another important factor affecting tenderness, as its content vary between muscle functions in the live animal (McKeith et al., 1985; Rhee et al., 2004). Post-mortem changes during conversion of muscle to meat can be influenced by muscle fiber type composition given the relationship between metabolic substrate availability, anaerobic metabolism and thereby lactic acid production (Klont et al., 1998). Muscle pH can also effect tenderization, as muscle tissue continues to undergo anaerobic respiration as it tries to maintain homeostasis accompanied with regeneration of ATP through creatine phosphate after slaughtering the animal, however the ATP produced per unit of glucose is less than during aerobic respiration. Stored glycogen is metabolized into pyruvate and then converted to lactic acid during anaerobic respiration. When lactic acid accumulates the intracellular pH declines and finally reaches a pH of about 5.4-5.7, 24 hours post-mortem. The pH value drop stops with the

Table 2
Number of Days Beef in Transit

Survey Year	Mean	Range
1991	17	3 - 90
1998	19	2 - 61
2006	23	3 -83
2011	21	1 - 358

Adapted from National Beef Tenderness Survey 1991, 1998, 2006 and 2011

depletion of glycolytic substrate. The rate of pH decrease post-mortem can vary between species, rate of ATP turnover, glycogen level and muscle (Lawrie, 1998). High ultimate pH meat (pH > 6.5) has reduced flavor, is typically dark and has higher vulnerability to bacterial spoilage (Watanabe et al., 1996). Dark, firm and dry (DFD) meat due lower availability of glycogen substrate that causes rapid depletion of ATP and early rigor is associated with variable tenderness (Watanabe et al., 1996). The PSE meat, defined as pale, soft exudative goes in the opposite direction as a result of accelerated rate of post-mortem glycolysis, that leads to a lower pH (5.4 to 5.6) immediately after slaughter) in the muscle while high temperature remains causing protein denaturation (Bowker et al., 2000). Generally PSE meat results in a poor eating quality product as the enzymes involved in post-mortem tenderization are inhibited by the low pH environment as well as high drip loss (Maltin et al., 2003). Temperature can also have a profound effect on the metabolism and rate of shortening of the muscle during the pre-rigor and post-rigor phase (Hertzman et al., 1993). The increase of temperature at an early rigor stage can make the development of meat tenderization prone to rapid pH decline, increasing meat toughness as the sarcomere length is shortened (Bruce and Ball, 1990). Cold shortening can also happen by an accelerated decline of temperature, muscle shortening is increased because of the reduced calcium ability by the sarcoplasmic reticulum as a result of energy compounds depletion which causes the muscle to contract and increase meat toughness (Huff Lonergan et al., 2010; Hannula and Puolanne, 2004). The ideal rigor temperature to optimize rate of tenderization and reduces rigor shortening without contributing to any detrimental effect on water holding capacity or color of the meat has been determined as being 15°C (Geesink et al., 2000).

2.3. Skeletal muscle structure and composition

After slaughtering the animal the skeletal muscle becomes meat, although it sounds simple it is a complex process where various factors can affect quality attributes, as tenderness post-mortem (Greaser, 1986). The skeletal muscle structure is a complex organization of proteins which have a hierarchy of components in the muscle. Muscle fibers or myofibers are multinucleated cells containing a range of quantities of components such as mitochondria, ribosomes, soluble protein, glycogen and lipids (Pearson & Young, 1989). Multiple long and thin cylindrical structures of protein filaments which lie parallel to each other, known as myofibrils are inside the muscle fibers. The contractile properties of the muscle is defined by the myofibril structure (Lawrence and Fowler, 2002) made by innumerous lined sarcomeres. A sarcomere is located between successive Z-disks (Taylor et al., 1995). Au (2004), in a review explains that the main sarcomere structural features are the longitudinal Z and M line (or known as disk or bands, Figure 1). Within the sarcomere are two distinct areas, the A-band [that has two alternating filaments, the thick (myosin) filament and the thin (actin) filament] and the I-band areas (that contains the thin actin filaments moored to the Z-disk,) which is between adjacent sarcomeres (Au, 2004)

When contraction occurs a crossbridge between the myosin and actin is formed, where the thick myosin and the thin actin filaments slide pass each other. As myosin and actin interact a complex is formed, it is called the actomysin complex (Rayment et al., 1993). During the contraction the sarcomere length is reduced, while the I-band and the H-zone will shorten, but not the length of the A-band (Rayment et al., 1993). When muscle is relaxed myosin is blocked from binding with actin by tropomysosin, a protein

regulator In addition to that there is another protein complex called the troponin complex, which is responsible for positioning of the tropomyosin on the actin filament (Au, 2004). Thus, when there is muscle stimulation, Ca^{2+} is released from the sarcoplasmic reticulum and binds troponin, that changes the position of tropomyosin on the actin filament and exposes the myosin binding site (Reece and Campbell, 2011). Energy is required as ATP form for the interaction between actin and myosin filaments to occur. The ATP binds to the myosin head and is hydrolyzed by ATPase to provide the energy for contraction (Holmes and Geeves, 2000).

2.4. Conversion of muscle to meat

Right after slaughter of the animal the conversion of muscle to meat starts. There are three important phases during post-mortem development which are the pre-rigor (or delay phase), the rigor phase (with onset and completion phases) and the post-rigor tenderization phase (Sentandreu et al., 2002). In a living muscle energy is required in the ATP form for the contraction and relaxation of muscle (Ebashi et al., 1969), after slaughter there is no more blood circulating, thus the oxygen and nutrient supply to the muscle are cut that unleashes the pre-rigor phase in the animal muscle. While in the pre-rigor phase muscle remains excitable as intracellular ATP is used and regenerated by reaction between creatine phosphate (CP) and adenosine diphosphate (ADP, Lawrie, 1998).To enable the interaction between actin and myosin energy is released during muscle contraction by conversion of ATP to ADP. Thus the ATP is needed in order to disconnect the crossbridge between actin and myosin by binding with Mg^{2+} (Rayment et al., 1993). The ATP amount in the cells will eventually deplete thus muscle cannot enter the relaxation state. When rigor starts to develop (onset phase) the actin myosin binding

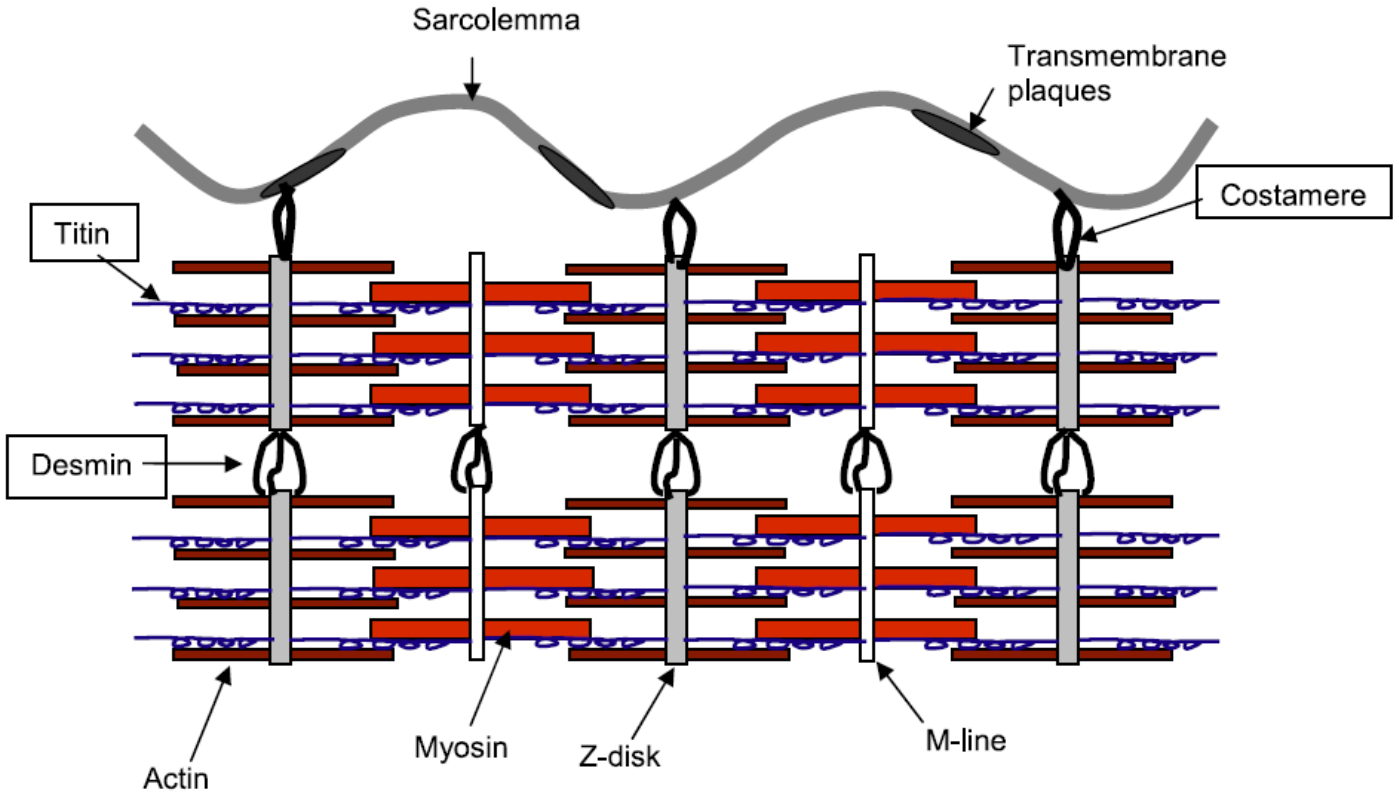

Fig.1. The key myofibril and associated proteins susceptible to proteolysis during post-mortem conversion of meat to skeletal muscle. Boxes indicate proteins that are cleaved during post-mortem tenderisation. Diagram of muscle myofibrilllar proteins adapted from Kemp et. al (2010)

starts to change from a weak binding to a strong and irreversible binding resulting in a permanent shortening of the sarcomere length (Koohmaraie, 1996). Rigor phase starts in order to maintain energy source homeostasis and subsequently ATP depletes, thus muscle glycogen is metabolized via anaerobic glycolysis to continue providing ATP production by phosphorylation of ADP by CP. Free Ca^{2+} concentration in the sarcoplasma increases as intracellular ATP depletes, thus the sarcoplasmatic reticulum role is to translocate Ca^{2+} across the membrane. For that an ATPase pump, dependent on ATP, is necessary (Robbins et al., 2003). From anaerobic glycolysis (needed to generate ATP) lactic acid is produced, which leads to a major reduction in intracellular pH during the process of muscle becoming meat. However as this process continues, CP will eventually become depleted resulting in a decline of ATP and thereby the availability of substrate required to maintain the contractile proteins actin/myosin in the relaxation state. Thus irreversible cross bridges between actin and myosin develop, as the sarcomeres are shortened and the muscle reaches its maximum toughness, being the tissue going through rigor mortis (completion phase; Goll et al., 1995). Time taken for the development is variable and depends on the animal species, chilling condition and the type of muscle (Sentandreu et al., 2002). In lamb and beef rigor development is found to be between 6 and 12 hours (Marsh, 1954) while pork rigor mortis can takes from 30 minutes to 3 hours (Honikel, 1987).

2.4.1. Post-rigor tenderization phase

The tenderization phase (or resolution) generally starts at 12 hours of storage post-mortem with the initiation of proteolysis (Wheeler and Koohmaraie, 1994). Post-mortem tenderness development in meat is caused by proteolytic activity which alters the

structure of muscle and associated proteins (Hopkins and Thompson, 2002). Meat gradually becomes tender after reaching the highest point of toughness during post-mortem, then the protease system(s) initiate the proteolysis in meat after rigor. The rate of tenderization during this phase, from 24 hours to 14 days, was demonstrated by Wheeler and Koohmaraie (1994) as being related to the rigor but other factors can also affect its development. Tissue types, pH and temperature are the major identified micro environmental factors that can play a role in the conversiton process of muscle to meat. Proteins such as desmin and titin are associated with the cytoskeleton and they maintain the myofibril's structure as well as the myofibril interaction with the sarcolemma (the costameres) which are cleaved during this phase by the protease system(s) (Kemp, et al., 2010; Figure 1). Koohmaraie (1988) stated that in order to be involved in this tenderization process the protease system must be endogenous to skeletal muscle cells, able to induce post-mortem changes in vitro under the optimal conditions similar to those seen *in situ* and have access to the myofibril. Several enzyme systems are described as being associated with the post-mortem proteolytic activity, these include the calpain, caspase, cathepsin lysosomal and the ATP-dependent proteasome system (Kemp et al., 2010). Many researchers have been investigating the role of each of these proteolytic systems in muscle post-mortem proteolysis, but there are essentially three models which have been asserted to be responsible for the proteolytic meat tenderization processes. The calpain system is the main protease responsible in meat tenderization, but some researchers suggest cathepsin and calpain to be equally involved in the process, as others suggest a multienzymatic process which includes proteasomes, calpains, cathepsins and caspases during post-mortem (Ouali et al., 2006). However, most of studies have agreed

that the calpain system plays a major role in post-mortem tenderization (Boehm et al., 1998; Koohmaraie, 1992a; Taylor et al., 1995). Post-mortem tenderization cathepsin system role is doubtful as its incubation with myofibrillar proteins results in a different degradation pattern than during the post-mortem period in situ. Also, it is unsure that lysosomes release these proteases (Koohmaraie, 1988).

2.5. The calpain-calpastatin enzyme system

The most important quality attribute contributing to meat quality in various farm species is tenderness; what is the result from the gradual changes occurring due the proteolytic activities of enzymes present in the muscle what influences protein turnover (Koohmaraie, 1994). The intracellular protein turnover has three proteolytic systems involved, they are: lysosomes, error-eliminating and black-box system (Goll et al., 1991). Although a pH range of 3.0 to 5.0 is necessary for an optimal activity of the proteases located in the lysosome, it is inactive at pH values in the cell cytosol (Goll et al., 1991). The fact that these proteins have to go through endocytosis in order to get into the lysossomes due to their size of plus the pH limitation suggest that lysossomal proteases are poor candidates for initiating myofibrillar protein turnover. (Goll et al., 1991; Goll et al., 1992). Also unlikely to play a role in the initiating the myofibrillar protein degradation are the error-eliminating system enzymes since they contains serine and cysteine proteases in the cell cytoplasm and that have optimal activity at pH of 7.5 and 8.0, above physiological pH (Goll et al., 1991). The black-box system contains serine, cysteine as well and some metalloproteinases also located in the cell cytoplasm and they have optimal active at pH 7.0 and 8.0, being calpain system part of this system (Goll et al., 1991), The calpain effects are well established as it is responsible for the degradation

of myofibrillar and associated proteins in postmortem muscle (Koohmaraie, 1996). The role of the calpain proteolytic system has been of scientific interest for a number of years and has been issued in review articles and book chapters (Wang, 2000; Zatz and Starling, 2005; Nixon, 2003). Calpains are important and play a role in several cellular processes as exocytosis, cell differentiation and fusion (Sazontova et al., 1999), also in controlling progression through the cell cycle, regulating gene expression and substrate degradation in some apoptotic pathways (Goll et al., 2003). The calpain system consists of two calcium-dependent enzymes that differ in their calcium requirement for activation, and a specific inhibitor, calpastatin. m-calpain requires millimolar calcium concentration and μ-calpain micromolar calcium concentrations for activation (Koomaraie, 1992b). Lastly calpastatin, the third component of the calpain system, which content varies in beef and inhibits calpain activity. High calpastatin content reduces the tenderization process once it limits the extend of postmortem proteolysis by the calpains. The highest calpains concentration is at the Z-disks and have optimal active at intracellular pH and ionic strength (Goll et al, 1992). Calpains singularity is due specific degradation of Z-disks and the fact calpains do not degrade undenatured myosin and actin (Goll et al., 1992) makes calpains the most likely candidates for initiating myofibrillar protein turnover. Dargelos et al. (2007) has observed the involvement of calpains in muscle aging when he saw a significant increase in the quantity and activity of μ-calpain, and at the same time a decreased amount of calpastatin in aged skeletal muscles. Thus by the degradation of myofibrillar and associated protein calpain system is believed to play a role in post-mortem tenderization of meat (Koohmaraie, 1992a). During the post-mortem period the protease system has been considered the main contributor of proteolytic activity, its

activity accounted for 95% of all the proteolytic activity during post-mortem (Delgado et al., 2001; Koohmaraie and Geesink, 2006; Sentandreu et al., 2002). The decline of myofibrilliar protein structural integrity is parallel to the decrease in meat toughness (Harper, 1999). This process occurs right after rigor mortis. Several researches have clearly described that the Z disk is the structural component of muscle which significantly changes post-mortem when troponin, titin, and desmin are selective degradated at specific sites. There are several calpain genes identified in mammals (Campbell and Davies, 2012) but the most studied and well known are μ- and m-calpain, where their activity is regulated in the presence of Ca^{2+} by their specific endogenous inhibitor calpastatin (Hanna et al., 2008). All of the proteolytic changes in myofibrillar proteins during postmortem storage are produced by calpains.

2.5.1 Calpain

Post-mortem meat tenderization is mainly due to protein degradation, with the calpain proteolytic system its principal contributor. However, calpain system is highly sensitive to calcium level, temperature and pH which are all prone to changes during post-mortem period (Melloni et al., 1992). Calpains are found in two main forms, m-calpain and μ-calpain, both of them are calcium dependent, thus the nomenclature refers to the calcium concentration required for each of them activation (Cong et al., 1989). Requirement for m-calpain goes from 300 μM to 1000 μM Ca^{2+} for half maximal activity while μ-calpain Ca^{2+} requirement is lower, being it between 5 μM and 65 μM for half maximal activity (Goll et al., 1992). Both of these calpains are able to cleave the same targets of myofibrillar protein but they are not able to degrade myosin and actin (Dayton et al, 1976; Koohmaraie, 1992a). Calpain was first purified from porcine skeletal muscle

when it was believed to be potentially involved in the turnover of myofibrilliar protein. Dayton et al. (1976) using SDS-PAGE, concluded that calpain exist as a two sub unit hetero dimer with 80kDA and 30kDA subunits. Dayton et al. (1976) also show calpain capacity to partially break the M line, disrupt the Z disk and to have some effect on troponin-T and troponin-I but not on the ultramuscular structure entirety. Taylor et al. (1995) found that a lot of muscle structure associated proteins were cleaved post-mortem, as Z-disk protein and costamere and Huff-Lonergan et al. (1996) showed calpains to degrade the key myofibrillar proteins including titin, nebulin, troponin-T and desmin. μ-calpain is of specific importance in post-mortem tenderization was shown in a μ-calpain knockout mice, as post-mortem proteolysis was significantly inhibited (Geesink et al., 2006). These knockout animals western blot analysis' show a much lower degradation level of myofibrillar structural proteins such as nebulin, desmin, vinculin, dystrophin and troponin when compared to control animals. Taylor et al. (1995) suggests that μ-calpain is activated post-mortem at an early stage (first 3 days; Taylor et al., 1995). Boehm et al. (1998) showed m-calpain not being active while μ-calpain was active during early post-mortem (1 to 8 hours after slaughter) due to limited Ca^{2+} concentration.

2.5.1.1 Calpain structure

As heterodimers m-calpain and μ-calpain are composed from two different subunits the catalytic, 80kDa subunit and a regulatory, 28kDa subunit. Identical in both m-calpain and μ-calpain is the small 28kDa subunit. The bigger 80kDa subunit is only 60% similar between isoforms in their amino acid sequence (Sorimachi et al, 1997). The 80kda has four structural domains (domain I, II, III and IV) while the regulatory (small) subunit contains Domain V and Domain VI. Domain I (NH_2 terminal domain) is known

as the autolytic domain as it is autolysed upon calpain activation. Domain II (cysteine catalytic site) acts as the proteolytic domain, being similar to other cysteine proteases (Goll et al., 2003). Domain III contains the characteristic C2 domain and is involved in structural changes during calcium binding (Storr et al., 2011). Domain IV is responsible for 'calmodulin like' binding capacity of calcium and has five EF hands. Referred as a hydrophobic domain is domain V subunit and has been reported to bind phospholipids (Fernández, 2006). Lastly is domain VI which has a lot of similarities to Domain IV in the catalytic subunit due to the four EF hands, homologous to domain IV (Suzuki et al., 2004).

2.5.2 Calpastatin

Calpastatin is an endogenous specific inhibitor for calpain proteinase and it is encoded by a single gene (CAST gene). Calpain activity is not only controlled by the presence of calpastatin inhibitor but also by the free calcium concentration. Calpastatin was first identified after calpain activity not being able to be recorded in crude porcine muscle homogenates even though calpain could be purified and isolated from the muscle after the precipitation of the calpain at pH 6.2 (Dayton et al., 1976). Though due to the native behaviour of calpastatin migration in SDS PAGE (estimated to be approximately 40 to 50% slower than their true molecular weight) it was hard to assess certain calpastatin isoforms and relate them to their molecular weight on a SDS PAGE (Maki et al., 1990).

2.5.2.1. Isolation of calpastatin

Purified bovine cardiac muscle calpastatin was stated as being a 115 kDa protein (Otsuka and Goll, 1987), therefore when calpastatin purified from different sources

calpastatin demonstrats its heterogeneity. Various calpastatin extraction techniques have demonstrated that this protein exhibits an anomalous behavior during SDS PAGE migration. Takano et al. (1988) when recombinant calpastatin protein expressed in E. coli were detected using a specific anti-calpastatin antibody, the migration rate was slow when compared to the calculated value from the amino acid sequence. Konno et al., (1997) found the explanation is that the calpastatin protein undergoes randomly conformational coiling, which was confirmed by circular dichroism. If isolated using size exclusion chromatography calpastatin turns out to have a bigger molecular weight than that observed on SDS PAGE which suggested that calpastatin exists as a dimer or tetramer in a non-denaturing solvent (Goll et al., 2003). Studies have tried to determine the relationship between the structure and function of the inhibitory domains of calpastatin polypeptide, results found all four repetitive regions to have inhibitory activity capability against calpain (Maki et al., 1987). Thus the calpastatin molecule is suggested to have the capacity to inhibit four calpains. Calpastatin significance in affecting meat tenderness and inhibitory role has been shown in callipgye lambs where high level of calpastatin was found to reduced proteolytic activity post-mortem what decreased meat tenderness (Geesink and Koohmaraie, 1999) as calpastatin inhibited protein degradation and allowed protein accretion resulting in muscle hypertrophy in the animals. Calpastatin role as inhibitor of calpain was also proved when mice overexpressing calpastatin showed decreased in proteolytic activity and also substrate degradation such as desmin and troponin-T however no affect was observed on the isolated activity of m-calpain and μ-calpain (Kent et al., 2004).

2.5.2.2. Calpastatin and meat tenderness

It has become well known that to a large extent the changes in meat tenderness during aging result from the calpain proteolytic system's ability to degrade cytoskeletal proteins, which are responsible for the structural integrity of muscle fibers (Geesink and Koohmaraie, 2000; Koohmaraie, 1996). Two of the Ca^{2+}-dependent cysteine proteinase (calpain) family members are present in all mammalian cells and are named calpain 1 and 2. They are also known as μ- and m-calpain (respectively), μ-calpain being active at micromolar concentration of Ca^{2+} and m-calpain that is only active at millimolar concentrations Ca^{2+}. These two calpain enzymes appear to keep at least part of their capacity when in vivo to degrade key structural proteins after death for hours or days, thus playing a great role to the tenderization process (Kemp et al., 2010; Koohmaraie, 1996). Calpastatin is a single gene in mammals and although many isoforms can be produced by alternative splicing the predominant isoforms all contain four calpain-inhibitory domains in the skeletal muscle (Parr et al., 2004). The existence of calpastatin occurs naturally in meat and has been proven its influence on calpain by inhibiting its activity. Koohmaraie (1995) found that differences in the rate and extent of postmortem tenderization were due to potential differences in proteolytic activity of the calpastatin system, indicating that proteolysis of key myofibrillar proteins mediated by calpain was responsible for increase in tenderness of carcasses or meat cuts during postmortem storage at refrigerated temperatures. After slaughter the first 24 hours are important for tenderness prediction in meat once biochemical factors regulating tenderness can be measured to predict tenderness. Scientific research results have shown for almost two decades that differences in calpastatin activity level measured 24 h after death can

explain a great proportion of the variation in bovine and ovine Longissimus dorsi (LD) tenderness 10 to 14 days after conditioning (Lorenzen et al., 2000; Shackelford et al., 1994), likely an early post-mortem period could be predictive of ultimate shear force (Whipple et al., 1990). Thus calpastatin activity measurement 24 h after slaughter is still one of the most promising targets for ultimate tenderness prediction. A higher level of calpastatin in pork in the first 2 hours after slaughter was associated with higher number of tough meat (Parr et al., 1999; Sensky et al., 1998), what is also in agreement with Koohmaraie et al. (1991) when concluding that variation of meat tenderness in beef, lamb and pork carcasses was due to calpastatin activity. Thereby calpastatin has been used as the best indicator for meat toughness as subsequent studies have identified calpastatin gene markers, in the form of polymorphisms, as a base for predicting tenderness in various meat animal breeds (Casas et al., 2006; Ciobanu et al., 2004). Loss of calpastatin activity is due to its degradation in postmortem muscle, while the cause of the loss of μ-calpain activity is still unknown (Camou et al., 2007) thus calpastatin activity measurement as a tool to measure tenderness has been used instead of μ-calpain activity.

2.6. Calpastatin Activity versus Quantity

While scientists agree that dominant biochemical factor on tenderization of bovine meat is the μ–calpain enzymatic and calpastatin inhibitory activities, it is also well known that their activity can be influenced by co-factors, being the main co-factors postmortem glycolysis and pH fall. These co-factors can variate due different dietary regimes, the stress of transport and slaughter and the use of growth promoters (Marsh et al., 1981; O'Halloran et al., 1997). The activity and quantity of calpastatin in skeletal

muscle were found to have a linear (Doumit et al., 1996), however sometimes quantity of calpastatin can be being measured instead of its activity. Measuring quantity of calpastatin using antibody–antigen recognition based sensors instead of activity is a new, faster, specific, reliable and low cost method (Zór et al., 2011). A lot of efforts in developing different immunosensors for calpastatin quantification have been done by many research groups as they emphasize the necessity of a faster test system (Bratcher et al., 2008; Geesink et al., 2005; Grant et al., 2005). The conventional biochemical method is expensive and consumes a lot of time to be used regularly on a slaughter line even though calpastatin activity measurement right after slaughter has been known to be a good way to predict tenderness of meat. Surface plasmon resonance (Geesink et al. 2005) immunoassay using enzymatic (Doumit et al. 1996) or fluorescent labels (Takano et al., 1984), and fluorescence resonance energy transfer (Grant el al. 2005) and capacitive immunosensor (Zór et al., 2009) are methods developed to measure calpastatin quantity, while calpain quantitative measurement is done using the sandwich (Yokota et al. 1991) and indirect non-competitive immunoassays methods (Hussain et al., 1998). Doumit et al. (1996) showed that there is a close correlation between the level of calpastatin activity and the quantity of calpastatin protein in muscle, meaning that the use of immunochemical assays for tenderness prediction is possible. Several label-free (Geesink et al., 2005; Grant, et al. 2005; Zór et al., 2009) and label-linked immunoassays (Doumit et al., 1996; Takano et al., 1984; Yokota et al., 1991; Zór et al., 2011) have been designed and applied for calpastatin quantification in meat samples. Widely used for complex matrices analysis such as the ones found in food products is the enzyme-linked immunosorbent assays (ELISA), especially using monoclonal antibodies (Bonwick and

Smith, 2004). Immunoassays are very prone to matrix effects, especially if used without prior sample extraction techniques even after being highly specific (Findlay et al., 2000). In order to decrease these matrix effects sample dilution is generally used (Cairoli et al., 1996; Martin et al., 1991; Nistor et al., 2004), but some form of matrix clean-up can be necessary is if this method fails (Findlay et al., 2000). Consisting mainly of full length Types I and III polypeptides calpastatin is a mixture of different isoforms, (Raynaud et al., 2005a; 2005b), both Type I and III include alternatively-spliced variable N-terminal XL- and L-noninhibitory domains upstream of four repetitive and relatively invariant inhibitory domains IIV (Raynaud et al. 2005a; 2005b). Besides that, calpastatin readily forms peptide fragments, some of them can retain activity and will help tenderization inhibition. Thus, the exact single standard antigen identification is not possible. The non-inhibitory XL- and L-calpastatin domains function is currently unknown, but may involve intracellular localization and more research is needed. Therefore more knowledge about these enzymes has become necessary in order to explain the tenderization mechanism rate in meat. Some questions are still unanswered, as in which rate does calpastatin activity decrease and if calpastatin activity found is actually due active enzymes or also due to fragments of them, for that more research is need.

2.7. Tenderness Prediction Methods

Research in tenderness prediction is not a new area of studies, several researchers have tried to develop a faster method and practicable directly on the slaughter line (Brøndum et al., 1998). Since no available method on the market can fast, accurately and objectively measure tenderness in any particular meat sample, tenderness measurements are usually made either by taste panels or by the Warner Bratzler Shear Force (WBSF)

method. Taste panels are time consuming and expensive, while WBSF evaluates meat resistance during slicing, what also does not provide a direct meat tenderness determination (Boccard et al., 1981). Once known that calpain system plays a big role in tenderness, its activity quantification and components quantity, mainly of μ-calpain and calpastatin, has become one of the big lines of tenderness research.

2.7.1. Traditional Calpastatin Assay

The knowledge of the role in postmortem storage of meat that the calpain proteolytic system and its inhibitor play drove researchers efforts to try to find a method able to quantify them in the meat. Thus the traditional method for calpastatin determination was first described by Koohmaraie (1990) and further refined by Shackelford et al. (1994) when a heating step was added to the method and resulted in smaller amount of meat needed. Koohmaraie (1990) found ion-exchange chromatography (DEAE-Sephacel) to be highly superior to the hydrophobic method when separating μ-calpain and calpastatin. Since then his method has been extensively used. Koomaraie (1995) indicates evidences that differences in proteolytic activity of the calpain system resulted in a different extent and rate of postmortem tenderization. Also, evidence was found showing calpastatin correlation with meat tenderness within species, 24 hr rather than at-death which is valuable information when trying to develop a tool for researchers and meat industry. It is estimated that calpastatin activity at 1 day post-mortem has a high (up to 40%) ability to explain beef tenderness, even though it can vary (Koomaraie, 1995).

2.7.2. ELISA

Enzyme linked immunosorbent assay (ELISA) has been a method commonly used in the diagnosis of various diseases (Johnson, 1987). It utilizes the chromogenic response given by the antigen–antibody reaction between an enzymatically labeled antibody and the antigen of interest. It provides selectivity, good sensitivity and at the same time is a fast and reliable method that requires low quantities of the analyte (Ikemoto et al., 2001). The ELISA calpastatin quantification method was developed by Doumit et al. (1996) and it has been shown to be linearly related to the calpastatin acitivity. However, the method is an indirect antibody procedure and time consuming as it requires calpastatin to be extracted from the muscle by the traditional calpastatin assay method developed by Koohmaraie (1990) and refined by Shackelford et al. (1994). Scientific research have shown calpastatin ELISA results to be linearly related to the traditional calpastatin assay of heated Longissimus dorsi for lamb (R^2 = 0.89) and beef aged for 24 to 48 hr (R^2 = 0.90) (Doumit et al., 1996). Existing ELISA protocols for detection of calpastatin and calpain are based on colorimetric or fluorimetric detection.

2.7.3. Western Blot

Enzymatic activity measurements coupled with ion-exchange chromatography (Pontremoli et al., 1988) or Western Blot used for indirect activity measurements (Imam et al., 2007; Gafni and Ellerby, 2002) are commonly used methods to study the role of the calpain proteolytic system in different pathological events. Still, they are laborious, costly, and time consuming. Beside activity measurements calpastatin (Pontremoli et al., 1988) and calpain (Gafni and Ellerby, 2002) detection can be

performed using Western Blot, which is semi quantitative, not giving a precise value of the amount of the target protein.

2.7.4. Newer research

Hildrum et al. (1994) has reported that NIR (Near Infrared Reflectance) is one of the methods spectra of beef muscles change during aging. Park et al. (1998) also researched tenderness predicting using NIR and found that it is correlated with tenderness of *longissimus dorsi* and as a nondestructive measurement method it could be performed at the processing plants. Geesink et al. (2005) using an optical biosensor for quantification of calpastatin found that the method correlated well with the activity measurement but the immunological amount of detectable calpastatin decreases faster than in the enzymatic assay, being the biosensor not suitable to measure evolution of calpastatin activity postmortem. Newer researches have tried to improve the procedures to the calpastatin quantification using ion-exchanged stepwise methods (Veiseth and Koohmaraie, 2001), but as it has been found by Grant et al. (2005) the improvements have refined the process for calpastatin activity determination but not for the actual quantification of calpastatin in the muscle. Bratcher et al. (2008) found that the most correlated measurements between the traditional calpastatin assay and the biosensors is at 48 hr postmortem, having the capillary biosensor more potential than the optical fiber biosensor, thus a possible valuable tool when measuring tenderness.

2.8 Sarcomere Length

Muscle shortening importance to meat tenderness is well established (Marsh, 1985) as sarcomere length have been recognized as a factor affecting meat tenderness for a long time (Herring et al. 1965). Numerous studies have found a positive correlation

between improved tenderness and longer sarcomere lengths (Wheeler et al., 2002). Although some researchers results suggest that the increase in tenderness during postmortem refrigerated storage depends on sarcomere length and proteolysis (Koohmaraie, 1996; Wheeler and Koohmaraie, 1994) other studies concluded that sarcomere length does not affect proteolysis extent (Wheeler & Koohmaraie, 1999). Weaver et al. (2008) discusses that the shortening of sarcomeres could hinder the ability of the proteases (like calpain) to access their substrates. The role of sarcomere length in regulating proteolysis remains intriguing. In order to clarify the mechanism between proteolysis postmortem tenderization and sarcomere length more research is necessary. However, comparisons between studies can be misled due the use of different methods by the researchers when preparing the samples previous the sarcomere readings. Preparation methods can vary in demand of time, amount of chemicals needed and also differ in sample storage and space required. The method which utilizes frozen samples prior homogenization is the most used but other preparation methods have been proposed as the powdering of frozen in liquid nitrogen samples. Our goal is to find out if there are differences within methods as far as sarcomere length is concerned.

Chapter III

3. Calpastatin Activity versus Quantity Overtime

3.1. Introduction

Meat tenderness variation is still one of the most critical quality problems facing the beef industry (Weaver et al., 2008) as several studies have pointed to tenderness as having great effect on consumer satisfaction and purchasing decisions when buying fresh beef (Lusk et al., 2001). More consistently tender beef is demanded by consumers as well as an improved and direct quality correlated grading system (Weaver et al., 2008). In order to achieve these goals a clear understanding of the tenderization mechanisms is required. Many researchers have shown that the increase in postmortem tenderness is due to calpain proteolysis of key myofibrillar and cytoskeletal proteins (Camou et al., 2007; Koohmaraie, 1994; 1996). The μ-calpain has been proven to be the major cause of postmortem tenderization by degrading structural proteins of the skeletal muscle (Geesink et al., 2005). Calpains activity is controlled by calpastatin, it endogenous inhibitor, being calpastatin activity responsible for a greater portion (~ 40%) of the variation in tenderness (Shackelford et al., 1994). Even though calpastatin activity and quantity in a skeletal muscle were found to be a linearly related, sometimes quantity of calpastatin can be being measured instead of its activity as discussed in Bratcher et al., (2008). Most commonly used, the standard calpastatin assay procedure is time consuming (Koohmaraie, 1995). Other faster methods are available to quantify calpastatin activity, as the enzyme-linked immunosorbent assay (ELISA) for example, developed by Doumit et al. (1996). Western Blotting (Sensky et al., 1999) is also an immunological quantification method used for calpastatin. However, even after refinements, these

methods available for calpastatin biological activity determination do not allow for an actual quantification of the enzyme (Grant et al., 2005) Several studies have been conducted in order to develop a tool that can accurately predict calpastatin concentration in meat using a biosensor. It is believed that a biological sensor could give us a more accurate assessment of tenderness at the time of grading carcasses (Grant et al., 2005). Labeling a graded meat as guaranteed would be a great recover for meat processors as they would be able to enhance their profitability. Bratcher et al. (2008) results when testing a capillary tube biosensor and an optical fiber biosensor showed capillary tube biosensor to be more useful for laboratory determination of differences in biologically active calpastatin concentrations. However more research is necessary in order to verify the presence of active versus quantity of calpastatin in meat samples using the present technology. The objectives of this study were to conduct a test over time (on day 0, 90 and 180 after the slaughter) to assess the degradation of calpastatin samples extracted from beef longissimus dorsi while comparing three different methods: traditional activity assay; ELISA and Western Blot and with that access differences on measuring activity versus quantity of calpastatin in meat solutions extracts.

3.2. Materials and Methods

3.2.1. Traditional Assay

Bovine *longissimus* muscle 25 g samples (n = 12) were collected from between the 12^{th} and 13^{th} rib at 0 h postmortem and used for calpastatin activity assay. The 25 g sample was divided in five of 5 g portions and each one was homogenized in 25 mL of pre-rigor extraction buffer (50 mM Tris, 10 mM EDTA). After centrifugation, the supernatant was heated to 95^{o}C and cooled in an ice bath. They were then dialyzed in

elution buffer (2 mM Tris, 0.025 mM EDTA) for at least 18 h. The supernatant was purified on a 10 mL ion-exchange DEAE-Sephacel column that was equilibrated with the elution buffer. The sample was eluted from the column using the elution buffer (200 mM NaCl). Calpastatin activity was determined according to procedures of Koohmaraie (1990), further modified by Shackelford et al. (1994) and Lorenzen et al. (2000). The fractions that were eluted were screened to determine which fractions were active for calpastatin. The active and partially active fractions were pooled. Each assay was run in triplicate. The assay reaction consisted of: the sample, elution buffer, purified m-calpain, assay media (100 mM Tris, 1 mM NaN3, 7 mg/mL Casein), and 100 mM $CaCl_2$. The reaction was then incubated in 25^{o}C water bath and the reaction was stopped with 2 mL of 5% TCA. After centrifugation, the supernatant was read on a Beckman Spectrophotometer Model DU-640 (Fullerton, CA) at a UV wavelength of 278 nm. The remaining sample of active calpastatin were kept under refrigeration (4^{o}C) until the next reading day. One unit of calpain activity was defined as an increase of 1.0 absorbance unit at 280 nm in 60 min at 25^{o}C and one unit of calpastatin activity was defined as the amount of calpastatin that inhibits one unit of m-calpain activity (Koohmaraie, 1990). For more detailed steps and descriptive of the solutions see Appendix I.

3.2.2. Western Blot

A 1 mL aliquot was taken and separated for each Western Blot and ELISA analysis on day 0, 90 and 180 and frozen using liquid nitrogen and then kept at -80^{o}C until the analysis could be performed, due to laboratory and logistic matters. Whatever sample left that was not separated for Western Blot or ELISA was kept under refrigeration until their next traditional assay readings. After the completion of the 180

days when all 1 mL of samples were collected and frozen for the Western Blot, they were left inside a cooler overnight to thaw. Before Western Blot could be performed we assayed the samples for a protein concentration using DC Bio-Rad (Lowery Assay). Purified calpastatin samples were used for SDS-PAGE and immunoblotting. Once the protein concentration in the purified calpastatin extract was determined we performed a protein concentration check using the Biuret method as modified by Robson et al. (1968) with a Molecular Weight Standard, in our case a BSA (bovine serum albumin). An image of the run gel was generated by using a 20-bit charge-coupled device camera (FluorChem8800; Alpha Innotech Corporation, San Leandro, CA) and FluorChem IS-800 software (Alpha Innotech Corporation) so bands densities could be compared. Calpastatin samples in sample buffer/tracking dye were run on acrylamide gels. Day 0 samples were loaded in the blotting gels with 8 µL, day 90 samples were loaded with 20µL and 180 days samples were loaded with 11.4 µL in order to standardize all the samples and days in the gel to a concentration of 0.5 µg of protein loaded in each well. The rabbit heart extract was loaded with 20 µL, day 0 beef standard was loaded with 10µL and molecular weight standard with 5 µL. After electrophoresis, samples were transferred onto transfer membrane according to the procedures of Lonergan et al. (2001). After transfer, membranes were placed in blocking solution 1 h at room temperature (23°C). After blocking, membranes were placed in their respective primary antibody diluted in PBS-Tween was diluted and incubated overnight at 4°C. After primary antibody incubations were complete, membranes were washed three times (10 min/wash,) using PBS-Tween at room temperature (23°C) before incubation with the secondary antibody. After completion of the secondary antibody incubation, all membranes were washed three

times (10 min/wash) using PBS-Tween at room temperature (23°C). Detection was initiated using premixed reagents. Chemiluminescence was detected using a 20-bit mega-pixel CCD camera FluorChem 8800 (Alpha Innotech Corp., San Leandro, CA) and FluorChem IS-800 software (Alpha Innotech Corp.). For more detailed steps and descriptive of the solutions see Appendix III

3.2.3. ELISA

A bovine calpastatin ELISA kit (BioSourse CAT# MBS932589, San Diego, CA) was used and procedures described on it were followed. Briefly, standards and samples are pipetted into the wells and any calpastatin present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for calpastatin is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of calpastatin bound in the initial step. The color development is stopped and the intensity of the color is measured." The pulled out 1mL calpastatin extracted samples were kept frozen for the ELISA assay, thus they were thawed inside a cooler overnight prior use. For more detailed see Appendix II

3.2.4 Statistical analysis

All statistical procedures were performed using Microsoft Office Excel program, data analysis: descriptive statistics and correlation tools. Correlations were generated within days 0, 90 and 180 results for the traditional calpastatin assays.

Table 3
Carcass Data

	Mean	Minimun	Maximun	Standard Deviation
HCW (Kg)	345.1	299.4	383.7	27.3
KPH (%)	2.1	1.5	3.0	0.5
12th Rib Fat (cm)	1.1	0.4	2.0	0.4
REA (cm^2)	82.4	71.6	90.3	5.9
USDA Yield Grade	2.9	2.2	4.2	0.7
Marbling score[1]	418.0	320.0	520.0	56.7
USDA Quality Grade[2]	708.0	617.0	783.0	45.2

[1]100 = Practically Devoid; 300 = Slight; 500 = Modest and 700 = Slightly Abundant.
[2]100 = Canner; 400 = Commercial; 600 = Select and 800 = Prime.

3.3. Results and Discussion

3.3.1. Traditional Assay

Cattle in this study were representative (Table 3) of those found in industry when we found this study carcasses grade to be an average Choice USDA grade, which fits in the US beef industry as found on the last National Beef Quality Audit (2011, Moore et al., 2012). The calpastatin activity traditional assay results (Table 4) for day 0 are in accordance with data found in previous research (Wulf et al., 1996; Shackelford et al. 1994). Wulf et al. (1996) reported mean calpastatin activities of 2.58 and Shackelford et al. (1994) reported meat calpastatin activities of 2.8 at 0 hr which is comparable to the 2.6 reported in this study even though there were much higher numbers of samples in these two papers, 114 and 555 respectively as compared to only 12 in the current research. Calpastatin had a reduction in activity within the 6 month period while stored under refrigeration, as expected, ranging from 1.7 to 3.3 on day 0; 0.7 to 1.5 on day 90 and 0.1 to 0.8 on day 180 (Table 4). This reduction was expected and is in agreement with other studies where it has been shown that calpastatin degrades over time of storage due to autolysis (Koohmaraie, 1995; Boehm et al., 1998; Kristensen et al., 2006). The correlation between day 0 and 90 was 77%, 49% between day 0 and 180 and 70% between day 0 and day 180 for the traditional assay (Table 5). The variation of calpastatin activity within samples might be explained by the fact that animals used in this study encompassed a variety of genetics. It is well known that calpastatin levels vary between species (Koohmaraie et al., 1991), different breeds within species (Shackelford et al., 1994; 1991), and between various muscles (Koohmaraie, 1988; Lorenzen et al., 2000).

Table 4

Means for Calpastatin Activity

Days	Mean	Min	Max	SD[1]	SE[2]
0	2.6	1.7	3.3	0.5	0.14
90	1.1	0.7	1.5	0.2	0.07
180	0.5	0.1	0.8	0.2	0.05

[1]SD = Standard deviation

[2]SE = Standard error

Table 5
Correlation between days for calpastatin activity

Days	0	90	180
0	1		
90	0.77	1	
180	0.49	0.70	1

3.3.2 Western Blot

Protein concentration found in the protein assay was as low as 0.1 µL/mL for day 0 samples, 0.04 µL/mL for day 90 samples and as low as 0.07 µL/mL for day 180, what was not expected and is in agreement with the SDS gel electrophoresis images showed on Figure 2, which includes the protein check gels. As seen, not much protein was found as almost all samples ran out of the gel.

As shown in the Figure 3 to Figure 6 there were no coherent results found as nothing could be seen in our gels, meaning that amount of protein (calpastatin) was too little, thus no immunological binding could occur.

It is hard to find a possible and plausible answer for why we had these results, since calpastatin is resistant to many denaturing conditions, even really low pH (Goll et al., 2003). Three possible explanations for the fact are that our calpastatin samples in this study lost their initial activity or were harmed in a way that made them lose their ability to act as the enzyme were found. One is that the decision of pooling out partially active fraction samples and mixing them with their respective active fraction samples made the protein (enzyme) content too diluted.There is also a concern about the NaCl used to wash off the unwanted protein and impurities out of the columns, which might have hurt or diminished calpastatin present in the samples or have worked against the ionic strength for calpastatin activity, even though literature is found about calpastatin being resistant to high salt concentration and we used the concentration recommended by the methodology (200 mM NaCl). The freezing step conducted in order to stop the decrease of activity in samples until the ELISA and Western Blot reading could be performed is although our

most likely explanation for these results with the additional too diluted samples. Ice crystals could have formed when freezing and might have the enzyme (protein)

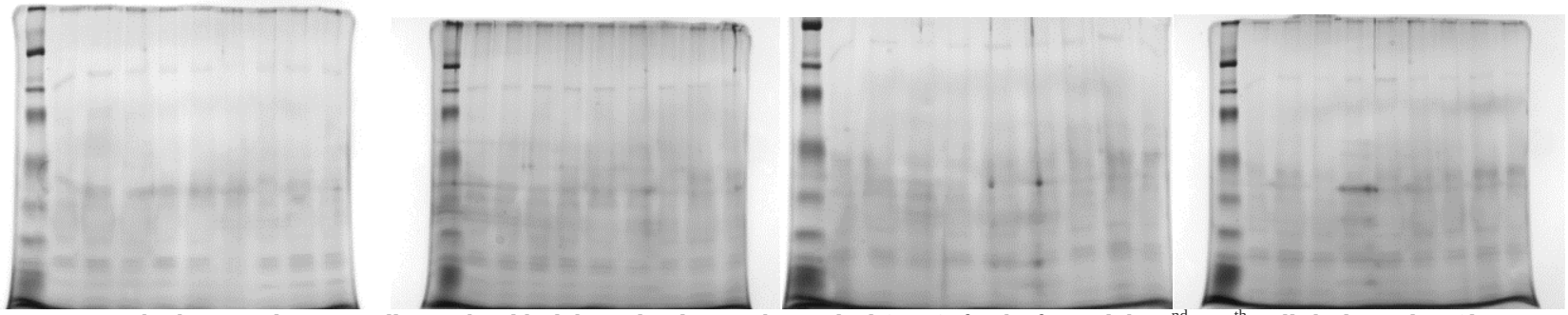

Fig. 2. Protein check SDS gels**.** First well in each gel had the molecular weight standard (BSA), for the first gel the 2nd to 4th wells had sample 1 (days 0, 90 and 180); wells 5th to 7th had sample 2 (days 0, 90 and 180) and wells 8th to 10th had sample 3 (days 0, 90 and 180). Same pattern is followed in the other gels. Gel 2 has samples 4 to 6, gel 3 has samples 7 to 9 and gel 4 has samples 10 to 12.

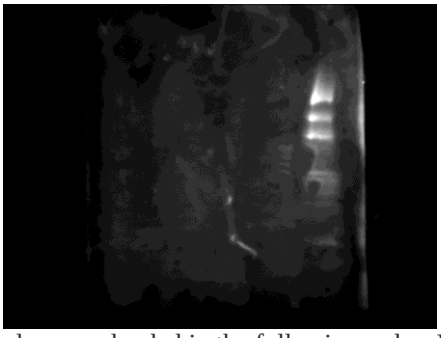

Fig. 3. Western Blot gel where samples were loaded in the following order: Molecular Weight Standard; Sample 9 (days 0, 90 and 180); Sample 10 (days 0, 90 and 180); Day 0 beef calpastatin; Rabbit heart extract.

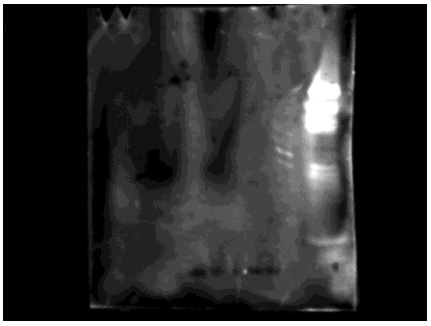

Fig. 4. Western Blot gel where samples were loaded in the following order: Molecular weight standard; Sample 11 (days 0, 90 and 180); Sample 12 (days 0, 90 and 180); Day 0 beef calpastatin; Rabbit heart extract.

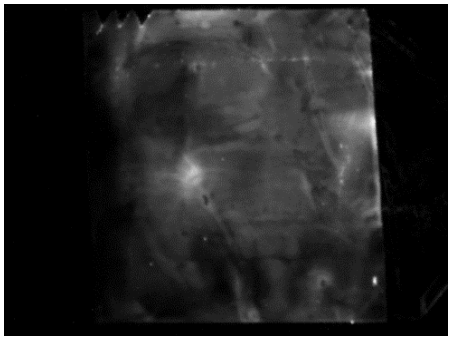

Fig. 5. Western Blot gel where samples were loaded in the following order: Day 0 samples 1 to 6; Day 0 Beef Calpastatin; Rabbit heart extract.

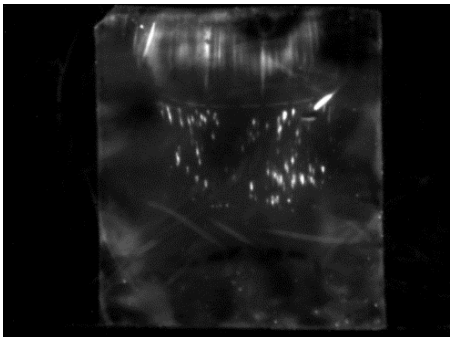

Fig. 6. Western Blot gel where samples were loaded in the following order: Day 0 samples 7 to 12; day 0 beef calpastatin..

structure, preventing a satisfactory enzyme-antibody interaction to occur in a measurable scale.Literature is still controversial when discussing the impact of freezing calpastatin and meat samples. Koohmaraie (1990) stated that calpastatin can be inactivated by freezing meat samples. Duckett et al. (1998) also found a reduction of 44% from day 0 to day 42 when samples were frozen, on the other hand Tantos et al. (2009) results affirm that intrinsically disordered proteins, including calpastatin, are resistant to cold treatments, such as freezing. Second, but less likely to be the explanation, is the NaCl solution used to wash off calpastatin from the columns altered the desired ionic strength causing damages to the protein structure even though papers like Camou et al. (2007) affirm calpastatin can be eluted from the column over a wide salt range. However µ-calpain looses activity if exposed to salt concentrations above 150 mM. So, it is less likely NaCl would be the reason for the lack of results, since normal calpastatin activity was determined by the traditional method, and the same calpastatin extracted sample was used for Western Blot and ELISA. However, it is found in literature discussions about oxidation changing calpastatin activity under varying envirolmental conditions. Carlin et al. (2006) results suggest for example that at a higher pH condition calpastatin may limit the possibility of oxidation induced inactivation of µ-calpain.

3.3.3. ELISA

The ELISA results showed a really small or even insignificant amount of protein, which did not allow any possible assessment of the calpastatin activity in our samples. As discussed previously ice crystals formed when we froze the samples is most likely reason for these results.

3.4. Conclusions

Meat tenderness is highly variable and it is extremely important to the meat industry since it is an important factor determining consumers choice. The beef industry seeks the ability to predict meat tenderness based on a faster, accurate method which could reduce cost and storage time required for meat conditioning. Therefore factors affecting meat tenderness and its development need to be better understood not only in order to improve consumer satisfaction but also in order to reduce waste involved in unacceptable quality carcasses. Calpastatin enzyme activity as an inhibitor of calpain has been demonstrated to have a great involvement in tenderness development. Thus, it is vital to understand the mechanisms and rate of calpain/calpastatin proteolytic system as it effects meat tenderness so that potential interventions, genetic selection, quality grading and more scientific research can be used to improve meat quality. Our study goals were met hence we were not able to get workable results from it. More research is necessary in order to be able to compare these methods and answer the question about calpastatin degradation over a long period of time, whereas its activity rate and quantity are diminished.

Chapter IV

4. Sarcomere Length

4.1. Introduction

The palability of meat products can greatly impact consumers decision when purchasing a product (Savell et al., 1987). Tenderness is ranked as one of the most important attributes by consumers and several studies have shown their wiliness to pay for a guaranteed tender meat (Boleman et al., 1997). The relationship between sarcomere length and tenderness has been well-established as classic scientific work have demonstrated that muscles with longer sarcomere lengths have lower resistance to shear force (Marsh and Carse, 1974; Herring et al., 1965), thus being more tender. Wheeler and Koohmaraie (1999) discussed longissimus tenderness as being largely dependent on the extent of rigor shortening of sarcomeres and postmortem proteolysis during aging, however the mechanism is unclear. Sarcomeres are the smallest unit of contraction in a muscle. Muscles are composed of tubular cells called myofibers, which are composed of tubular myofibrils (Figure 7). Myofibrils are composed of repeating sections of sarcomeres (Figure 7). Sarcomere structure stays within two Z-lines, which appear under the microscope as dark and light bands. Sarcomeres are composed of long, fibrous proteins (actin and myosin) that slide past each other when the muscles contract and relax (Rayment et al., 1993). The location of strong diffraction intensity maxima can be analyzed in a straightforward manner to provide the measurement of the average sarcomere length. Average sarcomere length of the muscle fibers varies considerably from animal to animal and muscle to muscle. Different meat species have different sarcomere length for *Longissimus dorsi* muscle fibers (DeVol et al., 1988),

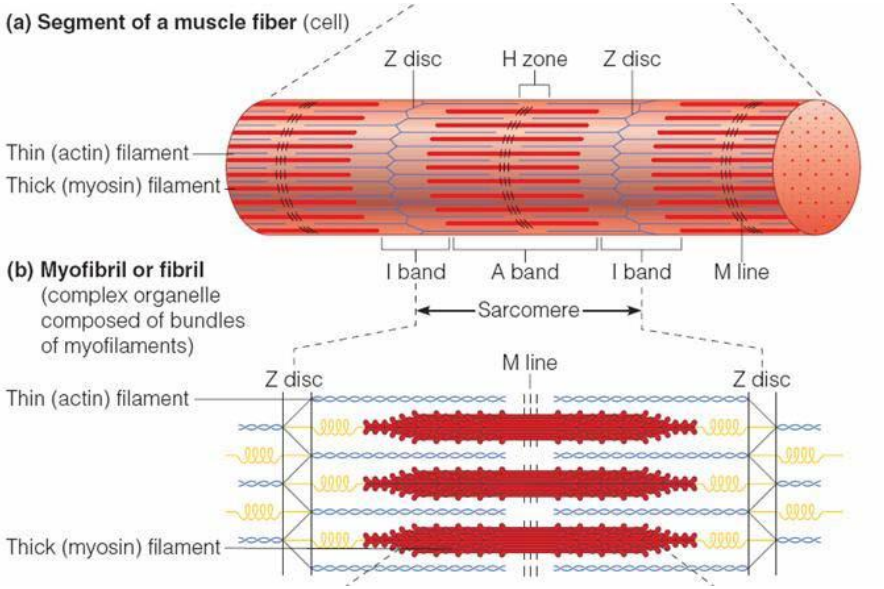

Fig. 7. The schematic representation of a sarcomere unit from the muscle fibre. (a) myofibril segment (b) the ultra-structure of the sarcomere of the a myofibril. Adapted from Seeley et al. (2002).

Sarcomere lengths are also affected during rigor mortis. Wheeler and Koohmaraie (1994) indicate the importance of sarcomere length to tenderness before extensive proteolysis and the decreased importance after proteolysis when finding that although shear force decreased to one-half from 24 to 72 h postmortem, sarcomere length did not change significantly while during the first 24 h postmortem, shear force increased 71% whereas sarcomere length decreased 25%. The objective of this study was to access the effectiveness of three different sample preparation methods in determining sarcomere length in livestock species.

4.2. Materials and Methods

In our study the sarcomere lengths were measured by light diffraction with a Helium-Neon laser, as Cross et al. (1981) described. Ten 7.5 g *Longissimus dorsi* samples from each specie: beef, pork and lamb were prepared using three different preparation methods: fresh, frozen conventionally (frozen), and frozen in liquid nitrogen and powdered (powdered). Beef and Lamb carcasses were aged for two weeks while pork was 2 days aged. Frozen and fresh 7.5 g samples were then homogenized in 50 mL of a 0.25 M sucrose, 0.002 M potassium chloride, and 0.005 M sodium iodoacetate solution at pH 7.0. Powdered samples were given only a drop of the same homogenization solution, applied on them when already in the glass slide. For more detailed steps and descriptive of the solutions see Appendix IV.

4.2.1. Statistical analysis

All statistical procedures were performed using SAS (SAS Inst. Inc., Cary, NC), code is included on appendix. Correlations were generated using PROC MIXED for

species (sp) beef, pork, lamb and for preparation method (trt), and specie and preparation method interaction. Code used is available in Appendix V.

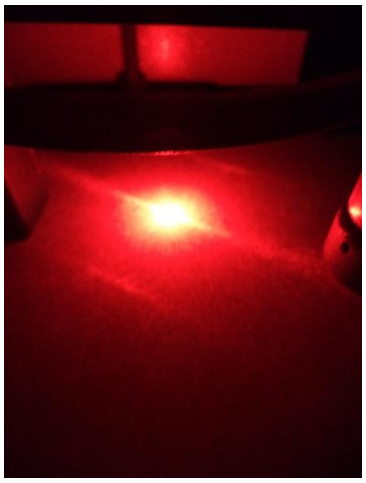

Fig. 8. Sarcomere measured by laser light difraction

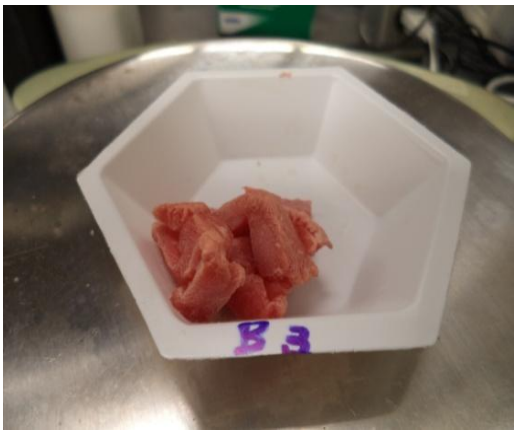

Fig. 9. Frozen pork sample

Fig. 10. Frozen in N_2 and powdered pork samples

4.3. Results and Discussion

Sarcomere lengths results for lamb and beef were in accordance with previous research where Wheeler and Koohmaraie (1999) reported lamb *Longissimus dorsi* sarcomeres ranged from 1.36 µm ± 0.04 µm to 1.67 ± 0.04 µm; Bendall and Voyle (1967) reported beef sarcomere length to be between 1.30 and 1.50 µm; however sarcomere length reading for pork samples were a little smaller than expected as we see when comparing to the literature as DeVol et al. (1988) finds a range of 1.66 to 2.00 µm and Wheeler et al. (2002) found a mean of 1.69 µm for pork sarcomere length. Statistical analysis showed that there is difference in sarcomere length within species, as expected. No difference was found ($P > 0.05$) within sample preparation methods other than for fresh and frozen beef samples (Table 6). This difference might be due to the fact to aging periods, as beef was aged for longer periods, allowing myofibrillar structure of sarcomere (Z lines) to be broken down by the proteolysis mechanism. Also other things might have influences, as in older animals the presence of more connective tissue and lowered tissue solubility after puberty can be expected to have an effect on sarcomere length. Differences on sarcomeres from frozen and fresh beef sample could be caused by the shortening of the muscle fiber when freezing meat samples. There is not much literature comparing preparation methods, however an important result was found as there was no differences between the three. The powdering method is unique since casual observations during this study showed the strength of the band for this method as being stronger and easier to visualize. The cost for powdered preparation method is much lower than for the other two considering it only takes a drop of the homogenization solution, against 50 mL for the other methods. The diminished amount used for powdering samples is also positive as

we need much less homogenization solution that contains sodium iodoacetate, a chemical hazard. Casual observations also found harder to visually find sarcomeres using light diffraction when samples were homogenized and some foam was formed. Frozen and fresh did differ much as far as the ability and facility to find the sarcomeres. As an overall casual observation, powdering preparation method seemed to have a considerable advantage against the other two methods used in this study.

Table 6

Significance Test of Fixed Effects for specie, treatment and specie/treatment interaction

	Pr> F
Specie[1]	<.0001
Treatment[2]	0.4715
Sp[1]*Trt[2]	0.1165

[1]Sp = Specie: beef, pork and lamb

[2]Trt = Treatment: fresh; frozen; frozen and powdered samples

($P < 0.05$)

Table 7

Least Square Mean Estimates of Sarcomere Length for Species, Treatment and Specie/treatment interaction

Species	Treatments			SE[1]	LSD[2]
	Fresh	Frozen	Powder	0.02092*	0.042*
Beef	1.46[a]	1.41[b]	1.44[a]		
Lamb	1.32[c]	1.33[c]	1.31[c]	0.02267**	0.045**
Pork	1.48[d]	1.49[d]	1.46[d]		

[1]SE = Standard Error

[2]LSD = Least Square Differences

* = within treatment ; ** = within species

[a,b,c,d]Means lacking a common superscript differ ($P < 0.05$)

4.4. Conclusions

Any of the methods used for sample preparation used in this experiment can be used as far as results are concerned, but laboratory work, safety, time and expenses can be diminushed by using the powdering method to prepare muscle samples for sarcomere length measurement.

Appendix I

HEATED CALPASTATIN EXTRACTION

Solutions:

Pre-rigor Extraction Buffer, 4°C, pH 8.3 (2 liters)
50 mM Tris (CAS 77-86-1) 12.11 g
10 mM EDTA (CAS 6381-92-6) 7.44 g

Adjust pH to 8.3 at 4°C with 6N HCl; qs to 2 liters. Store at 4°C. Day of use add 1 mL MCE (CAS 60-24-2) and inhibitors (except PMSF).

Post-rigor Extraction Buffer, 4°C, pH 8.3 (2 liters)
100 mM Tris (CAS 77-86-1) 24.22 g
10 mM EDTA (CAS 6381-92-6) 7.44 g

Adjust pH to 8.3 at 4°C with 6N HCl; qs to 2 liters. Store at 4°C. Day of use add 1 mL MCE (CAS 60-24-2) and inhibitors (except PMSF).

Inhibitors
100 mg/liter Ovomucoid (CAS 9035-81-8)
6 mg/liter Leupeptin (or 1 mg/liter E-64) (CAS 103476-89-7)
2 mM PMSF (CAS 329-98-6) (add to blender just before homogenizing; PMSF falls out of solution at 4°C)

20X Elution Buffer, 4°C, pH 7.35 (2 liters)
40 mM Tris (CAS 77-86-1) 192.00 g
0.5 mM EDTA (CAS 6381-92-6) 7.44 g
Adjust pH to 7.35 at 4°C; qs to 2 liters.
To make 1X: 100 mL 20X; qs to 2 liters.

100 mM CaCl2 (CAS 10035-04-8)
7.35 g/500 mL of deionized water. Store at 4°C.

50% TCA (Trichloroacetic acid) (CAS 76-03-9, RCRA D002)
1 kg TCA in 2 liters of water. Store at 4°C.
To make 5% TCA: dilute 50% stock 1:10 (10 mL stock + 90 mL water). Make fresh daily.

Casein Assay Media, pH 7.5 (2 liters)
100 mM Tris (CAS 77-86-1) 24.22 g
1 mM NaN3(CAS 26628-22-8, RCRA P105) 0.13 g or 2.0 mL from 1 M stock
7 mg/mL Casein (CAS 9000-71-9) 14.0 g
Dissolve Tris and NaN3 in 2 liter volumetric. Bring up to volume. Remove 130 mL of buffer and throw away. Transfer remaining buffer into a large mouth Erlenmeyer flask. Gradually add Casein-Hammerstein. Allow stirring for 1 to 2 hr after all the Casein is added. Using a 50 mL syringe fitted with a 14-gauge needle, slowly drip in 130 mL of 1N acetic acid (CAS 64-19-7, RCRA D001, D002). Once all the acetic acid is added, stir for 1 to 2 hr. Check the pH and adjust, if necessary, to 7.5 with 1N acetic acid. Store at 4°C. Add 4 µl/mL MCE (CAS 60-24-2) just before use. Make a fresh batch of working stock (media + MCE) daily.

200mM EDTA + 1mM NaN3 Neutralized (1L)
EDTA 74.44 g
1M NaN3 1.0 mL (65 g/L = 0.65 g/10 mL)

Dissolve in dd H20, it is hard to get this into solution so you may need to use heat. Neutralize to a pH of 7.0 with 1N NaOH. This solution has very little buffering ability. q.s. to 1L with dd H2O.

Sample Extraction and Heating:

- Use a 50 mL conical tube and a polytron 3 x 30 seconds with a 30 seconds. rest between each homogenation. Place the tubes on ice.
- Extract 5 g of longissimus dorsi tissue in 25 mL Pre-Rigor Extraction Buffer (with inhibitors added).
- Add 250 µL PMSF just before homogenation.
- Pour homogenate into 35 mL centrifuge tubes.
- Rinse the conical with 5 mL of Pre-Rigor Extraction Buffer (with inhibitors added) and add that to the corresponding centrifuge tube to bring the total volume to 30 mL of buffer and 5 g of sample.
- Using the Beckman JA-17 rotor, centrifuge samples at 16,500 rpm at 4°C for 1 ½hr.
- Prepare dialysis tubing while centrifuging. Use SpectraPor® Membrane MWCO: 12,000 to14,000 (Spectrum Medical Industries, Inc. Laguna Hills, CA), cut into 10 to 12 inch lengths, soak in cold deionized water. Make sure entire membrane is permeated with water before use.
- Prepare dialysis buffer (1X Elution Buffer) in dialysis container. Use one part sample to 40 parts buffer for dialysis, (make sure to have enough cold dd H_20).
- After centrifugation, filter supernate through a cheesecloth/glass wool sandwich.
- Dialyze against 1X elution buffer for approx. 18 hr at 4°C. Change buffer two times in the 18-hr period. (6 hr interval)
- After dialysis, transfer samples into centrifuge tubes and place into a 95°C water bath. Once the supernate reaches 95°C, heat the sample an additional 10 min. During the heating process, stir the coagulated protein with a glass stir rod every 5 min.
- Place tubes in an ice bath and chill tubes for at least 15 min.
- Centrifuge samples at 16,500 rpm for 30 min.
- Pass supernate through glass wool into a beaker. Samples can either be loaded on the columns or stored at 4°C in conicals until ready for columns.
- Record volume.

Column Packing & Regeneration:

Packing:

- Use column size 14 cm high, 1.5 x 12 cm polypropylene econo-pack columns (BioRad). The DEAE Sephacel resin is stored in 20% ethanol.
- To remove the ethanol and prepare the resin, wash the appropriate volume with a high Tris solution (1X + 20 g Tris => 2 liters). Wash the column with 200 mL of high Tris and then with 1X-MCE. When the effluent reaches a pH of ~7.5, the column is ready for use.
- Pack the columns with DEAE-Sephacel resin. Attach a funnel to the top of the column. Using a glass rod, stir the resin and slowly pour the slurry down a glass rod held against the wall of the funnel. This will help minimize the introduction of air bubbles. Allow the 1X to flow through the column and add more resin as needed to achieve a 10 mL total bed volume. Once the 10 mL volume is met, fill the remainder of the column with 1X to keep the resin from drying out. Cap and plug the columns until ready for use.

Regeneration:

- After the completion of the elution process, the column must be regenerated for th next extraction. To regenerate the column, wash it with 1X + 1M NaCl until no A278 is detected in the outflow (about two column volumes).
- Make sure the performance of the column is closely monitored. Since phosphate buffer is not used, the chance of microbial growth is low, assuming the quality of water is high. However, periodically wash the column with 20% ethanol to prevent microbial growth.

- If no samples are to be eluted for quite some time, the resin may be removed from the columns. The resin may be regenerated in a beaker and a Buchner funnel is used to remove the aqueous portion. Do not add MCE to the elution buffer. It is important that the resin is never allowed to dry out. Store the regenerated resin in 20% ethanol at 4°C.

Sample Elution:

- Load supernate on the column by pouring it on and allowing it to drip through the resin.
- Remove unbound proteins by washing the column with 100 mL 1X Elutio Buffer + 25 mM NaCl. While washing, label sample collection tubes 1 to 10 for each sample and place into a rack.
- Place a rack under each column in the 1 position and elute bound proteins with 1X Elution Buffer + 200 mM NaCl. Collect 10, 5 mL fractions, moving the rack down one position after each 5 mL fraction.

Salt Solutions: NaCl mw = 58.44, 1M = 58.44 g/l
1 mM = 0.05844 g/l, 25 mM = 1.46 g/l
150 mM = 8.77 g/l, 200 mM = 11.68 g/l
350 mM = 20.44 g/l, 400 mM = 23.36 g/l

Assay:

Localization of calpastatin activity:

*If no activity is present, the tubes will be cloudy.

- Assay all fractions in a single tube/fraction while blanks and + calpain are assayed in triplicate. Net activity of m-Calpain (+CDP) should be 0.30 to 0.40
- To each tube add, (in order):
  - ✓ 0.5mL fraction (or 1X for blanks & +CDP tubes)
  - ✓ 0.5 mL 1X (vortex)
  - ✓ Appropriate amount of m-calpain for activity of 0.30 to 0.40 (except blank)
  - ✓ mL assay media (with MCE just before use)
  - ✓ 100 µl 100 mM CaCl2 (vortex again)
- Mix and incubate at 25°C for 30 minutes. Tubes that are clear represent activity.
- Record the visual positive activity.

Pooling Fractions:

- Pool active fractions. Record total volume of pooled fractions (add 0.5 mL for the amount used in screening).
- Reassay (0.1 - 0.5 mL aliquots) Use 1X elution buffer to have sample equal 1 mL total fraction volume. Do ALL assays in triplicate (NOT THE EDTA).
- To each tube add, (in order):
  - ✓ 0.1 to 0.5 mL pooled fraction
  - ✓ 0.9 to 0.5 mL 1X (also 1 mL for Blanks & +CDP) (vortex)
  - ✓ Appropriate amount of m-calpain for activity of 0.30 to 0.40 (except blank)
  - ✓ 1 mL assay media (with MCE just before use)
  - ✓ 100 µl 100 mM $CaCl_2$ (tubes in triplicate) or 100 µl of EDTA (one single tube/dilution) (vortex again)
- Mix and incubate at 25°C for 1 hr.
- Stop reaction with 2 mL of 5% TCA. Mix.
- Centrifuge at 1500 to 2000 x g for 30 minutes at 4°C.
- Read absorbance at 278 nm.

Calculations: Total Calpastatin Activity =(((avg A278 positives – avg A278 blanks) – (avg A278 sample for each dilution – (A278 EDTA for each dilution – avg A278 blanks))) ÷ aliquot vol, mL) x (total vol of pooled fractions, mL ÷ grams muscle extracted).

Appendix II

ELISA

A bovine calpastatin (CAST) ELISA kit (Biosource CAT # MBS932589) was used and procedures described on it were followed. The principle of this ELISA kit is the quantitative sandwich enzyme immunoassay technique, where "antibody specific for calpastatin has been pre-coated onto a microplate.

Other supplies required:

- ✓ Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- ✓ An incubator which can provide stable incubation conditions up to 37^{o}C ±0.5^{o}C.
- ✓ Squirt bottle, manifold dispenser, or automated microplate washer.
- ✓ Absorbent paper for blotting the microplate.
- ✓ 100 mL and 500 mL graduated cylinders.
- ✓ Deionized or distilled water.
- ✓ Pipettes and pipette tips.
- ✓ Test tubes for dilution.

Assay:

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. It is recommended that all samples and standards be assayed in duplicate.

- Prepare all reagents, working standards, and samples as directed in the previous sections.
- Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.
- Add 100µl of standard and sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at 37°C. A plate layout is provided to record standards and samples assayed.
- Remove the liquid of each well, don't wash.
- Add 100µl of Biotin-antibody (1x) to each well. Cover with a new
- adhesive strip. Incubate for 1 hour at 37°C. (Biotin-antibody (1x) may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.)
- Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 2 minutes, complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash Buffer by aspirating ordecanting. Invert the plate and blot it against clean paper towels.
- Add 100µl of HRP-avidin (1x) to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C.
- Repeat the aspiration/wash process for five times as in step 6.
- Add 90µl of TMB Substrate to each well. Incubate for 15-30 minutes at 37°C. Protect from light.
- Add 50µl of Stop Solution to each well, gently tap the plate to ensure thorough mixing.
- Determine the optical density of each well within 5 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. Subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Appendix III

WESTERN BLOT

Protein concentration determination:

(DC - Bio-Rad protein assay Method)

- Make two protein sample dilution for every sample:
  - Dilution ratio: 10µL protein sample
    190µL DDI-H_20
- Vortex solutions
- Perform DC Assay as follows (using a well plate):
  Each well receives:
  - ✓ 5µL of either sample or standards
  - ✓ 25 µL of reagent A
  - ✓ 200µL of reagent B
- Let assay plate set for 15minutes before running in the spectrophotometer.

Preparation for SDS-PAGE:

- Decide what the concentration of the gel sample will be (i.e.5mg/mL)
- Calculate the amount of sample needed:

$$\frac{\text{(desired concentration) (total volume)}}{\text{(actual concentration)}} = \text{ml sample needed}$$

1 - mL sample = mL water needed

- Dilute samples to e.g. 6.4mg/mL with distilled deionized water.

Under hood:

- Combine samples with 0.5 mL of Wang's tracking dye and buffer
- Add 0.1 mL MCE (2-Mercaptoethanol) (CAS 60-24-2)

Makes a total of 1.6mL solution. Heat to 50^oC for 10-20minutes and load onto gels or freeze immediately.

Solutions:

Wang's Tracking Dye and Buffer (100mL)

Tris (30mM) (CAS 77-86-1) 3.6g
EDTA (3mM) (CAS 6381-92-6) 88mg
SDS (3%) (CAS 151-21-3) 3g

- Dissolve in 50mL DDI H_2O, adjust pH to 8.0 with HCl, then add:

Glycerol (30%) (CAS 56-81-5) 30g
Pyronin Y (0.001%) (CAS 92-32-0) 3mg *CARCINOGEN*

- Add DDI H_2O to 100mL

Acrilamide

Acrylamide (CAS 79-06-1) 30g

- Dissolved in DDI H_2O (50 mL)
  - 37:1 = 0.8g bis
  - 50:1 = 0.6g bis
  - 100:1 – 0.3g bis
- Stir til dissolved, fill to 100mL (put into beaker), add chunk charcoal and stir, filter through Whatman 541 or 542 filter paper (fast) into a dark bottle, store in refrigerator.

*Wear a dust mask, lab coat, double gloves and perform all operations under the hood.

20% SDS

20% SDS (CAS 151-21-3) in DDI H_2O

*Use dust mask

BioRad Gels

Stock Solutions:

*Store refrigerated in a brown plastic bottle

2M Tris-HCl (pH 8.8) 100mL

Tris (CAS 77-86-1) 24.2g

- Add to 50mL DDI H_2O
- Add concentrated HCl slowly to pH 8.8 (about 4mL)
- Add DDI H_2O to a total of 100mL

1M Tris-HCl (pH 6.8) 100mL

Tris (CAS 77-86-1) 12.1g

- Add to 50mL DDI H_2O
- Add concentrated HCl slowly to pH 6.8 (about 8mL)
- Add DDI H_2O to a total of 100mL .

Working Solutions:

*Store refrigerated in a brown plastic bottle. Stable for months in the refrigerator.

4X Separating Gel Buffe (100mL)

2M Tris (CAS 77-86-1) 75mL

- pH to 8.8 with HCl

20%SDS (CAS 151-21-3) 2mL

DDI H_2O 23mL

4X Stacking Gel Buffer (100mL)

1M Tris (CAS 77-86-1) 50mL

- pH to 6.8 with HCl

20% SDS (CAS 151-21-3) 2mL

DDI H_2O 48mL

Amounts of working solutions to use:

	10 % gel	**15% gel**
Acrylamide/ Bis solution	3.33mL	5 mL
4X Separating Gel Buffer (pH 8.8)	2.5 mL	2.5 mL
Distilled water	4.17 mL	2.5 mL
10% Ammonium Persulfate (CAS 7727-54-0)	50 µL	50 µL
TEMED (CAS 110-18-9)	5 µL	5 µL
Total Volume	10 mL	10 mL

Calculation for X% Separating Gel :

For 4 gels for the BioRad System you need to make a total volume of 40 mL

- Gently layer the top of each gel with distilled water (use the same amount for each gel)
- Allow all the gels to set for a minimum of 90 minutes before pouring the stacking gel

Stacking Gel (4 gels)

*It will have excess gel

Distilled water	9.2 mL
Acrylamide/Bis Solution	2.68 mL
4X Stacking Gel Buffer (pH 6.8)	4.0 mL
10% Ammonium Persufate	120 µL
TEMED (CAS 110-18-9)	20 µL

- First pour off the water on top of the separating gel.
- Insert the combs as soon as possible after pouring the stacking gel.
- Allow the gels to set for a minimum of 90 minutes before running.

Immunoblotting (Western Blotting)

Buffers:

10X Transfer Buffer (1L)

Tris (CAS 77-86-1) 30g
Glycine (CAS 56-40-6) 140g

- Add the above distilled, deionized water to make 1 liter.

1X Transfer Buffer (1L)

10X Transfer Buffer 100 mL
Methanol (CAS 67-56-1) 150 mL

- Fill to 1 liter and store in the cooler (cold room), prepare this buffer the night before as this buffer needs to be cold when used.

Phosphate Buffered Saline (PBS) pH 7.5 -- 1X Solution

Di-sodium hydrogen phosphate anhydrous [Na_2HPO_4] (80mM) (CAS 7558-79-4) 11.5g
Sodium dihydrogen phosphate [NaH_2PO_4] (20mM) (CAS 10049-21-5) 2.96g
Sodium chloride (CAS 7647-14-5) 5.84g

- Dilute to 1000mL with distilled water – Check pH

(in most cases make up a 5X solution of PBS instead – Recipe below)

5X PBS

Na_2HPO_4 57.5g
NaH_2PO_4 14.8g
Sodium chloride 29.2g

- Dilute to 1000mL with distilled water
- Before using dilute by 5 to make 1X working solution

PBS-Tween

Tween-20 (CAS 9005-64-5) 1 mL
1X solution of PBS 1L

Blocking Solution (100 mL)

Nonfat dry milk (NFDM) 5g
1X PBS-Tween 100mL

Preparing PVDF membranes for transfer:*

*Always wear clean gloves when handling PVDF membranes – this is to protect the membranes from contaminating protein, PVDF has a high affinity for protein, fingers and dirty gloves provide an excellent medium for contaminating protein).

- After cutting the membrane to the desired size, briefly wet the membrane in 100% methanol, then allow it to equilibrate in transfer buffer for a minimum of 5 minutes prior to putting together the membrane/gel sandwich for transfer.

Wash Buffer for myofibrils (Tris buffer) (2 L):

5mM Tris (CAS 77-86-1) 1.21g

- Dissolved in 1L DDI H_2O
- pH to 8.0 with HCl,
- Fill to 2L with DDI H_2O and store in cooler (cold room)

BSA

10.4% BSA (BioRad Cat#161-0318) 10.4g/100mL = 104mg/mL

- Use 10mL/100mL DDI H_2O to get 10.4mg/mL needed

1N Acetic acid

Acetic acid, Glacial (CAS 64-19-7) 57.4mL

- Fill to 1L with DDI H_2O

Tris-Glycine Buffer

- Dilute by 10 when running gel 100mL/L

e.g. For 6L:
Tris (CAS 77-86-1) 181.25g
Glycine (CAS 56-40-6) 864g
SDS (CAS 151-21-3) 60g

- Add EDTA amount 2mM,
- Fill to 6L with DDI H_2O

STAIN

Commassie Brilliant Blue R250 (CAS 6104-59-2) /100mL destain 0.1g

- Stain overnight

DESTAIN (2L)

7% Acetic acid	140mL
40% MeOH (CAS 67-56-1)	800mL
DDI H_2O	1060mL

- Destain minimum 6 hours changing every 2 hours

Supplies brands:

- FASTSilver ™ (Biosciences Cat# 786-30) used for Staining on SDS Page Gels
- Calpastatin Antibody (Thermo Scientific 2G11D6)
- Calpastatin (H-300): sc-20779 (Santa Cruz Biotechnology)
- Bovine Serum Albumine- BSA (BioRad Cat#161-0318)

Appendix IV

SARCOMERE LENGTH

Homogenization Solution:

Sucrose (CAS 57-50-1) (MW –342.3) 0.25 M
KCL (CAS 7447-40-7) (FW – 74.5) 0.002 M
Iodocetate (CAS 305-53-3) (MW – 207.9) 0.005 M
pH to 7.0 with KOH and acetic acid

Supplies:

- ✓ 50mL stainless steel Waring blender cup
- ✓ Glass stir rod
- ✓ Slide coverslip
- ✓ Helium-Neon Laser

Procedures:

- Approximately 7.5g of cubed (5-7mm) frozen/chilled tissue into 50mL of cold (4^{o}C) homogenization solution and homogenize on high for 10-15 sec.

- Place a drop of homogenate on a slide, cover with a coverslip and place the slide on the stage of laser stand, (distance from the top of the slide to base board of laser stand is set a 100mm). Place a piece of paper on the base board.

- Start at one edge of slide and move it past the laser light until a diffraction pattern Is seen on the base. Mark the origin and the first order diffraction band for 5 sarcomeres.

- Measure the distances and calculate the sarcomere length by the formula:

$$\text{Sarcomer length } (\mu m) = \frac{[632.8 \text{ X } 10\text{-}3 \text{ X D X } \sqrt{(T/D)2} + 1]/T}{}$$

D= distance in mm from top of slide to base board (100mm)
T= distance in mm from the origin to the first order band
632.8= wavelength of Helium-Neon Laser

Appendix V

STATISTICAL ANALYSIS

SAS Proc Mixed Analysis Code:

```
options ls=100 ps=70 formdlim ='-';
data beef; infile 'f:\beef.csv' dsd firstobs=1 missover;
input sp$ id trt$ x1-x5;

*proc print;
run;

data pork; infile 'f:\pork.csv' dsd firstobs=1 missover;
input sp$ id trt$ x1-x5;

*proc print;
run;

data lamb; infile 'f:\lamb.csv' dsd firstobs=1 missover;
input sp$ id trt$ x1-x5;

*proc print;
run;
data all; set beef pork lamb;
if id=. then delete;
proc print;

data all2; set all;

sac=x1; prep=1; output;
sac=x2; prep=2; output;
sac=x3; prep=3; output;
sac=x4; prep=4; output;
sac=x5; prep=5; output;
 drop x1-x5;
proc print;

proc mixed; class sp id trt;
model sac= sp|trt;
random id(sp) id(sp trt);
lsmeans sp|trt/pdiff;

run;
```

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