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Characterization of the skeletal muscle calpain/calpastatin system in growth models in swine and cattle

Shannon Michelle Cruzen

Iowa State University

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Characterization of the skeletal muscle calpain/calpastatin system in growth models in swine and cattle

by

Shannon Michelle Cruzen

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Major: Meat Science

Program of Study Committee:
Elisabeth Huff-Lonergan, Major Professor
Steven Lonergan
Philip Dixon
Ted Huiatt
Joseph Sebranek

Iowa State University
Ames, Iowa
2013

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smoothly and provided endless guidance and wisdom. A huge thanks goes to Kyle Grubbs, who has been my colleague, co-coach, and most importantly, one of my best friends through this process. I think we will be shooting ideas off each other for the rest of our lives.

To everyone who has helped me in completing my PhD, this has been an amazing experience, and I am ready to go out and show everyone how great Iowa State University has been. I can only hope to emulate the amazing examples, support systems, and friends that I have had the privilege of interacting with in my time here.
ABSTRACT

The overall objective of the work in this dissertation was to characterize μ-calpain, m-calpain, and calpastatin activities in order to learn more about the system in the live animal as well as its potential effects on postmortem proteolysis. Animal models of efficiency and growth were used to achieve this objective.

The first specific objective was to evaluate muscle protein turnover differences in pigs selected for residual feed intake (RFI). Muscle samples were collected, and insulin signaling, protein synthesis, and specific protein degradation proteins were analyzed for expression and activities in twelve gilts each from low (LRFI) and high (HRFI) RFI lines. LRFI (more efficient) pig muscle had greater calpastatin activity and a lower μ-calpain:calpastatin activity ratio compared to HRFI counterparts (P<0.05). LRFI pig muscle had less 20S proteasome activity (P<0.05). No differences in protein synthesis pathways were observed (P>0.05). Postmortem proteolysis was determined in the longissimus from generation 8 of the LRFI versus HRFI pigs (n=9). Less μ-calpain autolysis and troponin-T degradation were observed at 3 d postmortem in LRFI pigs (P<0.05), indicating slowed postmortem proteolysis during postmortem aging. These data provide significant evidence that less protein degradation occurs in the more efficient LRFI pigs, which may account for a significant portion of their increased efficiency.

The second specific objective was to determine calpain system activity and postmortem proteolysis in three muscles from growing and mature beef cattle (n=6 each). The μ-calpain:total calpastatin activity ratio was lesser in mature animals (P=0.08), suggesting less proteolytic potential compared to the younger animals. Muscles from the
mature group had greater calpastatin activity compared to calves at 6 days postmortem and had less μ-calpain autolysis and protein degradation over time (P<0.01). These data suggest that calpastatin activity in muscle from older animals is more persistent postmortem, partially explaining tougher meat from older animals, even after aging.

The third specific objective was to characterize two calpastatin activity peaks (Calpastatin I and Calpastatin II) detected during anion exchange chromatography. While there is less total activity in the Calpastatin I peak, both calpastatins inhibit μ- and m-calpain similarly. 2D immunoblotting for calpastatin detected anti-calpastatin immunoreactive proteins migrating at 145 and at 60 kDa in both Calpastatin I and Calpastatin II eluate samples. The 145 kDa spots were also identified as calpastatin via mass spectrometry. 2D gels were stained to detect phosphoprotein and total protein abundance. Calpastatin in the Calpastatin II peak stained more intensely with the phosphoprotein stain. We propose that Calpastatin II is more phosphorylated, resulting in its later elution from an anion exchange column. Phosphorylation may be a mechanism to regulate calpastatin inhibitory activity.

This work further elucidates the role that the calpain system plays in muscle growth and development, providing a greater understanding of muscle biology, efficiency, and meat quality.
CHAPTER 1. GENERAL INTRODUCTION

The calpains are a group of calcium-dependent neutral cysteine proteinases. Calpastatin is the specific endogenous inhibitor of the two ubiquitous conventional calpains, \(\mu\)-calpain and m-calpain. The calpain system, specifically \(\mu\)-calpain, m-calpain, and their specific endogenous inhibitor, calpastatin, play a regulatory role in muscle proteolysis in the live animal under normal conditions (Goll et al., 2003). Reduced proteolysis as a result of decreased calpain activity or increased calpastatin activity will result in less net protein turnover, all other factors being equal. This may lead to an animal which is more efficient, grows faster, is more heavily muscled, or simply better maintains its current muscle size (i.e., prevention of atrophy) (Goll et al., 1998). However, although the above achievements may be desirable in the live animal, an unintended consequence may be reduced postmortem proteolysis.

During the postmortem aging of meat, myofibrillar proteolysis results in tenderization. The majority, if not all, of this proteolysis is a result of \(\mu\)-calpain activity and is therefore inhibited by greater levels of calpastatin activity (Geesink et al., 2006; Koohmarai and Geesink, 2006). Thus, selection for more efficient, faster growing, or heavily muscled animals may have the unfortunate consequence of a tough, less desirable final product from these animals. Moreover, differing calpain system activities may also distinguish animals in distinct stages of growth, which may result in enhanced or reduced postmortem proteolysis and tenderization during aging (Northcutt et al. 1998; Ou and Forsberg, 1991; Ou et al. 1991). The overall hypothesis of this dissertation was that the calpain system plays a large role in animal growth and development, which translates to differences in postmortem tenderization. Therefore, the overall objective of this dissertation
was to characterize μ-calpain, m-calpain, and calpastatin activities in order to learn more about the system in the live animal as well as its potential effects on postmortem proteolysis. Different animal models of efficiency and growth were used to achieve this objective.

Efficiency

The world population is expected to reach 9 billion people by the year 2050 (United Nations, 2010). In order to feed this rapidly growing population, it has been estimated that 70 percent more food must be produced by that time (FAO, 2009). Therefore, as animal scientists, a major goal must be to increase production as efficiently and sustainably as possible by that time, while still maintaining quality. One way to increase animal efficiency is through selection based on residual feed intake (RFI). RFI is defined as the difference between an animal’s actual feed intake and their expected feed intake based on factors such as average daily gain and backfat. Therefore, a low RFI animal consumes less feed than expected and is more efficient, and a high RFI animal consumes more feed than expected and is less efficient. Thus, one objective of the current dissertation was to determine whether muscle protein turnover differences exist between low and high RFI pigs, which may account for some of the increased efficiency observed in the low RFI group.

Growth

Another objective of the current dissertation was to examine the differences in the calpain system and postmortem proteolysis in skeletal muscle between growing and mature cattle. It is well established that older, more mature cattle will have tougher meat. This toughness has been attributed primarily to an increase in insoluble collagen (Carmichael and
Lawrie, 1967; Hill, 1966). However, a few studies have provided evidence that postmortem proteolysis is decreased as well (Duarte et al., 2011; Huff-Lonergan et al., 1995; Xiong et al., 2007). It stands to reason that μ-calpain and calpastatin may play a role in the decreased postmortem proteolysis in muscle, but research in this area is lacking. A better understanding of proteolysis in muscle from animals of differing maturity and growth stages may allow for improved selection of animal endpoints, as well as offer ideas for tenderness improvement in meat from older animals.

**Further Characterization**

Finally, during the isolation process necessary for calpastatin activity measurement, a separation of two distinct “peaks” of calpastatin activity during its elution from an anion exchange column has been consistently observed in muscle from both pigs and cattle. The third study in this dissertation analyzes the two calpastatin activity peaks in an effort to determine what differs between them and causes separation during anion exchange chromatography. Possible explanations, as reported by others, include protein degradation (Geesink et al., 1998), alternative splicing (Gaarder et al., 2011; Geesink et al., 1998), or posttranslational modifications, such as phosphorylation (Averna et al., 2001; Pontremoli et al., 1992; Salamino et al., 1997; Salamino et al., 1994). The diversity of calpastatin seen in this dissertation research may be a previously undefined source of variability in the calpain system, explaining differences in postmortem tenderization in some systems. Differences in the calpastatin activity peaks may also be important in growth and protein turnover in the live animal, especially if one calpastatin form is more active or serves another purpose compared to the other.
The calpain system and its role in muscle protein turnover and postmortem tenderization may contribute to differences between groups of animals with differing growth or efficiency characteristics. Additionally, the existence of two separate forms of calpastatin may be an additional source of variability between animals which has not previously been documented. This research sets out to further elucidate the roles of µ-calpain, m-calpain, and the two forms of calpastatin in animal growth, development, and efficiency.

Dissertation Organization

This dissertation is organized into six chapters. The format of Chapters 1, 2, and 6 is in line with the style and form of Journal of Animal Science. Chapter 1 contains a general introduction. Chapter 2 is a review of the literature relevant to the dissertation topics. Chapter 3, titled “Evidence of decreased muscle protein turnover in gilts selected for low residual feed intake,” has been published in the Journal of Animal Science (Cruzen et al. 2013). Chapter 4, titled “Postmortem proteolysis in three muscles from growing and mature beef cattle” was submitted for review to Meat Science. Chapter 5 is titled “Analysis of calpastatin pools separated using anion exchange chromatography” and has been prepared for submission to the Journal of Agricultural and Food Chemistry. Chapter 6 is a general conclusion for the entire dissertation.

Literature Cited


CHAPTER 2. REVIEW OF LITERATURE

Introduction

Tenderness is one of the most important quality attributes consumers desire in meat products, and many are willing to pay a premium for meat that is guaranteed to be tender (Boleman et al., 1997; Platter et al., 2005). Tenderness may be affected by a variety of factors. Intramuscular fat is generally shown to be weakly correlated with tenderness in both beef and pork (DeVol et al., 1988; Gregory et al., 1995; Shackelford et al., 1994; van Laack et al., 2001). Connective tissue has a larger impact on tenderness, but this varies with muscle type or location, as well as age of the animal (Huff and Parrish, 1993). Collagen and elastin are the two primary connective tissue proteins which affect tenderness. Elastin is insoluble under normal conditions, but fortunately is not high in most meat cuts, one major exception being the semitendinosus muscle in beef (Bendall and Bendall, 1967; Rowe, 1986). Collagen, on the other hand may be either heat soluble or insoluble, and varies across muscles due to muscle type and location. Muscles which are more active and therefore subject to more mechanical stress have greater collagen concentration (Purslow, 2005). Additionally, muscles or portions of muscle which are closer to skeletal attachments typically have greater collagen (Ramsbottom et al., 1945). Sarcomere length also has a major impact on tenderness. Muscle with shorter sarcomeres is tougher than that with longer sarcomeres. This toughness is due to a greater number of actomyosin bonds, a more dense structure due to the greater overlap of myosin and actin in the sarcomere, more fibers per cross sectional area caused by lateral shrinkage, and potentially a greater concurrent contraction of collagen fibers (Lepetit et al., 2000; Tornberg, 1996). Sarcomere length is the main driver behind the toughening seen in the first hours postmortem before protein degradation begins to occur, and can be severely
affected by ambient temperature during this time (Koohmaraie et al., 1996; Locker and Hagyard, 1963). Finally, accounting for a relatively large proportion of variation in tenderness, and a major focus of this review, is postmortem proteolysis, the enzymatic degradation of meat proteins during the aging or conditioning period after death. Postmortem proteolysis is a significant driver of meat tenderization. During meat aging several cytoskeletal proteins are degraded, resulting in increased tenderness. Although other endogenous proteolytic enzymes may play a small role, the calpain system, specifically μ-calpain and its specific inhibitor calpastatin, accounts for the large majority of proteolysis (or lack thereof).

Unique to the calpain system in particular is its potential to influence both postmortem quality and live animal growth via its role in protein turnover. Reduced proteolysis in the live animal as a result of decreased calpain or increased calpastatin activity will result in less net protein turnover, all other factors being equal. This may lead to an animal which is more efficient, grows faster, is more heavily muscled, or simply better maintains its current muscle state. This literature review will specifically focus on models of efficiency and growth/maturity.

Efficiency and profit may be affected by costs of land, energy resources, management, feed, and the ability of the animal to efficiently convert that feed to muscle. While technological advances have improved efficiency from a management standpoint, feed costs are largely out of the industry’s control, and have risen exponentially over the past decade (USDA, 2013a). Therefore, animal efficiency has become an increasingly important way to improve efficiency of the entire production system. Already, great strides have been made in this area. A great example is beef production; in 2012, there were 21.3% fewer cattle
slaughtered than in 1977, but 2.8% more beef was produced (USDA, 1978, 2013b). Additionally, less feed, water, and land are required to produce 1 kg of beef in today’s beef production systems, compared to 1977 (Capper, 2011). Although many physiological factors may affect efficiency, a large percentage of variations in efficiency may be due to a combination of protein turnover and tissue metabolism (Richardson and Herd, 2004).

The physiology controlling growth and maturity are also important from the standpoint of both the meat industry and animal production. Because the calpain system is partly responsible for controlling protein turnover, animals in differing stages of growth may have varying meat quality based on their potential for postmortem proteolysis in muscle. This potential effect of postmortem protein degradation is in addition to already known factors that affect tenderness of meat from older animals, such as collagen solubility. Collagen cross-linking, which results in insolubility, is increased with animal age, culminating in increased toughness (Lepetit, 2007; Miller et al., 1983).

Together, efficiency and growth provide excellent models to discover more about the calpain system, especially μ-calpain, m-calpain, and their endogenous inhibitor calpastatin. Increased knowledge of the calpain system in the live animal may allow for a better understanding of the numerous factors which affect productivity and muscle hypertrophy. Equally, the concurrent effects on postmortem muscle will provide greater knowledge about the factors that affect meat quality and tenderness.
Muscle Protein Degradation and Synthesis

Proteases

Proteases are broadly defined as enzymes that conduct protein degradation by hydrolyzing the peptide bonds that link amino acids. Proteases can then be subdivided into either endopeptidases or exopeptidases. Endopeptidases, or proteinases, cleave peptide bonds within the polypeptide chain, whereas exopeptidases cleave amino acids at the ends of polypeptide chains. Proteinases can be classified into one of six groups, most of which are named for the amino acid central to their active sites. The cysteine, or thiol, proteases include the calpains, caspases, most cathepsins, and several fruit-derived proteases, including bromelin and papain. Serine proteases include the trypsin-like and chymotrypsin-like proteases. The threonine protease group includes the proteasome. Aspartyl proteases, also known as acid proteases, include cathepsins D and E, as well as rennin and pepsin. The glutamic proteases were not discovered until 2004 and appear to be limited to fungi (Sims et al., 2004). Metalloproteases are proteases which need a metal ion for functionality. Metalloproteases are extremely diverse, with over fifty families. One group of metalloproteases important in skeletal muscle is the matrix metalloproteases, which play a role in the maintenance of the extracellular matrix, the matrix of connective tissue in the interstitial space surrounding cells (Carmeli et al., 2004). With the exception of the calpains, many proteases are synthesized as zymogens, inactive precursors to the proteases. Activation occurs through cleavage of a specific portion of the protease. This prevents improper protein degradation before the proteases are in place and the cell is ready for it. In the skeletal muscle of the live animal, proteases play a variety of roles, including enzyme activation or deactivation, preparation for disposal of proteins that have exceeded their useful life,
programmed cell death, and others. During meat aging, proteases act on meat proteins, resulting in tenderization. Several proteases have proven or suspected action on meat during postmortem aging, including the calpains, the proteasome, the caspases, and the cathepsins.

The Calpain System

The calpains are neutral calcium activated cysteine proteinases. Guroff (1964) first discovered what would come to be known as calpain in rat brain. “Calcium-activated sarcoplasmic factor” (CASF) was later documented by Busch et al. (1972), who found that when strips of rabbit psoas major were incubated with a calcium solution over several hours, the Z-lines disappeared. The name for the protease was made less cumbersome a few years later when it was called “calcium-activated factor,” abbreviated “CAF” (Dayton et al., 1976).

Others began to call the enzyme “calcium-activated neutral protease” (CANP) to more accurately describe it (Toyo-Oka et al., 1978). In 1981, Dayton et al. discovered that two CANPs existed in skeletal muscle, each with different calcium requirements. Meanwhile, other authors were calling the enzyme “calcium-dependent protease,” abbreviated to CDP-1 and CDP-2, based on their order of elution from a DEAE-cellulose ion exchange column during purification, to represent the two protease forms (DeMartino et al., 1986; Inoue et al., 1977; Mellgren et al., 1982). Murachi et al. (1981) first gave the calpains (and their inhibitor, calpastatin) their current general name, cal standing for calcium, and pain coming from the similarities to the plant protease papain. Papain is also a cysteine protease and shares some sequence homology with the peptidase domain of the calpains. The two conventional ubiquitous calpains were termed calpain I and calpain II. However, this was not the final change in terminology; in 1989, these calpains were termed µ- and m-calpain, based on the fact that µ-calpain requires micromolar concentrations of calcium for activation and m-
calpain requires millimolar concentrations (Cong et al., 1989). The current official names for these two proteases are calpain 1 and calpain 2, although the three above terminologies (calpain I/II, µ/m-calpain, and calpain 1/2) are still used interchangeably by different authors.

**Structure and Properties**

To date, 15 isoforms of calpain have been documented, six of which are tissue-specific (Table 1). As previously discussed, µ- and m-calpain are ubiquitous and well expressed in muscle tissue. This, along with their earlier discovery, has led to µ- and m-calpain often being called the “conventional calpains.” Both µ- and m-calpain are composed of two subunits: an 80 kDa catalytic subunit and a 28 kDa regulatory subunit. The small subunit is identical for both µ- and m-calpain and is a separate gene product, called calpain small subunit 1, but previously referred to as calpain 4 (Campbell and Davies, 2012). A lack of either the 80 kDa m-calpain or the 28 kDa small subunit is embryonic lethal in knockout mice, but µ-calpain or calpastatin (the inhibitor of µ- and m-calpain) null mice appear healthy (Azam et al., 2001; Dutt et al., 2006; Takano et al., 2005; Zimmerman et al., 2000).

Another calpain isoform important to skeletal muscle is calpain 3, so named because it was the third calpain discovered. Calpain 3 was originally referred to as p94. Calpain 3 is a 94 kDa calpain protease with approximately 50% sequence homology with the large domain of µ- and m-calpain (Sorimachi et al., 1989). Calpain 3 is specific to skeletal muscle, and is also found in the retina and lens in an 82 kDa form. Calpain 3 requires only nanomolar levels of calcium for activation (García Díaz et al., 2006). Additionally, calpain 3 lacks the 28 kDa small subunit and may form homodimers in vivo (Ravulapalli et al., 2005). Mutations in p94 result in limb girdle muscle dystrophy type 2A (Richard et al., 1995). Calpain 3 knockout mice have abnormal sarcomere formation, where the A-bands are misaligned (Kramerova et
Early calpain 3 investigations were met with difficulty because calpain 3 is very unstable and degrades quickly under many purification conditions. This is likely because calpain 3 is susceptible to both sodium and calcium dependent autolysis (Ono et al., 2010). Additionally, calpain 3 has been demonstrated to have sodium dependent activity which results in different substrate specificity compared to when it is activated with calcium. For example, substrates for calpain 3 in the presence of calcium included troponin-I and troponin-T, whereas substrates in the presence of sodium included α-actinin-3 and tropomyosin (Ono et al., 2010). Calpain 3 may play a structural role in addition to its protease activity. Kramerova et al. (2008) demonstrated a structural role for calpain 3 in the triad regions composed of one T-tubule and two terminal cisternae in skeletal muscle fibers, which regulate calcium release during contraction. Lack of calpain 3 resulted in decreased calcium release in these fibers due to an impairment of the triad-associated protein complex. Calpain 3 is also known to bind with either the N2 or M-line region of the very large structural protein, titin, also known as connectin (Kramerova et al., 2004; Sorimachi et al., 1995). In fact, the association of calpain 3 with titin protects the protease against autodegradation, and its removal from titin results in rapid autolysis, which is one reason so many difficulties were encountered in the early attempts to purify it (Ono et al., 2006).

The structural role of calpain 3 and its interaction with titin have made the protease quite interesting from a meat science and postmortem tenderness perspective. Calpastatin, the endogenous inhibitor of μ- and m-calpain, does not inhibit calpain 3 activity, but is a substrate of calpain 3 in vitro, suggesting one mechanism by which calpain 3 could regulate the activity of the conventional calpains and therefore postmortem proteolysis if the same were true in vivo (Ono et al., 2004). However, when comparing postmortem muscle of
calpain 3 knockout mice and normal mice, Geesink et al. (2005) observed that there were no differences in degradation of desmin, vinculin, nebulin, dystrophin, or troponin-T, all of which are normally degraded postmortem. Therefore, the role calpain 3 may play, if any, appears to be minor.

**Table 1. Discovered calpain isoforms. Adapted from Campbell and Davies (2012).**

<table>
<thead>
<tr>
<th>Calpain</th>
<th>Aliases</th>
<th>PEF domain?</th>
<th>Tissue Specific?</th>
<th>Tissues With Greatest Expression</th>
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<tr>
<td>Calpain 1</td>
<td>µ-calpain, calpain I</td>
<td>Y</td>
<td>N</td>
<td>Placenta, esophagus, trachea</td>
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<tr>
<td>Calpain 2</td>
<td>m-calpain, calpain II</td>
<td>Y</td>
<td>N</td>
<td>Kidney, lung, trachea</td>
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<tr>
<td>Calpain 3</td>
<td>p94, nCL-1, Lp82</td>
<td>Y</td>
<td>Y</td>
<td>Skeletal muscle, lens, retina</td>
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<td>Calpain 5</td>
<td>nCL-3, htra-3</td>
<td>N</td>
<td>N</td>
<td>Brain, kidney, liver</td>
</tr>
<tr>
<td>Calpain 6</td>
<td></td>
<td>N</td>
<td>Y</td>
<td>Placenta</td>
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<td>palBH</td>
<td>N</td>
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<td>Calpain 8</td>
<td>nCL-2</td>
<td>Y</td>
<td>Y</td>
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<td>nCL-4</td>
<td>Y</td>
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</table>

As stated above, the two ubiquitous conventional calpains, µ- and m- calpain, are named for their required concentrations of calcium for activation (3-50 µM and 400-800 µM required for half-maximal activity, respectively) (Goll et al., 2003). These 80 kDa proteases are composed of five domains (Figure 1): the anchor domain (domain I), the protease core domains (domains IIA and IIB), the C2-like domain (domain III), and the penta-EF (PEF) domain (domain IV) (Campbell and Davies, 2012). EF hands are helix-loop-helix structures which bind calcium. Proteins in the PEF hand family are calcium dependent or play a role in regulating calcium homeostasis. Additionally, PEF proteins form homodimers or heterodimers (Maki et al., 2002). The PEF domain of µ- or m-calpain forms heterodimers.
with the PEF domain (domain VI) of the calpain small subunit 1 (Figure 1). When exposed to calcium levels required for activation, μ-calpain will undergo progressive autolysis of the anchor domain, resulting in progressive degradation of the 80 kDa μ-calpain to 78 kDa and eventually to 76 kDa. In contrast, m-calpain only undergoes autolysis to 78 kDa (Zimmerman and Schlaepfer, 1991). This autolysis lowers the calcium requirement for activity of both μ- and m-calpain, which may explain how μ- and m-calpain are active in cellular conditions which rarely, if ever, reach the documented calcium concentrations needed for activity. The autolyzed 76 kDa form of μ-calpain requires 92 percent less calcium for half-maximal activity, and autolyzed m-calpain requires 82 percent less calcium for half-maximal activity compared to their unautolyzed 80 kDa forms (Edmunds et al., 1991). The calpain small subunit will also autolyze with exposure to calcium, losing its glycine-rich domain and weighing approximately 18 kDa (Parkes et al., 1985). However, extended autolysis results in rapid loss of activity. Edmunds et al. (1991) reported that μ- and m-calpain retained only 16 percent and 6 percent, respectively, of their original specific activity after autolysis of both the large and small subunits. In this way, autolysis may limit the duration of μ- and m-calpain activity (Elce et al., 1997b). Because autolysis consistently accompanies μ- and m-calpain activity and proteolysis, measurement of calpain autolysis can often be used to provide evidence of how active the μ- or m-calpain has been up to the measured time point.

The endogenous inhibitor of μ- and m-calpain is calpastatin. Calpastatin does not inhibit calpain 3 (Ono et al., 2004). Calpastatin is a 70-80 kDa protein (except in erythrocytes, which have a smaller 46 kDa version of the protein) which is composed of the N-terminal an L domain and four repeating domains. Each repeating domain has the ability
to inhibit one calpain molecule (domains I-IV, Figure 1) (Campbell and Davies, 2012; Goll et al., 2003; Raynaud et al., 2005). Some calpastatin isoforms, especially in cardiac muscle, also contain a 68 amino acid XL N-terminal domain (Cong et al., 1998; Raynaud et al., 2005; Wendt et al., 2004).

![Calpain (µ- or m-) Domain Structures](image)

**Figure 1. Domain structures of calpains 1 (µ-calpain), 2 (m-calpain), and 4 (calpain small subunit 1), and calpastatin. (Not to scale)**

In early studies, reported calpastatin molecular weights ranged from less than 50 kDa to over 200 kDa, the result of many factors which have made calpastatin difficult to characterize (Goll et al., 2003). First, the disordered structure of calpastatin in solution makes it quite susceptible to proteolysis, which may have been exacerbated with heat and denaturing reagents used in purification. Second, the same disordered structure of calpastatin causes molecular weight to be greatly overestimated when using size exclusion chromatography because its unordered conformation gives it a large Stokes radius. This
explains several reported values over 200 kDa (Thompson and Goll, 2000). Third, molecular weight is also overestimated when using SDS-PAGE because calpastatin migrates anomalously (typically at 100-150 kDa for full-size calpastatin) (Goll et al., 2003; Wendt et al., 2004). Overestimation of molecular weight in SDS-PAGE may be due to anomalous electrophoretic migration caused by an abundance of charged amino acids (Takano et al., 1988). Finally, several isoforms of calpastatin exist based on alternative splicing or use of different promoters (De Tullio et al., 2007; Goll et al., 2003; Raynaud et al., 2005; Wendt et al., 2004). Some examples of known calpastatin isoforms are presented in Table 2.

Table 2. Calpastatin isoforms. Adapted from Goll et al. (2003).

<table>
<thead>
<tr>
<th>Nature of Transcript</th>
<th>Properties of Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I; contains 1xa, 1y, 1z, exons for domain L and all four repeats; 85-kDa polypeptide</td>
<td>Migrates at 145 kDa in SDS-PAGE (Raynaud et al., 2005); Other sites of tissue expression are unknown; 172 kDa in porcine skeletal muscle?</td>
</tr>
<tr>
<td>Type II; contains 1xb, 1y, 1z, exons for domain L and all four repeats; 84-kDa polypeptide</td>
<td>Migrates as 145 kDa in SDS-PAGE (Raynaud et al., 2005); XL domain contains three protein kinase A phosphorylation sites in some species</td>
</tr>
<tr>
<td>Type III; prototypical; contains exons for domain L and all four repeats; 77–78 kDa</td>
<td>Migrates as 135 or 125 kDa in SDS-PAGE; mammalian skeletal and cardiac muscle; other distribution not characterized (Parr et al., 2004; Raynaud et al., 2005)</td>
</tr>
<tr>
<td>Deletion of exon 3 in domain L; contains all four repeats; 74–75 kDa</td>
<td>Migrates as 125 kDa in SDS-PAGE; mammalian skeletal muscle; other distribution not characterized</td>
</tr>
<tr>
<td>Deletion of exons 3 and 6 in domain L; 72–73 kDa</td>
<td>Rate of migration in SDS-PAGE unknown (110–115 kDa?); tissue distribution has not been characterized</td>
</tr>
<tr>
<td>Lacks domains L and I; 46-kDa polypeptide</td>
<td>Migrates as 70 kDa in SDS-PAGE; human, pig, rabbit erythrocytes; possibly also in cells</td>
</tr>
<tr>
<td>Type IV; lacks domains L and I and part of domain II; 47 kDa</td>
<td>Migrates at 45 or 60 kDa (Li et al., 2000; Raynaud et al., 2005); transcript found only in testis</td>
</tr>
<tr>
<td>Lacks domain L and domains I, II, and III; 18.7 kDa</td>
<td>Rate of migration in SDS-PAGE unknown; found in human testis</td>
</tr>
</tbody>
</table>

Most (but not all) isoforms produced from alternative splicing or promoters vary in the XL or L domain of calpastatin, and therefore do not appreciably change calpain inhibitory ability (De Tullio et al., 2007; Geesink et al., 1998; Goll et al., 2003). Calpastatin
isoforms may be of much smaller molecular weight, as in erythrocytes or the testes (46 kDa
and 47 or 18.7 kDa, respectively) (UniProt Consortium, 2013c; Wendt et al., 2004), or have
seemingly unchanged molecular weight, as seen in Type I versus Type II (see Table 2 above)
calpastatin in bovine cardiac muscle (both 145 kDa in SDS-PAGE) (Raynaud et al., 2005).
Additionally, these isoforms may differ in isoelectric point (pI). Domain L has very basic
calculated pI (10.27), whereas domains I through IV each have a calculated pI between 4.26
and 4.90 (Lee et al., 1992). Therefore, alternative splicing or protein degradation which affect
domain L may cause a decrease in pI. Takano et al. observed that human erythrocyte
calpastatin, which lacks domains L and I, had a pI of 4.55, whereas calpastatin in other
tissues typically has a pI between 4.85 and 4.95 (Otsuka and Goll, 1987; Takano and
Murachi, 1982).

Mechanisms of Action of μ- and m-Calpain

During activation, μ- or m-calpain bind calcium at several locations. In total, μ-
calpain may bind approximately 5 to 8 calcium atoms per calpain molecule, whereas m-
calpain may bind 11 to 20 calcium atoms per calpain molecule (Goll et al., 2003). However,
analysis of the crystallized structure of m-calpain in the presence of calcium has shown that
m-calpain only binds 10 calcium ions in the presence of 5 mM calcium, contrary to earlier
reports (Hanna et al., 2008). The penta-EF domains IV and VI may each bind three calcium
molecules (perhaps four in the presence of excess calcium), resulting in a small
conformational shift and the dissociation of the small subunit (Elce et al., 1997a). However,
the small shift resulting from binding at the PEF domains is probably not enough to result in
activation of m-calpain. The cysteine (Cys105) residue in domain IIA and asparagine
(Asp262) and histidine (His286) residues in domain IIB, which form the proteolytic triad of
the molecule, are 10.5 Å apart from each other in the m-calpain protease. When calpain is active, the Cys105 and Asp262 residues are 3.7 Å apart. At this distance, the His residue binds the hydrogen atom on the Cys sulfur atom, decreasing the pKₐ of the sulfur and increasing its negative charge. This makes the Cys residue nucleophillic, resulting in hydrolysis of substrate peptide bonds (Hosfield et al., 1999). The conformational change brought on by calcium binding to the PEF hands is probably not large enough to bridge this gap (Hosfield et al., 1999). In domains IIA and IIB, there is each one calcium binding site. Moldoveanu et al. (Moldoveanu et al., 2002) showed that these protease core domains had no activity on their own. However, in the presence of calcium the domains undergo the conformational shift which allows protease activity. Therefore, calcium binding at the two protease core sites is essential for proteolysis. With calcium binding, salt bridges between the Glu333 and Arg104 residues are disrupted in domain II, allowing the catalytic triad to come together, resulting in activation (Benyamin, 2006; Moldoveanu et al., 2002). A salt bridge is a noncovalent interaction which is a combination of a hydrogen bond and an electrostatic interaction. A number of factors reduce the calcium requirement, including autolysis, phospholipid binding, or binding to activator proteins associated with membranes (Cong et al., 1989; Saido et al., 1992; Salamino et al., 1993).

Mechanism of Action of Calpastatin

Each of the four inhibitory domains of calpastatin contain three regions called A, B, and C. The B region of these domains is the inhibitory portion of the domains. Many studies have shown that µ- or m-calpain must first be in the active state before calpastatin can bind and inhibit the protease. Todd et al. (2003) have shown that the small structure shift resulting from calcium binding to the EF hands opens a large hydrophobic pocket in each domain that
calpastatin A and C regions can bind to. During binding to μ- or m-calpain, the disordered structure of calpastatin becomes more ordered. The A and C regions each form an amphipathic α-helix and bind to the PEF domains IV and VI of μ- or m-calpain. The B subdomain then binds to μ- or m-calpain on either side of the active site cleft. At the active site cleft, a glycine residue of the B subdomain causes a sharp twist in the molecule which keeps it just out of reach of the active cysteine residue of calpain (Hanna et al., 2008). It is also possible that calpastatin may control calpain activity by binding μ- or m-calpain in the absence of calcium. Melloni et al. (2006) reported that, in the absence of calcium, or at low physiological levels of calcium, the L domain of calpastatin may bind with inactive μ- or m-calpain, forming a complex which may prevent overactivation of calpain.

The L domain of calpastatin has no inhibitory function. In addition to the calpain binding mentioned above, other possible functions of the L domain include membrane binding or regulation of calcium channels. Mellgren et al. (1988; 1989) have shown that calpastatin binds to membranes, and this is largely due to the basic properties of the L domain. Indeed, calpastatin was shown to bind only to acidic phospholipids and not neutral phospholipids. Membrane binding of calpastatin may keep calpastatin close to membrane-bound μ- or m-calpain so it can quickly inhibit the protease if needed. Erythrocyte calpastatin, which has no L domain, cannot bind to membranes (Mellgren, 1988). Additionally, domain L restores calcium channel activity in cardiac myocytes following rundown, a common phenomena where calcium channel activity is lost soon after activation (Hao et al., 2000). Calcium channel regulation by domain L may is apparently achieved by the action of domain L as a partial agonist for calmodulin, which regulates channel activation (Minobe et al., 2011).
One modification documented in calpastatin is phosphorylation (Averna et al., 2001; Pontremoli et al., 1992; Salamino et al., 1997; Salamino et al., 1994). Bovine calpastatin has seven serine residues which may be phosphorylated, and porcine calpastatin has eight (Adachi et al., 1991; UniProt Consortium, 2013a, b). These phosphorylation sites are located on domains L (bovine, 1 residue; porcine, 2 residues), I (bovine, 2 residues; porcine 1 residue), II (bovine, 3 residues; porcine, 3 residues), and III (bovine, 1 residue; porcine 2 residues) (UniProt Consortium, 2013a, b). Calpastatin may also be phosphorylated on threonine residues (Adachi et al., 1991). Additionally, the XL domain of some calpastatin isoforms has three sites which are susceptible to phosphorylation by PKA (Takano et al., 1988). Reversible phosphorylation of calpastatin has been documented in several studies (Averna et al., 2001; Pontremoli et al., 1992; Salamino et al., 1997; Salamino et al., 1994). In these studies, phosphorylation or dephosphorylation changed the ionic properties of the calpastatin protein so that the elution pattern was changed during purification using ion exchange chromatography. Phosphorylation may be a mechanism by which calpastatin inhibitory activity is controlled. Averna et al. (2001) documented aggregation of human neuroblastoma LAN-5 cell phosphorylated calpastatin (inactive) and cytoplasmic diffusion of dephosphorylated calpastatin (active). Adachi et al. (1991) also speculated that phosphorylation may be a mechanism by which calpastatin attaches to membranes when it is not active. Interestingly, alternative splicing may result in increased or reduced number of phosphorylation sites on calpastatin (Cong et al., 1998). Therefore, alternative splicing and phosphorylation may work in tandem to regulate calpastatin activity.

*Calpain System Function in Skeletal Muscle*
The calpain system plays a significant role in a number of cellular processes in the live animal, including cell motility, skeletal muscle apoptosis, protein turnover, remodeling, myogenesis, gene expression, signal transduction, and metabolism (Campbell and Davies, 2012; Goll et al., 2003; Goll et al., 1998; Kar et al., 2010; Zhivotovsky and Orrenius, 2011). Although µ and m-calpain have many protein substrates and cleave at several different protein sequences, these calcium-activated proteases are quite specific for their substrates, unlike some other proteases, such as the cathepsins. Additionally, while µ- and m-calpain cleave these proteins, they do so at very specific sites and do not completely degrade the substrates. Cleavage specificity appears to be highly influenced by protein conformation rather than amino acid sequence, most commonly cleaving substrates that contain coils or loops (duVerle et al., 2011; Fan et al., 2013). However, recent studies have identified several substrate sequence specificities via computer modeling (Sorimachi et al., 2012).

The µ and m-calpains function in cell motility by the cleavage of cytoskeletal and membrane-associated proteins. Indeed, cytoskeletal proteins are some of the most susceptible proteins to calpain cleavage; some notable examples of cytoskeletal µ- and m-calpain substrates include titin (the largest known protein in muscle at 3,000 kDa), nebulin (also very large), desmin (an intermediate filament which binds myofibrils to each other as well as to the sarcolemma, nucleus, and mitochondria), filamin, and synemin (Goll et al., 2003).

In apoptosis, or programmed cell death, the calpains may act as either positive or negative regulators of apoptosis by cleaving the caspases. Chua et al. (2000) demonstrated that m-calpain cleaved caspase 9, an initiator caspase, which prevented activation of caspase 3 and subsequent apoptosis. Additionally, caspases 8 (initiator caspase) and 7 (effector caspase) were inactivated by m-calpain. However, m-calpain may also initiate apoptosis via
activation of caspase 12, an initiator caspase (Nakagawa and Yuan, 2000). Another way the calpains can initiate apoptosis is by mediating the release of apoptotic inducing factor. Apoptotic inducing factor (AIF) is located in the mitochondria; in apoptosis it is translated to the nucleus, initiating cell death independently of the caspases. An influx of calcium into the mitochondria has been shown to activate \( \mu \)-calpain which cleaves AIF, releasing it from the mitochondria and initiating apoptosis (Norberg et al., 2008). Calpain has also been shown to activate calcineurin (an upstream mediator of apoptosis) by cleaving its inhibitor, resulting in apoptotic signaling (Kim et al., 2002). Additionally, calpain activation may occur downstream of caspase activity during apoptosis (Wood and Newcomb, 1999).

Because of the role \( \mu \)- and \( m \)-calpain play in muscle protein turnover, greater calpastatin activity is often associated with decreased protein degradation and muscle hypertrophy (Goll et al., 1998). A prime example of this is the callipyge condition in lambs. The phenotype, which was named for the Greek word meaning “beautiful buttocks,” results in heavy muscling and greater feed efficiency. It was first discovered in 1983, but not heavily researched until the 1990s. The callipyge phenotype in lambs is caused by a single gene mutation located on chromosome 18 (Cockett et al., 1994). Interestingly, the expression pattern for callipyge was one never seen before. Only heterozygous offspring who received the callipyge mutation from the sire expressed the callipyge phenotype, in an inheritance pattern that would be called polar overdominance (Cockett et al., 1996). In the callipyge phenotype, several muscles are hypertrophied with greater glycolytic fiber type, particularly the muscles of the leg and loin (Koohmaraie et al., 1995). This muscle hypertrophy is thought to be the result of increased calpastatin expression and activity, which would result in less protein degradation (Duckett et al., 2000; Koohmaraie et al., 1995). Indeed, the muscles
which do not undergo hypertrophy have normal levels of calpastatin expression and activity, whereas hypertrophied muscles have calpastatin activities which are much greater than those of normal lambs (Duckett et al., 2000).

Continuing the discussion of calpastatin-mediated hypertrophy, livestock fed β-adrenergic agonists also experience muscle hypertrophy, and this is due in part to greater calpastatin activity (Bardsley et al., 1992; Sensky et al., 2006). Beta-adrenergic agonists act on β-adrenoreceptors in muscle to repartition fat and muscle, leading to reduced fat deposition and increased protein accretion. With β-adrenergic agonist supplementation, calpastatin and m-calpain activities increase, while µ-calpain activity decreases, resulting in limited net protein turnover. An increase in m-calpain (also noted in callipyge lambs (Geesink and Koohmaraie, 1999)) may be associated with an increased rate of skeletal muscle growth, as some protein degradation is needed to repartition muscle (Goll et al., 1998). Perhaps an m-calpain increase is a way to compensate for the increase in calpastatin in order to achieve protein turnover. Conversely, calpastatin expression that is too great may inhibit muscle growth by preventing the protein turnover necessary for fiber type shifts prior to hypertrophy (Douillard et al., 2012). During treatment with β-adrenergic agonists, muscle fibers undergo a slow to fast fiber type phenotypic shift (Criswell et al., 1996). Mice which were engineered to overexpress calpastatin through gene electrotransfection were unable to achieve this fiber type change and therefore did not have the same level of muscle hypertrophy as normal mice when exposed to the β-adrenergic agonist clenbuterol (Douillard et al., 2012). Protein synthesis is also increased in animals given β-adrenergic agonists, leading to a net protein accretion and growth (Kim and Sainz, 1992; Mersmann, 1998).

*Calpain System in Postmortem Muscle*
At death, meat begins to toughen due to sarcomere shortening during rigor onset (Koohmaraie et al., 1996). After rigor completion, tenderization begins to occur, which is known as the resolution of rigor (Wheeler and Koohmaraie, 1994). At this time point, postmortem proteolysis of several structural proteins, including desmin, titin, nebulin, and others, can be observed (Taylor et al., 1995b; Wheeler and Koohmaraie, 1994). A 30 kDa degradation product of troponin-T is another hallmark of postmortem proteolysis. Additionally, light and electron microscope studies have shown that Z-disks are degraded with a few days postmortem (Taylor et al., 1995b). These structural changes caused by postmortem proteolysis are the cause of tenderization during postmortem storage, commonly referred to as aging.

The sum of several studies has provided convincing evidence that the majority of postmortem proteolysis and tenderness development during postmortem storage is the result of µ-calpain activation. Koohmaraie (1992) listed several criteria for proteinases to be considered to be responsible for postmortem tenderization. First, the protease must be located within the skeletal muscle cell, and second, have access to the substrates in question. Third, the protease must be able to degrade the same proteins that are observed to be naturally degraded postmortem. Both µ-calpain and m-calpain fit these criteria. However, because calcium concentrations postmortem (maximal 210-230 µM) will generally never reach that needed to activate m-calpain, µ-calpain is considered to be of much greater importance for postmortem proteolysis and tenderness development (Ji and Takahashi, 2006; Koohmaraie et al., 1987). The fact that µ-calpain autolysis and loss of activity occurs relatively rapidly postmortem (most activity is lost within 7 days), while m-calpain autolysis and activity loss do not is further evidence of the limited activity of m-calpain postmortem (Camou et al.,
Geesink and Koohmaraie (1999) documented that m-calpain activity did not decrease in lamb biceps femoris from 0 to 56 days postmortem. In contrast, Camou et al. (2007) saw a loss in the majority of m-calpain activity by 6 days postmortem, but no autolysis had occurred.

Additional experiments that have provided evidence that μ-calpain is the primary protease involved in postmortem proteolysis are as follows. First, the proteolytic pattern observed when adding μ-calpain directly to myofibrils is very similar to that seen in postmortem aging of meat (Huff-Lonergan et al., 1996; Koohmaraie et al., 1986). This includes a lack of actin or myosin degradation. A two-dimensional electrophoresis study by Lametsch et al. (2003) showed an association of early (72 hours postmortem) actin degradation with tenderness in pork. Although levels of degradation were small, the size, abundance, and role of actin in the myofibril suggest that any actin degradation could affect tenderness. However, degradation of actin and myosin are generally not readily seen in early postmortem muscle, although some may occur at later time points (a week or more) postmortem (Geesink and Koohmaraie, 1999). Several other proteases, including the proteasome, caspases, and cathepsins, readily degrade actin and myosin and are therefore not likely candidates as the postmortem proteases responsible for degradation seen in early postmortem muscle. Second, the muscle of μ-calpain knockout mice undergoes very little proteolysis postmortem when compared to that of wild type mice (Geesink et al., 2006). Third, injection of postmortem muscle with calcium accelerates postmortem proteolysis and tenderization; of note is that m-calpain can be activated under these conditions (Koohmaraie et al., 1989; Lansdell et al., 1995; Wheeler et al., 1997).
Because the only known inhibitory function of calpastatin is against μ- and m-calpain, inhibitory activity of calpastatin has also provided convincing evidence for the role of μ-calpain in postmortem proteolysis. Muscle in animals with greater calpastatin activity will have decreased postmortem proteolysis during meat aging and increased toughness compared to that in animals with lesser calpastatin activity. Calpastatin activity is known to be influenced by species, breed, gender, and other genetic variants (Koohmaraie et al., 1995; Morgan et al., 1993a; Shackelford et al., 1994). From a species standpoint, post rigor calpastatin activity is greater in muscle from Bos indicus compared to Bos taurus cattle species and results in decreased postmortem proteolysis and tougher meat (Shackelford et al., 1995; Whipple et al., 1990). Whipple et al. (1990) demonstrated that calpastatin activity was similar between the two species prerigor, but at 24 hr postmortem calpastatin activity was lesser in muscle from Bos taurus cattle compared to Bos indicus influenced cattle. These results suggest that calpastatin activity is more stable postmortem in Bos indicus influenced breeds. Observed calpastatin stability could be due to lower activity of other proteases which might normally degrade calpastatin postmortem, such as caspase 3 (Wang et al., 1998). However, other data from Pringle et al. (1999) and Shackelford et al. (1991) showed no interaction between breed type and aging time, indicating no differences in rate of calpastatin activity loss postmortem.

In the callipyge phenotype, the same muscles that are hypertrophied have greater calpastatin activity (Duckett et al., 2000). Meat from callipyge lambs can be extremely tough and may undergo almost no tenderness development during postmortem aging when compared to muscle from normal lambs (Duckett et al., 2000; Koohmaraie et al., 1995). Geesink et al. (1999) investigated aged biceps femoris muscle from normal and callipyge
lambs for up to 56 days. At 56 days postmortem, proteolysis in the callipyge muscle was only equivalent to that of approximately 7 day aged normal muscle.

The increased calpastatin activity associated with the use of β-adrenergic agonists and subsequent meat toughness and resistance to aging has also provided evidence of the calpain system’s role in postmortem tenderization (Koohmaraie et al., 1991a; Wheeler and Koohmaraie, 1992). Additionally, greater calpastatin activity, probably due to androgenic hormones, has been associated with greater toughness seen in bull meat versus meat from steers (Morgan et al., 1993a; Morgan et al., 1993b). Finally, transgenic mice engineered to overexpress calpastatin have limited postmortem muscle proteolysis, further evidence that µ- or m-calcpain are the primary cause of postmortem protein degradation (Kent et al., 2004).

Although only one gene for calpastatin exists in most species, different genotypes within the calpastatin gene (CAST) have been discovered in several species. These calpastatin genotypes have often been shown to affect calpastatin activity. For example, differences in calpastatin genotype have been shown to influence drip loss, but not shear force, in pork. In intron 6 of the CAST gene in pork, Gandolfi et al. (2011) observed a guanine to adenine transition. Pigs carrying an AA genotype for calpastatin had greater skeletal muscle µ-calcain autolysis compared to those with GG or GA calpastatin genotypes, indicating that calpastatin probably limited the µ-calcain activity to a greater extent in the GG or GA genotypes. Muscle from pigs with the AA genotype also had less drip loss at 72 hours postmortem, which could be related to µ-calcain activity (Gandolfi et al., 2011; Huff-Lonergan and Lonergan, 2005). Ciobanu et al. (2004) investigated several calpastatin polymorphisms, revealing three different haplotypes in 8 Western commercial swine breeds. The first haplotype, which had 249Lys and 638Arg (versus 249Arg, 638Arg in haplotype 2,
frequency 0.07; and 249Arg, 638Ser in haplotype 3, frequency 0.43) had the highest frequency (0.50) and was associated with the best quality attributes, including higher pH, better subjective tenderness and juiciness, and lower shear force and cook loss in commercial crossbred pigs. Interestingly haplotypes 1 and 3 had different predicted phosphorylation sites due to the additional serine residue in haplotype 3, which may influence calpastatin activity. In beef, Casas et al. (2006) observed a guanine to adenine (C to T) transition in the 3’ untranslated region of the CAST gene. Longissimus samples from cattle with the CC or CT CAST genotype were tougher than those from cattle with the TT genotype. Other genetic variations in the CAST gene in beef cattle have been reported as a C/G substitution at intron 5 and C/T substitution in exon 3 (Gábor et al., 2012; Schenkel et al., 2006).

The activity of μ-calpain, m-calpain, and calpastatin may be affected by other environmental factors, including temperature, pH, and oxidation. Higher storage temperatures will result in increased activity of μ-calpain and greater postmortem protein degradation. Pomponio and Erbjerg (2012) stored porcine longissimus samples at 2, 15, 25 and 30 °C for 6, 24, 48, and 120 hours postmortem. With increasing temperature, μ-calpain activity decreased at a more rapid rate, indicating that it had been more active. Additionally, m-calpain activity did not decrease at 2 °C, which would be expected given that m-calpain is not normally active in fresh muscle, but at 30 °C m-calpain activity decreased until it was not detectable at 120 hours postmortem. The observed m-calpain activation may have been due to both the increased temperature and the increased free calcium concentrations that occurred in samples stored at 30 °C. Additionally, calpastatin activity and myofibril particle size decreased with increasing storage temperature. In contrast, Thomson et al. (2008) documented less total proteolysis in previously heated samples at 21 days postmortem, likely
due to the early loss of \( \mu \)-calpain activity. It is possible that longer aging times would dilute the effects of rapid proteolysis early postmortem, although from an industry standpoint, consumers may never see those longer aging times if rapid aging methods were put in place. Geesink et al. (2000) found that storage temperatures over 25 °C for one day negatively impacted lamb longissimus quality, but these results were confounded by the fact that the prerigor samples stored at higher temperatures underwent heat shortening. Of course, one of the major concerns with high temperature aging of meat is microbial safety, which is probably why high temperature aging is not a common practice today.

Calpain activity may be detrimentally affected by high temperature, low pH combinations. In pork, rapid pH decline has been shown to be associated with less \( \mu \)-calpain autolysis, as well as less desmin and talin degradation (Bee et al., 2007). This may be attributed to increased protein denaturation as a result of low pH, high temperature combinations. In contrast, Pomponio et al. (2010) observed increased \( \mu \)-calpain autolysis in the longissimus of pigs with a rapid pH decline, in addition to a more rapid loss of \( \mu \)-calpain activity and greater myofibrillar fragmentation at 24 hours postmortem. However, myofibrillar fragmentation was not different between rapid, intermediate, or slow pH decline groups at later time points. The data of Pomponio et al. (2010) indicate that \( \mu \)-calpain is activated more rapidly in the muscle of pigs with faster postmortem pH decline. The 3 hour pH cutoff for pigs to be assigned to the rapid group was lower in the Bee et al. study (5.7 compared to 6.0), which may explain differences in observations between the two studies. A similar effect to that of Bee et al. (2007) is seen in the deep semimembranosus when compared to the superficial semimembranosus in beef. The deep semimembranosus has a slower drop in temperature, which leads to more rapid glycolysis and pH decline. The
combined effect is greater protein denaturation, less µ-calpain autolysis, and less desmin and troponin-T degradation through 7 days postmortem (Kim et al., 2010).

Of course, a common example of high temperature, low pH which is not generally associated with toughness is in muscle undergoing electrical stimulation. Electrical stimulation of carcasses early postmortem results in an increased rate of glycolysis, exhausting ATP supplies and reducing pH. Much of the increased tenderness of meat from electrically stimulated carcasses can be attributed to prevention of cold shortening and physical disruption of myofibrils during contractions (Ho et al., 1997). However, electrical stimulation has also been documented to cause early increased calpain activity (and subsequent activity loss) postmortem, sometimes resulting in greater total proteolysis (Uytterhaegen et al., 1992). Nevertheless, high temperature, low pH conditions caused by slow chilling after electrical stimulation may negate gains in calpain activity, as the proteolytic activity is quickly lost. On the other hand, if rapid chilling is employed, µ-calpain and calpastatin activity remain more stable (Hwang and Thompson, 2001). The increase in µ-calpain activity seen early after electrical stimulation may be the result of activation by calcium during the rapid onset of rigor combined with high temperatures more favorable for activity.

The greatest µ-calpain activity levels in vitro have been observed at pH 6.5, compared to pH 7.5 or 6.0. The activity of m-calpain, however, was greater at pH 7.5. Inhibition of the calpains by calpastatin was generally not impacted by pH in non-oxidizing conditions (Carlin et al., 2006; Maddock et al., 2005). Kendall et al. (1993) made similar observations for m-calpain and calpastatin activities. Interestingly though, greater µ-calpain autolysis, which often indicates activity, occurred at pH 7.5 in those experiments (Maddock et al., 2005). This
may mean that μ-calpain was actually more active at pH 7.5, but was inactivated via autolysis more quickly at the higher pH than at pH 6.5. A pH near 6.5 may therefore decrease the rate of autolysis enough to result in greater total activity over time.

The activity of μ-calpain is also affected by oxidation. Incubation with 200 mM H$_2$O$_2$ results in a loss of μ-calpain activity, which is reversible under reducing conditions. This activity loss is the result of a disulfide bond formation between the active site Cys 115 and Cys 108 (Lametsch et al., 2008). At pH 6.5 μ-calpain activity was reduced by over 50% and m-calpain was reduced by over 80% with the addition of 200 mM H$_2$O$_2$. Addition of 200 mM H$_2$O$_2$ also resulted in decreased desmin degradation in vitro (Carlin et al., 2006). Irradiation may also increase protein oxidation. Rowe et al. (2004) showed that irradiation of beef longissimus resulted in increased shear force, decreased troponin-T and desmin degradation, and decreased μ-calpain autolysis. Additionally, μ-calpain activity in irradiated steaks was greater in casein zymograms, indicating that μ-calpain had been inactivated. The reducing conditions during zymography reactivated the μ-calpain, which resulted in greater activity observed in irradiated samples. Finally, calpastatin activity was greater in irradiated samples, likely because less degradation by μ-calpain had occurred. Although live animal vitamin E supplementation prior to slaughter did not completely negate the effects of irradiation, it did result in increased troponin-T and desmin degradation, demonstrating the effect of an antioxidant in preventing oxidative loss of μ-calpain activity. This antioxidant effect was also observed in the study of Harris et al. (2001), who documented that vitamin E supplementation reversed the pro-oxidative effects of calcium chloride injection in longissimus steaks. This resulted in steaks which were more tender and had greater troponin-T degradation at one day postmortem. The more rapid proteolysis was due to both increased
μ-calpain activation because of the calcium and reduced μ-calpain inactivation caused by oxidation.

**Proteasome**

Calpain cleavage products often become substrates for the ubiquitin-proteasome system (Smith and Dodd, 2007). Before proteins can be degraded by the proteasome, they must first be ubiquitinated. First, ubiquitin is prepared by ubiquitin-activating enzyme (E1) by the attachment of ubiquitin-conjugating enzyme, UBC (E2), creating a high-energy E2-ubiquitin thiol ester intermediate. This first step is ATP-dependent. Next, the protein substrate binds to a specific ubiquitin-protein ligase (E3). Ubiquitin is conjugated to a lysine residue or the NH$_2$-terminal amino group of the protein substrate. A polyubiquitin chain is formed by adding ubiquitin to a lysine residue on the previously conjugated ubiquitin molecule. Finally, the polyubiquitinated protein is targeted to the proteasome for degradation. Just before protein degradation by the proteasome, ubiquitin is released and recycled through the activity of deubiquitinating enzymes (Glickman and Ciechanover, 2002; Nandi et al., 2006; Weissman, 2001).

The 26S proteasome is a multicatalytic threonine protease complex in the nucleus and cytosol of all eukaryotic cells. This system breaks down ubiquitinated proteins into peptide fragments ranging from 3 to 30 residues in length (Kisselev et al., 1998). The 26S proteasome consists of the 20S catalytic core subunit and two 19S regulatory caps. The 19S regulatory caps have multiple ATPase active sites and ubiquitin binding sites to recognize polyubiquinated proteins to be transferred to the catalytic core, and the 20S core is where proteins are degraded in an ATP-dependent manner (Voges et al., 1999).
The role of the 26S proteasome in postmortem muscle proteolysis is unclear. With the depletion of ATP, the 20S and 19S proteasome dissociate, eliminating the requirements for ATP and ubiquitination (Peters et al., 1994). Activity of the 20S proteasome remains quite stable over at least seven days of aging in beef (Lamare et al., 2002). Taylor et al. (1995a) reported that, when incubated with purified myofibrils, the 20S proteasome preferentially degraded actin, myosin, and desmin before troponin-T, α-actinin, or tropomyosin. However, this degradation was slower, less specific, and dissimilar to the degradation normally observed in postmortem muscle with aging. When Houbak et al. (2008) incubated beef muscle samples with lactacystin, a proteasome inhibitor, at 24°C for 5 hours at neutral pH, less degradation of actin, nebulin, and fast-twitch myosin light chain was observed. However, the results of Houbak et al. (2008) should be interpreted with caution due to the differences between experimental and actual meat aging conditions over a longer period of time, including differences in pH, temperature, and muscle intactness.

**Caspases**

Caspases are cysteine proteases which cleave specifically after aspartic acid residues (Alnemri et al., 1996). The caspases are heavily involved in apoptosis and inflammation. To date, up to eighteen different caspases have been discovered, although some are species and/or tissue specific (Eckhart et al., 2008). Interleukin-1 converting enzyme (ICE) was the first caspase discovered, and is now more commonly known as caspase 1 (Nicholson and Thornberry, 1997). With the discovery that this enzyme was similar to the protein produced by *C. elegans* death gene 3 (CED-3), the search began for other caspases (Yuan et al., 1993). Caspases 1, 4, 5, 11, and 13 are involved in inflammatory response. Caspases 3, 6, 7, 8, 9, 10, 12, and 15 are apoptotic caspases (Eckhart et al., 2005). Caspase 14 appears to be involved in
Apoptotic caspases can generally be grouped into two categories: initiators and effectors. As the names imply, initiator caspases initiate apoptosis by activating the effector caspases (Denault and Salvesen, 2008). Skeletal muscle proteins potentially subject to degradation by the caspases include actin, myosin, desmin, troponin-T, and troponin-I (Kemp and Parr, 2008). Although there is little doubt that µ-calpain causes the majority of postmortem protein degradation in meat, evidence exists that the caspase system potentially plays a role in tenderization as well. Kemp et al. (2006) observed that the caspases were indeed activated in postmortem pork longissimus dorsi, and a negative relationship existed (r = -0.62) between caspase activities and shear force. Although not proof that the caspases cause tenderization, the fact that the caspases are active at the same time as µ-calpain during postmortem aging is worthy of note. Kemp and Parr (2008) also noted that myofibril incubations with recombinant caspase 3 and calpain inhibitors performed at 4°C, pH 5.8 produced protein degradation patterns similar to experiments where myofibrils were incubated with µ-calpain. It has been suggested that the caspases may influence postmortem tenderness by interaction with the calpain system. Kemp et al. (2009) demonstrated that calpastatin activity was negatively correlated (r = -0.65 and -0.68 at 0 and 2 days postmortem, respectively) with activity of the executioner caspases 3 and 7 in both normal and callipyge lambs. The caspases have been thought to possibly regulate µ-calpain activity through cleavage of calpastatin (Wang et al., 1998). However, recent work has shown that, despite significant degradation of calpastatin, caspase 3 addition did not cause an increase in proteolysis by µ-calpain in vitro (Mohrhauser et al., 2013). Postmortem proteolytic activity
of the caspases and interaction with the calpain system deserve further investigation to
determine their effect on tenderness development.

**Cathepsins**

Cathepsins are found in lysosomes and may be cysteine, aspartic, or serine proteases. The cathepsins are active at the acidic pH found in the lysosome, although several also have activity at slightly acidic, neutral, or even slightly basic pH ranges. A list of the cathepsins found in muscle tissue and their optimum pH ranges can be found in Table 3. Cathepsins have been thought to degrade proteins in the lysosome indiscriminately, but may also contribute in more specific ways. Cathepsins may contribute to apoptosis by the activation of caspases (Li et al., 2001) and may also play a role in myoblast differentiation (Jane et al., 2002). Activity of the cathepsins is controlled by the ratio of the precursor zymogen cathepsins (known as procathepsins) to mature cathepsins, as well as endogenous inhibitors, such as cystatin (Sentandreu et al., 2002).

Early in the investigations of which enzymes were active in postmortem proteolysis, the cathepsins were considered a prime suspect (Calkins and Seideman, 1988; Johnson et al., 1990). The pH of postrigor muscle is seemingly ideal for cathepsin activity. Studies since have shown little, if any role, of the cathepsins in postmortem tenderization (Koohmaraie et al., 1991b). One problem with the hypothesis that cathepsins are responsible for postmortem aging is that cathepsins degrade actin and myosin with ease, yet a relatively small amount actin and myosin is degraded postmortem (Schwartz and Bird, 1977; Whipple and Koohmaraie, 1991). Another issue is that cathepsins are located in lysosomes, away from muscle proteins degraded during aging. Some have speculated that with prolonged aging or electrical stimulation the lysosomes might be compromised, releasing cathepsins, while
others have found no relationship. In dry cured hams, which are held for up to 15 months, disappearance of myosin heavy chain and light chains is observed, suggesting that cathepsins B, H, and L may contribute to proteolysis (Toldra et al., 1993). Degradation of myosin may cause poor quality in hams, and prevention of cathepsin activity through the use of salts is recommended (Virgili et al., 1995). Pommier (1992) saw greater free cathepsin D activity in beef longissimus muscle with either electrical stimulation or longer aging. Cathepsins may also be released from lysosomes during high pressure processing, resulting in greater proteolytic activity (Jung et al., 2000).

**Table 3. Characteristics of the well known cathepsins with endopeptidase activity existing in skeletal muscle. Adapted from Sentandreu et al. (2002).**

<table>
<thead>
<tr>
<th>Name</th>
<th>Classification and type</th>
<th>Size (kDa)</th>
<th>Optimum pH range</th>
<th>pI</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin B</td>
<td>Cysteine Family C1 (papain family)</td>
<td>30 kDa (25+5)</td>
<td>5.5–6.5</td>
<td>4.5–5.5</td>
<td>Additional peptidyl dipeptidase activity</td>
</tr>
<tr>
<td>Cathepsin L</td>
<td>Cysteine Family C1</td>
<td>28 kDa (24+4)</td>
<td>5.5–6.5</td>
<td>5.0–6.3</td>
<td>Strong activity against proteins, less against synthetic substrate</td>
</tr>
<tr>
<td>Cathepsin H</td>
<td>Cysteine Family C1</td>
<td>28 kDa (23+5)</td>
<td>6.5–6.8</td>
<td>6.0–7.1</td>
<td>Strong aminopeptidase activity</td>
</tr>
<tr>
<td>Cathepsin S</td>
<td>Cysteine Family C1</td>
<td>24 kDa</td>
<td>6.0–6.5</td>
<td>6.3–7.0</td>
<td>Stable and active at basic pH (7.5)</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>Cysteine Family C1</td>
<td>29 kDa</td>
<td>6.0–6.5</td>
<td>—</td>
<td>High collagenolytic activity</td>
</tr>
<tr>
<td>Cathepsin F</td>
<td>Cysteine Family C1</td>
<td>—</td>
<td>5.2–6.8</td>
<td>—</td>
<td>High expression in skeletal muscle</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>Aspartyl Family A1 (pepsin family)</td>
<td>45 kDa (30+15)</td>
<td>3.0–5.0</td>
<td>6.8</td>
<td>One of the most abundant cathepsins in lysosomes</td>
</tr>
<tr>
<td>Cathepsin E</td>
<td>Aspartyl Family A1</td>
<td>42 kDa</td>
<td>3.0–3.5</td>
<td>4.1</td>
<td>Forms dimers of 84 kDa. Stabilized in presence of ATP. Present in muscle but only in macrophages.</td>
</tr>
</tbody>
</table>
Protein Synthesis Pathways

Protein synthesis in skeletal muscle is achieved primarily through the activation of the mammalian target of rapamycin (mTOR), although this can be accomplished in several ways (Figure 2). The first mechanism involves activation of Akt (also known as protein kinase B, or PKB) (Braun and Gautel, 2011; Otto and Patel, 2010; Vary and Lynch, 2007). First, insulin-like growth factor I (IGF-I) interacts with the IGF-I receptor, activating insulin receptor substrate 1 (IRS1) via phosphorylation. This action can be activated through either nutritional stimulation, particularly via amino acids, or mechanical action (Vary and Lynch, 2007; Zanchi and Lancha, 2008). Mechanotransduction in protein synthesis is achieved when a mechanical stimulus causes cell signaling events. Mechanical stimuli can cause the increase of growth factor production, including both IGF-I and mechanical growth factor (MGF). Increased IGF-I or MGF can be indicators of increased protein synthesis because they are directly upstream of the Akt/mTOR pathway.

Subsequent to IRS1 activation by the IGF-I receptor, phosphatidylinositol 3-kinase (PI3K), is phosphorylated. PI3K phosphorylation then leads to the phosphorylation of Akt. Akt suppresses activity of the tumor suppression complex (TSC1/TSC2) through phosphorylation. There is also some evidence that Akt inhibits AMP kinase activity by regulating intracellular ATP levels, preventing activation of TSC2 (Hahn-Windgassen et al., 2005). If activated, TSC1/TSC2 inhibits protein synthesis by acting on Ras homolog enriched in brain (Rheb). Rheb is responsible for activating mTOR via phosphorylation. Once mTOR is activated, it subsequently phosphorylates both the eIF4E-4EBP1 complex and S6K1 (also known as p70s6 kinase). Phosphorylation leads to a release of 4EBP1, which then allows eIF4E and S6K1 to each proceed in initiating protein translation (Vary and Lynch, 2007). In
total, protein synthesis signaling can consequently be measured by analyzing the phosphorylation of the many signaling molecules of the Akt/mTOR pathway, including PI3K, Akt, mTOR, 4EBP1, and S6K1.

Figure 2. Skeletal muscle protein synthesis signaling pathways.

Recent studies have also shown that mTOR can be activated without Akt when muscle undergoes acute stimulation via passive stretch (Hornberger and Chien, 2006; Hornberger et al., 2004). Unlike the signaling which causes greater production of growth factors, this activation of mTOR is almost immediate. One substance related to mechanical transduction which can activate mTOR is phosphatidic acid, produced by phospholipase D. Hornberger and Chien (2006) reported phosphatidic acid production and consequent S6K1 activation in response to stretching. Conversely, when phosphatidic acid production was
inhibited, S6K1 activation was neutralized, demonstrating the causal relationship between stretching, phosphatidic acid, and mTOR/S6K1 activation. The exact mechanisms which cause stretching-induced phosphatidic acid production are unknown, although some have speculated that α-actinin and/or β-actin may play a role based on their close proximity to phospholipase D (Zanchi and Lancha, 2008). Despite this protein synthesis pathway being considered Akt independent, AMP kinase must still be inactivated for mTOR activation to occur. AMP kinase may be inactivated by either Akt or intracellular amino acids, glycogen, or glucose (Viollet et al., 2010).

There is convincing evidence that protein synthesis and degradation are closely related and regulated by some of the same pathways. In addition to regulating protein synthesis, the Akt/mTOR pathway can also inhibit protein degradation. Akt activation negatively regulates transcription factors of the FoxO family, upregulators of atrogin-1 expression and therefore, muscle atrophy (Bonaldo and Sandri, 2013). AMPK, which is negatively regulated by Akt during protein synthesis, also has the capability to activate FoxO3 via phosphorylation, as well as promote autophagy (Mammucari et al., 2007; Romanello et al., 2010).

Altogether, protein synthesis can be achieved through several pathways, each of which could be influenced by genetics, activity, or nutrition. Conversely, synthesis may also be inhibited through genetic factors, or lack of activity and proper nutrition. In order for muscle hypertrophy to occur, synthesis must exceed degradation. A better understanding of both protein degradation and synthesis may allow researchers to optimize animal production while still maintaining a quality meat product for consumers in the future.
Efficiency and Residual Feed Intake

Efficiency

In recent years, much attention has been given to feed efficiency in livestock production due to the rising costs of feed and other inputs. Additionally, the United Nations has projected that the world population will exceed 9 billion people by 2050 (United Nations, 2011). This increase will make it imperative to increase efficiency, given the resources that will only become more limited as the population rises. Compounding the issue, global per capita income is also expected to rise during this time period (Maddison, 2003). As per capita income rises, consumption of livestock products also increases (Steinfeld et al., 2006). Without concurrent increases in production efficiency, this will place immense pressure on the industry to provide enough food to feed the world.

It can be argued that efficiency in livestock production has already greatly increased compared to that of years past. Major advancements have been made in livestock management. Key developments in management have included: housing systems, which have reduced the land input required for poultry and pig production, in particular; more effective land management in pasture systems; a better understanding of animal nutrition, which allows the most efficient use of feed resources possible; and better control of animal disease (Thornton, 2010). Additionally, advances in breeding and genetics have improved system efficiency. Cross-breeding and hybrid vigor produces more efficient animals to some extent, and selection for efficient animals within breeds has also gradually increased efficient production (Bishop and Woolliams, 2004). Selection itself has become more advanced through the use of technologies such as artificial insemination, genomics, and statistical techniques which focus on the relevant aspects of production (Thornton, 2010).
Feed is the single largest cost in meat animal production (Arthur and Herd, 2005). Therefore, improving feed efficiency is paramount in continuing to increase livestock production efficiency, especially given rising feed costs over the past several years. Feed efficiency can be measured (and then selected for) in numerous ways. Probably the most common feed efficiency measurement is a feed conversion ratio, which measures the feed intake for each unit of weight gained by the animal. A similar measurement may report the ratio of average daily gain to average daily feed intake. Alternatively, efficiency could be measured by comparing total carcass weight and feed consumption. A fourth feed efficiency measure is residual feed intake, or RFI.

**Residual Feed Intake**

One way to measure feed efficiency is residual feed intake. First described by Koch et al. (1963), residual feed intake (RFI) is defined as the difference between the observed feed intake and the expected feed intake of an animal, based on that animal’s performance attributes (Figure 3). These attributes can vary with the study being conducted, but may include characteristics such as average daily gain and backfat (Young et al., 2011). Among different studies, various production measures have been used to calculate the expected feed intake in RFI measurements. An animal with high RFI is less efficient, whereas an animal with low RFI is more efficient. Because the expected feed intake values are based on performance attributes, RFI is phenotypically independent of whatever attributes are used (Arthur et al., 2001). In other words, selection for low RFI, or a more efficient animal, is not necessarily selecting for animals with high performance, only reduced feed intake compared to other animals with the same performance. This results in improved statistical properties over traditional measurements of efficiency.
One goal of many RFI studies is to determine what physiological factors make the animals more efficient. The physiology defining differences in feed efficiency and RFI in livestock is complex, and no single biological mechanism is responsible for the observed increases in efficiency. Richardson and Herd (2004) broke down some of the physiological factors which influence RFI in cattle (Figure 4), including physical activity, protein turnover, digestibility, and stress. In poultry, biological factors identified that affect RFI include body composition, body temperature, physical activity, and metabolism (Luiting, 1990). The largest percentage of the variation in RFI in cattle is due to a combination of protein turnover, tissue metabolism, and stress (Richardson and Herd, 2004). Recently, similar calculations were made for pigs. Almost 13 percent of the variation in RFI was accounted for by the energetic cost of protein deposition (A.J. Harris, unpublished data). This great variation at the tissue level deserves further investigation and explanation of the specific mechanisms at play.

Figure 3. Adapted from Grubbs (2012). RFI is the difference between observed and expected feed intake.
Effects of RFI Selection on Livestock Species

Residual feed intake studies have been performed using every major livestock species. Although selection for RFI is theoretically independent of the production factors (such as backfat and average daily gain) included in the calculation of RFI, animal physiology which influences RFI will change with selection. Major alterations are seen in eating habits, digestibility, activity, body composition, and metabolism.

From a meat production standpoint, some of the most evident differences in animals selected for RFI are body composition differences. Low RFI is almost always accompanied by an increase in lean or decrease in fat content of carcasses of pigs. In the Iowa State University RFI Yorkshire pig selection project, pigs in the more efficient low RFI line had less carcass fat and greater carcass lean compared to control and high RFI lines after five generations of selection (Smith et al., 2011; Young et al., 2011). Boddicker et al. (2011a, b)
reported a trend for increased protein and decreased fat composition in carcasses from Yorkshire pigs selected for low RFI compared to the control line. These results have persisted through the seventh generation of selection of the project (Harris, 2012). Similar effects have been observed in Large White and Duroc pigs divergently selected for RFI (Faure et al., 2013; Hoque et al., 2009; Le Naou et al., 2012; Lefaucheur et al., 2011). In cattle, there is more variability as to whether RFI influences these traits. For example, after grouping cattle by their observed RFI phenotypes, Carstens (2011) found that one group of low RFI steers had greater ribeye area, less marbling, and lower yield grades, whereas another group of low RFI steers had no differences in these traits. A third group of low RFI heifers had less backfat, greater ribeye area, and lower yield grades and dressing percentages, but had no differences in marbling. McDonagh et al. (2001) found that after a single generation of divergent selection for RFI, low RFI cattle had less fat over the rib, but similar ribeye areas and marbling. Greater protein and less fat accretion may be more energetically favorable in growing animals. Interestingly, high RFI laying hens in the 18th generation of divergent selection for RFI have longer wattles than their low RFI counterparts, demonstrating lack of efficiency in energy partitioning during growth (Bordas and Minvielle, 1999).

Related to body composition, high and low RFI animals may also differ in metabolism and protein turnover. High RFI animals may produce energy less efficiently. Grubbs et al. (2013) demonstrated decreased mitochondrial reactive oxygen species production in low RFI pigs, reducing the energy requirement for cellular repair or autophagy. Low RFI steers and efficient broilers have also been reported to have better electron transport chain coupling compared to high RFI steers and inefficient broilers (Bottje et al., 2002;
Kolath et al., 2006; Lancaster et al., 2007). Additionally, high RFI animals lose more energy through heat production than low RFI animals (Herd and Arthur, 2009).

In general, low RFI animals have greater protein accretion rates. This has been demonstrated in cattle (Richardson et al., 1996) and pigs (Harris, 2012; Le Naou et al., 2012). Evidence points towards decreased protein degradation playing a large role in the decreased protein turnover observed in low RFI animals. McDonagh et al. (2001) found that activity of calpastatin, the specific inhibitor of the calpain proteases, was increased in muscle of low RFI cattle after one generation of divergent selection for low or high RFI. Additionally, postmortem proteolysis was decreased in low RFI animals. Greater calpastatin activity has also been documented in muscle from low RFI barrows (Smith et al., 2011). Greater plasma protein and blood urea, which indicates protein catabolism, are found in high RFI cattle (Richardson et al., 2004; Richardson et al., 1996). Protein synthesis may also be increased in low RFI animals. Le Naou et al. (2012) showed that fractional rates of protein synthesis were increased in Large White pigs selected over six to seven generations. Interestingly, concentration of insulin-like growth factor I (IGF-I) have been either positively correlated or shown no relationship with RFI in studies that have measured it (Bunter et al., 2010; Hoque et al., 2009; Le Naou et al., 2012). The protein IGF-I is upstream of the Akt pathway which is heavily involved in protein synthesis. With a positive correlation between IGF-I concentrations and RFI one would expect to observe less protein synthesis in low RFI animals.
Protein Turnover, Animal Maturity, and Meat Quality

Age and Protein Turnover

In order for skeletal muscle growth to occur, the rate of protein synthesis must exceed that of protein degradation. Protein synthesis may be upregulated due to hormones, nutrition, and exercise, whereas a degradative state (atrophy) may be stimulated through lack of nutrition. As adults age, synthetic capacity decreases. Several studies have reported slower rates of protein synthesis in old versus young adults, even following protein absorption (Guillet et al., 2004; Irving et al., 2012). This may be due in part to a decline in the mitochondrial production of ATP. Short et al. (2005) observed that as adults aged, mitochondrial DNA decreased, as did mitochondrial ATP production rate per gram of muscle, mitochondrial protein, and aerobic capacity. Despite the evidence that protein synthesis is decreased, Guillet et al. (2004) found no differences in mTOR signaling between young and old adults, except for S6K1 phosphorylation, which was lesser in elderly adults.

The decreased mitochondrial efficiency in aging brings about greater levels of oxidation with aging (Short et al., 2005). This phenomenon has long been described as the possible cause of aging itself (Harman, 1956). Oxidative damage of protein requires the use of mechanisms to unfold, refold, and repair damage to proteins. This is commonly achieved through molecular chaperones, including heat shock proteins. These processes do require energy. Interestingly, upregulation of heat shock proteins has been shown to result in increased longevity of Drosophila melanogaster, as damage is more easily repaired (Tatar et al., 1997).

However, if proteins are damaged beyond repair, they are sent to be degraded by the proteasome or lysosomes. Unfortunately, these degradation systems have age-related
problems of their own. The proteasome system has been shown to have decreased or unbalanced expression of its subunits, as well as damage and defects of the subunits in aging cells (Koga et al., 2011). Autophagy mechanisms of the lysosome also undergo a decline with age (Cuervo, 2008). The accumulation of damaged proteins that cannot be degraded can lead to further damage in the cell.

In total, both protein synthesis and protein degradation mechanisms are disrupted with advancing age, and net protein accretion is either neutral or negative. Overall, the entire system becomes less efficient as metabolic, synthetic, and degradative systems lose function due to oxidation, other damage, and down-regulation brought about by aging.

**The Calpain System**

Because the calpain system affects growth, it stands to reason that differences might exist between animals in varying stages of growth. However, few studies exist regarding calpain and calpastatin activity in muscles from cattle that are different ages. Huff-Lonergan et al. (1995) analyzed longissimus muscle of both cows (44 to 108 months old) and steers (14 months old) for nebulin and titin degradation. Less degradation of both nebulin and titin was seen in muscle from cows in their study. Xiong et al. (2007) have also observed decreased protein degradation (troponin-T) with advancing cow maturity. Finally, when comparing myofibrillar fragmentation index (an indicator of postmortem proteolysis based on fragmentation of homogenized myofibrils) between Nellore bulls with varying carcass maturity determined by dentition, longissimus postmortem proteolysis was lesser in older animals at 24 hours postmortem (Duarte et al., 2011).
Several studies have documented the effect of animal age on μ- and m-calpain and calpastatin in other species, finding that calpastatin activity decreases with age. However, these studies have generally used animals that are still considered “young” (Northcutt et al., 1998; Ou and Forsberg, 1991; Ou et al., 1991; Veiseth et al., 2004). In muscle samples taken from rabbits at ages 0, 1, 2, and 5 months, Ou and Forsberg (1991) found that calpastatin and calpain activities were highest at birth and declined thereafter. Calpain mRNA concentrations also declined after birth. However, calpastatin mRNA concentrations were decreased from birth to one month of age, and increased thereafter. In lambs, Ou et al. (1991) observed that muscle calpastatin and calpain activities decreased from birth to weaning and remained steady through market age in both wethers and rams. A decrease in calpastatin activity per gram of tissue through weaning in rabbits and lambs may have been related to increased muscle protein concentration and the accompanying dilution effect of the calpains or calpastatin (Ou and Forsberg, 1991; Ou et al., 1991). Decreasing calpastatin activity has also been documented through 10 months of age in lambs, while longissimus desmin degradation and the μ-calpain:calpastatin ratio increased (Veiseth et al., 2004). Northcutt et al. (1998) also documented decreasing activities of calpastatin, μ-calpain, and m-calpain in the early weeks of age (from 5 to 9 weeks) in turkeys. Interestingly, Ou and Forsberg (1991) observed two mRNA isoforms of calpastatin, one of which was not detectable at birth but was gradually increased thereafter. This provides some evidence that the various calpastatin isoforms may play different roles in growth and maturation.

Studies which have examined the effect of advanced maturity in animals have typically looked at disease states such as sarcopenia. In rats, calpastatin expression and inhibitory activity are decreased with advanced age, while μ-calpain expression and overall
calpain activity are increased (Dargelos et al., 2007). Increased calpain activity in sarcopenia may be related to both calpastatin expression and nitrosylation of m-calpain and caspase-3. Calpain in adult, non-sarcopenic muscle is S-nitrosylated, but sarcopenia results in a loss of S-nitrosylation due to a decrease in neuronal nitric oxide synthase (Samengo et al., 2012). Calcium regulation is also disrupted in the sarcopenic state, which would lead to greater calpain activation in the live animal, and potentially the carcass as well (Fraysse et al., 2006). It may be speculated, though, that calpastatin activity might be increased in this state in order to control calpain activity. However, it is unknown whether cattle are susceptible to sarcopenia, especially since most are slaughtered before they might potentially reach a sarcopenic state.

**Collagen**

It would be remiss to discuss tenderness of meat from the aging animal without at least briefly mentioning the contributions of collagen solubility. Collagen in meat is classified as either soluble or insoluble, as measured through the use of nondenaturing solvents, such as neutral salt solutions or weak acids. With moist heat, soluble collagen can be converted to gelatin, resulting in a more tender product. However, insoluble collagen cannot be converted and will remain tough. Collagen insolubility is the result of increased covalent crosslinking between collagen molecules (Bailey et al., 1974). It has been known since at least the 1960s that meat from older animals has higher levels of insoluble collagen, resulting in toughness (Carmichael and Lawrie, 1967; Hill, 1966). The increase in insoluble collagen has been shown to be related specifically to chronological age but not physiological age. For example, Field et al. (1997) showed that A and C maturity carcasses from heifers of the same chronological age had no difference in collagen amount or solubility. Generally, no
differences exist in the amount of total collagen, and in fact, some have found greater collagen in veal muscle compared to cow muscle (Loyd and Hiner, 1959; Wilson et al., 1954).

**Conclusion**

Protein turnover is the balance of protein degradation and synthesis to and from amino acids. Less muscle protein turnover when comparing one animal to another suggests that less combined protein synthesis and degradation occurs in that muscle. Therefore, when selecting for animals which are more efficient, have greater muscle hypertrophy, or faster growth, one may be in fact selecting for reduced protein turnover. However, reduced protein degradation, specifically reduced calpain activity, may have the unplanned result of decreased postmortem proteolysis and tenderness development, which must be closely monitored in order to ensure that quality is not being adversely affected by selection methods.

Creating a more efficient production system through the use of less feed is possible by selecting for residual feed intake. The combination of several physiological and behavioral factors bring about the increases in efficiency seen in low RFI animals, among them protein turnover. Along with increasing efficiency, there is an obligation to ensure the final product is not only safe, but also of high quality. Investigating differences in protein turnover, especially differences in μ-calpain, m-calpain, and calpastatin activity, may provide insights, not only to the role of skeletal muscle protein degradation in feed efficiency, but also to potential effects on meat quality.
The roles that µ-calpain, m-calpain, and calpastatin play in muscle growth and protein turnover also provide compelling reasons to investigate how the calpain proteases and their inhibitor affect muscle from animals in different stages of growth based on age. Although it is well established that decreased collagen solubility causes tougher beef from older animals, postmortem proteolysis may also be decreased, resulting in less tenderness development during aging. The involvement of the calpain system, especially µ-calpain and calpastatin, in postmortem proteolysis and tenderization provides further incentive to investigate possible differences in their activity between young growing animals and older mature animals.

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CHAPTER 3. EVIDENCE OF DECREASED MUSCLE PROTEIN TURNOVER IN GILTS SELECTED FOR LOW RESIDUAL FEED INTAKE

A paper accepted by the *Journal of Animal Science*


Abstract

The objective of this study was to evaluate the contribution of muscle protein turnover (synthesis and degradation) to the biological basis for genetic differences in finisher pigs selected for residual feed intake (RFI). RFI is defined as the difference between expected feed intake (based on the individual pig’s achieved rate of gain and backfat depth) and the individual’s observed feed intake. We hypothesized that protein turnover would be reduced in pigs selected for low RFI. Twelve gilts from a line selected for 7 generations for low RFI and twelve from a contemporary line selected for 2 generations for high RFI were paired by age and weight and fed a standard corn-soybean diet for 6 weeks. Pigs were euthanized, muscle and liver samples collected, and insulin signaling, protein synthesis, and protein degradation proteins were analyzed for expression and activities. Muscle from low RFI pigs tended to have less µ- and m-calpain activities (P = 0.10 and 0.09, respectively), and had significantly greater calpastatin activity and a lower µ-calpain:calpastatin activity ratio (P < 0.05). Muscle from low RFI pigs had less 20S proteasome activity compared to their high RFI counterparts (P < 0.05). No differences in insulin signaling intermediates and translation initiation signaling proteins (mTOR pathway) were observed (P > 0.05). Postmortem proteolysis was determined in the *longissimus* (LM) from the 8th generation of

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the low RFI pigs versus their high RFI counterparts (n=9 per line). Autolysis of μ-calpain was decreased in the low RFI pigs and less troponin-T degradation product was observed at 3 d postmortem (P < 0.05), indicating slowed postmortem proteolysis during aging in the low RFI pigs. These data provide significant evidence that less protein degradation occurs in pigs selected for reduced RFI, and this may account for a significant portion of the increased efficiency observed in these animals.

Introduction

The physiology defining differences in feed efficiency (FE) and residual feed intake (RFI) in livestock is complex and multifaceted. Many of these factors have been quantified in poultry and beef, and include physical activity, feed intake patterns and behavior, stress, digestion and metabolism (Herd and Arthur, 2009; Luiting, 1990). In recent years, much attention has been given to feed efficiency in livestock production due to the rising costs of feed and other inputs. Therefore, selecting for pigs that more efficiently utilize feed enables our industry to remain competitive and sustainable. Pigs selected for reduced RFI, which is the difference between the expected intake and the individual pig’s actual feed intake necessary to reach a given performance, may help the industry achieve these goals.

In the Iowa State University RFI Yorkshire pig selection project, pigs in the low RFI line are more efficient and tend to have less carcass fat, greater carcass lean, and lesser ADG compared to control and high RFI lines (Cai et al., 2008; Smith et al., 2011; Young et al., 2011). Boddicker et al. (2011a, b) reported a trend for increased protein and decreased fat composition in carcasses from Yorkshire pigs selected for low RFI compared to the control
line. In a similar swine RFI selection project, Barea et al. (2010) showed changes in metabolism and energy utilization, which may partially explain these divergences in FE. Richardson and Herd (2004) estimated that 37% of the variation in cattle for RFI was due to protein turnover, tissue metabolism, and stress, and it is reasonable to suggest that this may be true for swine as well. The Iowa State swine selection lines provide a useful model to study the genetics and physiology defining FE. The objective of this study was to evaluate the contribution of protein turnover pathways to FE in growing pigs. We hypothesized that protein turnover is reduced in pigs selected for reduced RFI versus the high RFI line.

**Materials and Methods**

*Experiment 1*

*Study Design and Animals.* All animal procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University (1-10-6862-S). Twelve Yorkshire gilts from the 7th generation of the low RFI line and twelve high RFI Yorkshire gilts (2nd generation of divergent selection for high RFI following 5 generations of random selection) were randomly chosen from the Iowa State University RFI project population. The total 24 gilts used came from 24 different litters and 15 different sires.

Gilts were paired by line, based on age and weight, and each pair was assigned to an individual pen. Pigs had free access to water and were fed ad libitum a standard corn-soybean diet that was formulated to meet or exceed the nutrient requirements for pigs of this physiological stage (NRC, 1998). Weekly body weights and feed intake were collected in order to calculate ADG. Ultrasound measurements of BF and loin eye area were performed on d 0 and d 42 on feed. These data were used to calculate RFI for each pig. RFI indices were
obtained as the residuals from analysis of ADFI using a model with BF and ADG included as covariates (Young et al., 2011). The difference in average RFI between the low and high RFI gilts was 0.19±0.07 kg/d (P = 0.06), indicating that the low RFI pigs required 0.19 kg less feed per d to achieve the same rate of growth and backfat values as high RFI pigs. At the end of the test period, pigs were euthanized by captive bolt and subsequent exsanguination (68.4±3.5 kg). Muscle (longissimus (LM) and red and white portions of semitendinosus (RST and WST, respectively)) and liver samples were collected and either analyzed immediately or frozen in liquid nitrogen for later analysis.

*Expression of Protein Synthesis Markers.* Whole muscle extracts (10mM phosphate, 2% SDS, pH 7.0) from the LM of 7 randomly chosen pigs from each line were prepared for immunoblotting. Protein concentration of the whole muscle extracts was determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Expression of total and phosphorylated Akt, insulin receptor, insulin-like growth factor receptor, total and phosphorylated Mammalian Target of Rapamycin (mTOR, Ser2448), S6K1 (Thr389), 4EBP1 (Thr70) were assessed using immunoblotting procedures (Kimball et al., 2003). In a 4-20% precast gradient gel (Lonza, Basel, Switzerland), 40 µg of protein was separated using standard techniques. Following separation, the protein was transferred to a nitrocellulose membrane (GE Water and Process Technologies, Feasterfille-Trevose, PA). The membrane was blocked for 30 min with 5% milk in PBS-Tween (0.05% Tween 20). The membrane was subsequently incubated with primary antibody diluted in 1% milk with PBS-Tween at 4°C overnight (see Table 1 for antibody information). The next day, the membrane was washed 3 times for 10 min in PBS-Tween and incubated in secondary antibody for 1 h at room
temperature and washed 3 more times. After the last wash, enhanced chemiluminescent substrate (Thermo Fisher, Rockford, IL) was added, exposed to film, and the film of the membrane was developed in the darkroom. To analyze protein abundance, the film was scanned and band density was measured using Carestream molecular imaging software version 5.0 (Carestream Health Inc., Rochester, NY).

**Protein Degradation Indicators.** Calpain and calpastatin activity were determined in extracts of the LM, RST, and WST (n=11 pigs from each line; one pig from each line was not used based on time constraints and equipment availability). Sarcoplasmic protein was extracted according to the method of Melody et al. (2004). Within 45 min post mortem, 10 g muscle samples were finely minced and 3 vol (wt/vol) of pre-rigor extraction buffer (100 mM Tris-HCl pH 8.3, 10 mM EDTA, 100mg/L trypsin inhibitor, 2 µM E-64, 0.1% 2-mercaptoethanol) were added. Samples were homogenized in pre-rigor extraction buffer using a Polytron PT 3100 (Lucerne, Switzerland), in three 30 s bursts. The homogenate was centrifuged at 25,000 x g for 20 min, and the supernatant was dialyzed against 40 vol of 40 mM Tris-HCl pH 7.4, 1 mM EDTA, and 0.1% 2-mercaptoethanol (TEM). The dialyzed sample was centrifuged at 25,000 x g for 20 min, and the supernatant was filtered through cheesecloth.

Supernatant protein samples were loaded onto a 20 ml Q-Sepharose Fast Flow (GE Healthcare Biosciences, Pittsburgh, PA) anion exchange column equilibrated with TEM (40 mM Tris, 1 mM EDTA, 0.1% 2-mercaptoethanol). After washing, calpastatin, µ-calpain, and m-calpain were eluted using a linear gradient of 0 to 400 mM KCl in TEM. Calpastatin eluted in two separate and distinct peaks (calpastatin I and II, at 50 to 90 mM KCl and 120 to
190 mM KCl, respectively, Figure 1), followed by μ-calpain (180 to 240 mM KCl) and m-calpain (300 to 400 mM KCl).

The activities of μ- or m-calpain or calpastatin-containing fractions were determined using casein as a substrate, using a modification of the method of Koohmaraie (1990). One unit of μ- or m-calpain activity was defined as the amount required to catalyze an increase of 1 absorbance unit at 278 nm in 1 h at 25°C. One unit of calpastatin activity was defined as the amount required to inhibit 1 unit of porcine lung m-calpain. Protein content of the original muscle sample was determined in order to calculate activity on a total protein basis. Crude protein (Nitrogen x 6.25) was estimated using an Automated LECO Nitrogen Analyzer (LECO-TruSpec® N, LECO Corp., St. Joseph, MI, USA).

Proteasome activity of the 20S subunit and ubiquitination was measured in the LM and liver of the same pigs used to measure protein synthesis. Muscle samples were homogenized and diluted with PBS, and protein content was determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Proteasome activity of the 20S subunit was then determined using a commercial kit (Chemicon International, Billerica, MA, USA). Additionally, samples were prepared for immunoblotting as described above and used to measure levels of ubiquitinated proteins (indicating protein tagging for proteasome degradation). Western blots were conducted as described above with the use of a polyclonal ubiquitin antibody (1:1000, #3936, Cell Signaling Technology, Inc.).

**Experiment 2**

*Study Design and Animals.* All animal procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University (1-11-7058-S). To further assess
protein degradation between the lines and its impact on meat quality, gilts (95.3±6.9 kg) from the 8th generation of selection for low RFI and the contemporary 3rd generation of divergent selection for high RFI were euthanized in pairs over 9 days (n=9 per line). The total 18 gilts used came from 17 different litters and 4 different sires per line.

Postmortem Proteolysis. The entire LM from one side of each pig was removed immediately after death, placed on ice, and cut into thirds. Each portion of the LM was placed in a sealed plastic bag and stored at 4 °C for 1, 3 or 7 days postmortem. Storage time for each LM portion was randomly assigned in order to minimize potential effect of muscle location. After storage, samples were removed from the bags and a 2.54 cm cube was removed from the center of each LM portion. This cube was minced, frozen in liquid nitrogen, powdered, and stored at -80 °C, as described by Melody et al. (2004). Samples for day 0 were collected immediately postmortem and powdered the same day. The potential impact of cold shortening and oxidation, resulting from removal of the muscle immediately postmortem and storage in aerobic packaging, was minimized by storing large portions of the LM and using only the approximate center of each portion for analyses.

Immunoblotting for μ-calpain autolysis and troponin-T degradation was performed on whole muscle extracts from the powdered samples, as previously described (Huff-Lonergan et al., 1996; Melody et al., 2004). For μ-calpain autolysis determination, an 8% polyacrylamide separating gel (acrylamide:N,N′-bis-methylene acrylamide = 100:1 [wt/wt],0.1% [wt/vol] SDS, 0.05% [vol/vol] tetramethylethylenediamine (TEMED), 0.05% [wt/vol] APS, and 0.5 M Tris HCl, pH 8.8) was used. To determine troponin-T degradation, 15% polyacrylamide gels were used. Hoefer 260 Mighty Small II units (Hoefer, Inc.,
Holliston, MA) were used to run all gels. Gels were loaded with 40 μg of protein and run at 20 V overnight. Gels were then transferred to polyvinylidene difluoride (PVDF) membrane, as described by Melody et al. (2004).

Membrane blocking was performed using 5% non-fat dry milk in a PBS solution containing 0.1% Tween-20 for 1 hour at room temperature. Incubation in primary antibodies was at 4 °C overnight at 1:20,000 and 1:10,000 for anti-troponin-T (JLT-12; Sigma, St Louis, MO), and anti-μ-calpain (MA3-940, Thermo Scientific, Rockford, IL), respectively. After washing with PBS-Tween, membranes were incubated in secondary antibody for 1 hour at room temperature (Goat anti-mouse-HRP, No 2554, Sigma, diluted 1:30,000 and 1:20,000 for troponin-T, μ-calpain respectively). Blots were developed using either ECL Plus (troponin-T) or ECL Prime (μ-calpain) Western Blotting Detection System (GE Healthcare, Piscataway, NJ). Images were captured using a ChemiImager 5500 (Alpha Innotech, San Leandro, CA) and Alpha Ease FC software (v 3.03 Alpha Innotech). Bands were quantified using densitometry; the 30 kDa troponin-T degradation product and the 80 (intact), 78, and 76 (autolysis products) kDa bands of μ-calpain were analyzed.

**Statistical Analysis**

Calpain and calpastatin activity data were analyzed using a split plot design with RFI line as the whole plot and muscle as the split plot. Analysis was done using the MIXED procedure in SAS (v. 9.2, SAS Inst, Cary, NC). All other data were analyzed as a complete randomized design using the MIXED procedure in SAS. The model included the fixed effect of line, the random effect of day, and the covariate of BW at euthanasia. Phenotypic correlations were computed based on residuals derived using the CORR procedure of SAS.
Results and Discussion

Experiment 1

Calpain System Activity. Protein degradation pathways, such as the calpain system and the ubiquitin-proteasome pathway, may play a role in FE (Bottje and Carstens, 2009) and are known to contribute to protein turnover through protein degradation in muscle (Smith and Dodd, 2007). Across muscles, μ- and m-calpain activities tended to be lesser (P = 0.10 and 0.09, respectively) in muscle from the more efficient low RFI pigs compared to high RFI pigs (Table 2). Further, total calpastatin activity was greater (P = 0.04) in muscle from low RFI pigs, which is consistent with previous findings in younger pigs (Smith et al., 2011). The difference in total calpastatin activity can be attributed primarily to differences in calpastatin II activity (P = 0.01), given that activity of calpastatin I was not different between lines (P = 0.79). Additionally, in the low RFI animals the ratio of μ-calpain:calpastatin activity was lower (P = 0.04) than in the high RFI line pigs (Table 2). This combination of reduced calpain activities and increased calpastatin activity in the low RFI pigs indicates the potential for decreased capacity for protein degradation in the muscles of these animals. Hence, gilts from the low RFI line are more efficient, at least in part, because of decreased protein degradation. These results are dissimilar to those of Le Naou et al. (2012), who documented no differences in total calpain activity in the LM or liver of either weaned or market weight Large White pigs selected for reduced or high RFI. This disparity may be due to differences in techniques used to measure activity; Le Naou et al. (2012) used a fluorometry technique which measured combined μ- and m-calpain activity of a sample extract, whereas the current
study used chromatography techniques to isolate the individual proteases (µ- and m-calpain) and their endogenous inhibitor (calpastatin), followed by determining their activities.

To our knowledge, the separation of the two isoforms of calpastatin using anion exchange chromatography has not been documented in porcine skeletal muscle, although other groups have separated calpastatin from other species and tissues in this manner (Averna et al., 2001; Pontremoli et al., 1992; Samanta et al., 2010). While there is general agreement that there are at least two isoforms of calpastatin, it is unclear if they result from posttranslational modification (Averna et al., 2001; Pontremoli et al., 1992) or alternative splicing (Geesink et al., 1998; Samanta et al., 2010).

Whereas the low RFI animals may be more efficient from a production perspective, decreased protein degradation has the potential to alter the normal tenderization process and impair meat quality. The protease µ-calpain and its inhibitor calpastatin play a large role in the proteolysis that occurs during meat aging; reduced postmortem proteolytic activity will result in less postmortem tenderization. Indeed, Smith et al. (2011) found positive correlations between RFI index value and pork tenderness ($P < 0.01$, $r = 0.24$), as well as negative correlations between RFI index value and star probe value ($P < 0.01$, $r = -0.26$) and chewiness ($P = 0.05$, $r = -0.15$) in the LM, which indicates that pigs with lower RFI have less tender meat.

Calpain and calpastatin activities for the LM, RST, and WST are reported in Table 3. Across RFI lines, the LM had the greatest ($P < 0.0001$) µ-calpain activity and the least ($P < 0.0001$) m-calpain activity across RFI lines. The RST and WST had similar µ-calpain activities, but the RST had greater ($P = 0.002$) m-calpain activity compared to the WST. Calpastatin I activity was less than calpastatin II activity in all muscles. The WST was
similar in calpastatin I activity to the LM (P = 0.08); both the LM and WST had lower calpastatin I activity compared to the RST (P < 0.0001 and 0.004, respectively). However, the WST and RST had similar calpastatin II activities, which were more than double that of the LM (P < 0.0001). These differences in “peaks” of calpastatin activity represent a previously undefined source of variation in calpastatin activity between muscles. Total calpastatin activity differed across muscles (P < 0.0001). The LM had the least total calpastatin activity, followed by the WST and the RST. The LM and the semitendinosus sections had vastly different calpain/calpastatin profiles. The LM had a μ-calpain:total calpastatin ratio which was much more conducive to proteolysis (P < 0.0001). No significant line by muscle interactions were observed for calpain system activity.

Improved FE and low RFI could be partially explained by a reduction in the amount of electron leakage from mitochondria isolated from muscle, liver and intestines, reactive oxygen species (ROS) production, and oxidative stress (Bottje and Carstens, 2009; Grubbs et al., 2013a). Decreasing ROS production could lead to a decrease in oxidative damage to DNA, lipids and proteins, leading to a decrease in mitophagy and protein turnover (Bottje and Carstens, 2009). This would then contribute to a shift in energy utilization from cellular repair to improved lean growth of pigs, which is the ultimate goal for improving production efficiency. A greater amount of oxidized proteins appears to be a hallmark of low FE animals. Breast muscle mitochondria, as well as gut, leg, heart, liver, and lymphocyte homogenates from low FE birds, consistently exhibit a greater pervasive total protein carbonyl content compared with high FE birds (Bottje and Carstens, 2009). This supports previous observations of greater mitochondrial ROS production in low FE broilers (Bottje et al., 2002; Iqbal et al., 2004; Lassiter et al., 2006; Ojano-Dirain et al., 2005; Ojano-Dirain et
al., 2007) and in our high RFI pig lines (Grubbs et al., 2013a). This is further supported by low FE steers having greater neck muscle mitochondria protein carbonyl content (Sandelin, 2005).

Interestingly, calpain activation appears to be associated with increased oxidative stress and ubiquitin-proteasome protein degradation (Barker and Traber, 2007). Oxidation of calpain can create a disulfide bond at the active site of the protease, resulting in inhibition of its activity, which is reversible in the presence of reducing conditions (Lametsch et al., 2008). However, oxidation also appears to decrease the efficacy of the inhibitor of calpain, calpastatin, at physiological pH (Carlin et al., 2006). Damaged proteins are also conveyed directly to the proteasome by molecular chaperones that include heat shock proteins such as heat shock protein 90 and alpha-crystallin B chain (Glickman and Ciechanover, 2002). This is supported by the fact that down-regulation of heat shock proteins (LM) and acute phase proteins (serum) is observed in the Iowa State low RFI pig line (Grubbs et al., 2013b; Mani et al., 2013). As protein degradation is a very energetically expensive cellular process, both in terms of ATP required to operate the proteasome system and the loss of energy expended in synthesizing the proteins in the first place, protein turnover could represent a major contributing process in the phenotypic expression of poor feed efficiency in animals.

Proteasome Activity and Ubiquitination. Calpain cleavage products often become substrates for the ubiquitin-proteasome system (Smith and Dodd, 2007). Proteins selected for degradation are poly-ubiquitinated in an ATP-dependent manner and then targeted to the proteasome for degradation (Nandi et al., 2006). The proteasome is a multicatalytic complex in the nucleus and cytosol of all eukaryotic cells. It is responsible for proteolysis of ubiquitin-
tagged proteins. This system breaks down proteins into peptide fragments ranging from 7 to 25 residues in length. The 20S subunit is the catalytic core of the 26S proteasome, with two 19S regulatory caps. The 20S core is where proteins are degraded and the 19S regulatory caps have multiple ATPase active sites and ubiquitin binding sites to recognize polyubiquitinated proteins to be transferred to the catalytic core (Voges et al., 1999).

No differences in 20S proteasome activity or protein ubiquitination were observed in liver samples of low versus high RFI pigs (Table 4, \( P = 0.12 \) and 0.86, respectively). However, liver 20S proteasome activity, while not significantly correlated with RFI index (\( r = 0.49, P = 0.10 \)), was significantly correlated with backfat thickness (\( r = 0.56, P = 0.05 \)) and ADFI (\( r = 0.59, P = 0.04 \)). Proteasome activity in the LM was decreased by 24% in the low RFI pigs (Table 4) compared to high RFI pigs and was positively correlated with RFI index values (\( r = 0.60, P = 0.04 \)) (Table 5). These results are in contrast to those reported by Le Naou et al. (2012), who observed that proteasome activity tended to be increased (\( P = 0.08 \)) in the liver of weaned (19 kg) Large White pigs selected for reduced RFI, although no differences were found in the LM of these pigs. However, market weight pigs (115 kg) from the same study had no differences in proteasome activity in either tissue. The pigs in the current study were intermediate to the above study at 65 kg BW; it may be that the differences observed by Le Naou et al. (2012) are only evident during the early stages of growth. It is also possible that differing methods allowed more sensitive characterization of proteasome activity in the current study; again Le Naou et al. (2012) used fluorometry techniques which determined overall proteasome activity, whereas the current study’s methods specifically measured 20S proteasome activity. Ubiquitination was similar between
RFI lines in the LM. Decreased LM 20S proteasome activity in the current study is further evidence of reduced protein degradation in pigs selected for low RFI.

Overall, the results of the current study are similar to those of Damon et al. (2012), in which gene expression of Large White and Basque pig breeds were compared. The Large White is a predominant breed known for greater lean, lower fat, and a high daily gain (similar to the low RFI pigs in this experiment), whereas the Basque breed is a breed local to France which grows slowly and has much lower lean and higher fat production (similar to what would be an extremely high RFI pig in this study). Damon et al. (2012) observed that the Large White pigs had greater expression of the CAST (calpastatin) gene and decreased expression of genes that code for ligase enzymes involved in the ubiquitin-proteasome system (TRIM63 and FBX032). While compelling, it is important to note that the results of Damon et al. (2012) are based on transcript expression, whereas the current data are based on protein activity, which may or may not mirror gene expression.

Protein Synthesis Pathway. Overall, the insulin signaling and protein synthesis markers measured were not significantly affected by RFI line (Table 6, P > 0.05 for all attributes). However, Akt phosphorylation on Thr308 and the ratio of Akt phosphorylation on either Thr308 or Ser473 to total Akt protein expression tended to decrease by approximately 30-60% in pigs selected for low RFI compared to high RFI pigs (P = 0.09, 0.07, and 0.10, respectively). As Akt is upstream of mTOR and tended to have decreased activation (as evaluated by reduced phosphorylation level), the expression and phosphorylation of several mTOR pathway components were also measured. Akt is upstream of several pathways involved in protein synthesis and prevention of protein degradation, including activation of
mTOR (Braun and Gautel, 2011; Otto and Patel, 2010). Reduced Akt phosphorylation should therefore result in decreased protein synthesis; however, no protein markers in the mTOR pathway were significantly different between the two lines (P > 0.05 for all attributes). Le Naou and others (2012) found that the total protein synthesis rate tended to be increased (P = 0.06) in livers of 6th and 7th generation weaned Large White pigs selected for reduced RFI; however, no differences were detected within the LM of these animals, supporting the data in the current study. Additionally, hepatic protein synthesis may have been upregulated due to greater insulin levels in the high RFI pigs at the time of measurement (Le Naou et al., 2012). Therefore, changes in protein synthesis do not appear to have a large impact on increased growth efficiency in low RFI pigs.

In aggregate, decreased protein degradation coupled with similar protein synthesis would be predicted to result in a net decrease in protein turnover and progressive protein accretion in low RFI pigs compared to high RFI pigs. Indeed, previous findings using pigs from these lines have measured greater nitrogen retention and greater protein accretion (Harris, 2012). Muscle from low RFI pigs has been shown to be composed of a greater proportion of type IIB fibers (Lefaucheur et al., 2011), which are known to exhibit decreased protein turnover when compared to oxidative fibers (Lewis et al., 1984; van Wessel et al., 2010). These data may explain a portion of the increased efficiency observed in low RFI pigs due to decreased energy required for protein degradation. In addition to the ubiquitin-proteasome pathway, which is ATP-dependent, the production of urea to dispose of excess nitrogen after amino acid breakdown, known as ureagenesis, also requires energy in the form of ATP. Slowing the degradation processes will therefore ultimately result in less energy expenditure by the animal from multiple sources.
Experiment 2

Postmortem Muscle Proteolysis. When exposed to calcium levels required for activation, μ-calpain will undergo progressive autolysis from 80 kDa to 78 kDa and eventually to 76 kDa. This autolysis lowers the calcium requirement for activity from approximately 1–50 μM to 0.5–2.0 μM (Edmunds et al., 1991; Goll et al., 2003). In pork from generation 8 of the low RFI line at day 3 postmortem, the 80 and the 78 kDa bands were more abundant (P = 0.046 and 0.08, respectively) and the 76 kDa band was less abundant (P = 0.03), compared to pork from high RFI pigs (Table 7, Figure 2). This indicates that the rate of calpain autolysis was slower in the low RFI lines compared to the high RFI line, which would lead to a slower rate of postmortem proteolysis and tenderization. These results are consistent with the calpastatin data because greater calpastatin activity inhibits calpain activity and therefore slows the rate of autolysis (Koohmaraie, 1992). At days 0 and 1 postmortem, μ-calpain autolysis was similar between the two lines (Table 7, Figure 2).

In support of the μ-calpain autolysis data, the presence of the 30 kDa degradation product of troponin-T was lesser in the day 3 postmortem samples of the low RFI line in generation 8 compared to the high RFI line (Figure 3, P = 0.04). No differences were observed at days 0 and 1 postmortem (P = 0.85 and 0.93, respectively). These data indicate a slower rate of postmortem protein degradation in the more efficient low RFI line (Figure 3). However, a significant difference in the extent of proteolysis at day 7 was not detected between the lines (P = 0.46). From a meat quality perspective, these results raise the possibility of slowed or impaired tenderness development in pork from the low RFI line, as troponin-T is thought to be a key indicator protein in the development of meat tenderness.
(Huff Lonergan et al., 2010). Again, these data are supported by Smith et al. (2011), who, while observing no differences in sensory tenderness between the 5th generation low RFI and control lines, documented a significant positive correlations between RFI value and both desmin degradation and tenderness score, which suggested that continued selection for RFI could result in decreased postmortem proteolysis and therefore impaired pork tenderness. Additionally, selection for lean growth efficiency in Duroc pigs has been shown to result in greater calpastatin activity in the semitendinosus, decreased troponin-T degradation in the LM, and greater Warner-Bratzler shear force values in both the LM and the semitendinosus after 5 d of postmortem storage (Lonergan et al., 2001). However, in another study conducted in France using French Large White pigs, no sensory tenderness differences have been observed between two lines of pigs after 6 generations of divergent selection for low or high RFI (Faure et al., 2013). Continued selection for low RFI merits monitoring of postmortem protein degradation and sensory attributes in order to identify whether tenderness has become a problem.

**Conclusion**

Overall, these results indicate that selection for low RFI in pigs may indirectly select for reduced protein degradation and turnover. Protein synthesis pathways in the context of RFI and FE have been poorly characterized in livestock and do not appear to be related to observed phenotype in these lines. Conversely, there is substantial evidence that protein degradation systems within muscle have decreased activity in low RFI versus high RFI line pigs. This overall reduced protein turnover could potentially be a cause of the greater efficiency in these pigs, resulting in decreased costs of production. However, due to the
reduced levels of µ-calpain and increased calpastatin activity, and given the evidence of slowed postmortem proteolysis in muscle from low RFI pigs, decreased tenderness and therefore reduced quality may be an unintended consequence of these changes. The benefits and potential adverse effects must be carefully considered as we strive for more efficient, sustainable production of meat animals.

Acknowledgements

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Tables and Figures

Figure 1. Representative example of calpastatin I and calpastatin II separation during elution using a Q-Sepharose ion exchange column. Dark line represents percent inhibition of porcine lung calpain and gray dashed line represents mM KCl at protein elution.
Table 1. Antibodies and dilutions used in immunoblotting to determine expression of protein synthesis markers

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<tr>
<td>Total Akt</td>
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<td>Rabbit polyclonal</td>
<td>Cell Signaling Technology, 9272</td>
</tr>
<tr>
<td>Insulin Receptor</td>
<td>1:1000</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz Biotechnology, sc-711</td>
</tr>
<tr>
<td>Insulin Receptor Substrate</td>
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<td>Santa Cruz Biotechnology, sc-7200</td>
</tr>
<tr>
<td>mTOR</td>
<td>1:1000</td>
<td>Rabbit polyclonal</td>
<td>Cell Signaling Technology, 9272</td>
</tr>
<tr>
<td>Phospho-mTOR (Ser2448)</td>
<td>1:1000</td>
<td>Rabbit polyclonal</td>
<td>Cell Signaling Technology, 2972</td>
</tr>
<tr>
<td>P70 S6K1</td>
<td>1:1000</td>
<td>Rabbit polyclonal</td>
<td>Cell Signaling Technology, 9202</td>
</tr>
<tr>
<td>Phospho-P70 S6K1 (Thr 389)</td>
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<td>Rabbit polyclonal</td>
<td>Cell Signaling Technology, 9205</td>
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<td>4EBP1</td>
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<td>Rabbit polyclonal</td>
<td>Cell Signaling Technology, 9452</td>
</tr>
<tr>
<td>Phospho-4EBP1 (Thr70)</td>
<td>1:1000</td>
<td>Rabbit polyclonal</td>
<td>Cell Signaling Technology, 9455</td>
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<tr>
<td>Actin</td>
<td>1:4000</td>
<td>Rabbit polyclonal</td>
<td>Abcam, ab15263</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Dilution</th>
<th>Product Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey anti-Rabbit IgG HRP</td>
<td>1:2000</td>
<td>GE, NA934V</td>
</tr>
</tbody>
</table>
Table 2. Calpain and calpastatin activities (units of activity/g protein)\(^1\) in muscle from gilts selected for low or high residual feed intake (RFI)\(^2\).

<table>
<thead>
<tr>
<th></th>
<th>High RFI</th>
<th>Low RFI</th>
<th>P-Value</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mu)-calpain</td>
<td>2.48</td>
<td>2.20</td>
<td>0.10</td>
<td>0.156</td>
</tr>
<tr>
<td>m-calpain</td>
<td>9.00</td>
<td>8.25</td>
<td>0.09</td>
<td>0.422</td>
</tr>
<tr>
<td>Calpastatin I</td>
<td>4.55</td>
<td>4.49</td>
<td>0.79</td>
<td>0.207</td>
</tr>
<tr>
<td>Calpastatin II</td>
<td>8.11</td>
<td>9.57</td>
<td><strong>0.01</strong></td>
<td>0.473</td>
</tr>
<tr>
<td>Total Calpastatin</td>
<td>12.67</td>
<td>13.99</td>
<td><strong>0.04</strong></td>
<td>0.563</td>
</tr>
<tr>
<td>(\mu) calpain:calpastatin(^3)</td>
<td>0.25</td>
<td>0.20</td>
<td><strong>0.04</strong></td>
<td>0.021</td>
</tr>
</tbody>
</table>

\(^1\) One unit of \(\mu\)- or m-calpain activity = amount required to catalyze an increase of 1 absorbance unit at 278 nm in 1 h at 25°C. One unit of calpastatin activity = amount required to inhibit 1 unit of porcine lung m-calpain

\(^2\) n = 11 gilts per RFI line

\(^3\) Ratio of \(\mu\)-calpain activity to total calpastatin activity
Table 3. Least square means for calpain and calpastatin activities (units of activity /g protein)\(^1\) in longissimus (LM), the red portion of the semitendinosus (RST), and the white portion of the semitendinosus (WST) muscles from gilts selected for low or high residual feed intake (RFI). Line by muscle interactions were not significant (P > 0.05)\(^2\).

<table>
<thead>
<tr>
<th></th>
<th>LM</th>
<th>RST</th>
<th>WST</th>
<th>P-Value</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>µ-calpain</td>
<td>3.53(^a)</td>
<td>1.84(^b)</td>
<td>1.66(^c)</td>
<td>&lt;0.0001</td>
<td>0.150</td>
</tr>
<tr>
<td>m-calpain</td>
<td>7.00(^c)</td>
<td>9.99(^a)</td>
<td>8.89(^b)</td>
<td>&lt;0.0001</td>
<td>0.260</td>
</tr>
<tr>
<td>Calpastatin I</td>
<td>3.99(^b)</td>
<td>5.16(^a)</td>
<td>4.41(^b)</td>
<td>0.0001</td>
<td>0.244</td>
</tr>
<tr>
<td>Calpastatin II</td>
<td>4.39(^b)</td>
<td>11.42(^a)</td>
<td>10.71(^a)</td>
<td>&lt;0.0001</td>
<td>0.562</td>
</tr>
<tr>
<td>Total Calpastatin</td>
<td>8.30(^c)</td>
<td>16.57(^a)</td>
<td>15.11(^b)</td>
<td>&lt;0.0001</td>
<td>0.666</td>
</tr>
<tr>
<td>µ calpain:calpastatin(^3)</td>
<td>0.46(^c)</td>
<td>0.12(^b)</td>
<td>0.11(^b)</td>
<td>&lt;0.0001</td>
<td>0.025</td>
</tr>
</tbody>
</table>

\(^{a,b}\) Values in a row with different superscripts differ (P < 0.05).
\(^1\) One unit of µ- or m-calpain activity = amount required to catalyze an increase of 1 absorbance unit at 278 nm in 1 h at 25°C. One unit of calpastatin activity = amount required to inhibit 1 unit of porcine lung m-calpain.
\(^2\) LM n = 22, RST n = 19, WST n = 22
\(^3\) Ratio of µ-calpain activity to total calpastatin activity
Table 4. Least squares means for 20S proteasome activity and ubiquitin protein expression in liver and *longissimus* (LM) samples from pigs selected for low or high residual feed intake (RFI)\(^1\).

<table>
<thead>
<tr>
<th></th>
<th>High RFI</th>
<th>Low RFI</th>
<th>P-Value</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20S Proteasome Activity, RFU(^2)</td>
<td>151.5</td>
<td>128.2</td>
<td>0.12</td>
<td>13.84</td>
</tr>
<tr>
<td>Ubiquitin, AU(^3)</td>
<td>1.30</td>
<td>1.26</td>
<td>0.86</td>
<td>0.280</td>
</tr>
<tr>
<td>LM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20S Proteasome Activity, RFU(^2)</td>
<td>63.8</td>
<td>48.3</td>
<td><strong>0.03</strong></td>
<td>5.90</td>
</tr>
<tr>
<td>Ubiquitin, AU(^3)</td>
<td>1.30</td>
<td>1.10</td>
<td>0.15</td>
<td>0.090</td>
</tr>
</tbody>
</table>

\(^1\) \(n= 12\) gilts per RFI line  
\(^2\) RFU = Relative fluorescence units.  
\(^3\) AU = Arbitrary units based on densitometry intensity of lanes expressing ubiquitin tagged proteins.
Table 5. Residual correlations between protein degradation markers, residual feed intake (RFI) index and performance traits in finisher pigs, including 20S proteasome activity, calpain and calpastatin activities, and protein ubiquitination, as determined by immunoblotting\textsuperscript{1,2,3}

<table>
<thead>
<tr>
<th></th>
<th>RFI index</th>
<th>Backfat Thickness</th>
<th>ADG</th>
<th>ADFI</th>
<th>FE\textsuperscript{4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFI index</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Backfat thickness</td>
<td>0.66</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADG</td>
<td>-0.20</td>
<td>-0.25</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADFI</td>
<td>0.93</td>
<td>0.82</td>
<td>-0.02</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>FE</td>
<td>-0.90</td>
<td>-0.80</td>
<td>0.53</td>
<td>-0.84</td>
<td>1.00</td>
</tr>
<tr>
<td>Liver 20S proteasome</td>
<td>0.49</td>
<td>0.56</td>
<td>-0.01</td>
<td>0.59</td>
<td>-0.45</td>
</tr>
<tr>
<td>LM\textsuperscript{4} 20S proteasome</td>
<td>0.60</td>
<td>0.34</td>
<td>-0.21</td>
<td>0.48</td>
<td>-0.50</td>
</tr>
<tr>
<td>LM\textsuperscript{4} ubiquitin</td>
<td>0.13</td>
<td>0.43</td>
<td>0.31</td>
<td>0.48</td>
<td>-0.22</td>
</tr>
<tr>
<td>LM\textsuperscript{4} m-calpain</td>
<td>-0.07</td>
<td>-0.03</td>
<td>0.40</td>
<td>0.04</td>
<td>0.15</td>
</tr>
<tr>
<td>LM\textsuperscript{4} (\mu)-calpain</td>
<td>0.07</td>
<td>0.17</td>
<td>0.15</td>
<td>0.14</td>
<td>-0.09</td>
</tr>
<tr>
<td>LM\textsuperscript{4} total calpastatin</td>
<td>-0.38</td>
<td>-0.25</td>
<td>0.10</td>
<td>-0.31</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.45</td>
<td>0.77</td>
<td>0.35</td>
<td>0.23</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Upper row = residual correlations. Bottom row = \(P\)-values.
\textsuperscript{2}n=12 gilts per RFI line
\textsuperscript{3}FE = Feed efficiency, defined as kg of gain per kg of feed
\textsuperscript{4}LM = longissimus
Table 6. *Longissimus* muscle expression of key insulin signaling and protein synthesis cascade proteins in pigs selected for low or high residual feed intake (RFI), as determined by immunoblotting.\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>High RFI</th>
<th>Low RFI</th>
<th>P-Value</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin Signaling Cascade</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-Ser Akt, AU(^2)</td>
<td>1.00</td>
<td>0.76</td>
<td>0.20</td>
<td>0.125</td>
</tr>
<tr>
<td>Phospho-Thr Akt, AU(^2)</td>
<td>1.00</td>
<td>0.32</td>
<td>0.09</td>
<td>0.256</td>
</tr>
<tr>
<td>Total Akt, AU(^2)</td>
<td>1.00</td>
<td>1.03</td>
<td>0.90</td>
<td>0.147</td>
</tr>
<tr>
<td>Ser:total Akt, AU(^2)</td>
<td>1.10</td>
<td>0.73</td>
<td>0.07</td>
<td>0.134</td>
</tr>
<tr>
<td>Thr:total Akt, AU(^2)</td>
<td>1.16</td>
<td>0.41</td>
<td>0.10</td>
<td>0.293</td>
</tr>
<tr>
<td>Insulin Receptor, AU(^2)</td>
<td>1.00</td>
<td>0.82</td>
<td>0.46</td>
<td>0.172</td>
</tr>
<tr>
<td>Insulin Receptor Substrate, AU(^2)</td>
<td>1.00</td>
<td>0.88</td>
<td>0.64</td>
<td>0.178</td>
</tr>
<tr>
<td><strong>Protein Synthesis Pathway</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTOR, AU(^2)</td>
<td>0.95</td>
<td>0.95</td>
<td>0.99</td>
<td>0.027</td>
</tr>
<tr>
<td>Phospho-Ser2448 mTOR, AU(^2)</td>
<td>0.97</td>
<td>0.97</td>
<td>0.99</td>
<td>0.012</td>
</tr>
<tr>
<td>S6K1, AU(^2)</td>
<td>0.95</td>
<td>0.95</td>
<td>0.99</td>
<td>0.027</td>
</tr>
<tr>
<td>Phospho-Thr389 S6K1, AU(^2)</td>
<td>0.97</td>
<td>1.04</td>
<td>0.09</td>
<td>0.036</td>
</tr>
<tr>
<td>4EBP1, AU(^2)</td>
<td>0.97</td>
<td>0.97</td>
<td>0.99</td>
<td>0.013</td>
</tr>
<tr>
<td>Phospho-Thr70 4EBP1, AU(^2)</td>
<td>0.98</td>
<td>1.02</td>
<td>0.14</td>
<td>0.025</td>
</tr>
<tr>
<td>Ser:total mTOR, AU(^2)</td>
<td>1.01</td>
<td>1.01</td>
<td>0.99</td>
<td>0.027</td>
</tr>
<tr>
<td>Thr:total S6K1, AU(^2)</td>
<td>1.03</td>
<td>1.09</td>
<td>0.28</td>
<td>0.059</td>
</tr>
<tr>
<td>Thr:total 4EBP1, AU(^2)</td>
<td>1.01</td>
<td>1.05</td>
<td>0.29</td>
<td>0.034</td>
</tr>
</tbody>
</table>

\(^1\) n = 7 gilts per RFI line  
\(^2\) AU = Arbitrary units based on densitometry intensity of lanes expressing ubiquitin tagged proteins.
Table 7. μ-calpain autolysis at days 0, 1, and 3 postmortem, measured by immunoblotting. Values represent the percent of the three bands present. Comparison between pigs genetically selected for high or low residual feed intake (RFI). Values indicated are a ratio of the sample band density to an in-gel reference.¹

<table>
<thead>
<tr>
<th></th>
<th>High RFI</th>
<th>Low RFI</th>
<th>P-Value</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80 kDa</td>
<td>28.24</td>
<td>35.57</td>
<td>0.50</td>
<td>7.45</td>
</tr>
<tr>
<td>78 kDa</td>
<td>29.97</td>
<td>27.52</td>
<td>0.55</td>
<td>2.75</td>
</tr>
<tr>
<td>76 kDa</td>
<td>41.64</td>
<td>37.06</td>
<td>0.63</td>
<td>6.64</td>
</tr>
<tr>
<td><strong>Day 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80 kDa</td>
<td>12.74</td>
<td>19.06</td>
<td>0.30</td>
<td>4.12</td>
</tr>
<tr>
<td>78 kDa</td>
<td>30.24</td>
<td>31.70</td>
<td>0.75</td>
<td>3.13</td>
</tr>
<tr>
<td>76 kDa</td>
<td>57.99</td>
<td>48.27</td>
<td>0.25</td>
<td>5.73</td>
</tr>
<tr>
<td><strong>Day 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80 kDa</td>
<td>5.89</td>
<td>14.98</td>
<td><strong>0.046</strong></td>
<td>2.91</td>
</tr>
<tr>
<td>78 kDa</td>
<td>19.47</td>
<td>27.19</td>
<td><strong>0.078</strong></td>
<td>4.08</td>
</tr>
<tr>
<td>76 kDa</td>
<td>74.97</td>
<td>57.51</td>
<td><strong>0.029</strong></td>
<td>7.20</td>
</tr>
</tbody>
</table>

¹ n = 9 gilts per RFI line
Figure 2. Representative Western blot of μ-calpain autolysis at days 0, 1, and 3 postmortem. Comparison between pigs genetically selected for high or low residual feed intake (RFI). Blot shows autolysis within the longissimus over time in one animal from each line (HRFI = High RFI and LRFI = Low RFI). n = 9 gilts per RFI line.
Figure 3. Troponin-T degradation (30 kDa) at day 0, 1, 3, and 7 postmortem. Comparison between pigs genetically selected for high or low residual feed intake (RFI). A) Values indicated are a ratio of the sample band density to an in-gel reference. Error bars reflect the standard error of the means. *Within a day, values differ significantly (P < 0.05). B) Representative Western blots show the troponin-T degradation in *longissimus* muscle from one animal from each line (HRFI = high RFI and LRFI = low RFI) over time. n = 9 gilts per RFI line.
CHAPTER 4. POSTMORTEM PROTEOLYSIS IN THREE MUSCLES FROM GROWING AND MATURE BEEF CATTLE

A paper submitted to *Meat Science*

Shannon M. Cruzen¹, Pedro V.R. Paulino², Steven M. Lonergan¹, Elisabeth Huff-Lonergan¹*

**Abstract**

The objective of this study was to determine calpain system activity and postmortem protein degradation in three muscles from growing (n = 6) and mature (n = 6) beef cattle. The ratio of μ-calpain:total calpastatin activity tended to be lesser in mature animals (P = 0.08), suggesting less potential for proteolysis. Additionally, muscles from the mature group had greater calpastatin activity compared to calves at 6 days postmortem and had less μ-calpain autolysis and troponin-T and titin degradation during the aging period (P < 0.01). Between the longissimus, semimembranosus, and triceps brachii muscles, the triceps brachii had the least postmortem proteolysis, with greater calpastatin activity and less troponin-T and titin degradation compared to other muscles (P < 0.01). These data suggest that calpastatin activity in muscle from older animals is more persistent postmortem. This difference is an explanation for decreased protein degradation and tougher meat from older animals, even after aging.

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* Corresponding author
1. Introduction

It is well established that beef from older cattle is less likely to undergo postmortem tenderization and is generally less tender than beef from younger animals (Huff-Lonergan, Parrish, & Robson, 1995). This has been primarily attributed to the amount of collagen cross-links in the muscle, which increase with age and result in lower solubility during cooking and greater strength and toughness (Bailey, 1985; Lepetit, 2007). However, few studies have investigated other mechanisms which may affect the tenderness of beef from older animals.

One additional factor that may change with age is the calpain system. The calpains are calcium activated cysteine proteases. Although 15 isoforms of calpain have been documented, the two ubiquitous conventional calpains, µ- and m-calpain, play a significant role in skeletal muscle apoptosis, protein turnover, remodeling, myogenesis, and metabolism (Campbell & Davies, 2012; Goll, Thompson, Li, Wei, & Cong, 2003; Goll, Thompson, Taylor, & Ouali, 1998; Kar et al., 2010; Zhivotovsky & Orrenius, 2011). These calpains are named for the required concentrations of calcium for activation (3-50 µM and 400-800 µM required for half-maximal activity, respectively) (Goll et al., 2003). Both µ- and m-calpain are composed of two subunits: an 80 kDa catalytic subunit and a 28 kDa regulatory subunit. A lack of either the 80 kDa m-calpain or the µ- and m-calpain 28 kDa small subunit (S1) is embryonic lethal in mice, but µ-calpain or calpastatin null mice appear healthy (Azam et al., 2001; Dutt et al., 2006; Takano et al., 2005; Zimmerman, Boring, Pak, Mukerjee, & Wang, 2000). The majority of postmortem proteolysis and tenderness development during postmortem storage (or meat aging) is thought to be the result of µ-calpain activation, in part because calcium concentrations postmortem will generally never reach that needed to activate m-calpain unless calcium is added to the meat (Geesink, Kuchay, Chishti, & Koohmaraie, 2006; Koohmaraie, 1992b; Koohmaraie & Geesink, 2006). Additionally, the...
proteolytic pattern observed when adding µ-calpain directly to myofibrils is very similar to that seen in postmortem aging of meat (Huff-Lonergan, Mitsuhashi, Beekman et al., 1996). Indeed, the muscle of µ-calpain knockout mice undergoes very little proteolysis postmortem when compared to that of wild type mice (Geesink et al., 2006). A muscle-specific calpain, calpain-3/p94, is also found in skeletal muscle, but has little, if any, known role in postmortem proteolysis (Gandolfi et al., 2011; Geesink, Taylor, & Koohmaraie, 2005).

The endogenous inhibitor of µ- and m-calpain is calpastatin. Bovine calpastatin is a 76 kDa protein which will migrate much higher (115 kDa) on sodium dodecyl sulfate (SDS) gels due to its large Stokes radius (Otsuka & Goll, 1987). Each calpastatin molecule contains a leader (L) domain and four inhibitory domains (I-IV), each of which can inhibit one calpain molecule (Goll et al., 2003). Inhibitory activity of calpastatin has also provided convincing evidence for the role of µ-calpain in postmortem proteolysis because the only known function of calpastatin is to inhibit µ- and m-calpain. Transgenic mice which overexpress calpastatin have limited postmortem muscle proteolysis, further evidence that calpain is the primary cause of postmortem protein degradation (Kent, Spencer, & Koohmaraie, 2004). Calpastatin activity is known to be influenced by species, breed, gender, and other genetic variants (Koohmaraie, Shackelford, Wheeler, Lonergan, & Doumit, 1995; Morgan, Wheeler, Koohmaraie, Crouse, & Savell, 1993; Shackelford et al., 1994). For example, sheep expressing the callipyge phenotype have greater size of specific muscles, and these same muscles have been observed to have greater calpastatin activity and increased meat toughness when compared with that of normal sheep (Koohmaraie et al., 1995). Androgenic hormones may play a major role in calpastatin activity, as demonstrated by increased calpastatin activity in bulls versus steers (Morgan, Wheeler, Koohmaraie, Crouse et al., 1993; Morgan,
Wheeler, Koohmaraie, Savell, & Crouse, 1993). This difference in calpastatin activity has been associated with the greater toughness seen in bull meat (Morgan, Wheeler, Koohmaraie, Savell et al., 1993). From a species standpoint, post rigor calpastatin activity is greater in muscle from Bos indicus compared to Bos taurus cattle species (G Whipple et al., 1990). These differences have been shown to result in longer postmortem aging requirements in beef from Bos indicus influenced cattle in order to achieve similar levels of tenderness when compared to beef from Bos taurus cattle (Casas et al., 2006; G Whipple et al., 1990). Finally, calpastatin activity and subsequent meat toughness may also be increased by the use of exogenous compounds, such as β-agonists used as growth promotants (Koohmaraie, Shackelford, Mugglicockett, & Stone, 1991; Wheeler & Koohmaraie, 1992).

Several studies have documented the effect of animal age on calpains and calpastatin, finding that calpastatin activity decreases with age. However, these studies have generally used animals that are still considered “young” (Northcutt, Pringle, Dickens, Buhr, & Young, 1998; Ou & Forsberg, 1991; Ou, Meyer, & Forsberg, 1991; Veiseth, Shackelford, Wheeler, & Koohmaraie, 2004). Therefore, there is a gap in the knowledge regarding calpastatin activity in muscles from cattle that are different ages. Studies which have examined the effect of advanced maturity have typically looked at disease states such as sarcopenia, where calpastatin expression and inhibitory activity against m-calpain are decreased (Dargelos et al., 2007). Calpain activity also appears to be increased in the sarcopenic state, which is related to both calpastatin expression and less nitrosylation of m-calpain and caspase-3 (Samengo et al., 2012).

Calpain and calpastatin activities also differ across muscles, likely due to differing turnover rates between muscles (Stolowski et al., 2006; G. Whipple & Koohmaraie, 1992).
For example, muscles with a more oxidative fiber type generally have greater calpain and calpastatin activities compared to those which are more glycolytic (G. Whipple & Koohmaraie, 1992). Additionally, muscles may change with age in their calpastatin activity at different rates. For instance, in 5 to 17 week old turkeys, calpastatin activity from breast muscle had reached its greatest level at 5 weeks of age and declined thereafter, whereas calpastatin from thigh muscle activity increased through 17 weeks of age (Northcutt et al., 1998).

The roles that μ-calpain, m-calpain, and calpastatin play in muscle growth and protein turnover provide compelling reasons to investigate how the calpain proteases and their inhibitor affect muscle from animals in growing and mature stages of development. Calpain and calpastatin involvement in postmortem proteolysis and tenderization of beef provide further incentive to investigate these possible differences. The objective of this project therefore was to determine the extent to which muscle and growth stage of beef cattle contribute to variation in the calpain system, including μ-calpain, m-calpain, and calpastatin.

2. Materials and Methods

Six weanling calves and six cull cows were slaughtered in pairs at the Iowa State University Meat Laboratory on 6 different days. Calves were 7 to 8 months in age (7.3±0.5 months) and weighed 235.3±38.0 kg at the time of slaughter; cows ranged from 56 to 162 months in age (106.7±43.1 months) and weighed 569.9±85.5 kg. Samples from the longissimus (LD), semimembranosus (SM), and triceps brachii (TB) were collected within 90 minutes post-exsanguination and placed on ice before extraction or packaging. Excluding
the first pair of cattle, muscle samples were randomly divided into 3 sections for analyses at either 0, 1, or 6 days of aging at 4°C.

2.1 Calpain System Activities

Two finely minced 5 g samples were taken from each day 0 muscle section and immediately extracted using 3 volumes (wt/vol) of ice-cold extraction buffer containing 100 mM Tris-HCl, 10 mM EDTA, pH 8.3. Immediately before use, 0.1% 2-mercaptoethanol (MCE), 2 µM E-64, and 500 mg/L trypsin inhibitor were added to the buffer. Samples were homogenized using a Polytron PT 3100 (Lucerne, Switzerland) in three 30 s bursts. The resulting homogenate was centrifuged at 25,000 x g for 20 min at 4°C, and the supernatant was filtered through cheesecloth and dialyzed in 40 volumes of TEM (40 mM Tris-HCl, 1 mM EDTA, pH 7.4, with 0.1% MCE). Once dialysis was complete, samples were again centrifuged at 25,000 x g for 20 min at 4°C and the supernatant filtered through cheesecloth.

Dialyzed and filtered samples were then loaded onto a 20 ml Q-Sepharose Fast Flow (GE Healthcare Biosciences, Pittsburgh, PA) anion exchange column previously equilibrated with TEM. After washing the loaded column with 10 volumes (200 ml) TEM, calpastatin, µ-calpain, and m-calpain were eluted using a linear gradient of 60 to 400 mM KCl in TEM. Calpastatin activity eluted in two separate, distinct peaks (calpastatin I and II, at 60 to 90 mM KCl and 120 to 190 mM KCl, respectively, Figure 1), followed by µ-calpain activity (180 to 240 mM KCl) and m-calpain activity (300 to 400 mM KCl).

The activities of µ- or m- calpain or calpastatin-containing fractions were determined using casein as a substrate, using a modification of the method of Koohmaraie (1990). Sample from each eluted fraction was brought to 1 ml with TE (40 mM Tris-HCl, 1 mM EDTA, pH 7.4). For calpastatin activity, approximately 0.4 units of previously purified
porcine lung m-calpain in TE were included. One milliliter of casein buffer (100 mM Tris-acetate 7 mg/ml casein, and 1 mM sodium azide, pH 7.5, with 0.2% MCE added just before use) was added, followed by 100 µl of 200 mM CaCl$_2$. Samples were briefly vortexed and incubated in a water bath at 25°C for 1 hr. Following incubation, the reaction was stopped with 2 ml of 0.5% trichloroacetic acid, vortexed, and centrifuged at 1,500 x g for 20 minutes at 25°C. The absorbance at 278 nm of the supernatant was measured and compared to blank (no eluant, no porcine lung m-calpain) and, in the case of calpastatin activity, positive control (no eluant, with porcine lung m-calpain) samples. One unit of μ- or m-calpain activity was defined as the amount required to catalyze an increase of 1 absorbance unit of the supernatant at 278 nm. One unit of calpastatin activity was defined as the amount required to inhibit 1 unit of porcine lung m-calpain (Koohmaraie, 1990).

2.2 Heated Calpastatin Assay

Calpastatin activity was determined on all muscle samples at 0, 1, and 6 d postmortem using the heated calpastatin assay originally described by Shackelford et al. (1994). Samples were extracted, centrifuged, and dialyzed as described above. Dialysates were then transferred to 50 ml tubes and heated in a 95°C water bath for 30 min, followed by chilling in an ice bath for 15 min. After chilling, the tubes were vortexed in order to break up the coagulated protein and to facilitate separation during centrifugation. Samples were then transferred to 30 ml centrifuge tubes and centrifuged at 25,000 x g for 20 min at 4°C. Following centrifugation, samples were filtered through cheesecloth and their volume was measured for subsequent calculation of calpastatin activity. Activity was determined using casein as a substrate, as described above.
2.3 SDS-PAGE and Immunoblotting

Muscle samples from cattle in the second through sixth slaughter groups were used for SDS-PAGE and immunoblotting to determine presence of calpastatin, μ-calpain autolysis, and troponin-T and titin degradation. Whole muscle sample extracts were prepared as described by Huff-Lonergan, Mitsuhashi, Parrish, and Robson (1996). An 8% polyacrylamide separating gel (acrylamide:N,N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.05% [vol/vol] TEMED, 0.05% [wt/vol] ammonium persulfate, and 0.5 M Tris-HCl, pH 8.8) was used to detect μ-calpain autolysis, and 10% polyacrylamide separating gels were used for calpastatin and troponin-T degradation. A 5% polyacrylamide gel (acrylamide:N,N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.125% [vol/vol] TEMED, 0.075% [wt/vol] ammonium persulfate, and 0.125 M Tris-HCl, pH 6.8) was used for the stacking gel. Gels (10 cm wide x 8 cm tall x 1.5 cm thick) were loaded with 40 µg protein per lane and run at a constant 120 volts in SE 260 Hoefer Mighty Small II (Hoefer, Inc., Holliston, MA) electrophoresis units. For titin analysis, a 5% polyacrylamide continuous gel was used. Gels (14 cm wide x 15 cm tall x 1.5 cm thick) were loaded with 160 µg protein and run at 5 mA for 48 hr in Hoefer SE 600 electrophoresis units before staining with colloidal Coomassie dye (34% methanol, 17% ammonium sulfate [wt/vol], 3% phosphoric acid, 0.1% Coomassie G-250 [wt/vol]).

Transfer of protein from SDS-PAGE gels to a PVDF membrane was performed as described by Melody et al. (2004) Membranes were blocked for 1 hr at room temperature in PBS with 0.1% Tween-20 and 0.5% nonfat dry milk and were then incubated in primary antibody overnight at 4°C. Primary antibodies and dilutions were as follows: μ-calpain, 1:10,000 (MA3-940, Thermo Scientific, Rockford, IL); calpastatin, 1:5,000 (MA3-945,
Thermo Scientific, Rockford, IL); and troponin-T, 1:40,000 (JLT-12; Sigma, St Louis, MO). Membranes were washed 3 times in PBS-Tween for 10 minutes each at room temperature. The membranes were then incubated in a goat anti-mouse horseradish peroxidase (No 2554, Sigma, St. Louis, MO) secondary antibody at 1:10,000 dilution for 1 hr at room temperature. After 3 additional 10 min washes in PBS-Tween, blots were developed using ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ) and imaged using a ChemiImager 5500 (Alpha Innotech, San Leandro, CA) and Alpha Ease FC software (v 3.03 Alpha Innotech). Bands were quantified by densitometry; the abundance of the 115 kDa calpastatin band at day 0 and the 30 kDa troponin-T degradation product at day 6 were calculated as a ratio of the sample band compared to a reference sample loaded on each gel. For µ-calpain autolysis at day 1, the intact catalytic subunit (80 kDa) and its autolyzed products (78 and 76 kDa) were each calculated as a percentage of all three bands within a sample. For titin degradation at day 6, T1 (intact titin band) was measured as a percentage of the total T1 and T2 (degraded) bands within a sample.

2.4 Statistical Analysis

Data were analyzed using split plot design with growth category (growing or mature) as the whole plot and muscle as the split plot. Data for the heated calpastatin assay used a repeated measures design to account for changes over the 6 day storage period. Analysis was done using the MIXED procedure of SAS (v. 9.3, SAS Institute, Cary, NC) and least squares means were separated (P < 0.05) using the PDIFF option. Additionally, the REG procedure of SAS was used for regression analysis of calpastatin activities obtained using the ion exchange chromatography versus the heated calpastatin assay.
3. Results and Discussion

3.1 Calpastatin Activity Separation

The discovery of the separation of two distinct pools of calpastatin activity using anion exchange chromatography is a recent development, likely due to improved separation protocols and materials. Molecular diversity of the calpastatin molecule is one explanation for this repeatable separation of the two peaks of activity. Diversity of calpastatin molecules has been described in porcine skeletal muscle with elution patterns similar to that seen in the current study (Cruzen et al., 2013). This variation has also been noted in bovine skeletal muscle (Geesink, Nonneman, & Koohmaraie, 1998), porcine cardiac muscle (Geesink et al., 1998), salmon skeletal muscle (Gaarder, Thomassen, & Veiseth-Kent, 2011) and rat brain, heart, kidney, and skeletal muscle (Averna et al., 2001; Pontremoli et al., 1991; Pontremoli et al., 1992; Franca Salamino et al., 1997; F. Salamino et al., 1994). This molecular diversity demonstrated by calpastatin activity elution peaks may be explained by calpastatin degradation (Geesink et al., 1998), alternative splicing resulting in different calpastatin isoforms (Gaarder et al., 2011; Geesink et al., 1998), or posttranslational phosphorylation differences (Averna et al., 2001; Pontremoli et al., 1992; Franca Salamino et al., 1997; F. Salamino et al., 1994). Although the reason for the separation was not investigated in the current study, it is evident that calpastatin I has less total inhibitory activity than calpastatin II, as assayed against porcine m-calpain (Tables 1 and 2).

3.2 Calpain and Calpastatin Activity

Comparisons of calpain system activity data between growth categories is presented in Table 1. Muscle from mature animals had greater levels (P = 0.04) of m-calpain activity compared to growing animals. Total calpastatin activity and μ-calpain activity did not differ
significantly at day 0 (P = 0.19 and 0.38, respectively) between growing and mature animals. However, the ratio of µ-calpain:total calpastatin activity tended to be greater (P = 0.08) in muscle from growing cattle. This indicates that there is a tendency for greater µ-calpain activity for every unit of calpastatin activity in the muscle from younger animals. A greater µ-calpain: total calpastatin ratio would predict greater protein turnover in living muscle and promote increased postmortem proteolysis in meat from those animals (Veiseth et al., 2004). Tissue with a greater µ-calpain:total calpastatin ratio is expected to allow more postmortem protein degradation than tissue with a lesser µ-calpain:total calpastatin ratio. Thus, it could be predicted that meat from growing animals compared to meat from those advanced in maturity would have a greater or more rapid development of tenderness. Lamb and rabbits have a sharp decrease in skeletal muscle calpain and calpastatin activities during the time between birth and weaning, but the cattle in this study were outside these stages of growth (Ou & Forsberg, 1991; Ou et al., 1991). The decrease in calpastatin activity observed in those studies is believed to partly be a dilution effect due to the accumulation of other proteins during growth (Ou & Forsberg, 1991). Again, in these previous studies, most animals were growing or just at the end of their growth period. As animals age, a decrease in muscle mass (sarcopenia) may be observed, with a decrease in both the number and size of muscle fibers. In 24 month old Wistar rats, µ-calpain expression is increased, and calpastatin expression is decreased compared to rats 3 months of age (Dargelos et al., 2007). It is unknown whether cattle in the age group in this study are subject to sarcopenia, but based on the results of this study, the cows selected did not have a sarcopenic condition characterized by decreased calpastatin or increased µ-calpain compared to their younger counterparts. The current study
is unique because it compares animals that are growing and those at the end of their growth period and approaching senescence.

Comparisons of calpastatin and μ- and m-calpain activities between muscles are presented in Table 2. While no differences were observed in μ-calpain or calpastatin I activities between different muscles (P = 0.23, and 0.54, respectively), the TB had the least m-calpain activity (P = 0.02) and the greatest calpastatin II (P < 0.0001) and total calpastatin activity (P < 0.001) compared to the other muscles. The TB had the lowest (P = 0.01) μ-calpain:total calpastatin ratio. These data indicate that the TB has the least potential for proteolytic activity during postmortem aging of the muscles analyzed. The LD and SM were similar in μ-calpain, m-calpain, and calpastatin activities (P = 0.40, 0.25, and 0.14, respectively). Stolowski et al. (2006) also found that calpastatin activity was greater in TB beef muscle when compared to LD or SM, which were similar in calpastatin activity. Muscles with a greater proportion of type I slow oxidative fibers are generally known to have greater calpastatin activity and exhibit a slower rate of postmortem proteolysis when compared with muscles that have more type II glycolytic fibers (Geesink, Koolmees, van Laack, & Smulders, 1995; Sazili et al., 2005). However, some exceptions exist, such as in the psoas major, which possesses a greater proportion of type I fibers and undergoes rapid proteolysis. This may be partially due to a rapid early postmortem chilling rate because in traditional harvest processes this muscle is exposed to ambient cooler temperatures. This may cause increased calcium release (Hunt & Hedrick, 1977; Melody et al., 2004). The SM and LD of beef cattle each typically have greater than 40% type IIb fibers and are therefore considered white, glycolytic muscles, whereas the TB is considered to be of intermediate fiber type, having approximately equal amounts of type I, IIa, and IIb fibers (Kirchofer,
Therefore, because the TB has more oxidative fibers compared to the LD or SM, it would be expected that the TB would have greater total calpastatin activity than the LD and SM in this and other studies. Calpastatin activity may partially explain the decreased tenderness previously observed in beef TB compared to the LD, but not the greater toughness observed in beef SM, which is likely largely a result of connective tissue abundance (Rhee, Wheeler, Shackelford, & Koohmaraie, 2004; Torrescano, Sánchez-Escalante, Giménez, Roncalés, & Beltrán, 2003). No interactions between growth category and muscle were observed for μ-calpain, m-calpain, or calpastatin activities.

3.3 Heated Calpastatin Assay

Calpastatin activity was also determined using a heated calpastatin assay, in order to observe differences in total calpastatin activity across several days of postmortem storage. Data from the heated calpastatin assay are presented in Figure 2. Differences between growing and mature animals were not observed until day 6 postmortem (P = 0.004), with mature animals having greater calpastatin activity at that time point. Calpastatin activity declined over time in all muscles in both growth categories (P < 0.0001). In growing cattle, day 6 calpastatin activity was only 53% of the original day 0 activity. However, calpastatin activity in muscle from mature cattle persisted more throughout the aging process, retaining 80% of its original activity at 6 days postmortem. Greater calpastatin stability in muscle from more mature cattle may limit the amount of postmortem tenderization that can occur in meat from older animals. These data support the day 0 μ-calpain:total calpastatin ratios observed in the calpain and calpastatin activity data. As muscle from mature animals tended to have a lower μ-calpain:total calpastatin ratio, it would be expected that less calpastatin degradation and loss of activity would occur in that muscle. Calpastatin itself is a substrate for μ-calpain.
as well as a substrate for other proteases, such as the proteasome, cathepsin B, and several caspases) (Doumit & Kooohmaraie, 1999; Goll et al., 2003; Wang et al., 1998). It is therefore reasonable to suggest that calpastatin may have been degraded more quickly in young versus growing animals, thus losing some of its inhibitory ability (Pontremoli et al., 1991; Wang et al., 1998).

Muscles differed (P < 0.0001) in their calpastatin activity, as measured by the heated calpastatin assay (Figure 2B). Overall, the TB had the greatest calpastatin activity, followed by the LD (P < 0.001). The SM had the least calpastatin activity (P = 0.002). Again, this signifies that the TB is likely to have limited postmortem proteolysis when compared to the LD or SM. These data also support the observed day 0 calpain and calpastatin activity data. However, while no differences in calpain or calpastatin activity were found between the LD and SM using the assay following chromatography (P = 0.14), less overall calpastatin activity was observed in the SM versus the LD using the heated calpastatin assay.

The heated calpastatin assay was originally developed by Shackelford et al. (1994), who observed that the procedure correlated very well (r² = 0.88) with a method using ion exchange chromatography, although the heated calpastatin assay tended to overestimate calpastatin by 1.2 units/g muscle. While the heated calpastatin assay in this study is very similar to that of Shackelford et al. (1994), calpastatin activity was actually underestimated compared to the ion exchange chromatography method at day 0 of the current study, possibly due to the improved protocols and materials in the ion exchange chromatography method. As shown in Figure 3, the heated calpastatin assay in this study was moderately correlated with total calpastatin activity (r² = 0.48, P < 0.001). However, when compared with only calpastatin II activity, this correlation was stronger (r² = 0.61, P < 0.0001). Calpastatin I
activity had no correlation with the heated calpastatin assay activity \( (r^2 = 0.00, P = 0.97) \), indicating that the heated assay may not include calpastatin I activity. One possible explanation for this could be lack of stability of calpastatin I; perhaps this calpastatin activity is lost during boiling, which would also assist in explaining the underestimation of activity seen in the heated calpastatin assay.

3.4 Calpastatin abundance and \( \mu \)-calpain autolysis

Measurement of calpain autolysis can be used to provide evidence of how active the calpain has been up the measured time point. As calpain is activated, the 80 kDa catalytic subunit is progressively autolyzed to the 78 kDa then 76 kDa form (Goll et al., 2003). Data for \( \mu \)-calpain autolysis and calpastatin abundance are presented in Table 3. Muscle from mature beef animals had less overall \( \mu \)-calpain autolysis at 1 day postmortem. This is evidenced by a significantly greater \( (P = 0.007) \) percentage of the intact 80 kDa subunit band present in muscle from mature animals versus muscle from growing animals, as well as a lower \( (P = 0.01) \) percentage of the 78 or 76 kDa autolysis products. Therefore, less activation of \( \mu \)-calpain activity had occurred in meat from mature animals at day 1. These data also corroborate the tendency for differences found in the \( \mu \)-calpain:calpastatin activity ratio, as the lower ratio found in the mature animals would indeed result in less \( \mu \)-calpain activity. Additionally, abundance of the 115 kDa calpastatin band at day 0 was greater \( (P = 0.04) \) in muscle from mature animals. These results support the \( \mu \)-calpain autolysis data, as greater calpastatin activity would inhibit \( \mu \)-calpain activity, and therefore autolysis (Koohmarai, 1992a). Autolysis of \( \mu \)-calpain did not significantly differ \( (P > 0.20\) for 80, 78, and 76 kDa bands) between muscles, but calpastatin abundance at day 0 was greater \( (P = 0.03) \) in TB
versus LD or SM, which is consistent with both the $\mu$-calpain and calpastatin activities observed in these muscles (Table 4).

3.5 Protein Degradation

Titin is the largest protein found in muscle at over 3,000 kDa, and extends from the Z-line to the M-line within sarcomeres. As an important structural protein, titin degradation is related to tenderness (Huff-Lonergan et al., 1995). Troponin-T is part of the troponin complex which regulates muscle contraction. Appearance of the 30 kDa degradation product of troponin-T is highly related to the development of tenderness of beef postmortem (Ho, Stromer, & Robson, 1994; Lonergan, Huff-Lonergan, Wiegand, & Kriese-Anderson, 2001). While some have speculated that degradation of troponin-T is only an indicator of overall proteolysis and therefore tenderness development, others believe that, because of its location and role in actomyosin cross bridge formation, the degradation of troponin-T may directly cause disruption of either the thin filament or the actomyosin bond itself, and result in increased tenderness (Huff-Lonergan, Zhang, & Lonergan, 2010). The data from early postmortem time points are shown in the day 6 protein degradation data. The percentage of titin that remained intact (T1) was greater (Table 3, $P = 0.004$) in muscle from mature animals. Huff-Lonergan et al. (1995) observed similar results when comparing titin degradation in muscle from cows and market steers. The troponin-T degradation product was also lesser (Figure 4, Table 3, $P < 0.008$) in mature beef muscle. Duarte et al. (2011) measured myofibrillar fragmentation index (MFI), an indicator of overall myofibrillar proteolysis (Olson, Parrish, & Stromer, 1976), and found that MFI decreased with increasing number of permanent incisors (from 2 to 8) in Nellore bulls. Both the titin and troponin-T
data are indicative of decreased postmortem protein degradation in muscle from mature animals, which would result in less tenderness development during aging.

Intact titin (T1) was more abundant (Table 4, P < 0.004), and troponin-T degradation product less abundant (Figure 4, Table 3, P < 0.0001) in TB versus LD or SM at 6 days postmortem. Anderson et al. (2012) also observed similar troponin-T and titin degradation between the LD and SM in beef. Although the SM is often observed to be tougher than either the LD or TB, this is probably due to greater levels of connective tissue in the SM, which may override the gains in tenderness that are realized from myofibrillar proteolysis (Rhee et al., 2004; Stolowski et al., 2006). The TB also has high levels of total and insoluble collagen which may influence tenderness (Seggern, Calkins, Johnson, Brickler, & Gwartney, 2005; Stolowski et al., 2006; Torrescano et al., 2003). Additionally, Rhee et al. (2004) observed that the TB had less desmin degradation over 14 days of aging versus either LD or SM in beef, and Geesink et al. (1995) have documented a slower rate of troponin-T degradation in TB versus LD beef muscle. An age by muscle interaction also existed for troponin-T degradation (P = 0.03, Figure 4). The SM had the greatest troponin-T degradation in mature but not growing animals. The LD had the greatest troponin-T degradation in growing but not mature animals, and was actually similar (P = 0.09) to the TB in mature animals. However, SM and LD troponin-T degradation were not significantly different from each other in either maturity group.

4. Conclusions

These data indicate growth stage (growing versus mature) may impact tenderness development during the postmortem conversion of muscle to meat. The lack of tenderness commonly observed in meat from older animals may be due to this lack of postmortem
proteolysis in addition to the increased insoluble collagen. Decreased protein degradation postmortem in muscle from older animals is the result of a lower µ-calpain:total calpastatin activity ratio, as well as calpastatin activity which is more stable throughout the postmortem aging process. Further, various muscles may go through the postmortem tenderization process at different rates or extents, and some muscles, such as the TB, are more resistant to this postmortem aging process than others. However, the relative extent of proteolytic activity and postmortem protein degradation between muscles does not appear to change with animal maturity. Methods other than postmortem aging may be needed to tenderize cuts from older animals or muscles with less proteolytic capacity to an acceptable level.

Acknowledgements

The authors would like to thank Dr. Ed Steadham for his support in completing this project. This project was funded by the Wise Burroughs Memorial Endowment in Animal Sciences research grant.

Literature Cited


Tables and Figures

Table 1. Calpain system activities in muscle from growing and mature beef cattle, expressed as units of activity per g muscle tissue. One unit of calpain activity is defined as the amount of enzyme required to catalyze an increase of 1.0 absorbance unit at 278 nm in 60 min at 25 °C. One unit of calpastatin activity is defined as the amount of calpastatin required to inhibit 1.0 unit of porcine lung m-calpain activity (Koohmaraie, 1990).

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<th>mature</th>
<th>SE</th>
<th>P-value</th>
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<td>0.68</td>
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<sup>a</sup> Calpastatin I plus calpastatin II

<sup>b</sup> Ratio of μ-calpain activity to total calpastatin activity within a sample
Table 2. Calpain system activities in *longissimus* (LD), *semimembranosus* (SM), and *triceps brachii* (TB) muscle from beef cattle, expressed as units of activity per g muscle tissue. One unit of calpain activity is defined as the amount of enzyme required to catalyze an increase of 1.0 absorbance unit at 278 nm in 60 min at 25 °C. One unit of calpastatin activity is defined as the amount of calpastatin required to inhibit 1.0 unit of porcine lung m-calpain activity (Koohmaraie, 1990).

<table>
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<td>1.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.44&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.30&lt;sup&gt;y&lt;/sup&gt;</td>
<td>0.086</td>
<td>0.02</td>
</tr>
<tr>
<td>calpastatin I</td>
<td>0.94</td>
<td>0.83</td>
<td>0.92</td>
<td>0.186</td>
<td>0.54</td>
</tr>
<tr>
<td>calpastatin II</td>
<td>1.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.245</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>total calpastatin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.358</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>µ-calpain:total calpastatin&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.050</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<sup>ab</sup> Within a row, values with different lowercase letters are significantly different (P < 0.05).
<sup>c</sup> Calpastatin I plus calpastatin II
<sup>d</sup> Ratio of µ-calpain activity to total calpastatin activity within a sample
Table 3. Analysis of µ-calpain autolysis, calpastatin abundance, and intact titin. Autolysis of µ-calpain, calpastatin abundance, and intact titin were determined using immunoblotting (µ-calpain and calpastatin) and SDS-PAGE (titin) in whole muscle extract from growing and mature beef animals.

<table>
<thead>
<tr>
<th></th>
<th>growing</th>
<th>mature</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>µ-calpain day 1(^a)</td>
<td>80 kDa</td>
<td></td>
<td>42.1</td>
<td>74.0</td>
</tr>
<tr>
<td></td>
<td>78 kDa</td>
<td></td>
<td>27.7</td>
<td>17.1</td>
</tr>
<tr>
<td></td>
<td>76 kDa</td>
<td></td>
<td>30.2</td>
<td>8.9</td>
</tr>
<tr>
<td>calpastatin day 0(^b)</td>
<td>0.09</td>
<td></td>
<td>0.31</td>
<td>0.054</td>
</tr>
<tr>
<td>titin T1 day 6(^c)</td>
<td>5.9</td>
<td></td>
<td>34.0</td>
<td>3.293</td>
</tr>
<tr>
<td>troponin-T day 6(^d)</td>
<td>1.06</td>
<td></td>
<td>0.32</td>
<td>0.105</td>
</tr>
</tbody>
</table>

\(^a\) Values are expressed as a percentage of the catalytic subunit present as the unautolyzed (80 kDa) or the autolysis products (78 and 76 kDa) of the catalytic subunit of µ-calpain.

\(^b\) Values are expressed as a ratio of intensity of the primary 115 kDa calpastatin band of the sample, compared to that of the primary band of an internal control.

\(^c\) Values are expressed as a percentage of the intact form of titin (T1) versus total titin (T1 + T2 (degraded form)) present within a sample.

\(^d\) Values are expressed as a ratio of the intensity of the 30 kDa degradation product compared to that of an internal control sample.
Table 4. Analysis of µ-calpain autolysis, calpastatin abundance, and titin degradation.

Autolysis of µ-calpain and calpastatin abundance were determined using immunoblotting (µ-calpain and calpastatin) and SDS-PAGE (titin) in longissimus (LD), semimembranosus (SM), and triceps brachii (TB) whole muscle extract from beef cattle.

<table>
<thead>
<tr>
<th></th>
<th>LD</th>
<th>SM</th>
<th>TB</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>µ-calpain day 1&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>78 kDa</td>
<td>20.7</td>
<td>24.4</td>
<td>22.2</td>
<td>2.757</td>
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<tr>
<td>76 kDa</td>
<td>20.2</td>
<td>13.7</td>
<td>24.6</td>
<td>5.290</td>
<td>0.20</td>
</tr>
<tr>
<td>calpastatin day 0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.059</td>
<td>0.03</td>
</tr>
<tr>
<td>titin T1 day 6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>15.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.297</td>
<td>0.004</td>
</tr>
<tr>
<td>troponin-T day 6&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.089</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<sup>a</sup>b Within a row, values with different lowercase letters are significantly different (P < 0.05).
<sup>c</sup> Values are expressed as a percentage of the catalytic subunit present as the unautolyzed (80 kDa) or the autolysis products (78 and 76 kDa) of the catalytic subunit of µ-calpain.
<sup>d</sup> Values are expressed as a ratio of intensity of the primary 115 kDa calpastatin band of the sample, compared to that of the primary band of an internal control.
<sup>e</sup> Values are expressed as a percentage of the intact form of titin (T1) versus total titin (T1 + T2 (degraded form)) present within a sample.
<sup>f</sup> Values are expressed as a ratio of the intensity of the 30 kDa degradation product compared to that of an internal control sample.
Figure 1. Representative chromatographic profile of calpastatin separation during elution using a Q-Sepharose Fast Flow ion exchange column. Calpastatin was eluted using a 60 to 200 mM KCl gradient with 2.5 ml fractions.
Figure 2. Calpastatin activity (determined on boiled extracts on days 0, 1, and 6 postmortem from the heated calpastatin assay) in A) muscle from growing versus mature beef cattle and in B) *longissimus* (LD), *semimembranosus* (SM), and *triceps brachii* (TB) muscles from beef cattle. Values are expressed as units of calpastatin activity per g muscle tissue. One unit is defined as the amount of calpastatin required to inhibit 1.0 unit of porcine lung m-calpain activity at 278 nm in 60 min at 25 °C (Koohmaraie, 1990). Within a day, values with different superscripts differ significantly (P < 0.05). Error bars are standard errors of the means.

A)

B)
Figure 3. Regression analysis of A) total calpastatin, B) calpastatin I, or C) calpastatin II, as determined on fractions from ion exchange chromatography methods, and on the boiled extract from the heated calpastatin assay. Values are expressed as units of calpastatin activity per g muscle tissue. One unit is defined as the amount of calpastatin required to inhibit 1.0 unit of porcine lung m-calpain activity at 278 nm in 60 min at 25 °C (Koohmaraie, 1990).

A)  
\[ y = 0.400x + 0.825 \]
\[ R^2 = 0.48 \]

B)  
\[ y = -0.005x + 1.963 \]
\[ R^2 = 0.0000 \]

C)  
\[ y = 0.619x + 0.749 \]
\[ R^2 = 0.61 \]
**Figure 4.** Troponin-T degradation at 6 days postmortem in *longissimus* (LD), *semimembranosus* (SM), and *triceps brachii* (TB) muscles from growing and mature beef cattle. A maturity group by muscle interaction was observed for troponin-T degradation. Values are expressed as a ratio of intensity of the 30 kDa degradation band of the sample, divided by the 30 kDa degradation band of an internal control. Means with different superscripts differ significantly (P < 0.05). Error bars are standard errors of the means.
Figure 5. Representative blots depicting A) μ-calpain autolysis, 1 day postmortem; B) presence of calpastatin, 0 days postmortem; and C) troponin-T degradation, 6 days postmortem in *longissimus* (LD), *semimembranosus* (SM), and *triceps brachii* (TB) muscles from growing and mature beef cattle. D) Representative gel depicting titin degradation, 6 days postmortem in the same muscles and animals.
CHAPTER 5. SKELETAL MUSCLE CALPASTATINS SEPARATED USING ANION EXCHANGE CHROMATOGRAPHY DIFFER IN PHOSPHORYLATION

A paper prepared for submission to the Journal of Agriculture and Food Chemistry

Shannon M. Cruzen, Steven M. Lonergan, Elisabeth Huff-Lonergan

Graphical Abstract

Abstract

The objective of the current work was to characterize two unique forms of calpastatin (calpastatin I and II) detected during elution from an anion exchange column. Our experiments show that while there is less total activity in the calpastatin I peak on either a total volume or per ml basis, both calpastatins inhibit μ- and m-calpain similarly. Two-dimensional immunoblotting for calpastatin resulted in detection of anti-calpastatin immunoreactive proteins that migrated at 145 and 60 kDa in both calpastatin I and II eluate samples. The 145 kDa spots were also identified as calpastatin with mass spectrometry. Two-

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*Corresponding author
dimensional gels were stained to detect phosphoproteins and total protein abundance. Compared to those in calpastatin I, the spots identified as calpastatin in the calpastatin II peak stained 3 times more intensely with the phosphoprotein stain when compared against total protein staining. It is proposed that calpastatin II is phosphorylated to a greater extent, resulting in its later elution from an anion exchange column.

**Introduction**

Calpastatin is the endogenous inhibitor of μ- and m-calpain. In skeletal muscle, μ- and m-calpain are two major calcium dependent cysteine proteinases. Calpain 3, or p94, is another muscle specific calpain which is not inhibited by calpastatin. In muscle, evidence supports the role of the calpain system in skeletal muscle protein turnover and hypertrophic muscle growth. Greater calpastatin activity results in decreased protein degradation, leading to greater total protein accretion. The callipyge condition in lambs is caused by a single gene mutation which results in heavy muscling and greater feed efficiency. In the callipyge phenotype, several muscles are hypertrophied, particularly the muscles of the leg and loin. These muscles are also characterized by decreased fractional protein synthesis and degradation rates, but increased calpastatin expression and activity. When livestock are fed β-adrenergic agonists as growth promotants, one reason for increased growth is greater calpastatin activity. With β-adrenergic agonist supplementation, calpastatin activity increases, limiting protein degradation. Additionally, protein synthesis is also increased, leading to a net protein accretion and growth. Conversely, calpastatin overexpression may inhibit muscle growth by preventing the protein turnover necessary for fiber type shifts prior to hypertrophy through reduction of calpain activity. During treatment with β-adrenergic
agonists, muscle fibers undergo a slow to fast fiber type phenotypic shift \(^9\). Mice which were engineered to overexpress calpastatin through electrotransfection were unable to achieve this fiber type change and therefore did not have the same level of muscle hypertrophy as normal mice when exposed to the β-adrenergic agonist clenbuterol \(^9\).

Because μ-calpain is considered to be one of the primary proteases responsible for the development of meat tenderness during postmortem aging \(^11-13\), calpastatin activity is also thought to be a primary regulator of tenderness development. Greater calpastatin activity results in decreased postmortem proteolysis during meat aging, leading to increased toughness. Indeed, greater calpastatin activity is indicated in several models involving limited postmortem proteolysis and meat toughness, including callipyge lambs, livestock fed beta-adrenergic agonists, and cattle with 37% or greater Bos indicus influence \(^3,14-16\). In the callipyge phenotype, the same muscles that are hypertrophied can be extremely tough and are 1.5 to 2.5 times tougher after postmortem aging when compared to muscle from normal lambs \(^3-4\). The hypertrophy resulting from β-adrenergic agonist supplementation also causes toughness in meat \(^15\). And finally, cattle with a high percentage of Bos indicus influence have greater poststrigor skeletal muscle calpastatin activity and generally produce tougher beef which does not undergo postmortem proteolysis to the extent that beef from Bos taurus cattle does \(^16-18\). Limited postmortem muscle protein degradation is similarly observed in mice that have been transgenically engineered to overexpress calpastatin \(^19\). Therefore, calpastatin activity has the potential to decrease meat quality by inhibiting postmortem proteolysis and tenderization.

Additionally, calpastatin genotype has been shown to account for increased drip loss, but not shear force, in pork \(^20\). In intron 6 of the CAST gene in pork, Gandolfi et al. \(^20\)
observed a guanine to adenine transition. Pigs carrying an AA genotype for calpastatin had greater skeletal muscle µ-calpain autolysis compared to those with GG or GA calpastatin genotypes, indicating that calpastatin may limit the µ-calpain activity to a greater extent in the GG or GA genotypes. Muscle from pigs with the AA genotype also had less drip loss at 72 hours postmortem, which could be related to µ-calpain activity. Ciobanu et al. investigated several calpastatin polymorphisms, revealing three different haplotypes in 8 Western commercial swine breeds. They found that the first haplotype, which had 249Lys and 638Arg (versus 249Arg or 638Arg in haplotype 2; and 249Arg, 638Ser in haplotype 3) was associated with the best quality attributes, including higher pH, better subjective tenderness and juiciness, and lower shear force and cook loss in commercial crossbred pigs. Interestingly haplotypes 1 and 3 had different predicted phosphorylation sites, which may influence calpastatin activity. Therefore, calpastatin activity in meat also has implications in meat quality beyond tenderness.

Calpastatin is a 70-80 kDa protein (except in erythrocytes, which have a smaller version of the protein) which is composed of an N-terminal an L domain, and four repeating domains which each have the ability to inhibit one calpain molecule (domains I-IV). Some calpastatin, primarily in cardiac muscle, also contains a 68 aa XL N-terminal domain. In early studies, reported calpastatin molecular weights ranged from less than 50 kDa to over 200 kDa, the result of many factors which have made calpastatin difficult to characterize. First, the disordered structure of calpastatin in solution makes it quite susceptible to proteolysis, which may have been exacerbated with heat and denaturing reagents used in purification. Second, the same disordered structure of calpastatin causes molecular weight to be greatly overestimated when using size exclusion chromatography.
(which explains the values over 200 kDa) \(^\text{26}\). Molecular weight is also overestimated when using SDS-PAGE because calpastatin migrates anomalously (typically 100-150 kDa for full-size calpastatin) \(^\text{1, 25}\). Finally, several isoforms of calpastatin exist based on alternative splicing or use of different promoters \(^\text{1, 24-25, 27}\).

Most (but not all) isoforms produced from alternative splicing affect the XL or L domain of calpastatin, and therefore do not appreciably change calpain inhibitory ability \(^\text{1, 28}\). Possible functions of the L domain include membrane binding or regulation of calcium channels \(^\text{29-32}\). Calpastatin isoforms may be of decreased molecular weight, as in erythrocytes or the testes (46 kDa and 18.7 kDa, respectively) \(^\text{25}\), or have seemingly unchanged molecular weight, as seen in Type I versus Type II calpastatin in bovine cardiac muscle (both 145 kDa in SDS-PAGE) \(^\text{24}\). Type II calpastatin with no XL domain is the isoform primarily seen in skeletal muscle, but some studies have observed expression of Type I and II calpastatin protein or RNA \(^\text{24, 33-34}\). Additionally, these isoforms may differ in isoelectric point (pI). Domain L has very basic calculated pI (10.27), whereas domains I through IV each have a calculated pI between 4.26 and 4.90 \(^\text{35}\). Therefore, alternative splicing or protein degradation which affect domain L may cause a change in pI. Takano et al. observed that human erythrocyte calpastatin, which lacks domains L and I, had a pI of 4.55, whereas calpastatin in other tissues typically has a pI between 4.85 and 4.95 \(^\text{36-37}\).

In most cases, calpastatin activity in muscle is reported as one value, the total calpastatin activity per g of protein or muscle tissue in the animal. However, during the purification process necessary for calpastatin activity measurement, our lab has consistently seen a separation of two distinct “peaks” of calpastatin activity during its elution from an anion exchange column, in both pork and beef muscle \(^\text{38-39}\) (Figure 1). Geesink et al. \(^\text{28}\)
observed similar separation in bovine skeletal muscle and porcine cardiac muscle. In bovine skeletal muscle, the first eluted calpastatin peak was believed to be a calpastatin degradation product, and in porcine cardiac muscle, Geesink et al. concluded that the two peaks were the result of alternative splicing. Calpastatin separation during ion exchange chromatography has also been documented in salmon skeletal muscle and rat brain, heart, kidney, and skeletal muscle.

An explanation other than degradation or alternative splicing is phosphorylation differences, which has been documented extensively. Calpastatin has several serine residues which may be phosphorylated, and calpastatin may be phosphorylated on threonine residues as well. Calpastatin containing the XL domain may have up to three additional phosphorylation sites. Reversible phosphorylation of two calpastatin activity peaks separated during ion exchange chromatography has been documented in several studies in several tissues, including rat brain, heart, kidney, and skeletal muscle, as well as human neuroblastoma cells. Phosphorylation may be a mechanism by which calpastatin inhibitory activity is controlled. Averna et al. documented aggregation of human neuroblastoma LAN-5 cell phosphorylated calpastatin and cytoplasmic diffusion of dephosphorylated calpastatin, which had greater activity. Adachi et al. also speculated that phosphorylation may be a mechanism by which calpastatin attaches to membranes when it is not active. Another possibility considered is that calpastatin in the first peak of activity has bound with inactive calpain, modifying its elution profile. Melloni et al. reported that, in the absence of calcium, or at low physiological levels of calcium, calpastatin may bind with inactive calpain, forming a complex which may prevent overactivation of calpain.
Because the current data concerning dual calpastatin activity elution peaks are conflicting, it is difficult to conclude what the molecular explanation for these observations may be. The objective of the current work was to characterize the two isolates of calpastatin activity seen in our laboratory. We hypothesized that the calpastatin separation during anion exchange chromatography was due to differences in phosphorylation and not degradation or calpain binding.

**Materials and Methods**

*Inhibitory activity against skeletal muscle μ- or m-calpain*

Calpastatin and μ- and m- calpain were isolated as previously described\(^{38-39}\). Briefly, 300 g of porcine longissimus muscle were obtained within 30 min postmortem and extracted using 3 vol ice cold extraction buffer containing 100 mM Tris-HCl, 10 mM EDTA, pH 8.3, with 0.1% 2-mercaptoethanol (MCE), 2 µM E-64, and 500 mg/L trypsin inhibitor added immediately before use. Samples were homogenized using a Waring blender (New Hartford, CT), followed by a Polytron PT 3100 (Lucerne, Switzerland) in three 30 s bursts. The resulting homogenate was centrifuged at 15,900 x g for 30 min at 4°C, and the supernatant was filtered through cheesecloth and dialyzed in 40 volumes of TEM (40 mM Tris-HCl, 1 mM EDTA, pH 7.4, with 0.1% MCE). After dialysis, samples were centrifuged at 15,900 x g for 20 min at 4°C and the supernatant was filtered through cheesecloth.

Filtered dialysates were then loaded onto a Q-Sepharose Fast Flow (GE Healthcare Biosciences, Pittsburgh, PA) anion exchange column equilibrated with TEM. After washing the loaded column with 10 vol TEM, calpastatin, μ-calpain, and m-calpain were eluted using a linear gradient of 60 to 400 mM KCl in TEM. Calpastatin activity eluted first (calpastatin I and II, at 60 to 90 mM KCl and 120 to 190 mM KCl, respectively), followed by μ-calpain
(180 to 240 mM KCl) and m-calpain activities (300 to 400 mM KCl). The calpains were further concentrated using centrifugal concentrators with a 10,000 Da MW cutoff. The inhibitory activities of the calpastatin peaks against the concentrated µ- or m-calpain from the same muscle were determined using casein as a substrate, using a modification of the method of Koohmaraie.

Sample from each eluted fraction was brought to 1 ml with TE (40 mM Tris-HCl, 1 mM EDTA, pH 7.4). Approximately 0.4 units of previously purified porcine lung m-calpain in TE were included. One milliliter of casein buffer (100 mM Tris-acetate 7 mg/ml casein, and 1 mM sodium azide, pH 7.5, with 0.2% MCE added just before use) was added, followed by 100 µl of 200 mM CaCl$_2$. Samples were briefly vortexed and incubated in a water bath at 25°C for 1 hr. Following incubation, the reaction was stopped with 2 ml of 5% trichloroacetic acid, vortexed, and centrifuged at 1,500 x g for 20 minutes at 25°C. Absorbance of the supernatant was read at 278 nm. All assays were performed in triplicate. One unit of calpastatin activity was defined as the amount required to inhibit 1 unit of calpain.

*Calpastatin purification*

In order to characterize further the calpastatin within the two peaks of activity, a more rigorous purification process was used. All purification steps were performed at 4 °C. Two kg of bovine longissimus was obtained within 90 minutes after exsanguination and extracted as described above, except that the tissue was first chopped briefly in a food processor (Black and Decker, Madison, WI) before 4 vol of ice cold extraction buffer were used. Also 0.2 mM phenylmethylsulfonyl fluoride was added to the buffer just before blending with the sample. After dialysis of the supernatant, the sample was fractionated through ammonium sulfate precipitation, first by bringing the solution to 23% saturation and discarding the pellet after
20 min of centrifugation (15,900 x g), then by bringing the solution to 50% saturation and discarding the supernatant after 20 min of centrifugation (15,900 x g). The resulting pellet was resuspended and dialyzed using 100 vol TEM.

Calpastatin was isolated as described by Thompson and Goll, with modifications 26. The dialyzed sample was brought to 150 mM KCl, 1 mM sodium azide by adding solid KCl and 1M sodium azide, before being loaded onto a Phenyl-Sepharose column equilibrated with TEM containing 1 mM sodium azide (TEMA) and 150 mM KCl. The column was washed with 10 vol TEMA plus 150 mM KCl. Calpastatin does not bind to the column at this salt concentration, and was collected immediately after the void volume. The collected calpastatin-containing eluant was dialyzed again in TEMA, loaded onto a Q-Sepharose column equilibrated with TEMA, and eluted using a linear gradient of 60 to 200 mM KCl in TEMA. At this step, calpastatin eluted in three separate peaks of activity (calpastatin 1: 40-55 mM, calpastatin 2: 60-100 mM, and calpastatin 3: 110-150 mM).

Each eluant peak was dialyzed once again in 40 volumes of TEMA overnight and brought to 1 M ammonium sulfate by slowly adding solid ammonium sulfate. The dialyzed calpastatins 1, 2, and 3 were further purified using a Phenyl-Sepharose column equilibrated with 1 M ammonium sulfate, plus TEMA. After washing, calpastatin was eluted using a decreasing ammonium sulfate gradient. In the calpastatin 1 and 2 samples, some calpastatin activity did not bind to the column and eluted during washing (calpastatin 1A and 2A). A second peak (and the only calpastatin 3 peak) eluted between 350 and 800 mM ammonium sulfate (calpastatin 1B, 2B, and 3). As a final step, eluant samples were dialyzed once more in TEMA and concentrated by loading onto a 10 ml Q-Sepharose column and eluting with 400 mM KCl in TEMA.
**SDS-PAGE and immunoblotting**

Eluant samples were prepared for loading onto SDS gels as described by Huff-Lonergan, Mitsuhashi, Parrish, and Robson. SDS-PAGE and immunoblotting were performed on the purified calpastatin samples using 12.5% polyacrylamide separating gels (acrylamide:N,N′-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.05% [vol/vol] TEMED, 0.05% [wt/vol] ammonium persulfate, and 0.5 M Tris-HCl, pH 8.8), with 0.4 units of calpastatin loaded in each lane. Gels were run at 120 V on SE 260 Hoefer Mighty Small II (10 cm wide x 8 cm tall x 1.5 mm thick, Hoefer, Inc., Holliston, MA) electrophoresis units. Gels were stained with SYPRO Ruby total protein stain and imaged using an Ettan DIGE Imager (GE Healthcare, Piscataway, NJ) with excitation wavelength of 480 nm and emission wavelength of 595 nm. Imaged gels were visualized using DeCyder 2-D Differential Analysis software (v6.5, GE Healthcare, Piscataway, NJ).

For immunoblotting, protein was transferred to a PVDF membrane using the method of Melody et al. After blocking for 1 hr at room temperature in PBS with 0.1% Tween-20 and 0.5% nonfat dry milk, membranes were incubated in primary antibody at 4°C overnight. The antibody was a polyclonal mouse anti-bovine skeletal muscle calpastatin, diluted 1:1,000 dilution in PBS-Tween. The next morning, membranes were washed 3 times in PBS-Tween for 10 minutes each at room temperature. The membranes were then incubated in a goat anti-mouse horseradish peroxidase (No 2554, Sigma-Aldrich Corp., St. Louis, MO) secondary antibody at 1:10,000 dilution for 1 hr. After 3 additional 10 min washes, blots were developed using ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ) and imaged using a ChemiImager 5500 (Alpha Innotech, San Leandro, CA) and Alpha Ease FC software (v 3.03 Alpha Innotech).
Casein zymography and calpain immunoblotting

In order to determine the extent to which μ- or m-calpain co-eluted with calpastatin and influenced its elution profile, casein zymography and immunoblotting for μ- and m-calpain were performed on samples from two peaks of calpastatin activity. A sample from bull longissimus (17 months old, 587 kg) was extracted and calpastatin was isolated as described above in section 2.1. To prepare the samples for casein zymography, 24 µl of either calpastatin I, calpastatin II, μ-calpain, or m-calpain obtained from pooled fractions after the chromatography step were combined with 16 µl (0.67 volumes) of tracking dye solution (20% [vol/vol] glycerol, 0.75% [vol/vol] MCE, 0.1% [wt/vol] bromophenol blue, and 150 mM Tris-HCl, pH 6.8) and loaded onto native gels prepared as described by Melody et al. \(^{51}\). In order to test the hypothesis that calpastatin would bind with calpain in the absence of calcium, 0.2 units of either a previously purified bovine skeletal muscle μ-calpain or porcine lung m-calpain were added alone or in conjunction with 20 µl (approximately 0.005 to 0.008 units) of the pooled calpastatin I or II from the ion exchange chromatography step and incubated on ice for 1 hr before mixing with 0.67 volumes of tracking dye solution and loading onto native gels. Native gels for casein zymography were run as described by Melody et al. \(^{51}\).

Immunoblotting for μ- and m-calpain was performed on the pooled fractions of calpastatin I and II, μ-calpain, and m-calpain obtained after the chromatography step. Immunoblotting was performed as described above, except that the primary antibodies were either mouse monoclonal anti-μ-calpain diluted 1:10,000 (MA3-940, Pierce, Rockford, IL) or rabbit polyclonal anti-m-calpain (OPA1-08805, Affinity Bioreagents, Golden, CO) diluted 1:1,000. Secondary antibodies were goat anti-mouse HRP (A2554, Sigma-Aldrich, St. Louis,
MO) diluted 1:10,000 and goat anti-rabbit IgG HRP (81-6120, Invitrogen Corp., Camarillo, CA) diluted 1:20,000, respectively.

Two-dimensional protein characterization of calpastatin

One bovine triceps brachii muscle was obtained within 90 min postmortem and prepared as described above in the calpain inhibition experiment except that eight 15 g samples were each prepared from the same muscle, then extracted, and eluted on separate columns. After identification of the two calpastatin activity peaks, calpastatin I and II fractions from each column were pooled. Eluants were then concentrated and desalted using centrifugal concentrators with a 10,000 MW cutoff. During this process, TEM buffer was replaced with buffer used for 2-dimensional electrophoresis (2DE) (8.3 M urea, 2 M thiourea, 2% CHAPS, 1 mM DTT, pH 8.5.) For calpastatin immunoblotting, 2DE was performed as described \(^{53}\), loading 150 µg of protein for a total concentration of 1 µg/ml in DeStreak rehydration solution (Amersham Biosciences Corp., Piscataway, NJ) onto 7 cm, pH 4-7 immobilized pH gradient (IPG) strips (GE Healthcare, Piscataway, NJ). Strips were focused for a total of 7,250 Vh. Equilibrated strips were loaded onto 12.5% SDS-PAGE gels (12 x 10 cm) using agarose as an overlay and run at 120 V on SE 280 Tall Mighty Small (Hoefer, Holliston, MA) electrophoresis units. Proteins were transferred as described by Melody et al. \(^{51}\) and immunoblotting for calpastatin was performed as described above.

A ratio of phosphorylation to total protein of protein spots was determined through phosphoprotein and total protein staining. Gels were first stained with a phosphoprotein stain (ProQ Diamond, Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and imaged using an Ettan DIGE Imager (GE Healthcare, Piscataway, NJ) to analyze protein phosphorylation (excitation 540 nm, emission 595 nm). Imaged gels were visualized using
DeCyder 2-D Differential Analysis software (v6.5, GE Healthcare, Piscataway, NJ). Gels were then stained with SYPRO Ruby total protein stain (BioRad, Berkeley, CA) and visualized (excitation 480 nm, emission 595 nm). Ratios of phosphorylated to total protein were determined for each spot, then normalized to that of a molecular weight standard loaded onto each gel in order to account for gel to gel variation.

For identification of protein spots, proteins (750 µg) were resolved using 2DE (First dimension: 11 cm, pH 4-7 IPG strips, 14,000 total volt-hour focusing; Second dimension: 12.5% acrylamide, 14 cm wide x 15 cm tall x 1.5 cm thick, Hoefer SE 600 electrophoresis units run at 100 V) and stained with Colloidal Coomassie Blue Stain (1.7% ammonium sulfate, 30% methanol, 3% phosphoric acid, and 0.1% Coomassie G-250) for a minimum of 24 h prior to destaining in water for a minimum of 24 h. To reduce potential for contamination, all reagents and buffers used during the protein identification step were filtered (0.22 µm pore size) prior to use. Spots corresponding to those identified in the Western blots were selected for identification. Selected spots were excised and sent to the Iowa State University Protein Facility for identification. In-gel Lys C digestion (P3428, Sigma-Aldrich Corp., St. Louis, MO) was performed. Peptides were dissolved in α-cyano-4-hydroxycinnamic acid (5 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid) and deposited to a MALDI target. MALDI mass spectrometry was performed using a QSTAR XL Quadrupole TOF mass spectrometer equipped with an oMALDI ion source (AB/MDS SCIEX, Toronto, Canada). Peak list was generated by Analyst QS Version 1.1 (AB/MDS SCIEX, Toronto, Canada). Spectra were processed by Mascot database search Version 2.2.07 (MatrixScience, London, UK). Search conditions used in Mascot included maximum one missed cleavage, fixed modification (carboxyamidomethyl cysteine), variable modification
(oxidation of methionine), peptide mass tolerance of \( \pm 100 \) ppm, and fragment mass tolerances of \( \pm 1 \) Da. Identification was based on a MOWSE score with a threshold of less than 0.05.

**Results and Discussion**

*Inhibitory activity against skeletal muscle \( \mu \)- or \( m \)-calpain*

Less inhibitory activity of both \( \mu \)- and \( m \)-calpain was observed from calpastatin I compared to calpastatin II in the one sample assayed (Table 1). However, calpastatin protein concentration was not standardized across calpastatin eluant samples. Because these samples were not normalized for calpastatin protein content, it is impossible to say based on these results whether calpastatin I is less active or simply less abundant in our samples. Salamino et al., who also observed two peaks of rat brain calpastatin activity elution from ion exchange columns, documented that the first calpastatin eluant had greater specific inhibitory activity against both \( \mu \)- and \( m \)-calpain \(^{42}\). Both calpastatin peaks in their study had almost two-fold greater inhibition of \( m \)-calpain compared to \( \mu \)-calpain, indicating some similarity in how each calpastatin inhibits the calpains. When using casein as a substrate, greater inhibition of \( m \)-calpain compared to \( \mu \)-calpain by calpastatin is well documented \(^{25,28,55}\).

*Calpastatin purification*

The protein profile in SDS-PAGE gels during purification steps up to the first Q-Sepharose Fast Flow anion exchange column is shown in Figure 2. During elution from the first Q-Sepharose column, calpastatin eluted in three separate peaks of activity (calpastatin 1, 2, and 3; Figure 3A). These three peaks were a development we had not seen previously during gradient elution from a Q-Sepharose column. The additional separation may have been due to protein degradation that had occurred during the time taken during the additional
steps of ammonium sulfate precipitation and hydrophobic chromatography, even though protease inhibitors were used. Approximately half of the total activity was found in the third peak, whereas each of the first two peaks composed about one fourth of the total sample activity.

Further purification using a second Phenyl-Sepharose column resulted in another separation of the first two peaks, for a total of 5 peaks of calpastatin activity (Figure 3B). When loading the calpastatin samples from the first two Q-Sepharose peaks onto the Phenyl-Sepharose column, some calpastatin activity eluted from the column during the washing step (calpastatin 1A and 2A). The remainder eluted normally (calpastatin 1B and 2B). The “subpeaks” that were not held by the Phenyl-Sepharose were calpastatin fragments, as confirmed in Western blot analysis of the protein profiles (Figure 3). Within the first original peak (calpastatin 1) of activity, slightly less than half of the activity was composed of the fragmented form (calpastatin 1A). Within the second original peak (calpastatin 2), only one fourth of the activity was from calpastatin fragments (calpastatin 2A). The later eluted calpastatin 1B and 2B were each composed primarily of a 41 kDa band (Figure 3C and 3D). In calpastatin 1B, this was the only band, but in calpastatin 2B, there were also less abundant 55 and 37 kDa bands. Calpastatin 3 was almost solely the larger 55 kDa band, but also had a trace of the 41 kDa band.

Because the calpastatin from each eluant activity peak differed in molecular weight, it may appear from these data that the separation of calpastatin during anion exchange chromatography was due to a change in ionic strength caused by protein degradation. However, all of the molecular weights seen in this purified calpastatin are below the 115 to 145 kDa molecular weight of calpastatin normally seen on SDS-PAGE gels (Figure 3).
Degradation had apparently occurred in these samples, despite the use of multiple protease inhibitors. The fact that none of the purified calpastatin was in its fully intact form suggests that these results should be interpreted with caution.

**Casein zymography and calpain immunoblotting**

Calpastatin was incubated with µ- or m-calpain in the absence of calcium in order to determine if calpastatin in either activity pool could bind with the calpains in a calcium-free environment. Many studies have consistently demonstrated that calcium is required for calpastatin-calpain binding. If binding occurred, it would be expected that calpain activity would be reduced in casein zymography gels. Melloni et al. observed this interaction using several calpastatin isoforms incubated with calpain in the absence of calcium. They also observed that domain L is required for interaction of calpain and calpastatin in the absence of calcium. However, those results go against multiple studies that have shown no calpain-calpastatin interaction in the absence of calcium. Interestingly, a synthetic peptide which mimics the inhibitory subdomain B of calpastatin has been shown to bind in the absence of calcium, but rate of binding was increased in the presence of calcium. In the current study, no differences in calpain activity on casein zymograms were observed in purified porcine skeletal µ-calpain and lung m-calpain samples after their incubation with calpastatin samples, indicating that the calpains did not bind to either form of calpastatin in the absence of calcium (Figure 4). Additionally, no calpain activity was detected in either the calpastatin I or calpastatin II samples alone (Figure 4). To further determine whether either calpastatin activity pool contained µ- or m-calpain, which might indicate that calpastatin was bound with inactive calpain, immunoblotting for µ- and m-calpain was performed. Western blots did not detect the presence of either µ- or m-calpain in the calpastatin samples (Figure 5). These
results provide additional support for the conclusion that the calpains did not bind or co-elute with either calpastatin activity peak. The m-calpain antibody was for domain III of m-calpain, which has approximately 60% homology with domain III of µ-calpain. The m-calpain antibody reacted with the µ-calpain sample, but this was likely due to cross-reactivity and not actual m-calpain presence, as there was wide separation between µ-calpain and m-calpain activity during elution. This is further demonstrated in the casein zymography gels because the calpains have distinctly different migration patterns in a native gel, and no m-calpain activity was detected in the µ-calpain sample (Figure 4).

*Two-dimensional calpastatin immunoblotting*

The calpastatin in each activity pool in the final multi-column preparation was visualized using immunoblotting techniques in order to identify potential differences in molecular weight and posttranslational modifications (Figure 6). Immunoblotting of 2D gels identified two anti-calpastatin reactive proteins in both activity pools, located at approximately 145 and 60 kDa. Additionally, in calpastatin II, a third set of spots was identified by the calpastatin antibody, at approximately 80 kDa. A low molecular weight spot (approximately 37 kDa) was also identified by the immunoblot of the calpastatin I sample. The lower molecular weight proteins identified as calpastatin may be degradation products.

Full bovine calpastatin is a 76 kDa protein with a pI of approximately 4.85 to 4.95. However, full length calpastatin tends to migrate anomalously at a much higher molecular weight on SDS gels, typically between 100 and 150 kDa. Raynaud et al. and Muroya et al. each observed three mRNA transcripts from alternative splicing in bovine skeletal muscle. The first, Type I, produced a full-size calpastatin isoform which migrated at approximately 145 kDa in heart muscle Western blots. However, Type I calpastatin was only...
visible in extracts from bovine skeletal muscle at 70 kDa. The second, Type II, produced a calpastatin isoform missing only a small part of the XL domain. Type II calpastatin also migrated at 145 kDa and 70 kDa in SDS gels, and both sizes were present in bovine skeletal muscle. The third transcript, Type III, produced a calpastatin isoform missing the entire XL domain and migrated at approximately 125 kDa in SDS gels. Type III calpastatin was also present in skeletal muscle. Based on the results of Raynaud et al., the high molecular weight calpastatin identified through immunoblotting in both calpastatin I and II in the current study could be the Type I or Type II calpastatins, which were of similar molecular weight on SDS-PAGE gels in their study\textsuperscript{24}. The 60 kDa calpastatin protein identified in the calpastatin I and calpastatin II eluants may be similar to the 70 kDa forms seen by Raynaud et al., which were speculated to be the result of posttranslational modifications such as phosphorylation, glycosylation, or proteolysis\textsuperscript{24}. Based on its lower pI (between 4.0 and 4.4), the 60 kDa protein may be missing part of, or the entire, domain L. The leader domain has the most basic pI of all the calpastatin domains, so its loss would result in a decrease in pI\textsuperscript{35}. It is also possible that the 60 kDa calpastatin form has some modification that causes it to migrate closer to its calculated molecular weight, although this has never been documented in calpastatin.

\textit{Two dimensional SDS-PAGE calpastatin analysis}

Calpastatin was also identified via mass spectrometry in the 145 kDa set of spots with a pI of approximately 4.9 identified as calpastatin by immunoblots in the calpastatin II gels (194 MOWSE score, 7% sequence coverage, 140 MOWSE score, 5% sequence coverage). Protein identification via mass spectrometry in calpastatin I or the other spots identified as calpastatin based on immunoblotting was inconclusive. Inability to identify protein spots may
have been due to low protein concentration, inadequate protein digestion, peptide fragments which were either too large or too small, or peptide fragments which did not match proteins in the database. Because the 145 kDa protein was found in both calpastatin I and II, degradation as the sole cause of protein separation during elution seems unlikely. If calpastatin I eluted earlier because it was a degradation product of calpastatin, as reported by Geesink et al., it is not likely that the 145 kDa calpastatin would be present in the pooled calpastatin I.

Analysis of the calpastatin spots migrating at 145 kDa revealed a 3 fold greater phosphorylation to total protein ratio in the calpastatin II sample compared to the 145 kDa spots from calpastatin I (Table 2). This area appeared as only one large spot with ProQ Diamond and SYPRO Ruby staining, but was actually composed of at least 6 to 8 spots, as visualized using colloidal Coomassie staining (Figures 7 and 8). A protein chain with 4 spots at approximately 60 kDa, with a pI between 4.0 and 4.4 and identified as calpastatin through immunoblotting was also analyzed for phosphorylation. The two 60 kDa spots with the lowest pI had a 2 to 3 fold greater phosphorylation to total protein ratio in the calpastatin II sample versus calpastatin I, while the two spots with greater pI were only about 90% as phosphorylated in the calpastatin II samples versus calpastatin I.

Bovine calpastatin has seven phosphorylation sites, whereas porcine calpastatin has eight phosphorylation sites. Phosphorylation would result in a lower pI. Phosphorylation would also result in later elution from an anion exchange column because of the same greater net negative charge. Averna et al. and Salamino et al. also observed that the second eluted peak of human neuroblastoma LAN-5 and rat brain cell calpastatin activity from an ion exchange column was more phosphorylated. Additionally, in their study,
dephosphorylation of the protein in the second peak through the use of a phosphatase resulted in an earlier ion exchange column elution pattern, similar to the first calpastatin activity peak. Averna et al. and Salamino et al. also found that the first eluted peak of calpastatin activity could be phosphorylated and would subsequently elute at the later elution point when ion exchange chromatography was employed. Therefore, they showed that phosphorylation of calpastatin is reversible, and results in differential elution from an anion exchange column. The observations in the current study involving skeletal muscle calpastatin are consistent with the observations made previously in other tissues.

Phosphorylation may be a mechanism by which calpastatin aggregation and cellular localization occurs. Averna et al. documented that introduction of a calcium ionophore caused calpastatin in human neuroblastoma LAN-5 cells to diffuse into the cytoplasm, concurrent with an increase in the first eluted peak of calpastatin during ion exchange chromatography. Additionally, when protein kinase A was activated, newly phosphorylated calpastatin aggregated near the nucleus of the cells, and an increase in the second eluted calpastatin peak was observed. Observations that phosphorylated calpastatin was more likely to be found in the cell membrane fraction of human hematopoietic system cells compared to unphosphorylated calpastatin provide additional support for the hypothesis that phosphorylation causes aggregation of calpastatin.

Calpastatin II comprises the majority of the calpastatin activity observed in bovine and porcine muscle samples, and is more phosphorylated than calpastatin I. Evidence pointing to this conclusion includes order of elution (the less phosphorylated calpastatin I elutes from an anion exchange column before the phosphorylated form) and a greater phosphorylation to total protein ratio in calpastatin II. Another possible explanation is
alternative splicing. If alternative splicing is the cause of the two calpastatins, only small changes have been made to the protein, such as in Type I and II calpastatin, where molecular weights on SDS-PAGE are still very similar. Alternative splicing might result in an increase or decrease in phosphorylation sites, as shown by Cong et al. 61, which might explain the differential elution of calpastatins I and II. Although protein degradation has been suggested to cause calpastatin separation during ion exchange chromatography, this explanation is unlikely given the close similarities in molecular weight and pI of the two calpastatins. These observed differences in calpastatin phosphorylation provide interesting insight into the biochemistry of the calpain system in skeletal muscle. Additionally, the two calpastatin forms seen in this study have the potential to elucidate many unanswered questions about variation in calpastatin activity between animals.

**Abbreviations Used**

- **IPG**: immobilized pH gradient
- **MCE**: 2-mercaptoethanol
- **PVDF**: polyvinylidene fluoride
- **SDS**: sodium dodecyl sulfate
- **SDS-PAGE**: sodium dodecyl sulfate – polyacrylamide gel electrophoresis
- **TE**: 40 mM Tris-HCl, 1 mM EDTA, pH 7.4
- **TEM**: 40 mM Tris-HCl, 1 mM EDTA, pH 7.4, with 0.1% MCE
- **TEMA**: 40 mM Tris-HCl, 1 mM EDTA, 1 mM sodium azide, pH 7.4, with 0.1% MCE
Acknowledgements

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Tables and Figures

Table 1. Inhibitory activities of calpastatin I and II from porcine longissimus muscle against μ- and m-calpain from the same muscle, performed in triplicate. One unit of calpastatin activity is defined as the amount of calpastatin required to inhibit 1.0 unit of porcine lung m-calpain activity, preventing an increase of 1.0 absorbance unit at 278 nm in 60 min at 25 °C.

<table>
<thead>
<tr>
<th>Calpastatin Peak</th>
<th>μ-calpain inhibition</th>
<th>m-calpain inhibition</th>
<th>Fold-difference (m/μ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calpastatin I</td>
<td>0.17 u/ml</td>
<td>0.33 u/ml</td>
<td>1.90</td>
</tr>
<tr>
<td>Calpastatin II</td>
<td>0.63 u/ml</td>
<td>1.18 u/ml</td>
<td>1.86</td>
</tr>
</tbody>
</table>
Table 2. Phosphorylation:total protein ratios of potential calpastatin protein spots in calpastatin I and II samples. Bovine longissimus (n=1) was subdivided into 8 samples which were each run over an anion exchange column to isolate calpastatin I and II, then repooled. The 60 kDa A, B, C, and D represent four calpastatin spots in order of increasing pI.

<table>
<thead>
<tr>
<th>Spot or Chain</th>
<th>Calpastatin I</th>
<th>Calpastatin II</th>
<th>Adjusted Calpastatin II&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fold Difference (adjusted)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>145 kDa</td>
<td>1.93</td>
<td>14.66</td>
<td>5.86</td>
<td>3.04</td>
</tr>
<tr>
<td>60 kDa – A</td>
<td>0.87</td>
<td>5.95</td>
<td>2.38</td>
<td>2.74</td>
</tr>
<tr>
<td>60 kDa – B</td>
<td>1.15</td>
<td>5.97</td>
<td>2.39</td>
<td>2.08</td>
</tr>
<tr>
<td>60 kDa – C</td>
<td>2.47</td>
<td>5.53</td>
<td>2.21</td>
<td>0.90</td>
</tr>
<tr>
<td>60 kDa – D</td>
<td>2.47</td>
<td>5.32</td>
<td>2.13</td>
<td>0.86</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calpastatin II ratios were adjusted based on the phosphorylation:total protein of molecular weight standards run the calpastatin I gel compared to the calpastatin II gel, in order to account for gel to gel variation.

<sup>b</sup> Fold difference of phosphorylation: total protein ratios between calpastatin I spots and adjusted calpastatin II spots.
Figure 1. Representative examples of calpastatin separation from A) porcine skeletal muscle and B) bovine skeletal muscle during elution using a Q-Sepharose Fast Flow ion exchange column. Calpastatin was eluted using a 60 to 200 mM KCl gradient.

A)

B)
Figure 2. SDS-PAGE gel showing protein profile during subsequent purification steps for calpastatin. MW = Molecular weight standard, Lane 1 – Sarcoplasmic extract, Lane 2 – After 23% ammonium sulfate precipitation, Lane 3 – After 50% ammonium sulfate precipitation, Lane 4 – Phenyl-Sepharose elution, Lanes 5-7 – Peaks 1, 2, and 3 of Q-Sepharose Fast Flow elution.
Figure 3. A) During the first anion exchange chromatography, calpastatin was eluted in three separate peaks of activity, labeled calpastatins 1, 2, and 3. B) Following that, hydrophobic interaction chromatography resulted in the further separation of calpastatins 1 and 2, into two peaks of activity each (calpastatins 1A, 1B, 2A, and 2B), where the first peaks (calpastatins 1A and 2A) were not retained by the hydrophobic column. Calpastatin 3 remained as one peak of activity with hydrophobic interaction chromatography. C) Western blot for calpastatin after purification steps. D) A SYPRO Ruby total protein stained gel of the same samples.
Figure 3, continued.

C) [Image of gel lane patterns with molecular weights 55 kDa, 41 kDa, 37 kDa, and 25 kDa]

D) [Image of gel lane patterns with molecular weights 55 kDa, 41 kDa, 37 kDa, and 25 kDa]
Figure 4. A) Casein zymograms of $\mu$-calpain, m-calpain, Calpastatin I or II (Cstn I, Cstn II) from the same bovine longissimus muscle (n=1). Concentrated calpain lanes were loaded with 0.2 units of calpain each, and 24 $\mu$l of each eluant sample were loaded in other lanes. B) Casein zymograms of 0.2 units of previously purified porcine skeletal muscle $\mu$-calpain or lung m-calpain alone (Control) or after incubation with Calpastatin I or Calapstatin II (n=1).
Figure 5. Immunoblots for A) μ-calpain and B) m-calpain. Gels were loaded as follows: Lane 1 – calpastatin I, Lane 2 – calpastatin II, lane 3 - μ-calpain, lane 4 – m-calpain.
Figure 6. Calpastatin immunoblots of two-dimensional electrophoresis gels of A) calpastatin I, and B) calpastatin II pooled samples.

A)

Calpastatin I

4  pI  7

145 kDa

60 kDa

37 kDa

B)

Calpastatin II

4  pI  7
Figure 7. Colloidal total protein stained, two-dimensional electrophoresis gels of calpastatin I (A and B) and calpastatin II (C and D) pooled samples. Images B and D are zoomed to show the location of calpastatin within the gel. Confirmed calpastatin spots are circled.

A)
Figure 8. Total protein (SYPRO Ruby) or phosphoprotein (ProQ Diamond) stained, two-dimensional electrophoresis gels of calpastatin I and calpastatin II pooled samples (n=1). Confirmed calpastatin spots are circled.

A)  

Calpastatin I Total Protein Stain
B) Calpastatin I Phosphoprotein Stain

250 kDa  
150 kDa  
100 kDa  
75 kDa  
50 kDa  
37 kDa  
25 kDa  

Calpastatin I Phosphoprotein Stain
Calpastatin II Total Protein Stain
D)

Calpastatin II Phosphoprotein Stain
CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS

The calpain system is an effective regulator of protein turnover and growth, as previously demonstrated in several animal models in the literature, including callipyge sheep and cattle supplemented with β-adrenergic agonists. The current results indicate the calpain system is also involved in reduced protein turnover in pigs selected for decreased residual feed intake. Conversely, protein synthesis does not appear to be related to observed phenotype in the low and high RFI pig lines. This overall reduced protein turnover resulting from decreased calpain activity, increased calpastatin activity, and decreased proteasome activity could be a cause of the greater efficiency in these pigs, resulting in decreased costs of production.

Additionally, the calpain system appears to differ in mature animals compared to those that are still growing. The increase in calpastatin activity and expression observed in mature cattle may be a mechanism to prevent calpain activity and protein degradation caused by a loss of calcium homeostasis with age, which is well documented in older adults (Fraysse et al., 2006; Squier, 2001). The current study found no age by muscle interaction in calpastatin activity. The intermediate fiber type triceps brachii had greater calpastatin activity than the predominately fast twitch longissimus and semimembranosus muscles, which is consistent with previous results showing that calpastatin activity is greater in muscles with slower fiber type (Geesink et al., 1995). The relative proteolytic activity differences between muscles do not appear to change with animal maturity.

Despite the clear advantages of increased calpastatin activity to muscle growth often observed in the live animal, a decrease in calpain-activated protein turnover may be
detrimental to the final product, meat. Pork from low RFI pigs has demonstrably slower postmortem proteolysis compared to the high RFI counterparts. Further, decreased postmortem protein degradation in beef from older animals compared to young, growing animals is the result of a lower \( \mu \)-calpain:total calpastatin activity ratio. In addition, in the older animals calpastatin activity was more stable throughout the postmortem aging process. The delayed or reduced tenderization which may occur in these products means that longer aging times, alternative methods of tenderization, or different uses may have to be employed in order to deliver high quality product from these animals. The benefits and potential adverse effects must be carefully considered as we strive for more efficient, sustainable production of meat animals, and as we seek to utilize animals past the normal market age.

During the investigations of calpain and calpastatin activity in both of the above studies, the observation that calpastatin eluted from an anion exchange column in two separate, distinct peaks presented itself as a matter worth exploring more deeply. A lab in Italy had published several papers, most of them about 20 years ago, with a similar observation and a conclusion that the second peak of calpastatin activity contained phosphorylated calpastatin (Averna et al., 2001; Pontremoli et al., 1991; Pontremoli et al., 1992; Salamino et al., 1997; Salamino et al., 1994). However, these results had not been reproduced. In fact, another group had found that an initial calpastatin peak when eluting bovine skeletal muscle calpastatin from an ion exchange column simply contained a calpastatin degradation product (Geesink et al., 1998).

The observations in the current study show that the second eluted calpastatin activity peak, calpastatin II, comprises the majority of the calpastatin activity observed in bovine and porcine muscle samples. It is concluded that calpastatin II is more phosphorylated. Evidence
pointing to this conclusion includes order of elution (the less phosphorylated calpastatin I elutes from an anion exchange column before the phosphorylated form because it has a lesser negative charge) and a greater phosphorylation to total calpastatin protein ratio in calpastatin II. Another possible explanation is alternative splicing. If alternative splicing is the cause of the two calpastatin peaks of activity, only changes which do not cause a discernible difference in molecular weight have occurred, as Raynaud et al. (2005) observed with Type I and II calpastatins. Alternative splicing could possibly change the number of phosphorylation sites. If Type II calpastatin were more phosphorylated, this might still explain the differential elution of calpastatins I and II. Although protein degradation has been suggested to cause calpastatin separation during ion exchange chromatography (Geesink et al., 1998), this explanation is unlikely given the close similarities in molecular weight and pI of the two calpastatins observed in this study.

It has been suggested that dephosphorylation of calpastatin may be a mechanism by which calpastatin is made more active, both through disaggregation and greater inhibitory ability (Averna et al., 2001; Salamino et al., 1997). Although not specifically measured, during assays of inhibitory activity, a difference in half maximal inhibitory concentration ($IC_{50}$) between calpastatin I and II was observed. With increasing volume of calpastatin eluant, inhibitory activity of calpastatin I increased at a much more rapid rate than that of calpastatin II. This supports the data of Salamino et al. (1997) who showed that dephosphorylation of calpastatin from rat heart or kidney resulted in decreased $IC_{50}$. Therefore, phosphorylation may be an important regulatory mechanism which puts calpastatin in a less active state when the inhibitor is not needed.
Overall, the calpain system, especially calpastatin, has consistently been shown to play a large role in muscle protein turnover and postmortem tenderization. Additionally, the existence of two forms of calpastatin, one more phosphorylated than the other, introduces many more avenues of meat and muscle research related to animal growth and muscle development, as well as postmortem meat tenderization. Future research related to this dissertation could include:

- Evaluation of animals over the spectrum of growth, in order to identify the point that calpastatin activity increases to a level which may adversely affect meat quality.
- Determining calpain activity differences in low and high RFI pigs at different stages of growth, in the interest of studying growth in an efficiency model and if efficiency might magnify the effects of the calpain system on growth.
- Examination of calpastatin differences in *Bos taurus* versus *Bos indicus* cattle. Some studies have shown that total calpastatin activity does not differ between species, but that calpastatin activity is more persistent in *Bos indicus* animals. It could be that there are differing expression levels of one calpastatin form versus the other.
- Detailed study of the inhibitory kinetics of calpastatin I compared to calpastatin II.
- Further purification and concentration of calpastatin I and II in order to confirm all of the calpastatin spots from 2D electrophoresis via mass spectrometry. Additionally, identify specific phosphorylation site differences between calpastatin proteins.

These potential future studies could further elucidate the role that the calpain system plays in growth and development, allowing a greater understanding of muscle biology, efficiency, and meat quality. Future applications of this knowledge could someday allow producers to effectively manage growth or efficiency using the calpain system in conjunction
with other proteases and synthesis pathways. For example, if calpastatin (and perhaps m-calpain) expression and activity could be upregulated during growth in order to achieve greater hypertrophy, then downregulated just before death, large amounts of high quality meat could be produced with greater efficiency compared to the current animal production systems. These increases in production efficiency could enhance profitability while realizing the gains in production necessary to feed the growing world population. Clearly, understanding of the calpain/calpastatin system is important to the future of animal production.

**Literature Cited**


