Effects of oxidation, pH, and ionic strength on inhibition of \([\mu]\)- and m-calpain by calpastatin

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Effects of oxidation, pH, and ionic strength on inhibition of μ- and m-calpain by calpastatin

by

Kasey Rae Maddock

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:
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GENERAL INTRODUCTION

The functions of the calcium activated cysteine proteinases, calpains, are not completely understood. Calpains have been linked to many cellular processes including signal transduction, apoptosis, cell division, and cell fusion. More specifically, in skeletal muscle, calpain activity has been linked with protein degradation and turnover, muscle growth, and also myoblast fusion during cell proliferation. Because important meat quality traits (meat tenderization and water-holding capacity) have been linked to postmortem proteolysis caused by calpain activity, the focus of much research is the role of calpains in postmortem muscle.

There are many changes in muscle intracellular environment that occur early postmortem. One of the most pronounced changes is the pH decline from near neutral pH in living muscle to approximately 5.6 in meat. Another change that occurs is the increase in ionic strength from an approximate equivalent of 165 mM NaCl in living muscle to approximately equivalent to 295 mM NaCl in meat (Winger and Pope, 1980-81). An additional change that occurs during postmortem aging of meat is an increase in protein oxidation (Rowe et al., 2004a; Martinaud et al., 1997).

The calpains are thought to be responsible for much of the postmortem proteolysis of myofibrillar and cytoskeletal proteins in muscle, a causative factor for an increase in
tenderness observed in meat during postmortem storage (Goll et al., 1992; Koohmaraie, 1992a; Koohmaraie et al., 2002). Calpain activity is influenced by intracellular environmental factors, including calcium concentration, pH, ionic strength, oxidation, and calpastatin (the endogenous inhibitor specific for calpains). Calpains have been determined to have a pH optimum of approximately 7.5 (Edmunds et al., 1991; Wang and Jiang, 1991). A decrease in pH has been shown to decrease μ- and m-calpain activity (Hwang and Thompson, 2001; Rowe et al., 2001). An increase in ionic strength also has been shown to decrease μ- and m-calpain activity (Tan et al, 1998; Elce et al., 1997; Geesink et al., 1999; Geesink et al. 2000; Li et al., 2004). Additionally, oxidation has been shown to decrease μ-calpain activity (Guttmann et al., 1997; Guttmann and Johnson, 1998; Rowe et al., 2004). The decrease in activity is hypothesized to be due to oxidation of the active site cysteine residue (Mehdi, 1991).

The endogenous inhibitor of calpains, calpastatin, is co-localized with calpains in cells. The regulatory mechanism of calpastatin on calpain activity is not completely understood. In postmortem muscle, high calpastatin activity has been shown to correspond to a decrease in postmortem tenderization (Koohmaraie et al., 1991; Koohmaraie et al., 1995; Lonergan et al., 2001). Experiments designed to determine the effects of changes in
environment have determined that pH does not affect calpastatin inhibition of calpains (Geesink and Koohmarie, 1999; Kendall et al., 1993). Kendall et al. (1993) determined that an interaction of lower pH and higher ionic strength did decrease m-calpain inhibition by calpastatin.

The effects of oxidation on calpastatin inhibition on µ- and m-calpain are not understood; however, greater postmortem calpastatin activity was observed in beef steaks that had been irradiated when compared to non-irradiated steaks (Rowe et al., 2004b). This higher calpastatin activity was attributed to a decrease in µ-calpain activity caused by irradiation-induced oxidation. µ-Calpain was inactivated by irradiation and thereby decreased µ-calpain proteolysis of calpastatin, a substrate for calpain proteolysis as well as an inhibitor.

As all of these intracellular environmental factors change in the conversion of muscle to meat, it is important to understand the impact each of these factors have on calpain activity and inhibition of calpain by calpastatin. The hypothesis of this project was that pH and ionic strength conditions observed in postmortem muscle, in addition to oxidation and the presence of calpastatin will decrease activity of µ- and m-calpain. The specific objectives designed to test this hypothesis include: 1) evaluation of µ- and m-calpain activity and their inhibition by calpastatin at pH and ionic strength conditions observed during postmortem conversion of
muscle to meat and 2) evaluation of μ- and m-calpain activity and their inhibition by calpastatin under oxidative conditions and 3) determine interactions of pH, ionic strength, and oxidation effects on μ- and m-calpain activity and their inhibition by calpastatin.

**Dissertation organization**

This thesis is in the alternate style format and consists of a general introduction, review of literature, a paper published in *Journal of Animal Science* 83: 1370-1376, a paper submitted to *Journal of Animal Science*, a paper prepared for submission to *Biochemical Journal*, and a general summary. The paper represents the work conducted by the first author to fulfill the requirements for a degree of Doctor of Philosophy. The first paper, "Effect of pH and ionic strength on μ- and m-calpain inhibition by calpastatin" and second paper, "Effect of oxidation, pH and ionic strength on calpastatin inhibition of μ- and m-calpain" were prepared according to the *Journal of Animal Science* Style and Form guide and include an Abstract, Introduction, Materials and Methods, Results and Discussion, Implications, and Literature Cited. The third paper, "Formation of the μ-calpain/calpastatin complex allows activation of μ-calpain under oxidizing conditions" was prepared according to the *Biochemical Journal* Instruction to Authors guide and includes a Synopsis, Introduction, Experimental, Results and Discussion, and Literature Cited.
INTRODUCTION OF LITERATURE REVIEW

The focus of this dissertation was to determine some of the effects intracellular environment has on the activity of calpains and on their inhibition by calpastatin. The pH, ionic strength, and oxidative conditions are known to change during cellular metabolism, particularly in postmortem muscle cells. The hypothesis was that as intracellular environmental conditions change, the activity of μ-calpain, m-calpain, and inhibition of calpains by calpastatin are affected. Each of the following topics addresses calpains and calpastatin, their mechanisms of action, and factors affecting their action.

I. Calpains

II. Classical calpain structure

III. Activation of calpain

IV. Autolysis of calpain

V. Physiological role of calpains

VI. Calpastatin

VII. Binding of calpastatin to calpain

VIII. Meat Quality

IX. Meat Tenderness

X. Calpain and Calpastatin involvement in Postmortem Tissue
XI. Oxidation in postmortem muscle

XII. Protein oxidation

XIII. Summary

Calpains

Calpains are a family of enzymes generally characterized as calcium-dependent cysteine proteinases with catalytic domain that resembles papain's. Two subclassifications of calpains have been suggested (Sorimachi et al., 1997). These include 'classical' or 'conventional' forms, which include μ-calpain and m-calpain. These classical forms are denoted as typical calpains as they are depend on calcium to be active, contain the calmodulin-like calcium binding domain, and they also have a papain-like catalytic domain. Currently, knowledge of the calpains applies predominantly to the typical calpains, μ- and m-calpain, and their naturally occurring inhibitor, calpastatin (Goll et al., 1999).

More recently, twelve other forms of calpain, have been identified (Goll et al., 2003), and are often considered ‘atypical’, as their structures deviate from the classical structure. Five of the atypical calpains have been identified as tissue specific. The atypical forms are reasoned to be involved in the many of the pathological conditions linked to calpain activity as discussed below.

The first tissue specific calpain discovered was the skeletal muscle specific calpain 3, or p94, (Sorimachi et al., 1989). Although the mRNA for p94 has been found, the protein
cannot be isolated, as it autolyzes very rapidly. Yeast two-hybrid analysis determined that p94 binds the myofibril in muscle, and more specifically to titin (Kinbara et al., 1997; Sorimachi et al., 1995). Calpain 3 is thought to be involved in muscle differentiation by interacting with the MyoD family (Weintraub, 1993). Various mutations in the calpain 3 gene has been identified in patients with limb-girdle muscular dystrophy (Ono et al., 1998) which is thought to results in a loss of proteolytic activity and loss of binding to titin. Genes for other atypical calpains have been determined, including smooth muscle specific calpain 8 (Sorimachi et al., 1993), calpain 6 in placental tissues, calpain 11 in testis, and calpain 12 in the skin.

Another atypical calpain, calpain 10, has received much attention as it has been linked to insulin resistance and type 2 diabetes (Horikawa et al., 2000). Calpain 10 is expressed in many tissues and it considered ubiquitous. The assumed structure of calpain 10 (based on domain architecture) determined that domains I-III are similar to μ- and m-calpain, (described in detail later). However, calpain 10 does not contain the EF-hand structures identified with the classical calpains, but in fact has a T domain at the COOH-terminal that does not have predicted calcium binding sites (Horikawa et al, 2000; Ma et al., 2001).

Calpains are located within the cell (Kumamoto et al., 1992). Prior to calcium binding, both subunits of calpain are homogenously distributed in the cytoplasm (Gil-Parrado
et al., 2003), with slight prevalence around the nuclear region. After activation, migration of calpains toward the cell membrane has been observed (Gil-Parrado et al., 2003) by identifying ionomycin activated μ-calpain at the cell membrane. This movement toward the cell membrane has been hypothesized to be involved in lowering the calcium requirement for calpain activation and autolysis (Mellgren, 1987 and Suzuki et al., 1987) or regulation of calpain activation through binding of phospholipids (Saido et al, 1992; Coolican and Hathaway, 1986) or proteins (Inomata and Kawashima, 1995; Inomata et al., 1990). In skeletal muscle, μ- and m-calpain have been found in greatest abundance associated with the myofibrils at the Z-disk (Goll et al., 1992; Spencer and Tidball, 1992).

**Classical calpain structure**

μ- and m-Calpain are heterodimers, with a large subunit of approximately 80 kDa and a smaller subunit of 28 kDa. The terms of μ-calpain and m-calpain refer to differing Ca^{2+} requirement for their activation (Cong et al., 1989). μ-Calpain requires 3-50 μM calcium ion concentration for half maximal rate of proteolytic activity (Edmunds et al., 1991) and m-calpain requires 400-800 μM calcium ion concentration for half maximal rate of activity (Goll, 1991). Because intracellular calcium concentrations fluctuate normally at submicromolar levels, μ-calpain is more likely to be functional in cells under physiological conditions.
The 80-kDa subunits of \( \mu \)- and m-calpain have 55-65% homology (Suzuki, 1991) and are the products of two separate genes (Ohno et al., 1990). The 28 kDa subunits of \( \mu \)- and m-calpain are identical and encoded by the same gene (Ohno et al., 1986). Recently, Hosfield et al. (1999) and Strobl et al. (2000) revealed the crystal structure of calcium-free m-calpain. The 80 kDa subunit of m-calpain was revealed to contain six domains (Strobl et al. 2000). \( \mu \)-Calpain has not been crystallized, so it is not known if the large subunit contains these six domains (Goll et al., 2003). Two domains, Domain I or the NH\(_2\)-terminal domain and the linker domain identified in m-calpain, are not typical domains, as they contain only 18 and 17 amino acid residues respectively. Discussion of domain structure of both calpains will be based on the crystal structure of m-calpain (domain structure shown below as modified from Goll et al., 2003).

![Domain Structure Diagram](image)

Domain I is known as the anchor domain and is proposed to connect with domain V of the small subunit (Strobl et al., 2000; Reverter et al., 2001). Domain II is where the active site is located. The cysteine, histidine, and asparagine residues make up the catalytic triad common to cysteine proteases. Domain II is divided into two functional subdomains, IIa and
IIb. Subdomains IIa and IIb are placed opposite of each other to form a cleft where substrates bind. In the inactive form of calpain, this cleft is open preventing proper alignment the active site needed for activation (Hosfield et al., 1999). Domain IIa is where the active site cysteine residue is located (Cys$^{105}$ for m-calpain and Cys$^{115}$ for μ-calpain).

Domain IIb contains the two other residues (His$^{262}$ and Asn$^{286}$ for m-calpain and His$^{272}$ and Asn$^{296}$ for μ-calpain) of the catalytic triad required for activation. The crystal structure of the Ca$^{2+}$-bound DII domain specifies binding of Ca$^{2+}$ ions that bridge the subdomains to form the catalytically competent active site (Moldoveanu et al., 2002).

Domain III has been hypothesized to aid in regulation of calpain activity via electrostatic interactions (Strobl et al., 2000) and Ca$^{2+}$-dependent binding of phospholipids (Tompa et al., 2001). Strobl et al. (2000) hypothesized that calcium binding to the negatively charged acidic loop in domain III could allow for a conformational shift of the catalytic site with domain IIa and IIb to moving closer towards each other, aligning the active site in proper conformation allowing catalytic activity to occur. Alexa et al. (2004) tested this hypothesis and determined that calpains with mutations of the acidic loop that Domain III can bind 3 Ca$^{2+}$ ions in the absence of liposomes, and two Ca$^{2+}$ in the presence of liposomes. Gil-Parrado et al. (2003) determined that when a calpain fragment contained only domain III;
it migrated to membranes upon exposure with ionomycin, suggesting a role for DIII in membrane targeting.

Domain IV is called the calmodulin like domain as it contains E-F hand Ca\(^{2+}\)-binding sites. The crystal structure of m-calpain (Strobl et al., 2000) determined that there are in fact five EF-hand structures. The first three N-terminal EF-hand structures bind Ca\(^{2+}\) (Blanchard et al., 1997). The fifth EF-hand structure is involved with dimerization of the two subunits (Strobl et al., 2000). Dutt et al. (2000) determined that the calcium binding mechanism of the EF-hand structures occurred based on order of importance for activation and determined that the third EF-hand structure from the NH\(_2\)-end of domain IV had the greatest affinity for calcium.

A linker region determined by the crystal structure of m-calpain connects domains III and IV may play a role in directing the conformational changes occurring in domains IV and VI that are essential for activation of calpains (Alexa et al., 2004).

The small subunits of \(\mu\)- and m-calpain are identical as they are encoded by the same gene (Ohno et al., 1986) and contain domains V and VI. Domain V, the NH\(_2\)-terminal region, is rich in glycine and often considered the hydrophobic domain. It has been hypothesized that domain V binds phospholipids (Imajoh et al., 1986), but Goll et al. (2003)
suggested that domain V may play a larger role in interactions with other molecules or structures as its hydrophobicity is not extreme.

Domain VI is the COOH-terminal portion of the small subunit. It is very similar to domain IV as it contains five EF-hand structures for binding of calcium and is thereby also considered a calmodulin-like domain. The X-ray crystallography of m-calpain also determined that a fifth E-F hand was located in domain VI, interacting with the fifth EF-hand structure in domain IV in the large subunit (Blanchard et al., 1997).

In addition to the calcium binding sites in domains IV and VI, the crystal structure of m-calpain revealed a calcium binding site located in domain III (Strobl et al. 2000) and two calcium binding sites have been identified in domains I and II (Moldoveanu et al., 2002). The “extra” binding sites probably have implications in the calcium mediated activation of calpains.

**Activation of calpain**

Both the large and small subunits play an important role in the activation and function of calpains. Because most of the information about structural changes occurring during calpain activation have been deduced from the m-calpain crystal structure, most of the following discussion relates to m-calpain. Mainly, inactive m-calpain is the result of the large cleft between domains IIa and IIb, causing a misalignment of the three amino acids
required for the catalytic triad (Strobl et al., 2002). The binding of calcium at the EF-hands in Domains IV and VI causes a small conformational change (Blanchard et al., 1997; Lin et al., 1997), leading to loss of contact between the N-terminal domain in the large subunit and domain VI of the small subunit and also between domains IV and VI. The initial phase of activation requires high Ca\(^{2+}\) concentration, which decreases after activation (Goll et al., 1992) and autolysis has occurred. Moldoveanu et al. (2002) determined that Ca\(^{2+}\) binding at two sites in an \(\mu\)-calpain protease core containing active site domains I and II causes conformational changes that bring the active site residues into a conformation that is fully active. Strobl et al. (2000) hypothesized that calcium binding to the negatively charged loop on domain III could release subdomain IIb to move closer to subdomain IIa for alignment of the active site.

As the conformational change due to binding of calcium to the EF-hand regions of domains IV and VI is quite small, it has been discussed whether this small change can have any effect in resolving the active site misalignment for calpain activation. Alexa et al. (2004) hypothesized that the linker region, or transducer region, could possibly act as a "stiff rope" that can help bring the conformational changes occurring in domain IV to domain II for activation. They suggested that the linker can act as an entropic spring mechanism, where it loses its upper region electrostatic interaction with domain IV when Ca\(^{2+}\) binds, causing the
molecule to shift to a more compact conformation. Further diagnosis of these hypotheses (Bozoky et al., 2005) determined that the transducer must break its interaction with domain IV, which could help to explain the large effect of the very small conformational changes occurring in domains IV and VI during Ca$^{2+}$ binding.

**Autolysis of calpain**

Autolysis of m-calpain was noted by Suzuki et al. (1981). During autolysis, the 80 kDa subunit autolyzes to a 79 kDa and further to a 78 kDa peptide in the presence of calcium. Autolysis of μ-calpain was determined later (Dayton 1982) where the 80 kDa subunit autolyzes to a 78 kDa and further to a 76 kDa subunit. The small subunit autolyzes from the 28 kDa subunit to an 18 kDa subunit in both μ- and m-calpain.

Autolysis has been determined to occur in a series of steps. Autolysis of μ-calpain occurs with the removal of 14 amino acids at the NH$_2$-terminal to produce the 78 kDa fragment, followed by removal of another 12 amino acids to produce the 76 kDa fragment (Zimmerman and Schlaepfer, 1991). Autolysis of m-calpain occurs by the removal of 9 amino acids from the NH$_2$-terminal to produce the 79 kDa fragment, followed by the removal of 10 additional amino acids to produce the 78 kDa fragment (Brown and Crawford, 1993). The small subunit in both μ- and m-calpain also autolyzes from the NH$_2$-terminal region on the 28 kDa subunit to a 26 kDa fragment with the removal of 26 amino acids, which is
followed by removal of 37 more amino acids to produce a 22 kDa fragment, and finally 28 more amino acids are removed to produce the 18 kDa fragment (McClelland et al., 1989). Autolysis of the 28 kDa subunit in m-calpain occurs more rapidly than the 80 kDa subunit, however in μ-calpain, autolysis of the large subunit occurs at the same or faster rate than the small subunit (Zimmerman and Schlaepfer, 1991).

Autolysis lowers the Ca\(^{2+}\) concentration required for half maximal activity of μ-calpain from 3-50 μM to 0.5-2.0 μM and m-calpain from 400-800 μM to 50-150 μM (Goll et al., 1995). μ- and m-Calpains require greater calcium for autolysis to occur than is required to activation after autolysis. The reason for this difference in calcium concentrations is still unclear. Regardless, the calcium concentration in living cells ranges from 50-300 nM (Maravell et al., 2000), which is not high enough to activate μ- and m-calpains.

As stated earlier, domain III of μ- and m-calpain may be a location for phospholipid binding, explaining observations that μ-calpain migrates towards the cell membrane after exposure to calcium. Phosphatidylinositol has been shown to lower the calcium concentration required to initiate autolysis of μ-calpain (Cong et al., 1993; Saido et al., 1992) and m-calpain (Coolican and Hathaway, 1986). However, the calcium concentration required to activate these calpains exposed to phospholipids is still higher that calcium concentration measured in living cells.
Autolysis has often been used as an indicator of calpain activation (Baki et al., 1996). Baki et al. (1996) observed that microtubule associated protein 2 (MAP2) degradation occurred after autolysis ofμ-calpain. They surmised that these results indicated autolysis must occur for proteolytic action ofμ-calpain to be activated. However, Molinari et al. (1994) determined thatμ-calpain did not require autolysis to cleave Ca^{2+}-ATPase and the calpain substrates erythrocyte Band 3 and Band 4.2. Migration of calpains to the cell membrane allowed for a reversible activation of calpain without the occurrence of autolysis (Molinari et al., 1994). These two ideas of calpain proteolytic activation have been the basis of much discussion. In a review by Goll et al. (2003), it was determined that with the current knowledge, autolysis can be used as an indicator of activation, but lack of autolysis does not guarantee that calpains have not been active. Clearly, autolysis ofμ- and m-calpain will be an area of much research in the future as it may play an important role in calpain activation.

**Physiological role of calpains**

The physiological roles of calpains in tissues are not completely understood. However, the detection ofμ- and m-calpain in most tissues suggest that they contribute to cellular processes that include signal transduction, apoptosis, cell division and cell fusion (Sorimachi et al., 1997; Carafoli and Molinari, 1998; Ono et al., 1998; Hosfield et al., 2001). Studies in transgenic mice indicate that the calpain small subunit is necessary for survival of
embryos (Arthur et al., 2000). A knockout of the 80 kDa subunit of \( \mu \)-calpain was not embryonically lethal (Azam et al. 2001) and mice were viable and fertile. \( m \)-Calpain was present in these mice and appeared to compensate for the lack of \( \mu \)-calpain, which may indicate that substrates of \( \mu \)- and \( m \)-calpain are similar.

The calpain system has also been implicated in several pathological conditions such as limb girdle muscular dystrophy type 2A which, as discussed previously, is caused by lack of calpain 3 activity. The loss of activity is attributed to fifteen nonsense, splice site, frameshift, or missense mutations in the calpain 3 gene (Richard et al., 1995). Duchenne and Beckers muscular dystrophy are also linked to calpain action, but are due to an increase in intracellular calcium due to a loss of calcium homeostasis, which allows calpain degradation of dystrophin (Tidball and Spencer, 2000). Type 2 diabetes is associated with a polymorphism in the calpain 10 gene expressed ubiquitously in tissues (Horiwaka et al., 2000) which may affect insulin action, secretion, or production. Paul et al. (2003) determined that inhibition of calpain 10 activity prevented the translocation of the insulin-response glucose transporter 4 (GLUT 4) to the plasma membrane. It was proposed that calpain 10 activity is required for actin reorganization responsible for the movement of GLUT 4, thus preventing glucose uptake into the cell. Loss of calcium homeostasis also occurs in cardiac and cerebral ischemia, which triggers uncontrolled \( m \)- and \( \mu \)-calpain...
activity causing tissue damage associated with apoptosis and necrosis (Blomgren et al., 2001). Cataract formation is due to activation of m-calpain upon a calcium influx into the lens (Shearer et al., 1999) where α-crystallin and β-crystallin are degraded and their degradation products accumulate to form the cataract. Studies have shown increased m-calpain activity in the brain of patients with Alzheimer’s disease (Tsuji et al., 1998). μ-Calpain autolytic fragments are also present at higher than normal levels in brain tissue (Saito et al., 1993).

m-Calpain was first purified in porcine skeletal muscle (Dayton et al., 1976). Additionally, many of the early reports on calpain were from studies in rabbit skeletal muscle (Azanza et al., 1979) or chicken skeletal muscle (Ishiura et al., 1978). Both μ- and m-calpain cleave myofibrillar and cytoskeletal proteins at a limited number of sites, resulting in large polypeptide fragments (Goll et al. 1999). Calpains do not target all cytoskeletal proteins, but μ- and m-calpain appear to have the similar substrate specificity. Calpain has been shown to degrade desmin (O’Shea et al., 1979), troponin T (Ho et al., 1994), titin (Suzuki et al., 1996), nebulin (Huff-Lonergan et al., 1996), talin (Hemmings et al., 1996) and vinculin (Evans et al., 1984). However, calpain does not degrade the main myofibrillar proteins, actin (Goll et al., 1991) and myosin (Pemrick and Grebanau, 1984), although recent evidence suggests the μ-calpain could cleave sites on actin, myosin heavy chain, and myosin light chain I.
Lametsch et al. (2004). Lametsch et al. (2004) attributed this discrepancy to \( \mu \)-calpain cleaving only small peptides off of intact actin and myosin, which is not detectable by SDS-PAGE. However, by using 2-D gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), actin, myosin heavy chain, and myosin light chain fragments were identified in \( \mu \)-calpain digested myofibrils that were no present in the control myofibrils (Lametsch et al., 2004).

One of main functions of calpains in skeletal muscle is thought to be involvement in protein turnover (Dayton et al., 1976). Myofibrillar protein breakdown into myofilaments occurs first, with subsequent breakdown to polypeptides and ultimately free amino acids. Goll et al. (1992) hypothesized that calpains are the proteinases involved in the specific cleavages required to release the myofilaments.

Another role of calpains in skeletal muscle is thought to be \( \mu \)-calpain involvement in myoblast fusion through degradation of cytoskeletal/plasma membrane attachments (Dedieu et al., 2004). When calpastatin was microinjected into \( C_2 C_{12} \) cells (Temm-Grove et al., 1999), fusion of the myoblasts was prevented. Western blot analysis indicated that only \( m \)-calpain was detectable in the cells before fusion, indicating that \( m \)-calpain is most likely the calpain involved in myoblast fusion. Dedieu et al. (2004) evaluated migratory ability of myoblasts with a four-fold increase in calpastatin and a two-fold decreased calpain activity.
It was observed that fusion of these cells into myotubes was completely prevented.

Morphological differences were observed, where in the cells overexpressing calpastatin presented a round morphology and the control cells had a spreading morphology. Differences were not observed in concentrations of known calpain substrates such as desmin, talin, and vinculin; however, increased levels of myristoylated alanine-rich C kinase substrate (MARCKS) were observed in cells overexpressing calpastatin. Myoblasts with MARCKS overexpression show decreased migratory capacity. MARCKS is a known actin binding protein and its degradation may be important in the cytoskeletal reorganization that occurs during myoblast fusion (Dedieu et al., 2004).

Schollmeyer (1988) monitored m-calpain localization throughout the cell cycle in PtK₁ cells. m-Calpain was associated with the chromosomes during anaphase. m-Calpain was observed at a bilateral location at the cell membrane and associated with the cytoplasmic bridge during telophase. At interphase, m-calpain was localized primarily at the cell membrane, indicating relocalization of m-calpain during the cell cycle. A second experiment was conducted in this study where m-calpain labeled with rhodamine siothiocyanate was injected near the nucleus of a cell in interphase. This injection of m-calpain increased the rate that the cell moved from interphase to metaphase. Injection of m-calpain into the cells at late metaphase also increased the rate of transition from metaphase to anaphase. It was
concluded that m-calpain could cause hydrolysis of microtubule-associated proteins, resulting in disassembly of microtubules of the mitotic spindle, helping to progress cells through the cell cycle. A recent study (Raynaud et al., 2004) evaluated calpain activity in myoblasts during the cell cycle and determined that cell cycle progression from G₀ to G₁ seems to be dependent on m-calpain activity. m-Calpain was localized in the nuclei during the G₁ phase, but if cells were treated with calpain specific inhibitor MDL 28170, m-calpain did not accumulate in the nucleus and G₀ cell cycle withdrawal did not occur. The role of calpains in muscle growth and myoblast proliferation and fusion is only starting to be understood. It is clear that calpains, particularly m-calpain, is a necessary for normal muscle development.

**Calpastatin**

An inhibitor of calpain activity was discovered in the purification of m-calpain (Dayton et al., 1976). Murachi (1989) named this inhibitor calpastatin. Calpastatin is ubiquitously expressed in cells (Ouali and Talmant, 1990) and is specific for μ- and m-calpain inhibition. Since the primary role of calpastatin is regulation of calpain, it follows that calpastatin is a regulator of calpain mediated processes.

Calpastatin is considered an IUP (Intrinsically Unstructured Protein) (Konno et al., 1997). IUPs have functional importance often related to regulatory roles in key cellular
processes (Tompa, 2002). These proteins are known for their lack of folded structure but highly flexible random-coil-like conformation (Wright and Dyson, 1999). The random coil formation of calpastatin was determined by circular dichroism and magnetic resonance (Konno et al., 1997) where no secondary or tertiary structure for calpastatin was apparent. Like most IUP’s, calpastatin is a very heat stable protein (Okitani et al., 1976) and is resistant of denaturing agents such as urea, SDS, 6 M guanidinium hydrochloride and trichloroacetic acid (Geesink and Koohmaraie, 1999; Otsaka and Goll, 1987).

Complementary DNAs encoding calpastatin have been cloned in human, monkey, mouse, rat, pig, bovine, and rabbit (As reviewed by Goll et al., 2003). The amino acid sequences of calpastatin consist of 713 residues for pig heart calpastatin (Takano et al., 1988), 706 residues for beef skeletal muscle (Killefer and Koohmaraie, 1994), and 718 residues for rabbit lung or liver (Emori et al., 1987). The structure of calpastatin (shown below) consists of four repeating inhibitory domains (I, II, III, and IV) of approximately 140 amino acids each, and an L-domain at the N-terminal (Emori at al., 1987; Takano et al., 1988). The inhibitory domains are approximately 22.5 to 36.0 % percent identical (Takano et al., 1988).
Purified calpastatin has an isoelectric point of 4.55 (Takano and Murachi, 1982). Interestingly, the isoelectric point of each inhibitory domain is similar (ranging from 4.26 to 4.90), but the isoelectric point of the L-domain is 10.27 (Lee et al., 1992). Each single inhibitory domain can inhibit the activity of \( \mu \) - or \( m \)-calpain, so theoretically, one calpastatin molecule can inhibit four calpain molecules (Emori et al., 1988, Maki et al., 1988). Domain I is more effective at inhibiting calpain than IV, which is greater than II, which is greater than III (Kawasaki et al., 1993) which is attributed to the location each domain on the calpastatin molecule. Within each inhibitory domain are three homologous subdomains A, B, and C. Subdomain B has been shown to be essential for inhibitory activity to occur (Kawasaki et al., 1993; Maki et al., 1988) and contains a conserved sequence, LGXK(R)D(E)XYIPPXYRLL, whereas removal of subdomains A and C does not prevent inhibition of calpains. The L-domain has no inhibitory activity (Takano et al., 1999) although phosphorylation by protein kinase C and protein kinase A of a serine site in the L-domain region increases the \( \text{Ca}^{2+} \) concentration required for binding to calpains, which decreases inhibitory activity on calpains (Averna et al., 1999). More recently, Averna et al. (2001) determined that phosphorylation of calpastatin was involved in intracellular localization of calpastatin, where dephosphorylated calpastatin was the active form and distributed throughout the cell. Once cAMP-dependent phosphorylation had occurred, calpastatin aggregated and relocalized
towards the nucleus. The L-domain region has also been reported to be involved with
binding of calpastatin to cell membranes (Mellgren et al., 1989), however, observed levels of
calpastatin bound to the sarcolemma in cardiac muscle is less than 2%. Lee at al. (1992)
hypothesized that the basic residues found in the L-domain explain binding of calpastatin to
membranes, and may play a role in stabilization of the calpastatin molecule.

The molecular weight of calpastatin has been reported to range from 34 to 300 kDa.
This variation in molecular weights is due to many different properties of calpastatin.
Overestimation of calpastatin molecular weight (> 200 kDa) occurs when subjected to size
exclusion chromatography due to calpastatin being a random coil (Konno et al., 1997;
Shannon and Goll, 1985). Calpastatin migration on SDS-PAGE gels also overestimates
molecular weight by 50-60 % (Maki et al., 1991). These high molecular weight estimations
may be explained by the amino acid composition as calpastatin contains a large number of
basic residues, which slow down gel migration. Another explanation of the variation in
molecular weight is because, although calpastatin is a single gene product, studies have
shown that different promoters and alternative splicing mechanisms cause a number of
different calpastatin isoforms (Imajoh et al., 1988; Cong et al., 1998). The mouse calpastatin
gene sequence shows that the calpastatin gene consists of 34 exons, of which five are
upstream from exon 2. Exon 2 encodes the L-domain NH2-terminal region (Takano et al.,
2000) for what is called “prototypical” calpastatin (Goll et al., 2003). Takano et al. (2000) classified four types of mammalian calpastatins with distinct N-terminal sequences based on these upstream exons. Type I calpastatin is based from mouse myoblasts and started at exon 1xa and has an extended region on the NH$_2$-terminal and predicts an 85 kDa polypeptide. Type II calpastatin is based from bovine heart and is started at exon 1xb and contains an extended region of the NH$_2$-terminal called the XL domain and predicts an 84-kDa polypeptide. Type III calpastatin starts at exon 1u and encodes the prototypical calpastatin. It is expressed in many tissues including all rabbit tissues tested, pig heart, bovine skeletal muscle, rat liver and brain and predicts a polypeptide with a range from 72-78 kDa. Type IV calpastatin starts at exon 14t, located downstream from exon 2, and determined to be initiated in domain II, after subdomain A and it is expressed in human testis and predicts a 35 kDa polypeptide. Takano et al. (2000) hypothesized the differences in transcribed calpastatins is due to different promoters specific for these exons. More recently, Raynaud et al. (2005) determined specific promoters (individually located upstream of exons 1xa, 1xb, 1u, and 14t) were functional which further validates the conclusion of Takano et al. (2000).

Calpastatin is susceptible to proteolytic degradation which also contributes to the variation in the molecular weight (Mellgren and Carr, 1983; Mellgren et al., 1986; Otsaka and Goll, 1987). Calpastatin not only inhibits calpain, but is also a substrate of calpain (Goll
et al., 1983). Calpastatin is cleaved by calpain in the interdomain regions which in turn
generates inhibitory peptides and can then inhibit more than one molecule of calpain. It has
been demonstrated that degradation of calpastatin by calpain does not result in the loss of
calpastatin’s ability to inhibit the calpains (Goll et al., 1983; Mellgren et al., 1986; Mellgren
and Carr, 1983). However, Doumit and Koohmaraie (1999) disagreed with this hypothesis
and showed that degradation of calpastatin by μ-calpain did reduce inhibitory activity. The
different results of this study were attributed to the fact that previous studies were based
qualitative assays, not quantitative. Doumit and Koohmaraie measured a lower calpastatin
activity after incubation with μ-calpain, whereas previous studies did not measure actual
calpastatin activity. It is possible that the extent of calpastatin proteolysis indicates whether
or not inhibitory activity is maintained.

**Binding of Calpastatin to Calpains**

The exact mechanism of calpastatin in binding to and inhibiting μ-calpain and m-
calpain is not defined (Goll et al., 2003). Calpastatin is co-localized intracellularly with
calpains (Kumamoto et al., 1992). Calcium is required for binding of calpastatin to calpains
(Cottin et al., 1981; Otsuka and Goll, 1987) and binding is reversible upon removal of
calcium. The Ca^{2+} concentration required for binding of calpastatin to μ- and m-calpain is
lower than the Ca^{2+} concentration required for calpain proteolytic activity at half-maximal
rate. This indicates that, if present, calpastatin will bind and inhibit calpain before it can become proteolytically active (Kapprell and Goll, 1989). Both μ- and m-calpain and the autolyzed form of m-calpain all require lower Ca$^{2+}$ concentrations for binding calpastatin, but the Ca$^{2+}$ requirement for autolyzed μ-calpain is approximately the same as the Ca$^{2+}$ requirement for activation of autolyzed μ-calpain (Kapprell and Goll, 1989). Calpastatin cannot inhibit the initial autolysis step of the 80 kDa subunit of μ-calpain, but it can further inhibit calpain autolysis from 78 kDa to 76 kDa (Johnson and Guttman, 1997; Zimmerman and Schlaepfer, 1991).

Calpastatin binds to three sites on the calpain molecule: subdomain A of calpastatin binds to domain IV in calpain, subdomain C of calpastatin binds to domain VI in calpain, then subdomain B of calpastatin binds to an area near the active site. Kinetic assays indicate that calpastatin is a competitive inhibitor of calpain (Croall and McGrody, 1994). However, evidence indicates that calpastatin does not bind at the active site of μ- or m-calpain (Nishimura and Goll, 1991, Croall and McGrody, 1994), but rather near the active site in a way that blocks access of substrates as hypothesized by Todd et al. (2003). As previously discussed, domain III of m-calpain contains an electrostatic switch (Strobl et al., 2000) and calcium binding sites (Alexa et al., 2003) that may play a role in the alignment of the calpain active site. Croall and McGrody (1994) determined that a calpastatin binding site is likely at
a highly conserved region in domain III of the calpain large subunit. Binding of calpastatin to this area in domain III is a possible mechanism behind calpain inhibition as calpastatin may prevent calcium binding from occurring at domain III of calpains, thus preventing the alignment of the active site required for activation.

Subdomains A and C appear to prevent calpain from associating with membranes when bound to calpain at domains IV and VI (Kawasaki et al., 1993). The interaction of subdomains A and C with calpains is calcium dependent. Tompa et al. (2002) observed an increase in \( \mu \)-calpain and \( m \)-calpain activity upon interaction with subdomains A and C in the absence of subdomain B. They suggested a role for calpastatin fragments in activation of calpains in vivo and hypothesized the subdomains A and C of calpastatin decrease the calcium requirement of calpains for activation and help to shift the conformation of calpains into the active form.

Localization of calpains and calpastatin in the cell may also explain activation of calpains in the presence of calpastatin. Gil-Parrado et al. (2003) observed that in LCLC 103 H cells both calpain and calpastatin were distributed in the cytoplasm, and calpastatin was also observed in the nucleus. When calpain was redistributed in the cell towards the membrane through activation with ionomycin, the nuclear localization of calpastatin was unchanged.
Meat Quality

Meat quality is a generic term used to describe properties and perception of meat. It includes composition and conformation, the eating quality such as juiciness, tenderness, flavor, and aroma, health issues associated with microbial contamination and other diseases, and also includes animal welfare and environment. All these attributes are described as important traits by the consumer (Boleman et al., 1997). The main source of consumer complaint with meat quality is the variability in eating quality, especially tenderness (Bindon and Jones, 2001).

The National Beef Tenderness Survey (Morgan et al., 1991) determined an unacceptable level of variation of tenderness in retail beef cuts. Boleman et al. (1997) determined that consumers can distinguish differences in tenderness and are willing to pay premiums for improved tenderness. The current system, U.S. Quality grade, is set up so that beef subprimals are priced according to their expected palatability. However, there has been much debate on whether or not quality grade can distinguish differences in palatability, particularly tenderness differences. Methods to predict tenderness quickly in a plant setting have not been successful, particularly in sorting out tender cuts in US Select carcasses. Shackelford et al. (2005) have recently evaluated the effectiveness of visible and near-infrared spectroscopy in predicting tenderness of US Select carcasses, and determined that
this technology was successful in sorting tender cuts from tough cuts based on shear force values and sensory panel evaluations and could be a step towards guaranteeing tenderness in retail sales.

Tenderness of meat is due to many different attributes including factors during the conversion of muscle to meat. Metabolic changes that occur as muscle is converted to meat include the switch from aerobic glycolysis to anaerobic metabolism of pyruvate in order to maintain a supply of phosphate to produce ATP. Lactate accumulation during this process lowers the intracellular pH to about 5.6. As these ATP levels are decreased and calcium regulation is decreased, irreversible cross-bridges form between myosin heads and actin, causing rigor mortis, which is associated with increased toughness of meat.

Another contributor to variation of tenderness is increasing connective tissue in relation to increasing animal age. Connective tissue is primarily composed of two proteins, collagen and elastin (Aberle et al., 2001). During animal maturation, the endomysium, perimysium, and epimysium thicken and cross-links form between collagen molecules (Robins et al., 1973). The effect of connective tissue on tenderness is called background toughness (Aberle et al., 2001). This background toughness increases with animal age and the insolubility of the connective tissue proteins makes resolving the increased background toughness difficult.
Changes in muscle fiber properties also affect variation in tenderness. The current evidence suggests that the proteolysis of cytoskeletal proteins is the cause of meat tenderization during aging. The proteins involved include 1) intermediate filaments such as desmin and vinculin 2) intra-myofibril linkages such as titin, nebulin, and troponin-T 3) costameric proteins such as vinculin and dystrophin and 4) proteins such as laminin and fibronectin are involved in attachment of muscle cells to the basal lamina (Koohmaraie et al., 2002). There are several endogenous proteolytic systems in muscle, including the cathepsin-lysosomal system, the ATP-dependent ubiquitin-proteosome system, the calpain-calpastatin system, and the matrix-metalloproteinases (Marsh, 1988). There is little evidence that the ubiquitin system is involved in postmortem proteolysis due to its ATP-dependency for activation (Marsh et al., 1988). The lysosomal system does not appear to be responsible, although the decrease in pH would allow for increased activation of cathepsins (Marsh et al., 1988). However, a lack of myosin degradation in the postmortem myofibril and the prevalence of myosin as a substrate for lysosomal enzymes (Koohmaraie, 1992) indicates that lysosomal enzymes are not a main protease involved in postmortem proteolysis. Postmortem proteolysis is primarily the result of calpain degradation of myofibrillar and cytoskeletal proteins (Koohmaraie, 1992; Taylor et al., 1995; Boehm et al., 1998; Wheeler et
Additionally, calpastatin, the endogenous inhibitor of calpains, can greatly affect calpain mediated degradation of cytoskeletal proteins in postmortem muscle.

**Calpain and Calpastatin Activity in Postmortem Tissue**

As previously stated, calpain degradation of myofibrillar and cytoskeletal proteins is responsible for tenderization that occurs during aging of meat postmortem (Koohmaraie, 1992). More specifically, it is agreed that μ-calpain, rather than m-calpain, plays the primary role in tenderization (Boehm et al., 1998; Ilian et al., 2001; Veiseth et al., 2001).

The first evidence supporting calpain mediated postmortem tenderization was an in vitro study that demonstrated the loss of Z-disk structure in calpain-treated skeletal muscle myofibrils (Busch et al., 1972). This deconstruction of the Z-disk was attributed to an increase in tenderness during aging. When evaluating calpain activity in carcasses, infusion of zinc (a calpain inhibitor) into ovine carcasses (Koohmaraie, 1992a) blocked postmortem proteolysis and tenderization. Whipple and Koohmaraie (1991) determined that zinc inhibited degradation of purified myofibrils that were incubated with m-calpain. Additionally, they compared degradation profiles of m-calpain and lysosomally degraded myofibrils and determined that the m-calpain degraded myofibrils more closely duplicated the degradation profiles of 14 day aged myofibrils.
Injection of calcium has been used to improve meat tenderness (Wheeler et al., 1993; Wheeler et al., 1997). Koohmaraie et al. (1989) determined that calcium infusion of ovine carcasses increased postmortem tenderization and correlated to increased proteolysis of myofibrillar proteins during postmortem storage. This was hypothesized to be due to activation of calpains. Harris et al. (2001) also observed increased sensory panel tenderness scores in CaCl₂-injected beef. They also saw accelerated tenderization in steaks injected with CaCl₂ from vitamin E fed cattle compared to CaCl₂-injected beef from cattle not supplemented vitamin E. This accelerated tenderization corresponded to an increase in troponin-T degradation at day 1 post-injection (48 hours postmortem).

Boehm et al. (1998) evaluated the changes that occur in activity and protein profiles of μ-calpain, m-calpain, and calpastatin during aging. They determined that m-calpain activity changed marginally between measurements taken at-death and 7 days postmortem. μ-Calpain activity decreased rapidly within the first day and showed almost no activity after 7 days postmortem. Calpastatin activity also decreased during the 7 day aging period. Western blot analysis determined that after 7 days postmortem, intact calpastatin was not detectable and the 80 kDa subunit of μ-calpain was almost completely in the 76 kDa form. m-Calpain was determined not to have autolyzed based on SDS-PAGE, but the small change in mass that occurs with m-calpain autolysis is difficult to determine using normal SDS-
PAGE. Proof of m-calpain autolysis must be done through direct sequencing or using an antibody that detects the NH$_2$-terminal sequence of m-calpain (Goll et al., 2003). N-terminal sequencing was attempted by Boehm et al. (1998) but the N-terminal region of m-calpain was blocked.

Much debate has occurred on the true action of calpastatin in postmortem muscle. Is variation in postmortem proteolysis related to inhibition of $\mu$-calpain by calpastatin or is it due to the fact that calpastatin is a known substrate for calpains (Doumit and Koohmaraie, 1999), thereby adding another target for $\mu$-calpain proteolysis? Activation of calpain causes inactivation of calpain, and if calpastatin is being targeted by calpain for proteolysis, degradation of key proteins involved in postmortem tenderization may be decreased. Additionally, calpastatin can act as a regulator of calpain activity as the interaction between calpain and calpastatin is irreversible. Results from Chapter 4 indicate that calpastatin can prevent $\mu$-calpain inactivation by oxidation, thus allowing for $\mu$-calpain to be active once conditions change to allow activation (i.e. disassociation of $\mu$-calpain/calapstatin complex and nonoxidating environment).

Transgenic mice overexpressing calpastatin were used to observe $\mu$-calpain activity in postmortem muscle (Kent et al., 2004). These mice had a 370-fold increase in calpastatin activity at death in comparison to control mice. Rate of $\mu$-calpain autolysis by day 3
postmortem was greater for control mice. Autolysis of the μ-calpain large subunit from the transgenic mice was completed only to the intermediate 78 kDa band. Additionally, desmin degradation was reduced in transgenic mice. They concluded from the results that calpastatin regulates calpain activity postmortem through inhibitory action as indicated by reduced autolysis and degradation of substrates.

A more recent interest in postmortem proteolysis focuses on its role in water-holding capacity. Kristensen and Purslow (2001) tested the hypothesis that water-holding capacity increases during aging due to the proteolytic change in the cytoskeletal structure of meat. More specifically, greater degradation of desmin, talin, and vinculin has been linked to decreased drip loss (Kristensen and Purslow, 2001). Lateral shrinkage that occurs in the myofibrils postmortem (Diesbourg et al., 1988) will, due to linkage of the myofibrils to the sarcolemma, shrink the muscle cell. This decrease in the muscle cell volume decreases the amount of intracellular space available for water. Additionally, shrinkage of the muscle cell will increase the pressure in the intracellular space as compared to the extracellular space, actually promoting water movement out of the cell. Kristensen and Purslow (2001) hypothesized that degradation of these cytoskeletal proteins, which are key in the linkage between myofibrils and linkages of costameres, relieve the pressure differences between
intracellular and extracellular water compartments and increase intracellular space for water storage through the release of the myofibrils from the sarcolemma.

Melody et al. (2004) observed a lower drip-loss percentage after 96 hours storage in porcine psoas major muscle in comparison with semimembranosus and longissimus dorsi. This lower drip loss corresponded to faster degradation of desmin observed in the psoas major. The psoas major muscle also had lower calpastatin activity by 6 hours postmortem and faster μ-calpain autolysis as early as 45 min postmortem, which corresponded to a faster pH decline, which explains the increased desmin degradation. These results closely relate to observations of Kristensen and Purslow (2001) in that greater degradation of cytoskeletal protein, particularly those involved in linkage of muscle fibers, created more space for water and decreased pressure in the intracellular compartments.

pH

One of the most pronounced changes in the conversion of muscle to meat is the pH decline from near neutral pH in living muscle to approximately 5.6 in meat. Calpains have a pH optimum of 7.5 (Edmunds et al., 1991; Wang and Jiang, 1991). Therefore, it is important to understand calpain activity at lower pH conditions as observed in meat. Many studies have been conducted to determine if calpain activity is affected by pH, particularly under low temperature conditions as observed in chilled carcasses. Koohmaraie et al. (1986) observed
that at pH 5.5 to 5.8 at 5°C, μ-calpain activity was 24-28 % of μ-calpain activity at pH 7.5 at 25°C after 90 min incubation. Although pH decreased μ-calpain activity, the results from this study indicated that μ-calpain can be active in conditions of pH and temperature similar to pH and temperature in a carcass.

Huff-Lonergan et al. (1996) evaluated degradation of five cytoskeletal proteins (titin, nebulin, filamin, desmin, and troponin-T) by purified μ-calpain from bovine skeletal muscle at pH 5.6 and 4°C and 165 mM NaCl. Immunoblot profiles of the degradation products from purified myofibrils were compared to immunoblot profiles of the degradation of the same proteins in aged beef steaks. It was determined that μ-calpain was active at conditions similar to meat and μ-calpain mediated degradation of these five proteins corresponded to the breakdown of these proteins in aged beef steaks. Additionally, steaks were divided into a high Warner-Bratzler shear force value and a low Warner-Bratzler shear force value group. Steaks from the low shear force value group clearly had faster proteolysis of these five proteins than steaks with higher shear force values.

Melody et al. (2004) observed that porcine muscles with low early postmortem pH (psoas major) had earlier μ-calpain autolysis and inactivation and earlier initiation and completed degradation of desmin than muscles with higher early postmortem pH (longissimus and semimembranosus). Although the mechanism behind the different pH
declines between muscles is not understood, they hypothesized that the differences in pH
decline occurred due to a faster temperature drop in the psoas major and a higher percentage
of the myosin heavy chain isoform type I, which may allow for increased calcium
concentrations in the sarcolemma, which in turn increased postmortem metabolism resulting
in a faster pH decline. They observed differences in tenderness, where the psoas major had
the lowest Warner-Bratzler shear force values when compared to the semimembranosus.

Geesink and Koohmaraie (2000) determined that pH affects stability of calpain. They
showed that autolyzed porcine skeletal μ-calpain activity was more stable (indicated by
greater activity) at pH values of 6.2 to 6.4 when evaluating pH ranges from 7.6 to 5.6.
Geesink and Koohmaraie (2000) determined that μ-calpain had approximately 70% of initial
activity after incubation for 30 min at pH 6.2 and approximately 22% of initial activity
remaining at pH 7.5 indicating a more stable molecule at the lower pH.

The change in pH has not shown to affect calpastatin inhibition of calpains. Geesink
and Koohmaraie (1999a) determined that calpastatin inhibition of μ-calpain activity was not
different under pH conditions of 5.7, 6.2, and 7.0 at 25°C. Kendall et al. (1993) determined
that pH alone did not affect calpastatin inhibition of m-calpain.
Ionic strength

Another change that occurs is the increase in ionic strength from an approximate equivalent of 165 mM NaCl in living muscle to approximately equivalent to 295 mM NaCl in meat (Winger and Pope, 1980-81).

Differences in ionic strength have been shown to affect μ-calpain activity by decreasing the stability of autolyzed μ-calpain (Geesink and Koohmarais, 2000, Li et al., 2004), thereby decreasing μ-calpain activity as ionic strength increases. Likewise, Li et al. (2004) observed loss of autolyzed μ-calpain activity at salt concentrations as low as 100 mM KCl at pH 7.5, and they determined that ionic strengths of 300 mM or greater decreased autolyzed μ-calpain activity by 50 to 55% within 5 min. The loss of μ-calpain activity due to high salt concentrations (500 mM) was not reversible once the salt had been removed (Li et al., 2004). The rate of activity loss was slower at lower ionic strengths (Li et al., 2004). Li et al. (2004) hypothesized an ionic strength equal to 100 mM KCl or greater caused disassociation of the two m-calpain subunits, allowing for inactivation of the proteinase due to the formation of dimers and trimers of the large subunit.

Kendall et al. (1993) determined that at lower pH conditions (i.e. 5.7) increasing ionic strength (200 mM NaCl or greater) decreased calpastatin inhibition of m-calpain. Tompa et al. (2001) through the use of overlay blotting techniques indicated the binding of
phospholipids to domain III is calcium dependent and influenced by ionic strength. At higher ionic strengths, decreased binding of phospholipids was observed, which negated the ability of lower calcium concentrations to activate calpains, indicating a decrease in \( \mu \)-calpain activation at higher ionic strengths.

**Other factors affecting calpain/calpastatin activity**

Large differences in tenderness have been observed between cattle containing \( bos \) *indicus* genetics compared to cattle with \( bos \) *taurus* genetics indicating that \( bos \) *indicus* cattle have less tender meat. Direct comparison of Brahman cattle (\( bos \) *indicus*) and Hereford cattle (\( bos \) *taurus*) at slaughter (Wheeler et al., 1990) indicated that there were not differences in pH decline, temperature, or ionic strength effects on myofibrillar protein solubility. However, Hereford cattle had greater \( \mu \)-calpain activity at harvest and lower \( \mu \)-calpain activity at day 1 postmortem, in addition to lower calpastatin activity at harvest. These measurements corresponded to differences in tenderness ratings from a sensory panel and Warner-Bratzler shear force values, where steaks from Hereford cattle were more tender than steaks from Brahman cattle. They concluded that \( \mu \)-calpain was more active postmortem due to the lower levels of calpastatin activity in the steaks from the Hereford cattle than was measured in the steaks from the Brahman cattle.
The callipyge condition in sheep results in increased muscle hypertrophy of skeletal muscle, particularly in the lamb hindquarters. Koohmaraie et al. (1995) evaluated postmortem differences between normal and callipyge sheep and observed that in normal lambs, shear force decreased throughout aging. Also during aging, degradation of titin, nebulin, desmin and troponin-T were observed. Conversely, in callipyge lambs, the shear force changes during aging were minimal, and the rate and extent of degradation of the aforementioned proteins were minimal. Additionally, they determined that callipyge lambs had greater calpastatin activities after 7 and 21 days postmortem. Delgado et al. (2001) observed similar results, where calpastatin activity was greater in callipyge muscle (biceps femoris and longissimus) than in normal sheep, but calpastatin was the same in the infraspinatus, which does not have increased hypertrophy in callipyge lambs.

Ilian et al. (2001) determined that fasting lambs for 1 day prior to harvest caused a significant improvement in longissimus tenderness. A correlation was calculated between shear force value at day 1 postmortem and μ-calpain activity, measured against casein after purification with DEAE ion-exchange chromatography from a sample taken at death. The negative correlation indicated that the higher the shear force value, the lower the μ-calpain activity. Correlations between shear force and m-calpain activity or calpastatin activity were
not significant. They concluded that μ-calpain and not m-calpain plays a role in postmortem
meat tenderization.

**Oxidation in postmortem muscle**

Oxidative processes are part of intracellular metabolic functions (Forman et al., 2002) including signal transduction pathways and second messenger systems. Oxidation also can occur due to pathological stimuli including radiation, air pollutants, pesticides, herbicides, and intracellular conditions such as hypoxia, ischemia, and antibiotic therapy (Van Steenhouse, 1987). Living cells balance oxidation with antioxidant systems to protect and retain cellular function (Thomas and Mallis, 2001). In postmortem tissue, as antioxidants are expended, that control is lost and affects the many mechanisms of oxidation important in protein functionality in meat products. The effect of oxidation on muscle protein functionality is very complex and a relationship exists between extent of oxidation and magnitude in the alteration of function of the effected proteins (Xiong, 2000). During aging, the protein sulphydryl content decreases while the protein carbonyl content increases in beef myofibrils (Martinaud et al., 1997; Srinivasan and Hultin, 1995) indicating oxidation is readily occurring.

Oxidative processes are known to be major causes of meat quality deterioration in traits such as flavor, color, and nutritional composition (Xiong, 2000). Oxidation of lipids
are known to be a primary cause of off flavor and autoxidation and oxygen consumption allow for the production of metmyoglobin to cause changes in color (Renerre, 1990). The effect of oxidation of proteins in relation to protein functionality is currently getting more attention (Martinaud et al., 1997). Ooizumi and Xiong (2004) determined that oxidation using a nonenzymatic hydroxyl radical generation system consisting of ascorbate, FeCl₃, and H₂O₂ in chicken myofibrils decreased ability of gel formation in myofibrillar proteins. Oxidation of myofibrils resulted in intramolecular cross-linking of the myosin heavy chain and modifications to thiol groups at the myosin ATPase active site, which then lead to aggregation of myosin molecules (Ooizumi and Xiong, 2004). Changes in gel formation can impact texture characteristics and binding strength of processed meats. Decker et al. (1993) observed similar effects of metal-catalyzed oxidation in turkey white muscle myofibrillar proteins where loss in protein solubility, decreased gel strength, and decreased water-holding capacity occurred. In addition of effects of oxidation on processed meat products, Xiong (2000) suggested that the oxidative changes in postmortem muscle proteins may contribute to postmortem proteolysis ultimately affecting fresh meat characteristics of tenderness and water-holding capacity.
Protein Oxidation

The oxidative process causes production of reactive oxygen species (ROS). Most ROS produced are free radicals, which by definition, are any molecules with an odd number of electrons (VanSteenhouse, 1987). Free radicals are formed by the splitting of an organic molecule and include superoxide anions, hydrogen peroxide, hydroxyl radicals, singlet oxygen, nitric oxide (NO), and peroxyl radicals. If not quenched by an antioxidant, these compounds will react with the nearest lipid, protein, carbohydrate, RNA, or DNA molecule, altering their structure and function.

In muscle tissue, high concentrations of oxidizable lipids, heme pigments, transitional metal ions, and other oxidative enzymes can serve as precursors or catalysts for the production of free radicals (Xiong, 2000). The production and action of these free radicals cause physical and chemical changes that can be beneficial to the host cell, particularly by killing microbial pathogens, but they are also implicated as mediators of tissue damage and cell aging processes that include amino acid destruction, decreases in protein solubility due to polymerization, enzyme activity loss, and increases in protein digestibility (Stadtman and Oliver, 1991; Agarwal and Sohal, 1994).

Oxidation of proteins in postmortem muscle tissue occurs primarily from lipid by-products and metal ions (Xiong, 2000). The reaction from lipid by-products starts with
initiation when a free radical associates with a lipid molecule. Polyunsaturated fatty acids are the most readily oxidized lipid due to the instability of its structure because of the reactivity of unstable double bonds. The second step of oxidation occurs with propagation of the lipid free radical where it interacts with an unstable oxygen molecule. The next step could occur with the lipid molecule interacting with a protein, removing a hydrogen from the protein side chain and passing the odd electron to the protein in a process called hydrogen abstraction. A different process could occur that involves the lipid molecule linking to the protein molecule in a process called addition. This lipid-protein molecule is still reactive and can link with another protein and form what is formally titled a “complex” (Xiong, 2000). These complexes can interact causing polymerization. Metal ions such as Fe$^{2+}$ and Cu$^{2+}$ are also strong catalysts of protein oxidation. This particular oxidation is site specific as side chains of aromatic and basic amino acids at the metal binding sites are specific targets (Stadtman and Oliver, 1991). Uchida et al., (1992) studied the effect of the copper/H$_2$O$_2$ and iron/H$_2$O$_2$ oxidizing systems and determined that these oxidizing agents caused the protein to polymerize or degrade. Copper mainly targeted aromatic and basic amino acids where effects of iron were more variable indicating possible difference in the oxidizing mechanism of the two metal ions.
Reactive oxygen species tend to target sulfhydryl containing amino acids on proteins such as cysteine (Freeman and Crapo, 1982). Cysteine is probably the most susceptible amino acid residue and one of the first to be oxidized (Xiong, 2000). The nucleophilic role of sulfhydryls on cysteines are well known and the redox state of active site cysteine in both transfer/addition reactions (S-transferases, glyceraldehydes 3-P dehydrogenase, glutathione reductase) and in peptidase activity (caspases, papain, and calpains) is critical for functionality (Giles et al., 2003). The nucleophilicity of these cysteines are usually increased by ionization of the thiol to the thiolate anion, which increases susceptibility of the residue to oxidation (Gilbert, 1990).

Normal cellular homeostasis helps to maintain fully reduced states of the cysteine residues in order to maintain full functionality, which includes regulation of proteins involved in catalysis, regulation, electron transfer, and protein structure. Therefore irreversible oxidation can cause complete loss of function of these proteins. However, reversible oxidation of a reactive cysteine residue can play a role in regulation and function of proteins (Ghezzi and Bonetto, 2003).

S-thiolation, S-nitrosylation, and irreversible oxidation are oxidative modifications of protein sulphhydryls that can occur (Gilbert, 1990). S-thiolation and S-nitrosylation may result from ROS action. Since both modifications are reversible, they may play important
regulatory roles in cell metabolism during times of increased oxidative stress. S-thiolation has long been thought to start with protein thiol disulfide-exchange.

1) Protein-S + RSSR ↔ protein-S-SR + RS-

However, this reaction occurs rather slowly (Gilbert, 1990) and requires higher RSSR concentrations, so is most unlikely in intact cells. Therefore other mechanisms have been proposed (Thomas and Mallis, 2001) that include:

2) Protein-SH- + R* → protein-S* + RH (thiolate anion)

3) Protein-S- + ROO• → protein-S- + ROO•- (thiyl radical)

4) Protein-SH + HOOH → protein-SOH + HOH (sulfenic acid)

These products of thiolation are reversible and can revert to the reduced form. Many regulatory proteins and enzymes form reversible disulfides and some signal transduction proteins and transcription factors determine the redox state of a cell by forming intramolecular disulfides.

Thiolation can continue to occur to the point where it is irreversible. Sulfinic and cysteic acids are formed (Thomas and Mallis, 2001) in the absence of antioxidants. These reactions are:

5) protein-SOH + O₂ → Protein-SO₂H (Sulfinic acid)

6) Protein-SO₂H + O₂ → Protein-SO₃H (Cysteic or sulfonic acid)
These irreversibly oxidized sulfhydryls can accumulate and affect normal cellular functions.

S-nitrosylation is a mechanism that is not well understood, but has been found to be quite similar to S-thiolation. It occurs when a nitric oxide group is transferred to a sulfhydryl on a protein, usually targeting cysteine and tyrosine residues (Stamler, 1994). Nitrosylation is an important mechanism of redox regulation and NO modulation of the activity of several proteins (Jaffrey et al. 2001; Hess et al., 2001). The primary mechanism proposed:

\[
\text{Protein-SH} + \text{NO}^+ \rightarrow \text{Protein-SNO} + \text{H}^+ 
\]

Nitrosothiols (RSNO) produced from this reaction are quite labile and can promote disulfide formation (Broillet, 1999). Nitrosothiols are formed under a wide variety of environments that include both aerobic and anaerobic conditions. Specificity for nitrosylation is thought to be through localization of nitric oxide synthase (NOS), which produces nitric oxide (NO) from the guanido nitrogen of L-arginine (Broillet, 1999). The location of NOS may be in proximity to targets of NO. Actual nitrosylation due to NO depends upon NO concentration, the redox status of the environment, and the susceptibility of target sites for modification, which helps explain the specificity of S-nitrosylation of cysteine residues to direct protein functionality. The reversibility of RSNO is largely unknown. It is hypothesized that reductases may be responsible for some of the detachment of NO from thiols (Hausladen et al., 1996).
Besides S-thiolation of sulphydryls to sulfonic or cysteic acid, other irreversible oxidation mechanisms of proteins include carbonylation and nitration (Ghezzi and Bonetto, 2003). Generally, irreversible modifications are responsible for permanent loss of function of the damaged proteins which are then degraded (Berlett and Stadtman, 1997; Dean et al., 1997).

Carbonylation is used as a marker of protein oxidation and its measurement gives an indication as to the extent of oxidation has occurred (Xiong, 2000). Carbonylation may occur via direct oxidation of some amino acid side chains, particularly histidine, arginine, and lysine, fragmentation of the peptide backbone, a reaction with reducing sugars, or by reaction with products of lipid oxidation. Carbonylation is named for a carbon atom bound to an oxygen molecule though a double bond. Cross-linking of proteins and peptide scission can occur readily after formation of carbonyls which can result in polymerization, aggregation, and possibly insolubilization of proteins. Polymerization and aggregation can occur when oxidatively induced unfolding of proteins can expose hydrophobic residues causing protein interactions (Gardner, 1979).

Nitration of tyrosine forms 3-nitrotyrosine (3-NT), which is used as a marker of irreversible protein damage. Increased levels of 3-NT has been linked to diseased states that include Alzheimer’s disease, Parkinson’s disease, and atherosclerosis (Ghezzi and Bonetto,
2003). A role for nitration in cell signaling is currently becoming better understood, as it is thought to affect protein kinase activities. Nitration has been shown to both inhibit (Hellberg et al., 1998) and stimulate (Li et al., 1998) tyrosine phosphorylation.

Oxidation by H$_2$O$_2$

A thiol (SH) does not react at physiologically significant rates with a hydroperoxide unless the reaction is catalyzed. However, thiolates (S-) react quickly with hydroperoxide under the general reactions series where sulfenic acid is the main product (Forman et al., 2004):

\[
\text{Protein-S-} + \text{H}_2\text{O}_2 \rightarrow \text{Protein-SO-} + \text{H}_2\text{O} + \text{H}^+ \quad \text{(Sulfenic acid)}
\]

\[
\text{Protein-SO-} + \text{Protein'}-\text{SH} \rightarrow \text{Protein-SS-Protein'} + \text{OH}^-
\]

Sulfenic acid modification is reversible, thus, the original thiolate can be restored by exchange with another thiolate (Saurin et al., 2004).

\[
\text{Protein-SS-Protein} + \text{Protein-S-} \rightarrow \text{Protein-S-} + \text{Protein-SS-Protein}
\]

This mechanism of H$_2$O$_2$ follows the basic principles of oxidation described previously with hydrogen abstraction and addition.

Since H$_2$O$_2$ is widely produced in cells, it is often used as the oxidant in research on oxidation-reduction mechanisms in cell biology. Saurin et al., (2004) evaluated sulfenic acid formation in mammalian tissues under elevated H$_2$O$_2$ conditions. It was determined that
many important proteins are modified in rat hearts upon H$_2$O$_2$ exposure, including myosin heavy chain, actin, tropomyosin 1 α chain, troponin-T, myosin light polypeptide 3, and myoglobin. However, when evaluation of concentration and duration effects of H$_2$O$_2$ was conducted, there was a gradual decrease in the amount of sulfenic acid as H$_2$O$_2$ concentration increased. It was discussed that the irreversible sulfinic and sulfonic acids were most likely being produced. Production of sulfenic acid was also decreased the greater the exposure time to H$_2$O$_2$, which they also attributed to a production of sulfinic and sulfonic acids. It was discussed that heart muscle may have a maximum concentration of H$_2$O$_2$ it can tolerate before irreversible tissue injury can occur.

**Oxidation of cysteine proteases and calpains**

As cysteine is a known target for oxidation, much of the research has shifted to the effects of oxidation on cysteine proteases. A cysteine residue in the active site of these proteases acts as a nucleophile working with a histidine residue acting as a proton donor resulting in a release of an amine product from its target. This active site cysteine can be readily oxidized as shown in archaeal GAPDH (Isupov et al., 1999) and pyrroline-5-carboxyl peptidase (Singleton et al. 1999). Implications of the active site cysteine oxidation have been shown to be important in caspase enzymes, which are a major component in the apoptotic pathway. Oxidative changes to caspase occur primarily through NO and free iron
Nitrosylation of the active site cysteine residue inhibits proteolytic activity (Zeigler et al., 2003). However, Mannick et al. (2001) suggest that this nitrosylation of caspase is not stable and is a possible method of reversible activation by denitrosylation indicating that oxidation of the active site cysteine residue is used to control enzymatic activity within the cell.

Oxidation of the active site cysteine has also been shown to be involved in direct activation of hydrolytic enzymes as Bourne et al. (2000) observed in agrobacterial esterase. A disulfide bond between two adjacent residues needs to be formed to allow for binding of the tetrahedral intermediate formed from the active site triad of cysteine, histidine, and asparagine which stabilizes that protein for further activation.

Since calpains are cysteine proteases, oxidation may affect calpain activity in various ways. Most research has determined that oxidation decreases the ability of µ-calpain to degrade its substrates (Guttmann and Johnson, 1998; Koh and Tidball, 2000; Michetti et al., 1995; Rowe et al., 2004b). Guttman et al. (1997) evaluated µ-calpain activity in the presence of H₂O₂ and observed that proteolytic cleavage of a fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC and microtubule associated protein tau by µ-calpain was strongly inhibited. They suggested that it was not activation of µ-calpain in oxidatively stressed diseased states, but in fact, inactivation of µ-calpain causing an increase in calpain substrates and accumulation of
autolyzed μ-calpain that was a mechanism behind some of the disease conditions that are observed in Alzheimer’s disease and ischemia.

Many studies have shown a stimulation of calpain activity following oxidative stress. Ray et al. (2000) observed increased m-calpain degradation of myelin associated protein after peroxide treatment in C6 glioma cells. Spectrin was degraded by μ- or m-calpains (unspecified) in oligodendrocytes after oxidative stress (McCracken et al., 2001). This activation of calpains may be dose dependent as Pronzato et al. (1993) observed that carbon tetrachloride, as an oxidizing agent, in low doses allowed for calpain activation, where higher doses inhibited calpain activity. It is also postulated that calpain protein substrates, when oxidized, can be preferentially degraded. No studies exist evaluating this hypothesis directly, but an in vitro study by Zolotarjova et al. (1994) demonstrated selective calpain degradation of oxidized Na⁺/K⁺-ATPase. Reversible inactivation has also been thought to occur in calpain, similar to caspases, where oxidation is used to regulate calpain activity. In ischemia/reperfusion injury, increases in calpain activity have been observed (Numar et al., 1996; Tolnai and Korecky, 1986). Kohli et al. (1997) observed increasing calpain activity in cold-preserved rat livers, livers going through a rewarming phase, and livers subjected to reperfusion in an experiment designed to mimic ischemia/reperfusion injuries.
Oxidative stress is often combined with a Ca\(^{2+}\)-influx into the cells (Hare, 2003) that is readily observed in anoxia/reoxygenation injury (Snowdowne et al., 1985). This influx of calcium is partially due to the effects of oxidation on the regulation of the ryanodine receptor and the sarcoplasmic reticulum calcium ATPase (Stamler and Meissner, 2001). In the ryanodine receptor, out of a total 364 cysteine residues, 84 provide free sulphhydryl groups indicating a large potential for oxidation (Xu et al., 1998). Calpains may be activated as a consequence of this increased intracellular calcium and not by oxidation effects.

Rowe at al. (2004a, b) evaluated how oxidative conditions in meat initiated by irradiation early postmortem caused inhibition of \(\mu\)-calpain activity slowing the rate of proteolysis occurring during aging, thereby decreasing tenderization. Carbonyl content of sarcoplasmic proteins and myofibrillar proteins was measured (Rowe et al., 2004a) up to 14 days post irradiation in beef steaks that were irradiated and compared against steaks that were not irradiated. Irradiated steaks had significantly higher carbonyl content in both sarcoplasmic and myofibrillar proteins up to day 7 post irradiation. Western blots for oxidized sarcoplasmic and myofibrillar proteins from irradiated and non-irradiated steaks indicated that carbonyl content was increased in the irradiated steaks as indicated by the detection of more bands. A comparison was made based on the animal diet containing vitamin E and a noticeable decrease in oxidized sarcoplasmic proteins was observed in
irradiated steaks from vitamin E supplemented animals. Vitamin E did not appear to affect oxidation of proteins in non-irradiated steaks. A positive correlation existed between the carbonyl measurements and the Warner-Bratzler shear force measurements, indicating higher carbonyl content was associated with a higher shear force. Calpastatin activity was higher in the irradiated steaks than in the non-irradiated steaks, which was attributed to a decrease in $\mu$-calpain activity resulting in less $\mu$-calpain mediated degradation of calpastatin. In addition to vitamin E supplementation decreasing protein oxidation of sarcoplasmic proteins, Rowe et al., (2004b) demonstrated that $\mu$-calpain activity was recoverable once reducing conditions were introduced, thereby providing a basis for antioxidant use in meats. Antioxidants are currently becoming more prevalently used in the meat industry to increase shelf life by preventing oxidation of lipids that cause rancidity. Harris et al. (2001) evaluated effects of antioxidants on tenderization and determined that increased levels of alpha-tocopherol in beef through supplemental vitamin E in the diet in combination with injecting steaks with CaCl$_2$ exhibited a faster rate of decline of Warner-Bratzler Shear Force values and a faster rate of troponin-T degradation. Additionally, Rowe at al. (2004b) observed an increase in the rate of troponin-T degradation in beef from calves fed a diet supplemented with vitamin E.
Summary

This literature review addressed the topics of calpains, calpastatin, meat quality, and oxidation. As the literature was collected, it is apparent that calpain activation is affected by many factors. Intracellular environment clearly plays a role in the extent of calpain activation, these factors of environment include pH and ionic strength. Clearly oxidation of calpains is an area that needs greater understanding, particularly in conditions that also affect calpain activity. Additionally, although calpastatin activity has been determined to play a role in many calpain processes, the mechanism of calpastatin inhibition of calpains needs to be elucidated in order to get a complete understanding of the activity of calpains and their roles in cellular metabolism and postmortem proteolysis.

Literature Cited


EFFECT OF pH AND IONIC STRENGTH ON µ- AND m-CALPAIN INHIBITION

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Abstract

The objectives of this study were to determine the extent to which pH and ionic
strength influence µ- and m-calpain activity and the inhibition of calpains by calpastatin.

Calpastatin, µ-calpain, and m-calpain were purified from at-death porcine semimembranosus.

µ-Calpain or m-calpain (0.45 U) were incubated with the calpain substrate Suc-Leu-Leu-Val-
Tyr-7-amino-4-methyl coumarin in the presence of calpastatin (0, 0.15, or 0.30 U of calpain
inhibitory activity) under the following pH and ionic strength conditions: pH 7.5 and 165
mM NaCl or 295 mM NaCl; pH 6.5 and 165 mM NaCl or 295 mM NaCl; and pH 6.0 and 165

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mM NaCl or 295 mM NaCl. The reactions were initiated with addition of 100 μM (μ-calpain) or 1 mM CaCl2 (m-calpain), and calpain activity was recorded at 30 and 60 min. μ-Calpain had the greatest (P < 0.01) activity at pH 6.5 at each ionic strength. Higher ionic strength decreased μ-calpain activity (P < 0.01) at all pH conditions. Inhibition percent of μ-calpain by calpastatin was not affected by pH; however, it was influenced by ionic strength. Inhibition of μ-calpain by calpastatin was higher (P < 0.01) at 295 mM NaCl than at 165 mM NaCl when 0.3 units of calpastatin were included in the assay. Activity of m-calpain was greater (P < 0.01) at pH 7.5 than at pH 6.5. m-Calpain activity was not detected at pH 6.0. Inhibition of m-calpain was greater (P < 0.01) when 0.15 and 0.3 U calpastatin were added at pH 6.5 than 7.5 at 165 mM NaCl, whereas percentage inhibition of m-calpain was greater (P < 0.01) at 295 mM than 165 mM NaCl at pH 7.5 and 6.5. These observations provide new evidence that defines further the influence of pH decline and increased ionic strength on μ-calpain, m-calpain, and calpastatin activity, thereby helping to more accurately define a role for these enzymes in the process of postmortem tenderization.

Key words: Calpain, Calpastatin, Ionic strength, Proteolysis, pH

Introduction

Changes in muscle intracellular environment occurring early postmortem are known to influence meat quality. One of the most pronounced changes is the pH decline from near
neutral pH in living muscle to approximately 5.6 in meat. Another change that occurs is the increase in ionic strength from an approximate equivalent of 165 mM NaCl in living muscle to an approximate equivalent of 295 mM NaCl in meat (Winger and Pope, 1980-81).

The calcium activated proteinases, calpains, are responsible for much of the postmortem proteolysis of myofibrillar and cytoskeletal proteins. This proteolysis is responsible for the increase in tenderness observed in meat during postmortem storage (Goll et al., 1992; Koohmaraie, 1992a; Koohmaraie et al., 2002). Calpain activity is influenced by intracellular environmental factors, including calcium concentration, pH, ionic strength, and calpastatin (the endogenous inhibitor specific for calpains). Calpains have a pH optimum of 7.5 (Edmunds et al., 1991; Wang and Jiang, 1991). An increase in ionic strength has been shown to decrease μ-calpain activity by decreasing the stability of autolyzed μ-calpain (Geesink and Koohmaraie, 2000, Li et al., 2004). The effects of the combination of pH and ionic strength on calpain activity in the presence of calpastatin have not been elucidated. Therefore, the hypothesis of this study was that postmortem pH and ionic strength affects the activity of μ- and m-calpain and the inhibition of calpains by calpastatin. For this reason, the first objective of this study was to determine the extent to which pH and ionic strength influence μ- and m-calpain activity. The second objective was to determine the extent to
which pH and ionic strength influence the ability of calpastatin to inhibit the activity of μ- or m-calpain.

**Materials and Methods**

*Purification of calpastatin, μ-calpain, and m-calpain.*

Calpastatin, μ-calpain, and m-calpain were purified from porcine skeletal muscle based on the procedures outlined by Thompson and Goll (2000) with minor modifications. A 2-kg porcine semimembranosus sample was taken from a market barrow approximately 25 min after exsanguination. The muscle was immediately ground and homogenized in six volumes of ice-cold buffer containing 10 mM EDTA, 0.1% (vol/vol) β-mercaptoethanol (MCE), and 100 mM Tris-HCl, pH 8.3. This buffer also included protease inhibitors (2.5 μM trans-Epoxysuccunyl-L-leucylamido- [4-guanidino] butane (E-64), 0.1 mg/mL ovomucoid trypsin inhibitor, and 0.2 mM phenylmethylsulfonylfluoride [PMSF]). The homogenate was centrifuged at 9750 x g at 4°C for 30 min. The supernatant was filtered through cheesecloth and proteins were salted out between 0 and 45% ammonium sulfate saturation. Proteins were collected at 9750 x g at 4°C for 30 min, resuspended in 1 mM EDTA, 0.1% MCE, 40 mM Tris-HCl, pH 7.4 (TEM), stirred overnight at 4°C, and dialyzed against TEM. The sample was loaded on a Q-Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ) anion-exchange column (900 mL) that had been previously equilibrated in TEM. Using a gradient
of 0 to 500 mM KCl in TEM (total volume 4,500 mL), porcine calpastatin, μ-calpain, and m-
calpain were eluted in three separate peaks from the column (calpastatin eluted between 90
and 150 mM KCl, μ-calpain between 160 and 190 mM KCl, and m-calpain between 250 and
280 mM KCl).

**Purification of calpastatin.**

Fractions containing calpastatin activity were pooled and further purified using
methods described by Thompson and Goll (2000) with modifications from Geesink and
Koohmaraie (1998). The sample was heated at 100°C for 20 min, chilled on ice, and
centrifuged at 9750 x g at 4°C for 30 min. Calpastatin was further purified using successive
chromatography over Phenyl Sepharose 6 Fast Flow (Amersham Biosciences, Piscataway,
NJ), Blue Sepharose 6 Fast Flow (Amersham Biosciences, Piscataway, NJ), and EMD
TMAE 650 S (EM Science, Gibbstown, NJ). Purified calpastatin consisted only of a 68 kDa
band when analyzed by SDS-PAGE and had a specific activity of 365 U/mg protein. One
unit of calpastatin activity was defined as the ability to inhibit one unit of m-calpain
caseinolytic activity (Koohmaraie et al., 1995).

**Purification of μ- and m-calpain.**

μ-Calpain and m-calpain were purified according to the methods of Thompson and
Goll (2000) with minor modifications. Fractions from the Q-sepharose column containing μ-
calpain activity were pooled. Pooled μ-calpain was purified using successive chromatography over a Phenyl Sepharose 6 Fast Flow (Amersham Biosciences, Piscataway, NJ), Butyl Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ), EMD TMAE 650 S (EM Science, Gibbstown, NJ), and DEAE-TSK Toyopearl (Supelco, Bellefonte, PA). Purified μ-calpain had a specific activity of 75 U/mg protein. One unit of calpain was defined as the amount of calpain required to increase the absorbance at 278 nm of the supernatant by one unit due to the release of trichloroacetic acid-soluble polypeptides resulting from the digestion of casein (Koohmaraie, 1990). Fractions from the Q-sepharose column containing m-calpain activity were pooled and purified using successive chromatography over a Phenyl Sepharose 6 Fast Flow (Amersham Biosciences, Piscataway, NJ), Reactive Red 120 (Sigma, St. Louis, MO), and DEAE-TSK Toyopearl (Supelco, Bellefonte, PA). Purified m-calpain had a specific activity of 186.4 U/mg protein. The purified μ-calpain, m-calpain, and calpastatin were stored in TEM with the addition of 1 mM sodium azide at 4°C.

**Calpain Activity Assays**

The technique used in this experiment is a sensitive activity assay using a calpain substrate, Suc-Leu-Leu-Val-Tyr-7-amino-4-methyl coumarin (Bachem; Torrence, CA; 10 mg/mL in dimethyl sulfoxide) (Sasaki et al., 1984). Although the use of this specific peptide
as a substrate for \( \mu \)-calpain and \( m \)-calpain allows for highly controlled experiments, it does not represent the diversity of all the potential calpain substrates. It should be noted that the substrate concentration used is quite close to the \( K_m \) for \( \mu \)-calpain (Saski et al., 1984). Therefore, the reaction was occurring under first order conditions, rather than zero order conditions that occur with protein substrates. The \( K_m \) of protein substrates for calpains tends to be in the low \( \mu M \) ranges (Goll et al., 2003). Highly purified calpains (\( \mu \)- or \( m \)-calpain; 0.45 units caseinolytic activity) were incubated with 170 \( \mu M \) Suc-Leu-Leu-Val-Tyr-AMC in either the presence of highly purified calpastatin (0.15, or 0.30 units measured against 0.45 units of \( m \)-calpain caseinolytic activity) or 0 units of calpastatin under the following conditions: 1) pH 7.5, 165 mM NaCl; 2) pH 7.5, 295 mM NaCl; 3) pH 6.5, 165 mM NaCl; 4) pH 6.5, 295 mM NaCl; 5) pH 6.0, 165 mM NaCl; or 6) pH 6.0, 295 mM NaCl. The buffers were either 50 mM HEPES (pH 7.5 and 6.5) or 50 mM 2-(4-morpholino)-ethane sulfonic acid (MES) (pH 6.0). Dithiothreitol (DTT; 1 mM final concentration) was added before the addition of calcium to ensure fully reduced conditions. Reactions were initiated with the addition of 100 \( \mu M \) CaCl\(_2\) (\( \mu \)-calpain) or 1 mM CaCl\(_2\) (\( m \)-calpain). Three replications of each pH, ionic strength, and calpastatin combination were conducted. Calpain activity and percent inhibition by calpastatin (percent inhibition = 1-(calpain activity without calpastatin \( \div \) calpain activity with calpastatin) \( \times \) 100) were measured at regular intervals from 0
(immediately prior to the addition of CaCl₂) to 60 min in a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Standard curves were generated for each experiment using 7-amino-4-methyl coumarin (AMC) of known concentrations. A control with calcium (without the addition of calpain) for each pH and ionic strength condition was conducted to determine if calcium affected the fluorescence of the peptide. An EDTA control (20 mM EDTA final concentration added prior to addition of calpain and calcium) was included for each pH and ionic strength condition and used for the baseline.

In order to ensure that pH and ionic strength did not directly influence the peptide, assays were also conducted with the enzyme carboxypeptidase Y (Calbiochem, La Jolla, CA). Carboxypeptidase Y has a pH optimum of pH 5.5 to 6.5, stability up to pH 8.0, and Suc-Leu-Leu-Val-Tyr-7-amino-4-methyl coumarin is a substrate for this enzyme (Stennicke et al., 1994). In these assays, carboxypeptidase Y was added in place of μ- or m-calpain at 0.5 μL (0.0045 μM final concentration). Comparison of activity measured in the assays at pH 7.5, 6.5, and 6.0, as well as ionic strength of 165 mM NaCl and 295 mM NaCl, was done to determine if these environmental conditions changed the susceptibility of the peptide to proteolysis.
SDS-PAGE Gel System and Western Blotting

Samples used for western blotting of μ-calpain autolysis were prepared by removing 20 μL from the fluorescence assay (pH 7.5, 6.5, and 6.0; 165 mM NaCl; 0 units calpastatin) at 5-min increments for 25 min. This experiment included two replications. Each aliquot was added to 10 μL of sample buffer tracking dye solution (3 mM EDTA, 3% SDS, 20% glycerol, 0.003% pyronin Y, and 30 mM Tris-HCl, pH 8.0 (Wang, 1982) and 0.1% β-mercaptoethanol. Gel samples were heated at 50°C for 20 min, and subsequently frozen to −80°C until analysis. Samples were fractionated using 9% polyacrylamide separating gels and transferred to a membrane (Melody et al., 2004). A sensitive chemiluminescent system (ECL Plus kit; Amersham Biosciences, Piscataway, NJ) was used to detect labeled protein bands using a charged coupled device (CCD) camera (FluroChem 8800; Alpha Innotech Corporation, San Leandro, CA) and FluorChem IS-800 software (Alpha Innotech Corporation, San Leandro, CA).

Statistical Analysis

Data were analyzed using a split-split plot design. The whole plot was pH (7.5, 6.5, or 6.0). The split plot was ionic strength (165 mM NaCl or 295 mM NaCl). The split-split plot was calpastatin (0, 0.15, or 0.30 units). The experimental unit was each cuvette placed in the fluorometer. Calpain activity at 30 and 60 min was analyzed using PROC MIXED of
SAS (SAS Institute, Inc., Cary, NC). Least squares means were separated using tests of effect slices and significance was defined as $P < 0.05$.

**Results and Discussion**

*Carboxypeptidase Y activity control*

Ionic strength and pH conditions used in these experiments did not alter susceptibility of the calpain substrate to proteolysis. Fluorescence at pH 7.5 and 165 mM NaCl was defined as 100% of carboxypeptidase Y activity. Fluorescence at pH 6.5 and 165 mM NaCl was 97.2% of activity, pH 6.0 and 165 mM NaCl was 99.2%, pH 7.5 and 295 mM NaCl was 100.3%, pH 6.5 and 295 mM NaCl was 97.3%, and pH 6.0 and 295 mM NaCl was 99.7% after 60 min. It was concluded that the calpain substrate was not affected ($P > 0.50$) by the differing pH and ionic strength conditions and was appropriate to use for our study.

*μ-calpain activity*

At both 165 mM NaCl and 295 mM NaCl, μ-calpain activity was greater ($P < 0.05$) at pH 6.5 than 7.5 or 6.0 (Table 1). The slope of the rate of hydrolysis of the peptide by μ-calpain during the first five minutes indicates that, at pH 6.5, μ-calpain was more active than at pH 7.5. This is consistent with Geesink and Koohmaraie (2000), who found that autolyzed porcine skeletal μ-calpain activity was more stable (indicated by greater activity) at pH values of 6.2 to 6.4 when evaluating pH ranges from 7.6 to 5.6. In order to understand the
mechanism underlying the greater activity of μ-calpain at pH 6.5, μ-calpain autolysis was examined using western blots. Appearance of the 78 and 76 kDa immunoreactive bands (Figure 1) indicated that autolysis occurred in all pH conditions; yet the rate of autolysis within the first 5 min was slowest at pH 6.5. μ-Calpain was almost completely autolyzed by 20 min as shown by the less intense 80 kDa band at pH 7.5 than in samples incubated at pH 6.5. In contrast, the intact 80 kDa band of μ-calpain in the pH 6.5 incubation was still relatively more intense at 25 min, indicating less autolysis had occurred during the entire 25-min incubation. The more limited autolysis at pH 6.5 most likely contributed to the greater μ-calpain activity remaining at 30 and 60 min compared to the other pH incubations. This is important because autolysis of μ-calpain has been shown to lead to a loss of activity (Koohmaraie, 1992b; Edmunds et al., 1991). Geesink and Koohmaraie (2000) determined that μ-calpain had approximately 70% of initial activity after incubation for 30 min at pH 6.2 and approximately 22% of initial activity remaining at pH 7.5. In the current study, μ-calpain autolyzed more rapidly at pH 7.5, which resulted in less active μ-calpain present in the assay to degrade the peptide at the later time points.

Autolysis observed at pH 6.0 shows greater intensity of the 78 kDa autolysis product. Very little 78 kDa autolysis product was detected at pH 7.5 or 6.5 at all time points. This contrast indicates that pH affects the accumulation of the 78 kDa autolysis product.
Koohmaraie et al. (1986) observed that, in conditions of pH 5.5 to 5.8 at 5°C, μ-calpain had 24-28% of its activity after 90 min at pH 7.5 at 25°C, indicating less proteolytic activity at a lower pH. A rapid pH decline in postmortem muscle also reduces μ-calpain activity (Claeys et al., 2001), and has been shown to decrease the degradation of troponin-T (Lonergan et al., 2001). Melody et al. (2004) observed that porcine muscles with low early postmortem pH (psoas major) had earlier μ-calpain autolysis and inactivation and earlier degradation of desmin than muscles with higher early postmortem pH (LM and semimembranosus).

Additionally, moderate rates of postmortem pH decline (pH of 5.8 to 6.2 at 3 h) have been shown to produce the most tender beef loin steaks, whereas rapid rates (pH of 5.5 at 3 h) and slow rates (pH 6.8 at 3 h) of postmortem glycolysis produced less tender meat (Marsh et al., 1987). The novel observations in the current study indicated greater μ-calpain activity at the intermediate pH (6.5) than pH 7.5 and 6.0 could help to explain the tenderization effects reported by Marsh et al. (1987). If the pH decline is rapid, μ-calpain activity is diminished due to the lower pH. If postmortem glycolysis is slow and the pH does not decline as rapidly, μ-calpain may autolyze earlier postmortem, thereby losing proteolytic activity earlier and not allowing for maximal proteolysis. Thus, intermediate pH decline allows more proteolysis and slower completion of autolysis, therefore ultimately allowing for greater postmortem protein degradation and increased tenderization.
µ-Calpain was more active ($P < 0.01$) at 165 mM NaCl than at 295 mM NaCl under all experimental conditions. Thus, it is expected that µ-calpain activity will be reduced as intracellular ionic strength increases in postmortem muscle. Geesink and Koohmaraie (2000) also demonstrated that increased ionic strength decreased activity and stability at concentrations of NaCl as low as 100 mM. Likewise, Li et al. (2004) observed irreversible loss of autolyzed µ-calpain activity at salt concentrations as low as 100 mM KCl at pH 7.5. They determined that ionic strengths of 300 mM or greater decreased autolyzed µ-calpain activity by 50 to 55% within 5 min. The rate of activity loss was slower at lower ionic strengths (Li et al., 2004).

*m-calpain activity*

In contrast to µ-calpain, m-calpain activity was greater ($P < 0.05$) at pH 7.5 than pH 6.5 (Table 2). Dayton et al. (1976) and Edmunds et al. (1991) determined the optimum pH of porcine muscle m-calpain to be 7.5. In the current study, no measurable activity of m-calpain was observed at pH 6.0 at either ionic strength. m-Calpain was more active ($P < 0.01$) at 165 mM NaCl than 295 mM NaCl (Table 2). This ionic strength effect was greater than the differences observed for µ-calpain. At pH 7.5, the increase in ionic strength decreased µ- and m-calpain activity by 43.1% and 83.7% respectively. Li et al. (2004) observed similar effects of ionic strength on autolyzed m-calpain, and they hypothesized an ionic strength
equal to 100 mM KCl or greater caused disassociation of the two m-calpain subunits, allowing for inactivation of the proteinase due to the formation of dimers and trimers of the large subunit.

The activities of μ- and m-calpain observed with regard to pH may be evidence for a difference in the roles of μ- and m-calpain in postmortem muscle. It has been hypothesized that μ-calpain is the protease primarily responsible for the postmortem degradation of myofibrillar and cytoskeletal proteins resulting in tenderization of meat, whereas the role of m-calpain may be limited based on its high calcium requirement. The results from this study also indicate a more important role for μ-calpain in postmortem muscle, as μ-calpain activity was measured at pH 6.0, whereas no m-calpain activity was detected. Additionally, although the higher ionic strength did decrease μ-calpain activity, m-calpain activity was diminished to a greater extent by the increase in ionic strength.

μ-calpain inhibition by calpastatin

In porcine muscle, the ratio of calpastatin activity to μ-calpain activity is approximately 1.5:1 (Ouali and Talmant, 1990). In the current study, the ratio of calpastatin to calpain added to the assays was 1:3 and 2:3 in order to evaluate the effect of calpastatin on calpain without the occurrence of complete inhibition. Calpastatin inhibited \( P < 0.001 \) μ-calpain activity (Table 3), but percent inhibition was not altered by pH. This is consistent
with results observed by Geesink and Koohmarie (1999). Percent inhibition of μ-calpain by calpastatin was greater at 295 than at 165 mM NaCl at pH 7.5, 6.5, and 6.0 when the higher level of calpastatin (0.3 units) was added to the assay. An increase in ionic strength in postmortem muscle was predicted to increase the inhibitory efficiency of calpastatin, however there were no significant differences in percent inhibition between 165 and 295 mM NaCl when 0.15 units of calpastatin were added to the assay. In general, the percent inhibition is numerically greater at 295 than 165 mM NaCl when the lower level of calpastatin was present. The lack of significance could be due to the higher variance of percent inhibition between replications in the assays. The observation that calpastatin is a substrate for calpain (Mellgren et al., 1986; Doumit and Koohmarie, 1999) may contribute to this variation.

*m-calpain inhibition by calpastatin*

Inhibition of m-calpain by calpastatin was greater (P < 0.01) at pH 6.5 than 7.5 at both 165 and 295 mM NaCl (Table 4). Otsuka and Goll (1987) determined that calpastatin had a broad optimum pH, and concluded that inhibition of m-calpain by calpastatin was not affected by pH when casein was a substrate. Differences in results between these two studies could be due to differences in substrates used. As mentioned previously, the proteolytic susceptibility of the peptide used as a substrate in this study was not affected by pH or ionic
strength, whereas pH may affect the solubility of casein used to determine calpain activity because the pI of casein is 4.6. In addition, the lowest ionic strength condition used in the current study was 165 mM NaCl. In the Otsuka and Goll (1987) study, the ionic strength conditions used were much lower. In the current study, inhibition of m-calpain was greater at 295 than at 165 mM NaCl at both 30 and 60 min and with the addition of both 0.15 and 0.3 units of calpastatin. The high percent inhibition of m-calpain by calpastatin at the lower pH and higher ionic strength provides further evidence against a role for m-calpain in postmortem muscle. The observations made for μ-calpain inhibition were different, in that μ-calpain inhibition was not as great as m-calpain inhibition at 295 mM NaCl and μ-calpain was not affected by pH, indicating that μ-calpain could be more active at conditions found in postmortem muscle.

In early postmortem muscle, μ-calpain inactivation, in response to either a rapid pH decline or by rapid autolysis, has the potential to decrease proteolysis of myofibrillar proteins and subsequent postmortem tenderization. Yet, intermediate pH decline allows for proteolytic activity of μ-calpain, but a slower rate of autolysis could explain a portion of the variation in meat tenderness. A major role for m-calpain in postmortem proteolysis is not supported because the high ionic strength and low pH conditions, similar to conditions observed in postmortem muscle, appear to limit m-calpain activity. Additionally, the inhibition of μ-
calpain and m-calpain by calpastatin was greater at the higher ionic strength, indicating that calpastatin inhibits calpains to a greater extent in conditions similar to meat than in conditions found in living muscle.

**Implications**

Rate of pH decline in postmortem muscle has the potential to affect the rates of activation and subsequent autolytic inactivation of μ-calpain. In addition, an increase in ionic strength in postmortem muscle may allow for more efficient inhibition of calpains by calpastatin. Therefore, rates of pH decline and increases in ionic strength are important variables to be considered when examining the variations in calpain-induced proteolysis of meat proteins in postmortem muscle. Studies centered on the question of how intracellular environmental factors affect μ- and m-calpain will lead to a greater understanding of the role of calpain and calpastatin in the conversion of muscle to meat.

**Literature Cited**


Table 1. Least squares means (± SE) of μ-calpain activity at 30 and 60 min after addition of CaCl$_2$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity$^a$</th>
<th>165 mM NaCl</th>
<th>295 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30 min</td>
<td>60 min</td>
</tr>
<tr>
<td>pH 7.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 units calpastatin</td>
<td>94.21 ± 2.50$^{ex}$</td>
<td>152.53 ± 1.54$^{cw}$</td>
<td>55.55 ± 2.51$^{dz}$</td>
</tr>
<tr>
<td>0.15 units calpastatin</td>
<td>62.67 ± 1.05$^{dx}$</td>
<td>113.79 ± 4.61$^{dw}$</td>
<td>38.05 ± 1.96$^{ez}$</td>
</tr>
<tr>
<td>0.30 units calpastatin</td>
<td>44.99 ± 1.84$^{ez}$</td>
<td>79.83 ± 1.65$^{ew}$</td>
<td>21.00 ± 0.63$^{fz}$</td>
</tr>
<tr>
<td>pH 6.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 units calpastatin</td>
<td>131.65 ± 6.79$^{by}$</td>
<td>208.18 ± 8.87$^{bw}$</td>
<td>100.62 ± 3.79$^{bz}$</td>
</tr>
<tr>
<td>0.15 units calpastatin</td>
<td>87.01 ± 5.69$^{cy}$</td>
<td>146.95 ± 7.51$^{cw}$</td>
<td>69.19 ± 6.03$^{cz}$</td>
</tr>
<tr>
<td>0.30 units calpastatin</td>
<td>60.44 ± 1.87$^{dy}$</td>
<td>112.38 ± 4.16$^{dw}$</td>
<td>33.29 ± 2.33$^{dz}$</td>
</tr>
<tr>
<td>pH 6.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 units calpastatin</td>
<td>46.47 ± 3.20$^{ex}$</td>
<td>68.12 ± 4.48$^{fw}$</td>
<td>18.35 ± 1.63$^{fz}$</td>
</tr>
<tr>
<td>0.15 units calpastatin</td>
<td>29.91 ± 2.50$^{fx}$</td>
<td>49.39 ± 2.79$^{gw}$</td>
<td>12.28 ± 0.92$^{gz}$</td>
</tr>
<tr>
<td>0.30 units calpastatin</td>
<td>24.08 ± 0.39$^{gx}$</td>
<td>38.68 ± 0.61$^{hw}$</td>
<td>6.91 ± 1.34$^{hz}$</td>
</tr>
</tbody>
</table>

$^a$Activity = fluorescence units with CaCl$_2$ - fluorescence units with EDTA (n = 3).

$^{b,c,d,e,f,g,h,i,j}$ Within a column, least squares means lacking a common superscript differ (P < 0.01).

$^{w,x,y,z}$ Within a row, least squares means lacking a common superscript differ (P < 0.05).
Table 2. Least squares means (± SE) of m-calpain activity taken at 30 and 60 min after addition of CaCl₂

<table>
<thead>
<tr>
<th>Treatment</th>
<th>165 mM NaCl</th>
<th>295 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>60 min</td>
</tr>
<tr>
<td>pH 7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 units calpastatin</td>
<td>98.12 ± 2.98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>120.74 ± 6.85&lt;sup&gt;bw&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.15 units calpastatin</td>
<td>72.92 ± 2.64&lt;sup&gt;c&lt;/sup&gt;y</td>
<td>88.71 ± 3.19&lt;sup&gt;c&lt;/sup&gt;x</td>
</tr>
<tr>
<td>0.30 units calpastatin</td>
<td>50.11 ± 1.01&lt;sup&gt;d&lt;/sup&gt;y</td>
<td>61.32 ± 1.74&lt;sup&gt;d&lt;/sup&gt;x</td>
</tr>
<tr>
<td>pH 6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 units calpastatin</td>
<td>36.37 ± 0.52&lt;sup&gt;e&lt;/sup&gt;x</td>
<td>58.45 ± 2.15&lt;sup&gt;d&lt;/sup&gt;w</td>
</tr>
<tr>
<td>0.15 units calpastatin</td>
<td>22.92 ± 1.39&lt;sup&gt;f&lt;/sup&gt;y</td>
<td>37.59 ± 2.81&lt;sup&gt;e&lt;/sup&gt;x</td>
</tr>
<tr>
<td>0.30 units calpastatin</td>
<td>10.56 ± 1.96&lt;sup&gt;g&lt;/sup&gt;y</td>
<td>20.89 ± 1.66&lt;sup&gt;f&lt;/sup&gt;x</td>
</tr>
</tbody>
</table>

<sup>a</sup>Activity= fluorescence units with CaCl₂- fluorescence units with EDTA (n=3).
<sup>b,c,d,e,f,g</sup> Within a column, least squares means lacking a common superscript differ (P < 0.01).
<sup>w,x,y,z</sup> Within a row, least squares means lacking a common superscript differ (P < 0.05).
Table 3. Least squares means (± SE) of percent inhibition of μ-calpain by 0.15 and 0.3 units calpastatin\textsuperscript{a} calculated from μ-calpain activity measured at 30 and 60 min after the addition of CaCl\textsubscript{2}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>165 mM NaCl</th>
<th>295 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent inhibition</td>
<td></td>
</tr>
<tr>
<td>pH 7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 units calpastatin</td>
<td>33.48 ± 1.12\textsuperscript{cy}</td>
<td>25.39 ± 1.59\textsuperscript{cz}</td>
</tr>
<tr>
<td>0.30 units calpastatin</td>
<td>52.25 ± 1.96\textsuperscript{by}</td>
<td>47.66 ± 2.96\textsuperscript{bz}</td>
</tr>
<tr>
<td>pH 6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 units calpastatin</td>
<td>33.90 ± 4.32\textsuperscript{cy}</td>
<td>29.41 ± 3.61\textsuperscript{cy}</td>
</tr>
<tr>
<td>0.30 units calpastatin</td>
<td>54.09 ± 1.42\textsuperscript{bx}</td>
<td>46.02 ± 2.00\textsuperscript{by}</td>
</tr>
<tr>
<td>pH 6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 units calpastatin</td>
<td>35.63 ± 5.37\textsuperscript{cx}</td>
<td>27.49 ± 4.10\textsuperscript{cy}</td>
</tr>
<tr>
<td>0.30 units calpastatin</td>
<td>48.19 ± 1.41\textsuperscript{bz}</td>
<td>43.21 ± 0.89\textsuperscript{by}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Inhibition was calculated as the percent inhibition = 1-(calpain activity without calpastatin / calpain activity with calpastatin; \textit{n}=3).
\textsuperscript{b,c} Means in a column without a common superscript differ (\textit{P} < 0.01).
\textsuperscript{x,y,z} Within a row, means without a common superscript differ (\textit{P} < 0.05)
Table 4. Least squares means (± SE) of percent inhibition of m-calpain by 0.15 and 0.3 units calpastatin\textsuperscript{a} calculated from m-calpain activity at 30 and 60 min after the addition of CaCl\textsubscript{2}.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH 7.5</th>
<th>pH 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>165 mM NaCl</td>
<td>295 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>30 min</td>
<td>60 min</td>
</tr>
<tr>
<td>0.15 units calpastatin</td>
<td>25.68 ± 2.69\textsuperscript{c}\textsuperscript{z}</td>
<td>26.53 ± 2.63\textsuperscript{c}\textsuperscript{z}</td>
</tr>
<tr>
<td>0.30 units calpastatin</td>
<td>48.92 ± 1.78\textsuperscript{e}\textsuperscript{z}</td>
<td>49.21 ± 1.44\textsuperscript{e}\textsuperscript{z}</td>
</tr>
<tr>
<td>0.15 units calpastatin</td>
<td>36.98 ± 3.83\textsuperscript{c}\textsuperscript{z}</td>
<td>35.70 ± 4.81\textsuperscript{d}\textsuperscript{z}</td>
</tr>
<tr>
<td>0.30 units calpastatin</td>
<td>70.92 ± 5.36\textsuperscript{b}\textsuperscript{y}</td>
<td>64.26 ± 2.83\textsuperscript{b}\textsuperscript{z}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Inhibition was calculated as the percent inhibition = 1-(calpain activity without calpastatin / calpain activity with calpastatin; n=3).
\textsuperscript{b,c,d,e} Within a column, least squares means lacking a common superscript differ (P < 0.01).
\textsuperscript{x,y,z} Within a row, least squares means lacking a common superscript differ (P < 0.05).
<table>
<thead>
<tr>
<th>pH</th>
<th>Incubation time (min)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>0 15.5±0.07&lt;sup&gt;b&lt;/sup&gt; 32.6±1.20&lt;sup&gt;b&lt;/sup&gt; 49.1±1.75&lt;sup&gt;b&lt;/sup&gt; 65.3±2.13&lt;sup&gt;b&lt;/sup&gt; 80.6±2.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>0 19.1±1.44&lt;sup&gt;a&lt;/sup&gt; 43.9±3.60&lt;sup&gt;a&lt;/sup&gt; 70.1±5.16&lt;sup&gt;a&lt;/sup&gt; 92.8±6.19&lt;sup&gt;a&lt;/sup&gt; 113.1±6.60&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>0 10.4±0.78&lt;sup&gt;‡&lt;/sup&gt; 18.8±1.45&lt;sup&gt;‡&lt;/sup&gt; 27.1±2.02&lt;sup&gt;‡&lt;/sup&gt; 34.3±2.40&lt;sup&gt;‡&lt;/sup&gt; 40.8±2.78&lt;sup&gt;‡&lt;/sup&gt;</td>
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</tbody>
</table>
EFFECT OF OXIDATION, pH AND IONIC STRENGTH ON CALPASTATIN

INHIBITION OF μ- AND m-CALPAIN

A paper submitted to the Journal of Animal Science

K.R. Maddock, E. Huff-Lonergan, L.J. Rowe, and S.M. Lonergan*

Abstract

The objective of this study was to evaluate the effect of oxidation on μ- and m-calpain activity at varying pH and ionic strength conditions in the presence of calpastatin. In two separate experiments, purified porcine skeletal muscle μ- or m-calpain (0.45 units of caseinolytic activity) was incubated in the presence of calpastatin (0, 0.15, or 0.30 units) at pH 7.5, 6.5, or 6.0 with either 165 mM NaCl or 295 mM NaCl. The reactions were initiated with addition of CaCl₂ (100 μM for μ-calpain; 1 mM for m-calpain). In experiment 1, μ- or m-calpain were incubated with the calpain substrate Suc-Leu-Leu-Val-Tyr-AMC (170 μM). Either 0 or 0.16 μM H₂O₂ was added to each assay. Activity was measured at 60 min. In experiment 2, calpain was incubated with highly purified porcine myofibrils (4 mg/ml) under conditions described. Either 0 or 100 μM H₂O₂ was added immediately prior to the addition of calpain. Degradation of desmin was determined on samples collected at 2, 15, 60, 120
Calpain activity measured in experiment 1 indicated that oxidation decreased ($P < 0.01$) activity of $\mu$-calpain. $\mu$-Calpain had the greatest ($P < 0.01$) activity at pH 6.5 and $m$-calpain had the greatest ($P < 0.01$) activity at pH 7.5 at 60 min. No activity of $m$-calpain was detected at pH 6.0. $\mu$- and $m$-Calpain activity were lower ($P < 0.01$) at 295 mM NaCl than at 165 mM NaCl at all pH conditions. Oxidation lowered ($P < 0.01$) calpastatin inhibition of $\mu$- and $m$-calpain at all pH and ionic strength combinations. In experiment 2, oxidation decreased proteolytic activity of $\mu$-calpain against desmin at pH 6.0 ($P < 0.05$ at 15, 60, and 120 min) and $m$-calpain at all pH conditions. However, inhibition of $\mu$-calpain degradation of desmin by calpastatin was decreased by oxidation at pH 7.5 and 6.5 at both ionic strengths ($P = 0.03$ at 60 min). This is consistent with the results from the activity assays that indicated oxidation decreased percent inhibition of $\mu$-calpain by calpastatin. In conclusion, these studies provide evidence that oxidation influences calpain activity and inhibition of calpains by calpastatin differently under varying environmental conditions. The results suggest that, at the higher pH conditions used, calpastatin may limit the possibility of oxidation-induced inactivation of $\mu$-calpain.

Keywords: Calpain, calpastatin, ionic strength, oxidation, proteolysis, pH
Introduction

The significant changes in muscle cellular environment that occur early postmortem are known to affect meat quality (Huff-Lonergan et al., 1996). One of the most pronounced changes is the pH decline from near neutrality in living muscle to approximately 5.6 in meat. Another change that occurs is the increase in ionic strength from an approximate equivalent of 165 mM NaCl in living muscle to approximately 295 mM NaCl in meat (Winger and Pope, 1980-81). μ-Calpain is a main factor in postmortem proteolysis of cytoskeletal proteins and subsequent tenderization of meat (Goll et al., 1992; Koohmaraie, 1992). Another calpain, m-calpain is also present in muscle, and its action in postmortem proteolysis and meat tenderization is highly debated (As reviewed by Geesink et al., 2000). Calpains are regulated by several factors including calcium concentration, pH, and the endogenous inhibitor, calpastatin. An increase in ionic strength has been shown to decrease μ-calpain activity by decreasing the stability of autolyzed μ-calpain (Li et al., 2004; Geesink and Koohmaraie, 2000). Another change that occurs during postmortem aging of meat is an increase in protein oxidation (Rowe et al., 2004a; Martinaud et al., 1997). Oxidation has been shown to decrease the ability of μ-calpain to degrade its substrates (Rowe et al., 2004b; Guttmann and Johnson, 1998). Therefore the objective of this study was to determine the
extent to which oxidation influences calpain activity and calpain inhibition by calpastatin under pH and ionic strength conditions observed in early postmortem muscle.

**Materials and Methods**

*Purification of calpastatin, µ-calpain, and m-calpain.*

Calpastatin, µ-calpain, and m-calpain were purified from porcine skeletal muscle based on the procedures outlined by Thompson and Goll (2000) with minor modifications as described by Maddock et al. (2005). Porcine semimembranosus sample (2 kg) was taken from a market barrow approximately 25 min after exsanguination, prepared as previously described, and loaded on a Q-Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ) anion-exchange column. Porcine calpastatin, µ-calpain, and m-calpain were eluted in three separate peaks off this column.

*Purification of calpastatin.* Fractions containing calpastatin activity were pooled and further purified using methods described by Thompson and Goll (2000) with modifications taken from procedures of Geesink and Koohmaraie (1998) as described by Maddock et al. (2005). Purification was completed using successive chromatography over Phenyl Sepharose 6 Fast Flow (Amersham Biosciences, Piscataway, NJ), Blue Sepharose 6 Fast Flow (Amersham Biosciences, Piscataway, NJ), and EMD TMAE 650 S (EM Science, Gibbstown, NJ).

Purified calpastatin consisted only of a 68 kDa band when analyzed by SDS-PAGE and had a
specific activity of 451 U/mg protein. One unit of activity of calpastatin was defined as the ability to inhibit one unit of m-calpain caseinolytic activity (Koohmaraiie et al., 1995).

Purification of \( \mu \)- and m-calpain. \( \mu \)- and m-Calpain were purified according to the methods of Thompson and Goll (2000) with minor modifications as described by Maddock et al. (2005). Fractions from the Q-sepharose column containing \( \mu \)-calpain activity were pooled and m-calpain activity were pooled. The \( \mu \)-calpain was purified using successive chromatography over a Phenyl Sepharose 6 Fast Flow (Amersham Biosciences, Piscataway, NJ), Butyl Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ), EMD TMAE 650 S (EM Science, Gibbstown, NJ), and DEAE-TSK Toyopearl (Supelco, Bellefonte, PA). The purified \( \mu \)-calpain had a specific activity of 71.3 U/mg protein. One unit of calpain was defined as the amount of calpain required to increase the absorbance at 278 nM of the supernatant by one unit in 1 h incubation at 25°C due to the release of trichloroacetic acid-soluble polypeptides resulting from the digestion of casein (Koohmaraiie, 1990).

Fractions containing m-calpain activity were pooled and purified using successive chromatography over a Phenyl Sepharose 6 Fast Flow (Amersham Biosciences, Piscataway, NJ), Reactive Red 120 (Sigma, St. Louis, MO), and DEAE-TSK Toyopearl (Supelco, Bellefonte, PA). Purified m-calpain had a specific activity of 147 U/mg protein. The
purified \( \mu \)-calpain, \( m \)-calpain, and calpastatin were stored in TEM with the addition of 1 mM sodium azide at 4\(^\circ\)C.

**Calpain Activity Assays**

In order to ensure that pH, ionic strength, and \( H_2O_2 \) did not directly influence the peptide, assays were also conducted with the enzyme carboxypeptidase Y (Calbiochem, La Jolla, CA). Carboxypeptidase Y has a pH optimum of pH 5.5-6.5, stability up to pH 8.0, and Suc-Leu-Leu-Val-Tyr-7-amino-4-methyl coumarin is a substrate for this enzyme (Stennicke et al., 1994). In these assays, carboxypeptidase Y was added in place of \( \mu \)- or \( m \)-calpain at 0.5 \( \mu l \) (0.0045 \( \mu M \) final concentration). Comparison of activity measured in the assays at pH 7.5, 6.5, and 6.0, ionic strength of 165 mM NaCl and 295 mM NaCl, and 0 \( \mu M \) or 0.16 \( \mu M \) \( H_2O_2 \) was done to determine if these environmental conditions changed the susceptibility of the peptide to proteolysis.

The technique used in this experiment is a sensitive activity assay using a calpain substrate, Suc-Leu-Leu-Val-Tyr-7-amino-4-methyl coumarin (Bachem; Torrence, CA; 10 mg/ml in dimethyl sulfoxide), which is a known substrate of \( \mu \)- and \( m \)-calpain (Sasaki et al., 1984). The highly purified calpains (\( \mu \)- or \( m \)-calpain; 0.45 units caseinolytic activity) were incubated with 170 \( \mu M \) Suc-Leu-Leu-Val-Tyr-AMC in either the presence of highly purified calpastatin (0.15, or 0.30 units measured against 0.45 units of \( m \)-calpain caseinolytic activity).
or 0 units of calpastatin under the following conditions: 1) pH 7.5, 165 mM NaCl 2) pH 7.5, 295 mM NaCl 3) pH 6.5, 165 mM NaCl 4) pH 6.5, 295 mM NaCl 5) pH 6.0, 165 mM NaCl 6) pH 6.0, 295 mM NaCl. The buffers were either 50 mM HEPES (pH 7.5 and 6.5) or 50 mM 2-(4-morpholino)-ethane sulfonic acid (MES) (pH 6.0). Oxidation treatments consisted of the addition 0.16 μM H₂O₂ immediately prior to addition of calpain. The reactions were initiated with the addition of 100 μM CaCl₂ (μ-calpain) or 1 mM CaCl₂ (m-calpain). Calpain activity and percent inhibition by calpastatin (percent inhibition = 1-(calpain activity without calpastatin + calpain activity with calpastatin) x 100) were measured at 60 min in a TD-700 fluorometer (Turner Designs, Sunnyvale, CA). A control with calcium (without the addition of μ- or m-calpain) for each pH and ionic strength condition was conducted to determine if calcium affected the fluorescence of the peptide. An EDTA control (20 mM EDTA final concentration added prior to addition of calpain and calcium) was included for each pH and ionic strength condition and used for the baseline.

Myofibril Isolation

Porcine semimembranosus (100 g; 45 min postmortem) was homogenized in ice cold extraction buffer (10 mM EDTA, 2 μM E-64, 100 mg/L trypsin inhibitor, and 2 mM phenylmethylsulfonylfluoride [PMSF], 100 mM Tris-HCl, pH 8.3) using a polytron PT 3100 (Kinmetica AG, Littau, Switzerland) set at 10,000 rpm. Samples were centrifuged (20,000 x
g) for 30 min at 4°C. Pellets were homogenized in 10 vol of standard salt solution (100 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM NaN₃, 20 mM K₂HPO₄, pH 7.0). Purification of myofibrils was completed by differential centrifugation (Goll et al., 1974), and protein concentration was determined using the Biuret method as modified by Robson et al. (1968).

Digest of myofibrils

The highly purified calpains (0.45 units caseinolytic activity/ml in the assay) were incubated with highly purified porcine myofibrils in suspension (4 mg/ml buffer) in the presence of calpastatin (0.15, or 0.3 units measured against 0.45 units of m-calpain caseinolytic activity/ml in the assay) or 0 units calpastatin under the following conditions: pH 7.5; 165 mM NaCl, 295 mM NaCl, pH 6.5; 165 mM NaCl, 295 mM NaCl, and pH 6.0; 165 mM NaCl, 295 mM NaCl. The buffers were either 50 mM HEPES (pH 7.5 and 6.5) or 50 mM MES (pH 6.0). Oxidation treatment consisted of the addition of 100 μM H₂O₂ (Saurin et al., 2004). The reactions were initiated with the addition of CaCl₂ at a final concentration of 100 μM (μ-calpain) or 1 mM (m-calpain). Aliquots were removed at 2, 15, 60, and 120 min after the addition of calpain. The digests were terminated by the addition 20 mM EDTA (final concentration). The aliquots were centrifuged at 6000 x g for 15 min at 4°C. A portion of the pellet was used for SDS-PAGE and immunoblotting to determine changes in desmin.
SDS-PAGE and Western Blotting

SDS-PAGE and membrane transfer were conducted as described by Rowe et al. (2004b) using 10% polyacrylamide separating gels and a desmin primary antibody (polyclonal rabbit anti-desmin antibody, V2022; Biomedia, Foster City, CA) for desmin. A sensitive chemiluminescent (ECL Plus kit; Amersham Biosciences, Piscataway, NJ) system was used to detect labeled protein bands using a charged coupled device (CCD) camera (FluroChem 8800; Alpha Innotech Corporation, San Leandro, CA) and FluorChem IS-800 software (Alpha Innotech Corporation, San Leandro, CA). Densitometry was completed using the AlphaEaseFC software (Alpha Innotech Corporation, San Leandro, CA). A reference sample was used on each blot in order to standardize densitometry data to compare differences between blots.

Oxidation of calpastatin

Calpastatin (10 units) was incubated with an irreversible sulphhydryl blocking agent N-ethylmaleimide (NEM; 0, 4, 8, or 12 mM final concentration; n=2) for 1 h at 4°C. Samples were exhaustively dialyzed in 1 mM EDTA, 40 mM Tris-HCl, pH 7.5 to remove NEM. Activity of calpastatin after dialysis was determined by measuring inhibition of m-calpain caseinolytic activity (Koohmarai et al., 1995). A protein assay (Bradford et al., 1976) was conducted on each sample to determine specific activity of each calpastatin sample.
Statistical Analysis

Data were analyzed using a split-split plot design. The whole plot was pH/Ionic strength (7.5-165, 7.5-295, 6.5-165, 6.5-295, 6.0-165, or 6.0-295). The split plot was calpastatin (0, 0.15, or 0.30 units). The split-split plot was H$_2$O$_2$ (0 or 0.16 µM/100 µM). Calpain activity at 60 min and densitometry of intact desmin was analyzed using PROC MIXED procedure in SAS (SAS Institute, Inc., Cary, NC). Least squares means were separated using Tukey's Honestly Significant Difference test. Significance was defined as $P < 0.05$.

Results

Carboxypeptidase Y activity control

Ionic strength, pH, or H$_2$O$_2$ conditions (Table 1) used in these experiments did not alter susceptibility of the peptide to proteolysis. Fluorescence measured at 60 min at pH 7.5, 165 mM NaCl and 0 µM H$_2$O$_2$ was defined as 100% of carboxypeptidase Y activity. It was concluded that the synthetic peptide was not affected ($P > 0.5$) by the differing pH, ionic strength, and oxidation conditions and was appropriate to use for our study.

Calpastatin oxidation

Irreversibly blocking sulfhydryls with NEM did not alter the specific activity of calpastatin. The specific activity of control calpastatin was 416 units of activity/mg protein.
The mean specific activity of the oxidized calpastatin is 406.5, 410.5, and 398 units of activity/mg protein for 4, 8, and 12 mM NEM respectively.

**Calpain Activity Assays**

**μ-Calpain activity.** Fluorescence measurements at 60 min indicate that the addition of H$_2$O$_2$ significantly decreased ($P < 0.05$; Table 2) μ-calpain activity regardless of pH and ionic strength conditions. μ-Calpain had greater activity at pH 6.5 ($P < 0.01$) than pH 7.5 or 6.0. μ-Calpain activity was lower ($P < 0.05$) at 295 mM NaCl than at 165 mM NaCl.

**m-Calpain activity.** Similar to μ-calpain, addition of H$_2$O$_2$ significantly decreased ($P < 0.01$; Table 3) activity of m-calpain at all pH and ionic strength conditions. Furthermore, the presence of H$_2$O$_2$ in the assays completely prevented proteolytic activity of m-calpain at pH 6.5 and 295 mM NaCl, with or without the presence of calpastatin. In contrast to μ-calpain, m-calpain had greater activity at pH 7.5 ($P < 0.01$) than pH 6.5. Higher ionic strength decreased ($P < 0.05$) m-calpain activity. No measurable activity of m-calpain was detected at pH 6.0 which was previously observed by Maddock et al. (2005).

**μ-Calpain inhibition by calpastatin.** Calpastatin inhibited ($P < 0.05$; Table 4) μ-calpain activity at all pH and ionic strength conditions. Percent inhibition of μ-calpain activity by calpastatin was not affected by pH ($P > 0.05$), but it is noteworthy that at pH 6.5 and 165 mM NaCl, only 12.77 % inhibition occurred when 0.15 units of calpastatin was added to the
assay. This lower percent inhibition of \( \mu \)-calpain activity by calpastatin was not observed when the higher ionic strength was used at pH 6.5. However, in the presence of \( \text{H}_2\text{O}_2 \) at pH 7.5 and 295 \( \text{mM} \) NaCl, 0.15 units of calpastatin only inhibited 5.99% of \( \mu \)-calpain activity. Higher ionic strength increased \((P < 0.05)\) percent inhibition of \( \mu \)-calpain activity by calpastatin at pH 6.5 and 6.0, but not at pH 7.5. The addition of \( \text{H}_2\text{O}_2 \) not only caused a decrease in activity of \( \mu \)-calpain, as discussed previously, but also caused a decrease \((P < 0.05)\) in the percent inhibition of \( \mu \)-calpain activity by calpastatin at pH 7.5 and 6.5 at both ionic strengths and at pH 6.0 and 295 \( \text{mM} \) NaCl. The effect \((P > 0.05)\) of oxidation was not significant at pH 6.0 and 165 \( \text{mM} \) NaCl.

**m-Calpain inhibition by calpastatin.** Calpastatin inhibited \((P < 0.05; \text{Table 5})\) m-calpain activity at all pH and ionic strength conditions. There was no main effect \((P > 0.05)\) of pH on percent inhibition of m-calpain by calpastatin. Inhibition percent was not reported at pH 6.5 and 295 \( \text{mM} \) NaCl because no m-calpain activity was detected, even when 0 units of calpastatin was added to the assay. An ionic strength effect \((P < 0.05)\) was observed where at 295 \( \text{mM} \) NaCl, percent inhibition of m-calpain by calpastatin was greater than at 165 \( \text{mM} \) NaCl, with the exception of assays conducted at pH 7.5 and 0.3 units of calpastatin. Interestingly, the same effect of \( \text{H}_2\text{O}_2 \) causing a decrease \((P < 0.05)\) in percent inhibition of m-calpain by calpastatin was observed with m-calpain that was observed for \( \mu \)-calpain. This
effect was observed at pH 7.5 and 165 mM NaCl at both calpastatin levels and pH 7.5 and 295 mM NaCl when 0.15 units of calpastatin was present.

**Digest of Myofibrils and Western Blots of Desmin degradation**

*μ-Calpain myofibril digests.* Differences in desmin degradation due to oxidation and calpastatin are observed. At pH 6.0, calpastatin decreased degradation of desmin as indicated by detection of intact desmin at 60 min and 120 min incubation with increasing levels of calpastatin at both 165 mM NaCl and 295 mM NaCl. An effect of oxidation was observed where the addition of H$_2$O$_2$ to the digest decreased the degradation of desmin, particularly obvious at 295 mM NaCl when 0 units of calpastatin were added to the digests and intact desmin is detected at all time points.

The combination of calpastatin and H$_2$O$_2$ appear to work synergistically to limit the activity of μ-calpain at pH 6.0. The inhibition of μ-calpain by calpastatin was very different at pH 6.5 and 7.5. The addition of H$_2$O$_2$ appears to decrease the degradation of desmin under the conditions when 0 units of calpastatin were added to the digests. This is apparent at both pH and ionic strength conditions and is consistent with assays with the calpain substrate. Unexpected results occurred when calpastatin was added to the digests. These results are best evaluated when observing the blots from the pH 6.5 and 165 mM NaCl digests. It appears that the addition of H$_2$O$_2$ in the presence of calpastatin results in greater degradation
of intact desmin. When H$_2$O$_2$ is added to the digests in the presence of 0 units of calpastatin, intact desmin is detected as late as 120 min incubation. When 0.15 units of calpastatin were added, the degradation of intact desmin is almost complete by 60 min as indicated by the very light band. Finally, when 0.3 units of calpastatin were added to the assay, degradation of intact desmin appears to be almost complete by 15 min. A similar pattern is observed at 295 mM NaCl and at pH 7.5 at both ionic strength conditions. These effects are similar to the results observed in the calpain assays where percent inhibition of $\mu$-calpain was decreased in the presence of H$_2$O$_2$.

Densitometry data describing intact desmin degradation (n=2) are shown in Table 6 and statistical main effects and interactions are shown in Table 7. After 2 min incubation, no significant main effects are observed. There is a trend toward an effect of pH ($P = 0.08$) where, at pH 6.5, degradation of desmin is less than pH 7.5 and 6.0 at all three levels of calpastatin. After 15 min incubation, an effect of oxidation ($P = 0.04$) is observed. The addition of H$_2$O$_2$, in general, causes a decrease in the degradation of desmin, as indicated by higher densitometry measurements. At 60 min incubation, many effects ($P < 0.05$) are observed. Oxidation decreased ($P < 0.001$) desmin degradation as indicated by more intact desmin. The interactions of pH x oxidation, calpastatin x oxidation, and pH x calpastatin x oxidation are significant. At pH 6.0 the combination of H$_2$O$_2$ and calpastatin appeared to be
additive in its inhibitory effects on μ-calpain activity versus pH 7.5 and 6.5 where the combination appeared to decrease inhibition. After 120 min incubation, many of the significant effects observed at 60 min are not apparent. This is due to the almost complete degradation of intact desmin that occurs by 60 minutes under the different environmental conditions. An effect of oxidation \( (P = 0.04) \) is still observed, where in general, the presence of \( \text{H}_2\text{O}_2 \) decreased degradation of desmin by μ-calpain.

\textit{m-Calpain myofibril digests.} Visual analysis of western blots for desmin from the myofibril digests with m-calpain showed greater proteolysis of intact desmin at pH 7.5 than 6.5, and less proteolysis at 295 mM NaCl than 165 mM NaCl. More intact desmin was observed when calpastatin and \( \text{H}_2\text{O}_2 \) were present. In these samples, the addition of calpastatin and \( \text{H}_2\text{O}_2 \) appear to be additive in the inhibitory effect on m-calpain at pH 6.5 and 7.5.

Densitometry data describing intact desmin degradation are shown in Table 8 \( (n=2) \) and main effects and interactions are shown in Table 9. After 15 min incubation, the pH x calpastatin interaction \( (P = 0.013) \) indicated there was more degradation of desmin at pH 7.5 and 0 units calpastatin than at 0.15 units and 0.3 units calpastatin at pH 7.5 and at all levels of calpastatin at pH 6.5. By 60 min incubation, a main effect of calpastatin \( (P = 0.002) \) appears indicating calpastatin is inhibiting degradation of intact desmin by m-calpain. An interaction of pH x calpastatin \( (P < 0.001) \) occurs because adding more calpastatin decreased desmin
degradation at pH 7.5 but not at pH 6.5. A main effect of oxidation does not appear in the analysis of densitometry measurements, but there is an interaction of pH x oxidation ($P < 0.001$) and calpastatin x oxidation ($P = 0.005$) results in the pH x calpastatin x oxidation interaction ($P = 0.002$), where at pH 7.5, the addition of H$_2$O$_2$ inhibited desmin degradation when 0 units of calpastatin was added at both ionic strength conditions and at pH 6.5, there is not a significant effect of oxidation. The ionic strength x calpastatin interaction ($P = 0.013$) indicates that calpastatin was a better inhibitor of desmin degradation by m-calpain at 295 mM NaCl than at 165 mM NaCl, which is consistent with the results observed in the fluorescent assays (Table 5). By 120 min incubation, there is a reduction of the significance of the particular main effects and interactions observed at 60 min.

**Discussion**

During the conversion of muscle to meat, many changes occur within the environment of the muscle (Winger and Pope, 1980-81). Loss of homeostasis and depleted oxygen ultimately allow for these changes that include a pH decline from near neutrality to approximately 5.6 and a continued increase in ionic strength. These changes, when combined with the temperature decrease that occurs during harvest, can affect other postmortem processes that affect meat quality. These specific environmental changes have been shown to affect activity of calpains (Huff-Lonergan et al., 1996; Kendall et al., 1993).
Another change in environment that occurs with the loss of homeostasis is an increase in oxidative conditions (Martinaud et al., 1997; Harris et al., 2001). Oxidation can also affect calpain activity. A study by Guttman et al. (1997) evaluated \( \mu \)-calpain activity when exposed to \( \text{H}_2\text{O}_2 \) and observed that proteolytic activity of \( \mu \)-calpain was strongly inhibited. Rowe et al. (2004a) used irradiation to demonstrate that inactivation of \( \mu \)-calpain resulted in a decrease in postmortem proteolysis and decrease in tenderization. Based on these observations, it is important to understand how environmental conditions of pH, ionic strength, and oxidation interact to affect calpain activity and additionally how the presence of calpastatin adds to these effects.

In the current study, the objective was to determine the extent to which oxidation influences \( \mu \)- and m-calpain activity and calpain inhibition by calpastatin under the pH and ionic strength conditions observed in early postmortem muscle. Two different methods were used to evaluate the sum of the effects of pH, ionic strength, and oxidation of \( \mu \)- and m-calpain activity in the presence or absence of calpastatin. The first method used a calpain substrate in order to precisely measure activity of \( \mu \)- and m-calpain and their inhibition by calpastatin. The second method used purified myofibrils as a substrate and evaluation of degradation of the protein desmin. This allowed definition of the effects of environment on the activities of \( \mu \)- and m-calpain and their inhibition by calpastatin using a substrate found in
meat. It was concluded that both the calpain activity and inhibition of calpain by calpastatin can be dramatically affected by pH, ionic strength, and oxidation. The effects of environment were profoundly different between μ- and m-calpain.

**pH and Ionic strength**

A previous study was conducted using the calpain substrate to evaluate μ- and m-calpain activity in the presence of reducing agents (Maddock et al., 2005). Ionic strength and pH effects similar to calpain activity assay results in the current study were observed on the activities of m- and μ-calpain and their inhibition by calpastatin. As observed in this study, m-calpain activity was also greatest at pH 7.5, and activity was least at pH 6.0. Additionally μ-calpain activity was also greater at pH 6.5 versus 7.5 or 6.0. Western blotting of μ-calpain autolysis revealed that autolysis occurred at a slower rate at pH 6.5 than at 7.5. It was hypothesized that the observed activity differences were not due to a faster activation of μ-calpain, but rather a slower autolytic inactivation of μ-calpain at pH 6.5. The ionic strength effects observed in Maddock et al. (2005) and the current study indicate that both μ- and m-calpain activities are decreased with increasing ionic strength. Geesink and Koohmaraie (2000) observed that μ-calpain activity decreased with increasing ionic strengths and indicated that the decrease in activity of μ-calpain is due to a decrease in the stability of the molecule. Li et al. (2004) hypothesized that an elevated ionic strength (equal to 100 mM
KCl) caused disassociation of the two calpain subunits, allowing for formation of dimers and trimers of the large subunit, which irreversibly inactivated the proteinase. These results indicate that pH and ionic strength have the potential to affect activity of μ- and m-calpain through autolytic inactivation and dissociation.

In the current study, inhibition of μ-calpain and m-calpain by calpastatin were not affected by pH. This is consistent with reports by Geesink and Koohmaraie (1999) and Otsuka and Goll (1987). Calpastatin is the endogenous inhibitor of μ- and m-calpain and does not inhibit any other protease that it has been tested against (Goll et al., 2003). The presence of Ca\(^{2+}\) is required for calpastatin to bind to the calpains in order to inhibit their activity (Cottin et al., 1981). In porcine muscle, the ratio of calpastatin activity to μ-calpain activity is approximately 1.5:1 (Ouali and Talmant, 1990). The ratio of calpastatin to calpain used in this study was 1:3 and 2:3 in order to evaluate the effect of calpastatin on calpain without the occurrence of complete inhibition. Maddock et al. (2005) reported an effect of pH on inhibition of m-calpain was observed where inhibition was greater at pH 6.5 than 7.5. The discrepancy between this study and the previous study is likely due to the presence of a reducing agent in Maddock et al. (2005) when the activity of m-calpain was maximized, where in this study no reducing agents were used in order to maximize the effect of oxidation on activity and inhibition.
**Oxidation**

Since μ- and m-calpain are cysteine proteases, their activity requires the exchange of electrons between the active site cysteine and histidine residues (Mehdi, 1991). The cysteine residue site is highly susceptible to oxidation by H₂O₂ (Neumann, 1972). Oxidation of calpain with H₂O₂ can allow for inactivation of calpain (Guttmann et al., 1997) indicating that the active site cysteine residue may be oxidized. Oxidation decreased the proteolytic activity of μ- and m-calpain (Table 2 and Table 3) in the activity assays. When using myofibrils as a substrate, the main effect of oxidation (Table 7) was specifically noted on μ-calpain. The effect of oxidation on m-calpain (Table 9) in this system was detected only when also considering interactions with pH and ionic strength in combination with calpastatin. Previous studies observed similar effects of oxidation on μ-calpain activity in meat (Rowe et al., 2004a) and in vitro (Guttmann et al., 1997; Guttmann and Johnson, 1998).

Evidence that cysteine residues on calpastatin are not affected by oxidation is provided in this study by incubations with NEM, which covalently blocks sulfhydryl groups, resulting in effects similar to oxidation. This important observation provides evidence that oxidation of calpastatin does not affect its action on inhibition of calpains.
Effect of treatment combinations

Many of the singular effects of environment did not appear to be significant on \( \mu \)- or m-calpain activity. However, the specific objectives of these experiments were to evaluate the interactions between environmental conditions. The interaction of oxidation and calpastatin indicates that these environmental combinations do have an effect on the proteolytic activity of \( \mu \)- and m-calpain, therefore having implications in postmortem muscle and also in living muscle tissue.

The presence of \( \text{H}_2\text{O}_2 \) decreased the percent inhibition of both \( \mu \)- and m-calpain by calpastatin when using the calpain substrate. This corresponds to the effects observed in the \( \mu \)-calpain degraded myofibrils where the addition of \( \text{H}_2\text{O}_2 \) in the presence of calpastatin allowed for greater degradation of desmin at the higher pH of 7.5 and 6.5. These observations were unique to \( \mu \)-calpain in the digests of myofibrils.

The role of calpastatin in binding to and inhibiting calpain is an area of research that is currently getting some attention as the exact mechanism is not understood (Goll et al., 2003). As previously discussed, \( \text{Ca}^{2+} \) is required for binding of calpastatin to calpains (Cottin et al., 1981). The \( \text{Ca}^{2+} \) concentration required for binding of calpastatin to \( \mu \)- and m-calpain is lower than the \( \text{Ca}^{2+} \) concentration required for proteolytic activity at half-maximal rate. This indicates that, if present, calpastatin will bind and inhibit calpain before it can
become proteolytically active (Kapprell and Goll, 1989). Calpastatin is considered a competitive inhibitor of calpains based on kinetic evidence (Croall and McGrody, 1994). However, evidence indicates that calpastatin does not bind at the active site of calpain (Nishimura and Goll, 1991, Croall and McGrody, 1994), but rather near the active site in a way that blocks access of substrates as hypothesized by Todd et al. (2003). In the current study, when calpastatin/\(\mu\)-calpain complex was formed, and then exposed to \(\text{H}_2\text{O}_2\), greater degradation of desmin was observed at pH 6.5 and 7.5. Oxidation of the active site cysteine residue decreases calpain activity (Guttmann et al., 1997). It is possible that the interaction of calpastatin with \(\mu\)-calpain occurs in a way that blocks \(\text{H}_2\text{O}_2\) from accessing and oxidizing the active site cysteine residue, thus preventing oxidative inactivation of \(\mu\)-calpain. Doumit and Koohmaraie (1999) observed that calpastatin is also as substrate for calpains in addition to its role as an inhibitor. If calpastatin is preventing oxidation of the \(\mu\)-calpain cysteine residue, it may be possible for activated calpain to degrade calpastatin present, particularly at the levels used in these assays, and allow for greater proteolytic activity to occur.

The significant interaction of pH x oxidation on desmin degradation for both \(\mu\)- and m-calpain are different for the two proteinases. Oxidation decreased desmin degradation by \(\mu\)-calpain at pH 6.5 to a greater extent than at pH 7.5 and 6.0. Oxidation decreased desmin degradation by m-calpain to a greater extent at pH 7.5 than pH 6.5. \(\mu\)-Calpain was most
active at pH 6.5 and m-calpain was most active at pH 7.5. Oxidation appears to have a
greater affect when each calpain was in the environment where it was most active. The
oxidized calpain activity observed may be the maximum activity attainable, regardless of pH
conditions. When calpastatin was included, an interaction of pH x calpastatin x oxidation
was observed for both calpains. In the μ-calpain myofibril digests, when calpastatin levels
increased from 0.15 units to 0.3 units, the addition of H₂O₂ allowed for greater desmin
degradation. When m-calpain was the proteinase used in the assays, the addition of
calpastatin decreased degradation of desmin, but this decrease in degradation was larger at
pH 7.5 than 6.5. μ- and m-Calpain are very similar in structure as they consist of an identical
small subunit and a large subunit with 55-65% homology (Suzuki, 1991). The large subunit
is where the active site is located. The observed differences in pH are likely due to the
variation between the two calpain structures and the differences in how calpastatin interacts
with these molecules at the varying pH conditions.

The interaction of ionic strength x oxidation was significant for μ-calpain degradation
of desmin, but this interaction was not significant for m-calpain. In general, the higher ionic
strength decreased the oxidation effects on μ-calpain activity and allowed for greater
degradation of desmin particularly when calpastatin was added to the assays, which explains
the significant interaction of ionic strength x calpastatin x oxidation. When calpastatin was
included in the interaction analysis, the ionic strength x calpastatin x oxidation interaction was significant with m-calpain but at higher ionic strength, desmin degradation decreased with oxidation in the presence of calpastatin.

When all four environmental factors were evaluated, the interaction of these factors did not significantly affect μ-calpain activity. There was a significant interaction of all four factors on m-calpain. To explain this, m-calpain was more active at pH 7.5 than 6.5 and at 165 mM NaCl than at 295 mM NaCl. Calpastatin inhibited m-calpain to a greater extent at pH 7.5 than 6.5, and the addition of H2O2 decreased desmin degradation to a greater extent at the higher ionic strength.

The results of this study indicate that μ-calpain and m-calpain react differently to different environments. Interestingly, the environmental conditions that are observed in postmortem muscle are the conditions that appear to be most favorable to activation of μ-calpain. This is consistent with previous hypotheses (as reviewed by Geesink et al., 2000) that indicate μ-calpain and not m-calpain is responsible for the proteolysis that occurs in postmortem muscle and therefore subsequent tenderization.

The effects of oxidation observed in this study have implications in meat quality. Rowe at al. (2004a) evaluated how oxidative conditions initiated by irradiation early postmortem caused inhibition of μ-calpain activity slowing the rate of proteolysis occurring during aging,
thereby decreased tenderization. Antioxidants are currently becoming more prevalently used in the meat industry to increase shelf life by preventing oxidation of lipids that cause rancidity. Previous research has also evaluated the use of antioxidants as a feed supplement and how their use can affect meat tenderization. Harris et al. (2001) evaluated increased levels of alpha-tocopherol in beef through supplemental vitamin E in the diet in combination with injecting steaks with CaCl₂. They found that beef with increased levels of alpha-tocopherol exhibited a faster rate of decline of Warner-Bratzler Shear Force values and a faster rate of troponin-T degradation. Additionally, Rowe at al. (2004a) observed an increase in the rate of troponin-T degradation in beef from calves fed a diet supplemented with vitamin E.

Oxidation has been shown to decrease activity of calpains, but in contrast, calpains have also been shown to play a role in protein degradation when oxidative conditions are higher than normal. A relationship between protein oxidation and proteolysis has been clearly established (as reviewed by Mehlhase and Grune, 2002) where it has been established that oxidation of proteins can enhance susceptibility to proteolytic degradation, but at the same time, the excessive oxidation may cause a decrease in susceptibility to degradation. Oxidative stress, in combination with observed intracellular Ca²⁺ concentration in a mouse embryonal carcinoma cell line (Ray et al., 2000) and a rat adrenal gland cell line (Ishihara et
al., 2000) have shown an increase in activation of calpains. Pronzato et al. (1993) observed the level of oxidative stress can affect calpain activity, where moderate levels of oxidation enhance activity but increased oxidative exposure can cause inactivation of calpains. These observations could provide an explanation of how the involvement of calpastatin regulates oxidation of μ-calpain and allow activation to occur.

In summary, activity differences were observed due to pH where μ-calpain had greater activity at pH 6.5 than pH 7.5, while the opposite was true for m-calpain. Oxidation of m-calpain with H₂O₂ resulted in a decrease of proteolytic activity and the addition of calpastatin decreased proteolytic activity even further as demonstrated by the decrease in desmin degradation (Figure 2). Oxidation of μ-calpain with H₂O₂ did decrease proteolytic activity, but oxidation of the calpastatin/μ-calpain complex with H₂O₂ resulted in increased proteolysis of desmin (Figure 1) and therefore appeared to increase activity of μ-calpain in the presence of H₂O₂. This novel observation is important, because it demonstrates that inhibition of μ-calpain by calpastatin is diminished by oxidation.

**Implications**

Oxidative conditions in postmortem muscle could have an effect on proteolytic activity that occurs during the aging process. The differences observed between oxidation of μ-calpain versus m-calpain provide further evidence of the role of μ-calpain in postmortem
proteolysis. The results from may also explain some of the variation observed in tenderness, as the interactions of pH, ionic strength, and oxidation in addition to the presence of calpastatin cause μ-calpain to act differently than if examined under each environmental factor alone. These results also indicate a need for greater understanding of the interaction of calpastatin with the calpains in order to understand how these proteins function in the muscle.

**Literature Cited**


Table 1. Carboxypeptidase Y control activity
Percentage is based on measurements taken after 60 min at pH 7.5 and 165 mM NaCl and no H₂O₂ added is 100 %

<table>
<thead>
<tr>
<th>pH</th>
<th>Con 165 mM NaCl</th>
<th>H₂O₂ 165 mM NaCl</th>
<th>Con 295 mM NaCl</th>
<th>H₂O₂ 295 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>100</td>
<td>95.9</td>
<td>106</td>
<td>102</td>
</tr>
<tr>
<td>6.5</td>
<td>99.3</td>
<td>102</td>
<td>92.1</td>
<td>92.7</td>
</tr>
<tr>
<td>6.0</td>
<td>96.4</td>
<td>95.6</td>
<td>93.4</td>
<td>98.1</td>
</tr>
</tbody>
</table>
Table 2. Least squares mean (± SE) of \( \mu \)-calpain activity at 60 min after addition of \( \text{CaCl}_2 \)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>165 mM NaCl</th>
<th>295 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con ( \text{H}_2\text{O}_2 )</td>
<td>Con ( \text{H}_2\text{O}_2 )</td>
</tr>
<tr>
<td>7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>63.99 ± 0.69(^{bw})</td>
<td>36.73 ± 2.48(^{sv})</td>
</tr>
<tr>
<td>0.15</td>
<td>50.16 ± 0.66(^{cx})</td>
<td>32.15 ± 4.19(^{sy})</td>
</tr>
<tr>
<td>0.30</td>
<td>35.22 ± 2.13(^{cx})</td>
<td>22.98 ± 4.42(^{by})</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>117.72 ± 5.02(^{bw})</td>
<td>50.07 ± 1.98(^{sy})</td>
</tr>
<tr>
<td>0.15</td>
<td>102.68 ± 1.43(^{bw})</td>
<td>43.72 ± 1.97(^{sx})</td>
</tr>
<tr>
<td>0.30</td>
<td>78.12 ± 3.74(^{sw})</td>
<td>36.23 ± 0.46(^{cx})</td>
</tr>
<tr>
<td>6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>11.85 ± 0.17(^{by})</td>
<td>6.45 ± 0.36(^{cz})</td>
</tr>
<tr>
<td>0.15</td>
<td>8.94 ± 0.54(^{by})</td>
<td>4.54 ± 1.10(^{bx})</td>
</tr>
<tr>
<td>0.30</td>
<td>7.97 ± 0.28(^{ix})</td>
<td>4.59 ± 0.36(^{by})</td>
</tr>
</tbody>
</table>

\(^{a,b,c,d,e,f,g,h,i}\) Within a column, least squares means lacking a common superscript differ \( (P < 0.05; n=3) \)

\(^{w,x,y,z}\) Within a row, least squares means lacking a common superscript differ \( (P < 0.05) \).
Table 3. Least squares means (± SE) of m-calpain activity at 60 min after addition of CaCl₂

<table>
<thead>
<tr>
<th>Activity</th>
<th>165 mM NaCl</th>
<th>295 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Con</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>93.14 ± 7.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.59 ± 2.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.15</td>
<td>46.34 ± 1.86&lt;sup&gt;e&lt;/sup&gt;</td>
<td>15.06 ± 1.17&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.30</td>
<td>21.38 ± 1.90&lt;sup&gt;i&lt;/sup&gt;</td>
<td>7.92 ± 1.00&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>18.00 ± 0.41&lt;sup&gt;m&lt;/sup&gt;</td>
<td>7.82 ± 0.81&lt;sup&gt;n&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.15</td>
<td>8.27 ± 0.33&lt;sup&gt;q&lt;/sup&gt;</td>
<td>2.95 ± 1.54&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.30</td>
<td>6.24 ± 0.40&lt;sup&gt;u&lt;/sup&gt;</td>
<td>2.92 ± 0.17&lt;sup&gt;v&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c,d,e,f</sup> Within a column, least squares means lacking a common superscript differ (P < 0.05; n=3)
<sup>w,X,Y,Z</sup> Within a row, least squares means lacking a common superscript differ (P < 0.05). Value in parentheses = standard error of the mean
Table 4. Least squares means (± SE) of percent inhibition of μ-calpain by 0.15 and 0.3 units calpastatin\textsuperscript{a} calculated from fluorescence measurements taken at 60 min after the addition of CaCl\textsubscript{2}.

<table>
<thead>
<tr>
<th>Percent Inhibition</th>
<th>165 mM NaCl</th>
<th></th>
<th>295 mM NaCl</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Con H\textsubscript{2}O\textsubscript{2}</td>
<td>H\textsubscript{2}O\textsubscript{2}</td>
<td>Con H\textsubscript{2}O\textsubscript{2}</td>
<td>H\textsubscript{2}O\textsubscript{2}</td>
</tr>
<tr>
<td>7.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>21.61 ± 2.16\textsuperscript{dx}</td>
<td>12.47 ± 6.19\textsuperscript{byxz}</td>
<td>18.62 ± 2.16\textsuperscript{ezy}</td>
<td>5.99 ± 6.18\textsuperscript{exz}</td>
</tr>
<tr>
<td>0.30</td>
<td>44.96 ± 2.95\textsuperscript{by}</td>
<td>37.44 ± 6.95\textsuperscript{bz}</td>
<td>49.29 ± 2.95\textsuperscript{by}</td>
<td>33.83 ± 6.95\textsuperscript{ez}</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>12.77 ± 1.21\textsuperscript{ex}</td>
<td>12.67 ± 3.93\textsuperscript{bx}</td>
<td>32.44 ± 3.04\textsuperscript{ey}</td>
<td>24.37 ± 5.38\textsuperscript{ex}</td>
</tr>
<tr>
<td>0.30</td>
<td>33.64 ± 3.18\textsuperscript{cw}</td>
<td>27.63 ± 0.93\textsuperscript{bx}</td>
<td>55.23 ± 1.15\textsuperscript{bz}</td>
<td>45.65 ± 1.42\textsuperscript{by}</td>
</tr>
<tr>
<td>6.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>24.54 ± 4.62\textsuperscript{dx}</td>
<td>29.56 ± 15.70\textsuperscript{byyz}</td>
<td>32.53 ± 1.51\textsuperscript{ey}</td>
<td>25.19 ± 0.94\textsuperscript{dx}</td>
</tr>
<tr>
<td>0.30</td>
<td>32.69 ± 2.39\textsuperscript{cx}</td>
<td>28.73 ± 5.55\textsuperscript{bx}</td>
<td>53.52 ± 2.66\textsuperscript{by}</td>
<td>33.29 ± 3.35\textsuperscript{ex}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Inhibition was calculated as the percent inhibition = (1-(calpain activity without calpastatin ÷ calpain activity with calpastatin)) x 100 (n=3).

\textsuperscript{b,c} Within a column, least squares means lacking a common superscript differ (P < 0.05; n=3).

\textsuperscript{x,y,z} Within a row, least squares means lacking a common superscript differ (P < 0.05).
Table 5. Least squares mean (±SE) of percent inhibition of m-calpain by 0.15 and 0.3 units calpastatin calculated from fluorescence measurements taken at 60 min after the addition of CaCl₂

<table>
<thead>
<tr>
<th>Treatment</th>
<th>165 mM NaCl</th>
<th>295 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>50.24 ± 2.01&lt;sup&gt;dx&lt;/sup&gt;</td>
<td>36.15 ± 4.96&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.30</td>
<td>77.05 ± 2.04&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>66.41 ± 4.23&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>54.07 ± 1.84&lt;sup&gt;dx&lt;/sup&gt;</td>
<td>62.23 ± 19.71&lt;sup&gt;by&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.30</td>
<td>65.35 ± 2.25&lt;sup&gt;cy&lt;/sup&gt;</td>
<td>62.74 ± 2.22&lt;sup&gt;by&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Inhibition was calculated as the percent inhibition = (1-(calpain activity without calpastatin ÷ calpain activity with calpastatin))×100 (n=3).

<sup>b,c</sup> Within a column, least squares means lacking a common superscript differ (P < 0.05; n=3).

<sup>x,y</sup> Within a row, least squares means lacking a common superscript differ (P < 0.05).
### Table 6. Relative intensity (±SE) of intact desmin after incubation with μ-calpain (n=2).

<table>
<thead>
<tr>
<th>Time x Oxidation x ionic strength</th>
<th>165 mM NaCl</th>
<th>295 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH x calpastatin</td>
<td>Con</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>pH 7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 units calpastatin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 units calpastatin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 units calpastatin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Within a column, least squares means lacking a common superscript differ (P < 0.05)

Within a row, least squares means lacking a common superscript differ (P < 0.05)
Table 7. *P*-values of main effects and interactions of environmental conditions on μ-calpain degradation of desmin

<table>
<thead>
<tr>
<th>Treatment effects</th>
<th>2 min</th>
<th>15 min</th>
<th>60 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>0.08</td>
<td>0.45</td>
<td>0.78</td>
<td>0.74</td>
</tr>
<tr>
<td>Ionic strength</td>
<td>0.44</td>
<td>0.48</td>
<td>0.97</td>
<td>0.92</td>
</tr>
<tr>
<td>Calpastatin</td>
<td>0.94</td>
<td>0.48</td>
<td>0.41</td>
<td>0.58</td>
</tr>
<tr>
<td>Oxidation</td>
<td>0.77</td>
<td>0.04</td>
<td>0.001</td>
<td>0.04</td>
</tr>
<tr>
<td>pH x Ionic strength</td>
<td>0.18</td>
<td>0.46</td>
<td>0.80</td>
<td>0.89</td>
</tr>
<tr>
<td>pH x calpastatin</td>
<td>0.36</td>
<td>0.61</td>
<td>0.23</td>
<td>0.33</td>
</tr>
<tr>
<td>pH x oxidation</td>
<td>0.35</td>
<td>0.22</td>
<td>0.02</td>
<td>0.21</td>
</tr>
<tr>
<td>Ionic strength x calpastatin</td>
<td>0.72</td>
<td>0.52</td>
<td>0.87</td>
<td>0.88</td>
</tr>
<tr>
<td>Ionic strength x oxidation</td>
<td>0.79</td>
<td>0.15</td>
<td>0.008</td>
<td>0.13</td>
</tr>
<tr>
<td>Calpastatin x oxidation</td>
<td>0.99</td>
<td>0.24</td>
<td>0.007</td>
<td>0.05</td>
</tr>
<tr>
<td>pH x Ionic strength x calpastatin</td>
<td>0.29</td>
<td>0.32</td>
<td>0.66</td>
<td>0.71</td>
</tr>
<tr>
<td>pH x Ionic strength x oxidation</td>
<td>0.66</td>
<td>0.24</td>
<td>0.07</td>
<td>0.48</td>
</tr>
<tr>
<td>pH x calpastatin x oxidation</td>
<td>0.91</td>
<td>0.56</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>Ionic strength x calpastatin x oxidation</td>
<td>0.98</td>
<td>0.42</td>
<td>0.06</td>
<td>0.17</td>
</tr>
<tr>
<td>pH x ionic strength x calpastatin x oxidation</td>
<td>0.89</td>
<td>0.31</td>
<td>0.19</td>
<td>0.36</td>
</tr>
<tr>
<td>pH x</td>
<td>2 min</td>
<td>15</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td>------</td>
<td>-------</td>
<td>----</td>
<td>----</td>
<td>-----</td>
</tr>
<tr>
<td>Con</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.5</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0 units</td>
<td>0.93$^{ab}_v$</td>
<td>0.5$^{bc}_v$</td>
<td>0$^{c_*}$</td>
<td>0$^c$</td>
</tr>
<tr>
<td>calpastatin</td>
<td>±0.13</td>
<td>±0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 units</td>
<td>0.94$^{bvw}_v$</td>
<td>0.93$^{bcw}_v$</td>
<td>0.84$^{bcw}_v$</td>
<td>0.17$^v$</td>
</tr>
<tr>
<td>calpastatin</td>
<td>±0.79</td>
<td>±0.09</td>
<td>±0.21</td>
<td>±0.06</td>
</tr>
<tr>
<td>0.3 units</td>
<td>1.12$^a$</td>
<td>1.14$^a$</td>
<td>0.99$^a$</td>
<td>0.66</td>
</tr>
<tr>
<td>calpastatin</td>
<td>±0.19</td>
<td>±0.25</td>
<td>±0.44</td>
<td>±0.65</td>
</tr>
<tr>
<td>pH 6.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 units</td>
<td>0.93$^v$</td>
<td>1.07$^{av}$</td>
<td>0.24$^{av}$</td>
<td>0$^c$</td>
</tr>
<tr>
<td>calpastatin</td>
<td>±0.03</td>
<td>±0.22</td>
<td>±0.02</td>
<td>±0.33</td>
</tr>
<tr>
<td>0.15 units</td>
<td>0.83$^b$</td>
<td>0.67$^{b}_v$</td>
<td>0.48$^{bc}_v$</td>
<td>0.38</td>
</tr>
<tr>
<td>calpastatin</td>
<td>±0.03</td>
<td>±0.26</td>
<td>±0.33</td>
<td>±0.38</td>
</tr>
<tr>
<td>0.3 units</td>
<td>0.95$^{by}$</td>
<td>0.93$^{by}$</td>
<td>0.66$^c$</td>
<td>0.40$^c$</td>
</tr>
<tr>
<td>calpastatin</td>
<td>±0.12</td>
<td>±0.14</td>
<td>±0.04</td>
<td>±0.33</td>
</tr>
</tbody>
</table>

*abc Within a column, least squares means lacking a common superscript differ ($P < 0.05$)

vwxyz Within a row, least squares means lacking a common superscript differ ($P < 0.05$)
Table 9. *P*-values of main effects and interactions of environmental conditions on m-calpain degradation of desmin

<table>
<thead>
<tr>
<th>Treatment effects</th>
<th>2 min</th>
<th>15 min</th>
<th>60 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>0.08</td>
<td>0.08</td>
<td>0.20</td>
<td>0.55</td>
</tr>
<tr>
<td>Ionic strength</td>
<td>0.99</td>
<td>0.99</td>
<td>0.49</td>
<td>0.24</td>
</tr>
<tr>
<td>Calpastatin</td>
<td>0.46</td>
<td>0.11</td>
<td>0.002</td>
<td>0.003</td>
</tr>
<tr>
<td>Oxidation</td>
<td>0.38</td>
<td>0.54</td>
<td>0.12</td>
<td>0.15</td>
</tr>
<tr>
<td>pH x Ionic strength</td>
<td>0.32</td>
<td>0.37</td>
<td>0.55</td>
<td>0.41</td>
</tr>
<tr>
<td>pH x calpastatin</td>
<td>0.10</td>
<td>0.01</td>
<td>0.0006</td>
<td>0.002</td>
</tr>
<tr>
<td>pH x oxidation</td>
<td>0.19</td>
<td>0.21</td>
<td>0.047</td>
<td>0.09</td>
</tr>
<tr>
<td>Ionic strength x calpastatin</td>
<td>0.60</td>
<td>0.21</td>
<td>0.012</td>
<td>0.001</td>
</tr>
<tr>
<td>Ionic strength x oxidation</td>
<td>0.71</td>
<td>0.95</td>
<td>0.36</td>
<td>0.08</td>
</tr>
<tr>
<td>Calpastatin x oxidation</td>
<td>0.72</td>
<td>0.36</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>pH x Ionic strength x calpastatin</td>
<td>0.29</td>
<td>0.07</td>
<td>0.011</td>
<td>0.003</td>
</tr>
<tr>
<td>pH x Ionic strength x oxidation</td>
<td>0.57</td>
<td>0.71</td>
<td>0.24</td>
<td>0.29</td>
</tr>
<tr>
<td>pH x calpastatin x oxidation</td>
<td>0.32</td>
<td>0.11</td>
<td>0.002</td>
<td>0.001</td>
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<tr>
<td>Ionic strength x calpastatin x oxidation</td>
<td>0.87</td>
<td>0.64</td>
<td>0.047</td>
<td>0.006</td>
</tr>
<tr>
<td>pH x ionic strength x calpastatin x oxidation</td>
<td>0.76</td>
<td>0.48</td>
<td>0.05</td>
<td>0.006</td>
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</tbody>
</table>
### Figure 1

#### pH 7.5-165 mM NaCl

<table>
<thead>
<tr>
<th>Calpastatin</th>
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<th>H₂O₂</th>
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<tbody>
<tr>
<td>0 U</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>0.15 U</td>
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<tr>
<td>0.30 U</td>
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#### pH 6.5-165 mM NaCl

<table>
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<th>Calpastatin</th>
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<th>H₂O₂</th>
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</thead>
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<tr>
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<tr>
<td>0.30 U</td>
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<td><img src="image12.png" alt="Image" /></td>
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#### pH 6.0-165 mM NaCl

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#### pH 7.5-295 mM NaCl

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<td>0.15 U</td>
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<tr>
<td>0.30 U</td>
<td><img src="image23.png" alt="Image" /></td>
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#### pH 6.5-295 mM NaCl

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<td><img src="image29.png" alt="Image" /></td>
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#### pH 6.0-295 mM NaCl

<table>
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<th>H₂O₂</th>
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<tbody>
<tr>
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<tr>
<td>0.15 U</td>
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</tr>
<tr>
<td>0.30 U</td>
<td><img src="image35.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 2

pH 7.5-165 mM NaCl

Calpastatin | 0 U | 0.15 U | 0.30 U | Control | H₂O₂
---|---|---|---|---|---
S | 2 | 15 | 60 | 120 | 2 | 15 | 60 | 120

pH 7.5-295 mM NaCl

Calpastatin | 0 U | 0.15 U | 0.30 U | Control | H₂O₂
---|---|---|---|---|---
S | 2 | 15 | 60 | 120 | 2 | 15 | 60 | 120

pH 6.5-165 mM NaCl

Calpastatin | 0 U | 0.15 U | 0.30 U | Control | H₂O₂
---|---|---|---|---|---
S | 2 | 15 | 60 | 120 | 2 | 15 | 60 | 120

pH 6.5-295 mM NaCl

Calpastatin | 0 U | 0.15 U | 0.30 U | Control | H₂O₂
---|---|---|---|---|---
S | 2 | 15 | 60 | 120 | 2 | 15 | 60 | 120
FORMATION OF THE \( \mu \)-CALPAIN/CALPASTATIN COMPLEX ALLOWS ACTIVATION OF \( \mu \)-CALPAIN UNDER OXIDIZING CONDITIONS

A paper to be submitted to Biochemical Journal

Kasey R. Maddock*, Elisabeth Huff-Lonergan, Steven M. Lonergan

Synopsis:

Calpains are cysteine proteinases responsible for many biological roles in muscle that include protein degradation and muscle growth [1], and myoblast fusion [2]. Calpains are inhibited by calpastatin, an endogenous inhibitor, as well as pH, ionic strength, and oxidation within the cell. The influence of pH and ionic strength has been well characterized, but the effect of oxidation on calpain activity is relatively uncharacterized. The objective of this study was to determine if, in the presence of calpastatin, \( \mu \)-calpain can be active under oxidizing conditions. Results from this study indicate that autolysis of \( \mu \)-calpain occurred when the \( \mu \)-calpain/calpastatin complex was exposed to an oxidant or cysteine modifier such as N-ethylmaleimide (NEM). However, when \( \mu \)-calpain was exposed to the cysteine modifier prior to calpastatin, autolysis of \( \mu \)-calpain did not occur. Additionally, irreversible modification of cysteine residues prevented activation of \( \mu \)-calpain in the absence of calpastatin as was observed with NEM, but if the cysteine modification is potentially reversible (H\(_2\)O\(_2\)), \( \mu \)-calpain
activity can be recovered. Results from this study indicate that when calpastatin is bound to \( \mu \)-calpain, activation of \( \mu \)-calpain can occur when exposed to a cysteine modifier (NEM) or oxidizing conditions (H\(_2\)O\(_2\)). When domain I calpastatin or calpain inhibitor peptide (consisting of similar peptide sequence as subdomain B of calpastatin) was used to evaluate inhibition of \( \mu \)-calpain by calpastatin, no effect of oxidation was observed indicating that calpastatin must consist of more than one inhibitory domain to be affected by NEM or H\(_2\)O\(_2\).

\( \mu \)-Calpain is not tagged with maleimide-polyethyleneglycol (MAL-PEG) in the presence of calpastatin indicating that calpastatin prevents access of \( \mu \)-calpain to cysteine modification. Exposure of the \( \mu \)-calpain/calpastatin complex with a cysteine modifier allows activation of \( \mu \)-calpain indicating that the inhibitory action of calpastatin is compromised. These results indicate a regulatory role for calpastatin that is not inhibitory, but protective for \( \mu \)-calpain.

**Keywords:** \( \mu \)-calpain, calpastatin, oxidation, H\(_2\)O\(_2\), cysteine protease

**Introduction**

Calpains are calcium activated cysteine proteinases that require the exchange of electrons between the active site cysteine and histidine residues for activation [3]. \( \mu \)-Calpain is a heterodimer with a large catalytic subunit of approximately 80 kDa and a smaller subunit of 30 kDa. \( \mu \)-Calpain is expressed in most mammalian and avian cells. The biological role for calpains is still under investigation and has been since the purification of m-calpain in 1976.
It is known that calpains cleave specific sites in native proteins and produce large polypeptide fragments. Substrates of calpain have been placed into various categories including cytoskeletal proteins, kinases and phosphatases, membrane associated proteins, and some transcription factors [5]. This broad array of proposed substrates provides evidence that calpains are involved in diverse cellular process including signal transduction, apoptosis, cell division and cell fusion [5-8].

The calpain inhibitor, calpastatin, binds to the calpain heterodimer in the presence of calcium at three sites, domain VI on the 28-kDa subunit and domain IV on the 80-kDa subunit [9] and also to or near the active site of calpain [10]. One calpastatin molecule has 4 inhibitory domains and within each domain are three subdomains (A, B, and C). Each subdomain interacts with calpain; therefore it is possible that one calpastatin molecule is able to interact with four different calpain molecules [11-12]. Whether four interactions do occur has not yet been determined.

Maddock et al. [13] demonstrated that when the μ-calpain/calpastatin complex was exposed to oxidizing conditions, greater proteolytic activity was observed than when the complex was not exposed to oxidizing conditions. However, a specific mechanism is not yet defined. The cysteine residue in the active site of cysteine proteases is highly susceptible to oxidation by H₂O₂ [14]. Oxidation of calpain with H₂O₂ causes inactivation of calpain [15],
however if reducing conditions are introduced, \( \mu \)-calpain activity can be recovered. Hydrogen peroxide is widely produced in cells and tissues, thereby the common oxidant used when studying oxidation biology [16]. The main product produced between a protein and \( \text{H}_2\text{O}_2 \) is sulfenic acid. Sulfenic acid is unstable and can easily be converted back to a sulfhydryl under reducing conditions [16] thus explaining the irreversible effects of \( \text{H}_2\text{O}_2 \) on calpain activity.

Previous research found that a) calpastatin decreased \( \mu \)-calpain degradation of desmin and b) the presence of oxidizing conditions clearly decreased desmin degradation by \( \mu \)-calpain [13]. However, increased degradation of desmin was observed in the presence of \( \text{H}_2\text{O}_2 \) as increased amounts of calpastatin were added to the digests. Thus, it is notable that oxidation of the \( \mu \)-calpain/calpastatin complex resulted in greater desmin degradation [13].

These observations lead to the hypothesis that calpastatin, when interacting with \( \mu \)-calpain, may protect \( \mu \)-calpain from being inactivated by oxidation. Therefore, the objective of this study was to determine if, in the presence of calpastatin, \( \mu \)-calpain can be active under oxidizing conditions.

**Experimental**

**Purification of calpastatin and \( \mu \)-calpain**

Calpastatin and \( \mu \)-calpain were purified from porcine skeletal muscle based on the procedures of Thompson and Goll [17] with minor modifications. Porcine semimembranosus
(~ 2-kg) was removed from a market barrow approximately 25 min after exsanguination. The muscle was homogenized in ice cold buffer (six volumes buffer: tissue) containing 10 mM EDTA, 0.1% (vol/vol) β-mercaptoethanol (MCE), and 100 mM Tris-HCl, pH 8.3. Protease inhibitors (2.5 μM trans-Epoxysuccunyl-L-leucylamido-[4-guanidino] butane (E-64), 0.1 mg/mL ovomucoid trypsin inhibitor, and 0.2 mM phenylmethylsulfonylfluoride [PMSF]) were added to the buffer prior to homogenization. The homogenate was centrifuged at 9750 x g at 4°C for 30 min. The supernatant was filtered through cheesecloth and proteins were salted out between 0 and 45% ammonium sulfate saturation. Proteins were pelleted at 9750 x g at 4°C for 30 min, resuspended in 1 mM EDTA, 0.1% MCE, 40 mM Tris-HCl, pH 7.4 (TEM), stirred overnight at 4°C, and dialyzed against TEM. The sample was loaded on a Q-Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ) anion-exchange column (900 mL) equilibrated in TEM. Using a gradient of 0 to 500 mM KCl in TEM (total volume 4,500 mL), porcine calpastatin and μ-calpain were eluted in two separate peaks from the column (calpastatin eluted between 90 and 150 mM KCl and μ-calpain between 160 and 190 mM KCl).

Purification of calpastatin

Fractions containing calpastatin activity were pooled and further purified using methods described by Thompson and Goll [17] with modifications from Geesink and Koohmaraie [18]. The sample was heated at 100°C for 20 min, chilled on ice, and centrifuged
at 9750 x g at 4°C for 30 min. Using successive chromatography over Phenyl Sepharose 6 Fast Flow (Amersham Biosciences, Piscataway, NJ), Blue Sepharose 6 Fast Flow (Amersham Biosciences, Piscataway, NJ), and EMD TMAE 650 S (EM Science, Gibbstown, NJ), calpastatin was further purified. Purified calpastatin consisted of a single 68 kDa band when analyzed by SDS-PAGE and had a specific activity of 365 U/mg protein. One unit of calpastatin activity was defined as the ability to inhibit one unit of m-calpain caseinolytic activity [19].

**Purification of μ-calpain**

μ-Calpain was purified according to the methods of Thompson and Goll [17] with minor modifications. Fractions from the Q-sepharose column containing μ-calpain activity were pooled. μ-Calpain was further purified using successive chromatography over a Phenyl Sepharose 6 Fast Flow (Amersham Biosciences, Piscataway, NJ), Butyl Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ), and EMD TMAE 650 S (EM Science, Gibbstown, NJ). Purified μ-calpain had a specific activity of 67 U/mg protein. One unit of calpain was defined as the amount of calpain required to increase the absorbance at 278 nm of the supernatant by one unit due to the release of trichloroacetic acid-soluble polypeptides resulting from the digestion of casein [20].
Incubation of μ-calpain with N-ethylmaleimide

N-ethylmaleimide (NEM) targets protein thiol groups on cysteine residues working as an irreversible alkylating agent [21]. Alkylation is irreversible in the presence of reducing conditions. Purified porcine μ-calpain (0.6 units) was incubated in 165 mM NaCl, 50 mM HEPES, pH 6.5, on ice for 1 h under each treatment group (n=3). Treatment groups consisted of 1) 100 μM CaCl₂; 2) 4 mM NEM 3) 4 mM NEM with 100 μM CaCl₂; 4) 1.2 units purified porcine calpastatin and 100 μM CaCl₂; and 5) 1.2 units calpastatin, 100 μM CaCl₂, then 4 mM NEM; or 6) 4 mM NEM, then 1.2 units calpastatin, 100 μM CaCl₂. Calpastatin was used in this experiment at a ratio of 2:1 calpastatin to μ-calpain. Units of calpastatin were determined by measurement against 0.45 units of m-calpain caseinolytic activity/ml in the assay [19]. The excess of calpastatin was used to increase the efficiency of μ-calpain interaction in the presence of calcium. A control of μ-calpain incubated in 50 mM HEPES; pH 6.5 on ice for 1 h was used. After incubation, reactions were stopped with 20 mM EDTA. Samples were aliquoted for casein zymography and SDS-PAGE analysis. Comparisons of treatment groups were made on the basis of μ-calpain activity and autolysis.

Incubation of μ-calpain with H₂O₂

Hydrogen peroxide is produced in many cells and tissues and reacts with protein thiols forming sulfenic acid [22]. Sulfenic acid is a reversible product of H₂O₂ oxidation. Purified
porcine \( \mu \)-calpain (0.6 units) was incubated in 50 mM HEPES, pH 6.5, on ice for 1 h at 165 mM NaCl under each treatment group (\( n = 3 \)). Treatment groups consisted of 1) 100 \( \mu \)M CaCl\(_2\); 2) 0.170 \( \mu \)M H\(_2\)O\(_2\) 3) 0.170 \( \mu \)M H\(_2\)O\(_2\) with 100 \( \mu \)M CaCl\(_2\); 4) 1.2 units purified porcine calpastatin and 100 \( \mu \)M CaCl\(_2\); and 5) 1.2 units calpastatin, 100 \( \mu \)M CaCl\(_2\), then 0.170 \( \mu \)M H\(_2\)O\(_2\); or 6) 0.170 \( \mu \)M H\(_2\)O\(_2\), then 1.2 units calpastatin, 100 \( \mu \)M CaCl\(_2\). Calpastatin was used in this experiment at a ratio of 2:1 calpastatin to \( \mu \)-calpain. Units of calpastatin were determined by measurement against 0.45 units of \( \mu \)-calpain caseinolytic activity/ml in the assay [19]. The excess of calpastatin was used to increase the efficiency of \( \mu \)-calpain interaction in the presence of calcium. A control of \( \mu \)-calpain incubated in 50 mM HEPES; pH 6.5 on ice for 1 h was used. After incubation, reactions were stopped with 20 mM EDTA. Samples were aliquoted for casein zymography and SDS-PAGE analysis.

*Ionic strength comparisons*

Purified porcine \( \mu \)-calpain (0.6 units) was incubated in 50 mM HEPES, pH 6.5, on ice for 1 h at 165 mM NaCl or 295 mM NaCl. Previous experiments [23] indicate the ionic strength affects \( \mu \)-calpain activity and inhibition by calpastatin. Treatment groups consisted of 1) 100 \( \mu \)M CaCl\(_2\); 2) 0.170 \( \mu \)M H\(_2\)O\(_2\) 3) 0.170 \( \mu \)M H\(_2\)O\(_2\) with 100 \( \mu \)M CaCl\(_2\); 4) 1.2 units purified porcine calpastatin and 100 \( \mu \)M CaCl\(_2\); and 5) 1.2 units calpastatin, 100 \( \mu \)M CaCl\(_2\), then 0.170 \( \mu \)M H\(_2\)O\(_2\); or 6) 0.170 \( \mu \)M H\(_2\)O\(_2\), then 1.2 units calpastatin, 100 \( \mu \)M CaCl\(_2\). A
control of µ-calpain incubated in 50 mM HEPES; pH 6.5 on ice for 1 h was used. After incubation, reactions were stopped with 20 mM EDTA. Samples were aliquoted for SDS-PAGE analysis.

*Incubation of µ-calpain with calpastatin, domain I of calpastatin, and a calpain inhibitor peptide*

Purified porcine µ-calpain (0.6 units) was incubated in 50 mM HEPES, pH 6.5, on ice for 1 h at 165 mM NaCl. Three different forms of calpastatin were used to compare effects of H$_2$O$_2$. The calpastatin forms consisted of the purified calpastatin from porcine semimembranosus, as described above, the second was highly purified domain I of human calpastatin (14 kDa; Takara Bio Inc., Otsu, Shiga, Japan), and the third was a calpain inhibitor consisting of the 26 amino acids DPMSSTYIEELGKREVTIPPKYRELL (Takara Bio Inc., Otsu, Shiga, Japan) based on the amino acid sequence of subdomain B in human calpastatin. Activity of all three calpain inhibitors were standardized based on inhibitory activity against µ-calpain where one unit of calpastatin activity was defined as the ability to inhibit one unit of µ-calpain caseinolytic activity [19]. Treatment groups consisted of 1) 1 mM CaCl$_2$; 2) either 1.14 units porcine calpastatin, domain I calpastatin, or calpain inhibitor, all treatments contained 1 mM CaCl$_2$. Oxidation treatments consisted of 3) 1.14 units porcine calpastatin, domain I calpastatin, or calpain inhibitor, 1 mM CaCl$_2$ was added, and finally 0.170 µM H$_2$O$_2$;
or 4) 0.170 μM H2O2, 1.14 units porcine calpastatin, domain I calpastatin, or calpain inhibitor, 1 mM CaCl2. A μ-calpain control incubated in 50 mM HEPES; pH 6.5 on ice for 1 h was used. After incubation, reactions were stopped with 20 mM EDTA. Samples were aliquoted for casein zymography and SDS-PAGE analysis. Comparisons were made between treatment groups on μ-calpain activity and autolysis.

Casein zymography and native gels

Casein zymography [24] was used to determine remaining μ-calpain activity after incubation. Native gels were used to confirm μ-calpain was present in the sample. Gel samples were made for casein zymography and nondenaturing polyacrylamide gels by diluting the sample 70:30 (sample: electrophoresis sample buffer [20% glycerol, 0.1% bromphenol blue, 0.75% 2-mercaptoethanol (MCE), 150 mM Tris-HCl, pH 6.8]. Samples were loaded onto nondenaturing acrylamide gels containing casein and identical nondenaturing gels that did not contain casein. Gels were run at a constant voltage of 75 V for approximately 18 hours. Casein gels were incubated in 5 mM CaCl2, 0.1% MCE, 50 mM Tris-HCl, pH 7.5 solution overnight at room temperature to active any potentially active μ-calpain. Both casein and native gels were stained in 0.1% Coomassie brilliant-blue R-250, 40% methanol, and 7% acetic acid solution and then destained in a 40% methanol, 7% acetic acid solution. Clear zones on the casein zymograms indicated activation of μ-calpain. Stained protein on the
native gels confirmed the presence of μ-calpain in the gel sample that was used in the casein zymograms when no active μ-calpain was detected. Images were taken using a 16-bit megapixel CCD camera FluorChem880 (Alpha Innotech Corp., San Leandro, CA) and FluorChem IS800 software (Alpha Innotech Corp.).

SDS-PAGE

Gel samples were prepared for SDS-PAGE [25] by diluting samples 70:30 (sample: buffer tracking dye solution [3 mM EDTA, 3% SDS, 20% glycerol, 0.003% pyronin Y, and 30 mM Tris-HCl, pH 8.0]. Samples were run on a 10% polyacrylamide separating gel at a constant voltage of 120 V for approximately 3.5 hours. Gels were stained in 0.1% Coomassie brilliant blue R-250, 40% methanol, and 7% acetic acid solution, and destained in a 40% methanol, 7% acetic acid solution. Differences in autolysis of the 80 kDa subunit of μ-calpain to a 78-kD and a 76-kDa autolysis product were evaluated. Images were taken using a 16-bit megapixel CCD camera FluorChem880 (Alpha Innotech Corp.) and FluorChem IS800 software (Alpha Innotech Corp.).

Incubation of μ-calpain with MAL-PEG

Purified porcine μ-calpain (0.4 units) was incubated in 165 mM NaCl, 50 mM HEPES, pH 6.5. Treatment groups consisted of 1) 1 mM CaCl₂; 2) 0.170 μM H₂O₂ 3) 0.8 units purified porcine calpastatin and 1 mM CaCl₂. Units of calpastatin were determined by
measurement against 0.45 units of m-calpain caseinolytic activity/ml in the assay [19].

Samples were treated with NEM and MAL-PEG according methods of Makmura et al. [26]. Duplicates of each treatment were treated with 20 mM NEM; all samples were incubated on ice for 1 hour. Samples were dialyzed in 50 mM HEPES, pH 6.5 at 4°C for approximately 2 hours (> 1000 x vol) to remove excess NEM. Maleimide-polyethyleneglycol (MAL-PEG; 4 mM final conc.; MW=5000) was added to each treatment and incubated for 1 h on ice. Controls of μ-calpain and μ-calpain and MAL-PEG were incubated in 50 mM HEPES; pH 6.5 on ice for 1 h was used. Samples were aliquoted for SDS-PAGE and western blot analysis.

SDS-PAGE and membrane transfer were conducted as described by Rowe et al. [27] using 9% polyacrylamide separating gels and a μ-calpain primary antibody (monoclonal anti μ-calpain antibody, MA3-940; Affinity Bioreagents, Inc., Golden, CO) for μ-calpain diluted 1:40000 and a secondary antibody (goat anti-mouse conjugated with horseradish peroxidase, catalog no. A2554; Sigma Chemical Company) diluted 1:10000. A sensitive chemiluminescent (ECL Plus kit; Amersham Biosciences, Piscataway, NJ) system was used to detect labeled protein bands using a charged coupled device (CCD) camera (FluroChem 8800; Alpha Innotech Corporation, San Leandro, CA) and FluorChem IS-800 software (Alpha Innotech Corporation, San Leandro, CA). Comparisons were made between treatment groups on molecular weight shifts of μ-calpain.
Results and Discussion

Previous research [13], using purified porcine myofibrils as a substrate, determined that μ-calpain proteolytic activity was inhibited by calpastatin and oxidation as determined by degradation of desmin. Calpastatin inhibited μ-calpain activity in the samples that had not been exposed to H_{2}O_{2}, as shown by an increase in intact desmin. Oxidation with H_{2}O_{2} clearly inhibited desmin degradation by μ-calpain, particularly when no calpastatin was used in the experiment. However, inclusion of H_{2}O_{2} with μ-calpain and calpastatin at pH 6.5 and 7.5 proved to stimulate proteolysis of desmin, indicating increased μ-calpain activity. This lead to the hypothesis that calpastatin, when bound to μ-calpain, can at least partially prevent μ-calpain from being oxidized.

In the NEM treated calpain (Figure 1, Lane 2), the lack of clear zones in casein zymograms indicated that μ-calpain was no longer active, which corresponds to results of Inomata and Kawashima [28], where NEM was used as a calpain inactivator. In the H_{2}O_{2} treated samples (Figure 2, Lane 7), clear zones on the casein zymograms indicate that active μ-calpain was present in the samples, which demonstrates that μ-calpain in the experiments was not irreversibly inactivated, either by autolysis or oxidation. The use of the two different reagents to alter cysteine residues determined if the inhibition of calpain activity occurred and if the inhibition was reversible. The stained native gels (Figures 1A and 2A) correspond to the
casein gels and indicate the presence of μ-calpain in gel samples loaded onto the casein zymograms. SDS-PAGE gels (Figures 1A and 2A) indicate activation of μ-calpain based on autolysis of the large 80 kDa subunit degrading to a 78 kDa and 76 kDa subunit. Autolysis is often used as an indicator of μ-calpain activation and can also directly cause inactivation of μ-calpain [29] as shown in Figure 2A (Lane 10). Incubation of μ-calpain with NEM resulted in loss of μ-calpain proteolytic activity (Figure 1A [Lane 2] and 1B). NEM binds irreversibly to reduced cysteine residues on proteins [30], acting as an alkylating agent. Therefore NEM may bind the active site cysteine residue or possibly to one of the other 10 cysteine residues on μ-calpain, irreversibly preventing activation. When NEM was incubated with μ-calpain in the presence of calcium, autolysis of μ-calpain did not occur (Figure 1A [Lane 2] and 1B), indicating that μ-calpain was not activated and did not become active in the casein zymograms.

Predictably, calpastatin inhibited μ-calpain autolysis. μ-Calpain activity was observed on casein zymograms (Figure 1A [Lanes 5, 7, 9], 1B, and Figure 2A [Lane 1], 2B) after incubation with calpastatin and calcium, and autolysis occurred at a much slower rate than μ-calpain incubated with calcium alone. When NEM was added after the formation of the μ-calpain/calpastatin complex (Figure 1A [Lanes 6, 8, 10] 1B and Figure 2A [Lane 3]), autolysis of μ-calpain did occur to a greater extent compared to when only calpastatin and calcium were
present. Therefore, calpastatin alone inhibited \( \mu \)-calpain autolysis, but the addition of NEM to the reaction after the formation of the \( \mu \)-calpain/calpastatin complex caused autolysis to occur to a greater extent and autolytic inactivation of \( \mu \)-calpain did occur. This is an interesting observation in that NEM alone caused complete loss of \( \mu \)-calpain activity and prevented autolysis, calpastatin prevented autolysis, but after formation of the \( \mu \)-calpain/calpastatin complex, incubation with NEM created conditions that promoted autolysis of \( \mu \)-calpain to occur. In Maddock et al. [13], calpastatin was incubated with NEM (0, 4, 8, or 12 mM final concentration) to determine the effect of a cysteine modifier on calpastatin activity. Specific activity of calpastatin was not changed by exposure to NEM. Therefore calpastatin, if not in formation with calpain, is not affected by exposure to an irreversible cysteine modifier [13].

Incubation of \( \mu \)-calpain with \( H_2O_2 \) resulted in no irreversible loss of proteolytic activity as shown in casein zymograms after reduction of samples (Figure 2A [Lane 7] and 2B), which is in contrast with the NEM incubations, where proteolytic activity was not observed in the casein zymograms. Oxidation with \( H_2O_2 \) is reversible when exposed to a reducing agent, as was done in the casein zymogram samples. When calcium was used in the incubation of \( \mu \)-calpain with \( H_2O_2 \), autolysis did occur (Figure 2 [Lane 2] and 2B) which is similar to results observed by Guttmann et al. [16] and autolytic inactivation of \( \mu \)-calpain activity was observed. Addition of \( H_2O_2 \) after formation of the \( \mu \)-calpain/calpastatin complex resulted in \( \mu \)-calpain
autolysis (Figure 2 [Lanes 5 and 9] and 2B). The result is consistent with the result when NEM is added to the \(\mu\)-calpain/calpastatin complex. Conversely, when \(H_2O_2\) was added to \(\mu\)-calpain before the addition of calpastatin and calcium (Figure 2 [Lanes 6 and 8] and 2B), no autolysis of \(\mu\)-calpain was observed and proteolytic activity was still apparent on the casein zymograms, which in combination indicated that \(\mu\)-calpain had not been irreversibly inactivated. Collectively, the results indicated that oxidation of the \(\mu\)-calpain/calpastatin complex promotes autolysis, activation, and subsequent autolytic inactivation of \(\mu\)-calpain. This conclusion is consistent with the results discussed in Maddock et al. [13] that indicated greater proteolytic activity and autolysis of \(\mu\)-calpain occurred in the presence of calpastatin and \(H_2O_2\). Conversely, exposure of \(\mu\)-calpain to a cysteine modifier (NEM or \(H_2O_2\)) before exposure to calpastatin inhibits autolysis of calpain. Interestingly, the reagents used in the study appeared to affect the \(\mu\)-calpain/calpastatin complex similarly, but when used alone, NEM causes complete and irreversible loss of proteolytic activity, whereas \(H_2O_2\) inactivation of \(\mu\)-calpain is reversible and proteolytic activity is recovered if reducing conditions are introduced. The reversibility of \(H_2O_2\) oxidation indicates that the oxidation that occurred was probably conversion of a cysteine thiol to sulfenic acid [16]. Sulfenic acid is a product of \(H_2O_2\) oxidation that is easily reduced back to a sulfhydryl group if proper reducing conditions are introduced.
Oxidation has been shown to decrease activity of calpains, but in contrast, calpains have also been shown to play a role in protein degradation in vivo when oxidative conditions are higher than normal. A relationship between protein oxidation and proteolysis has been clearly established [31] where oxidation of proteins can enhance susceptibility to proteolytic degradation, but at the same time, excessive oxidation may cause a decrease in susceptibility to degradation. The increase in susceptibility has been discussed in regard to the proteosome system, which has a preference to bind hydrophobic residues [32]. Oxidation of proteins causes an exposure of hydrophobic residues [33] increasing its susceptibility to the proteosome system. It is hypothesized that the proteosome is necessary in the removal of oxidatively modified or damaged proteins [31]. So far, no studies exist showing direct recognition of oxidized substrates causing increased susceptibility to calpain [31]. However, oxidative stress, in combination with observed increased intracellular Ca\(^{2+}\) concentration in a mouse embryonal carcinoma cell line [34] and a rat adrenal gland cell line [35] have shown an increase in activation of calpains in general. The activation of calpains in these instances is possibly linked to the increase in intracellular calcium, rather than an increase in the susceptibility of the calpain substrates to degradation. However, Pronzato et al. [36] observed the level of oxidative stress can affect calpain activity, where moderate levels of oxidation enhance activity but increased oxidative exposure can cause inactivation of calpains. Based on the
results of Saurin et al. [16], moderate levels of oxidation is defined as a high enough concentration of H$_2$O$_2$ to affect protein activity through formation of sulfenic acid, which is a reversible modification. Higher concentrations or increased exposure of H$_2$O$_2$ to proteins allows for production of sulfinic acid and sulfonic acid [16]. The formation of these acids is irreversible, thus irrevocably changing protein functionality. These observations could provide an explanation of how the involvement of calpastatin regulates oxidation of μ-calpain and allows activation to occur.

Previous research has evaluated the effect of ionic strength on μ-calpain activity [22] where it was determined that higher ionic strength decreased μ-calpain activity. A comparison of treatments at two different ionic strengths, 165 mM NaCl and 295 mM NaCl, is shown in Figure 3. Results indicate that the higher ionic strength slowed autolysis of μ-calpain in the presence of H$_2$O$_2$ and calcium (Figure 3, Lanes 5A and 5B). The higher ionic strength also caused greater autolysis to occur in the presence of calpastatin (Figure 3, Lanes 3A and 3B). Geesink and Koohmaraie [37] indicated that a decrease in activity of μ-calpain at higher ionic strengths is the result of a decrease in the stability of the molecule. Li et al. [38] hypothesized a higher ionic strength causes dissociation of the heterodimeric subunits of μ-calpain, which allow for formation of dimers and trimers of the large subunit, subsequently inactivating the proteinase. These results indicate that differences in ionic strength have the potential to affect
activity of μ-calpain through autolytic inactivation and dissociation. Differences in ionic strength did not have any apparent effects on the effects of oxidation on μ-calpain and calpastatin.

To more closely evaluate the role of calpastatin in the activation of μ-calpain in the presence of H₂O₂, three different calpastatins were evaluated (Figure 4). The first is the purified porcine calpastatin used in the previous experiments, the second is an expressed peptide fragment from domain I of human calpastatin, and the third is synthetic peptide consisting of 26 amino acids that replicate the homologous sequence of amino acids found in subdomain B of the inhibitory calpastatin domains. Uemori et al. [39] determined that subdomain B is essential for inhibitory activity of calpastatin to occur. A similar peptide to the one in this study was determined to bind to a highly conserved region of domain III of μ-calpain [40] indicating that this peptide does not block to active site where the cysteine residue is located. The three forms of calpastatin were used on an equal activity basis, which was determined using a casein assay [20] against μ-calpain. Calpastatin was added to the incubations at twice the amount of μ-calpain activity to maximize calpastatin interaction with μ-calpain. Autolysis was observed (Figure 4, Lanes 2, 12) when μ-calpain was incubated with porcine calpastatin, whereas domain I calpastatin (Figure 4, Lane 4) completely inhibited autolysis of μ-calpain. Autolysis stopped at the 78 kDa band (Figure 4, Lane 14) when μ-
calpain was incubated with the calpastatin peptide 26mer. When these calpastatins were tested similarly to the previously described experiment with H$_2$O$_2$ added after the calpastatin in order to allow formation of the $\mu$-calpain/calpastatin complex (Figure 4, Lanes 5 and 15), autolysis of porcine $\mu$-calpain occurred and $\mu$-calpain activity was decreased. When H$_2$O$_2$ was added before calpastatin (Figure 4, Lanes 8 and 18), autolysis of $\mu$-calpain did occur but was slower than when H$_2$O$_2$ was added after the calpastatin. However, no differences were observed due to order of H$_2$O$_2$ addition in $\mu$-calpain autolysis or activity when the domain I (Figure 4, Lanes 6, 9, 16, 19) or calpastatin peptide (Figure 4, Lanes 7, 10, 17, and 20) were used regardless of when or if H$_2$O$_2$ was added to the assay. This is consistent with the results described by Croall and McGrody [40] where the peptide used in their study does not bind at the active site and the results described by Guttmann et al.[15], where H$_2$O$_2$ did not inhibit autolysis and proteolytic activity is recoverable. To summarize, the porcine calpastatin prevented autolysis, but complete autolysis did occur if H$_2$O$_2$ was added, the domain I calpastatin prevented any autolysis from occurring, even when exposed to H$_2$O$_2$, and the peptide fragment permitted autolysis to occur to only the 78 kDa band regardless of H$_2$O$_2$ treatment. The differences observed in the different calpastatin molecules could be due to the interaction of calpastatin with $\mu$-calpain at sites other than the subdomain B inhibitory region. Subdomains A and C in the calpastatin molecule were not present in the peptide fragment. The lack of these
subdomains could explain why autolysis occurred when the peptide fragment was used. The roles of subdomains A and C in calpain inhibition have been attributed to stabilizing the calpain molecule by binding to domains IV and VI [9] and increasing calpain inhibition [41]. However, the peptide fragment only allowed autolysis of μ-calpain to the 78 kDa form.

Melloni et al. [42] determined that calpastatin was a better inhibitor of autolysis of μ-calpain from the 78 kDa to the 76 kDa form. The differences observed with the purified porcine calpastatin and domain I calpastatin may be contributed to the fact that the purified porcine calpastatin is a larger molecule, which may affect how H₂O₂ interacts with μ-calpain when compared to the smaller domain I calpastatin. The larger molecule may physically prevent oxidation of another cysteine residue that is required to be reduced for activation in comparisons to smaller domain I of calpastatin. The larger calpastatin molecule may also be a target for NEM and H₂O₂ when bound to μ-calpain, thus altering their interaction, thereby decreasing the inhibition of μ-calpain.

The use of MAL-PEG is to evaluate tagging of reduced cysteine residues as indicated by a molecular weight shift when run on SDS-PAGE. Maleimides covalently bind to free sulfhydryl groups on proteins [26], and have the highest affinity for cysteine residues. Results indicate the MAL-PEG will tag μ-calpain with or without the presence of calcium (Figure 5, Lanes 2 and 3). Additionally, μ-calpain oxidized with H₂O₂ was also tagged with MAL-PEG,
indicating that not all cysteine residues are altered by H₂O₂. It has been determined that H₂O₂ does inhibit μ-calpain activity, but it does not inhibit autolysis (Figure 2, Lane 2), which corresponds to Guttmann et al. [15]. Therefore, it is likely that cysteine residues other than the active site cysteine are the targets of oxidation, and may affect proteolytic function of μ-calpain by altering its ability to target its substrates or interaction to calpastatin, but not preventing activation, which allows for autolysis to occur. Calpastatin prevented MAL-PEG from tagging μ-calpain (Figure 5, Lane 5) indicating that calpastatin blocks or essentially “protects” μ-calpain from being targeted by cysteine modifiers NEM or MAL-PEG.

Oxidative processes of living cells are balanced with the antioxidant systems in cells, but the oxidative processes can cause production of reactive oxygen species. These ROS will react with lipids and proteins found in the tissues. Specifically, oxidation of proteins can alter function, but prevention or regulation of oxidation of proteins like calpain can regulate calpain activity. Results from this study indicate that calpastatin may play a role in the regulation of calpain oxidation. Calpastatin prevents cysteine modification of μ-calpain, thus preventing inactivation of μ-calpain. Additionally, exposure of the μ-calpain/calpastatin complex to a cysteine modifier decreases the inhibition of μ-calpain by calpastatin, indicating that calpastatin, when bound to μ-calpain may also be a target of NEM and H₂O₂. Further research must be conducted to elucidate the mechanism of the interaction of μ-calpain with calpastatin,
and additionally, the importance of a reducing environment on calpain activation needs to be clarified as cysteine residues other than the active site cysteine may be involved. The data from this study indicate that irreversible modification of a μ-calpain cysteine residue prevents μ-calpain activation and autolysis. Additionally calpastatin, in an intact form containing more than one inhibitory domain, allows for activation of μ-calpain when exposed to a cysteine modifier. Calpastatin, therefore, may regulate calpain activity related to pathological conditions where oxidative stress is prevalent such as ischemia, cataract formation, and Alzheimer’s disease.

References


14 Neumann, N.P. (1972) Oxidation with hydrogen peroxide. Meth. Enzym. 25, 393-400


Figure 1. \( \mu \)-calpain incubations with NEM at pH 6.5 (n=3).

<table>
<thead>
<tr>
<th>Treatment (Lane on gels)</th>
<th>Proteolytic activity</th>
<th>Autolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>C) ( \mu )-calpain (1) B</td>
<td>+++</td>
<td>---</td>
</tr>
<tr>
<td>1) ( \mu )-calpain + CaCl(_2) (3)</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>2) ( \mu )-calpain + NEM (2)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3) ( \mu )-calpain + NEM + CaCl(_2) (4)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>4) ( \mu )-calpain + calpastatin + CaCl(_2) (5,7,9)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>5) ( \mu )-calpain + calpastatin + CaCl(_2) + NEM (6,8,10)</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

\( ^{a,b,c} \) Proteolytic activity of \( \mu \)-calpain is depicted by casein zymogram and autolysis of \( \mu \)-calpain is depicted by SDS-PAGE gel. Native gel is used to show location of protein on casein zymograms.
Figure 2. μ-calpain incubations with H₂O₂ at pH 6.5 (n=3)

<table>
<thead>
<tr>
<th>Treatment (Lane on gels)</th>
<th>Proteolytic activity</th>
<th>Autolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>C) μ-calpain (4)</td>
<td>+++</td>
<td>---</td>
</tr>
<tr>
<td>1) μ-calpain + CaCl₂ (10)</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>2) μ-calpain + H₂O₂ (7)</td>
<td>+++</td>
<td>---</td>
</tr>
<tr>
<td>3) μ-calpain + H₂O₂ + CaCl₂ (2)</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>4) μ-calpain + calpastatin + CaCl₂ (1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5) μ-calpain + calpastatin + CaCl₂ + NEM (3)</td>
<td>--</td>
<td>++</td>
</tr>
<tr>
<td>5) μ-calpain + calpastatin + CaCl₂ + H₂O₂ (5, 9)</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>6) μ-calpain + H₂O₂ + calpastatin + CaCl₂ (6, 8)</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

a,b,c Protolytic activity of μ-calpain is depicted by casein zymogram and autolysis of μ-calpain is depicted by SDS-PAGE gel. Native gel is used to show location of protein on casein zymograms.
Figure 3. SDS-PAGE of μ-calpain incubations at A) 165 mM NaCl and B) 295 mM NaCl.
Figure 4. Casein zymograms and SDS-PAGE of μ-calpain incubations with three forms of calpastatin at pH 6.5.

<table>
<thead>
<tr>
<th>Treatment (Lane on gels)</th>
<th>Proteolytic activity</th>
<th>Autolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>C) μ-calpain (1,11)</td>
<td>+++</td>
<td>---</td>
</tr>
<tr>
<td>1) μ-calpain + CaCl₂ (2, 12)</td>
<td>-/sl. +</td>
<td>+++</td>
</tr>
<tr>
<td>2) μ-calpain + calpastatin + CaCl₂ (3, 13)</td>
<td>+/-</td>
<td>++</td>
</tr>
<tr>
<td>3) μ-calpain + domain I + CaCl₂ (4)</td>
<td>+++</td>
<td>---</td>
</tr>
<tr>
<td>4) μ-calpain + calpain inhibitor peptide + CaCl₂ (14)</td>
<td>+++</td>
<td>+ to 78</td>
</tr>
<tr>
<td>5) μ-calpain + calpastatin + CaCl₂ + H₂O₂ (5, 15)</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>6) μ-calpain + H₂O₂ + calpastatin + CaCl₂ (8, 18)</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>7) μ-calpain domain I + CaCl₂ + H₂O₂ (6, 16)</td>
<td>++</td>
<td>---</td>
</tr>
<tr>
<td>8) μ-calpain + H₂O₂ + domain I + CaCl₂ (9, 19)</td>
<td>+</td>
<td>---</td>
</tr>
<tr>
<td>9) μ-calpain + calpain inhibitor peptide + CaCl₂ + H₂O₂ (7, 17)</td>
<td>++</td>
<td>+ to 78</td>
</tr>
<tr>
<td>10) μ-calpain + H₂O₂ + calpain inhibitor peptide + CaCl₂ (10, 20)</td>
<td>-/+</td>
<td>+ to 78</td>
</tr>
</tbody>
</table>
Figure 5 Western blot of μ-calpain tagged with MAL-PEG.

1 2 3 4 5 6 7 8 9

80 kDa
78 kDa
76 kDa
GENERAL SUMMARY

The intracellular environment of postmortem muscle changes dramatically in the first 24 hours due to loss of homeostasis. These cellular environmental changes include a pH decline from near neutral to approximately 5.6, an increase in ionic strength from an equivalent of 165 mM NaCl to approximately 295 mM NaCl and an increase in lipid and protein oxidation. Previous studies have determined the extent to which effects of pH, ionic strength, and oxidation influence calpain activity. Many of these studies have included the effects of calpastatin on calpain under these varying environments. The majority of these studies only evaluated one or two environmental conditions, but as more is understood about the calpain system, the interactions of these environments can impact calpain activity in a much different way.

Our μ- and m-calpain activity assays using a fluorescent peptide as a substrate demonstrate that there are differences in activation of μ-calpain and m-calpain and their inhibition by calpastatin. μ-Calpain activity was greatest at pH 6.5 and m-calpain activity was greatest at pH 7.5, and m-calpain activity was not detected at pH 6.0. Inhibition of μ-calpain by calpastatin was higher ($P < 0.01$) in 295 mM NaCl than 165 mM NaCl when 0.3 units of calpastatin were included in the assay. Inhibition of m-calpain was greater ($P < 0.01$) when calpastatin was added at pH 6.5 than 7.5 at 165 mM NaCl, whereas percent inhibition of m-
calpain was greater \( (P < 0.01) \) at 295 mM than 165 mM NaCl at pH 7.5 and 6.5. The effects of pH and ionic strength on calpain activity and calpain inhibition by calpastatin support the hypothesis that \( \mu \text{-calpain} \) and not \( \mu \text{-calpain} \) is most likely to be active in postmortem muscle. Oxidation decreased activity of both \( \mu \)- and \( \mu \)-calpain, but oxidation decreased inhibition of \( \mu \) - and \( \mu \)-calpain by calpastatin indicating that \( \text{H}_2\text{O}_2 \) was either not affecting calpain in the presence of calpastatin, or \( \text{H}_2\text{O}_2 \) was directly affecting calpastatin inhibition of calpains.

When the same conditions of pH, ionic strength, and oxidation on \( \mu \)- and \( \mu \)-calpain activity in the presence of calpastatin were evaluated in degradation of desmin, similar effects of oxidation in the presence of calpastatin were observed for \( \mu \)-calpain at pH 7.5 and 6.5. Oxidation decreased proteolytic activity of \( \mu \)-calpain against desmin at pH 6.0 \( (P < 0.05 \text{ at } 15, 60, \text{ and } 120 \text{ min}) \) and \( \mu \)-calpain at all pH conditions. However, inhibition of \( \mu \)-calpain degradation of desmin by calpastatin was decreased by oxidation at pH 7.5 and 6.5 at both ionic strengths \( (P = 0.03) \). The results suggest that, at the higher pH conditions used (pH 7.5 and 6.5), calpastatin may limit the possibility of oxidation-induced inactivation of \( \mu \)-calpain.

The results from these first experiments described in Chapter 2 and Chapter 3 lead to the hypothesis of the third paper that calpastatin was protecting \( \mu \)-calpain from \( \text{H}_2\text{O}_2 \) oxidation of the active site cysteine residue, thereby allowing \( \mu \)-calpain to become active. Experiments were summarized in Chapter 4 used cysteine modifiers N-ethylmaleimide and \( \text{H}_2\text{O}_2 \) on \( \mu \)-
calpain at pH 6.5. In evaluation of activity and autolysis of μ-calpain, calpastatin was either incubated with μ-calpain before or after NEM or H₂O₂. Results indicated that the cysteine modifiers will inhibit μ-calpain activity completely. NEM inhibition of μ-calpain activity is irreversible, while H₂O₂ inhibition was reversible. If calpastatin was present before the addition of the cysteine modifiers, activation of μ-calpain occurred. Maleimide tagged with polyethyleneglycol (MAL-PEG) was used in the incubations with μ-calpain in order to determine if there is an available cysteine residue for tagging that causes a molecular weight shift in a SDS-PAGE. Calpastatin prevented tagging of μ-calpain with MAL-PEG indicating that calpastatin is essentially protecting a μ-calpain cysteine residue from being altered and thus preventing μ-calpain inactivation. When two alternate forms of calpastatin were used to evaluate differences due to H₂O₂, the calpastatin containing only domain I did prevent autolysis regardless of the oxidative conditions, and the calpastatin peptide fragment containing subdomain B allowed autolysis to occur to the 78 kDa fragment of μ-calpain regardless of the oxidative conditions, but complete autolysis of μ-calpain was not observed. The purified porcine calpastatin used in this experiment and all previous experiments is a much larger molecule than the two alternative calpastatins used. The MAL-PEG experiment determined that the larger form calpastatin blocks μ-calpain cysteine modification, however, calpastatin did not prevent μ-calpain activity when incubated with NEM or H₂O₂, indicating
that calpastatin is probably affected by these cysteine modifiers when in a complex with μ-calpain.

These studies show that cellular environment have a large effect on μ-calpain activity. Conditions used in these experiments are relevant in both living and postmortem muscle. The differences evaluated due to interactions of pH, ionic strength, and oxidation on μ-calpain activity in the presence of calpastatin provide a basis for understanding mechanisms controlling μ-calpain activity in not only postmortem muscle, but the action of μ-calpain in living cells. The role of calpastatin in μ-calpain activity appears to be more regulatory rather than inhibitory under oxidizing conditions. Calpastatin prevented targeting of μ-calpain cysteine residues and allowed μ-calpain activity under oxidizing conditions of NEM or H₂O₂. The regulatory role of calpastatin on calpain activity starts to explain calpain action in conditions where oxidation is a factor such as ischemia, cataract formation, and Alzheimer’s disease.
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