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The relationship between the calpain enzyme system and the postmortem degradation of selected myofibrillar proteins

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The relationship between the calpain enzyme system and the postmortem degradation of selected myofibrillar proteins.

by

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1995
EFFECTS OF THE PROTEASE μ-CALPAIN ON PURIFIED MYOFIBRILS AT PHYSIOLOGICAL TEMPERATURE UNDER SELECTED CONDITIONS OF CALCIUM CONCENTRATION, PH, AND IONIC STRENGTH ................................................................. 194

Summary .................................................................................................................. 195
Introduction ............................................................................................................. 196
Materials and methods ........................................................................................ 197
Results .................................................................................................................... 203
Discussion .............................................................................................................. 211
Conclusions ........................................................................................................... 217
Acknowledgments ................................................................................................. 217
Literature cited ....................................................................................................... 217

GENERAL SUMMARY ............................................................................................. 250

ACKNOWLEDGMENTS ........................................................................................... 254
GENERAL INTRODUCTION

The postmortem degradation of skeletal muscle proteins and their relationship to meat tenderness and quality has been the subject of much research. Several proteins that have been examined include: titin (Lusby et al., 1983; Anderson and Parrish, 1989; Fritz and Greaser, 1991; Huff-Lonergan et al., 1995), nebulin (Anderson and Parrish, 1989; Fritz and Greaser, 1991; Taylor et al., 1995), filamin (Uytterhaegen et al., 1992), desmin (Whipple et al., 1990; Koohmaraie et al., 1991) and troponin-T (Olson et al., 1977; Whipple and Koohmaraie 1992; Ho et al., 1994). While most of these proteins have been shown to undergo postmortem proteolysis, specific degradation products of these proteins (with the exception of troponin-T) have not always been carefully identified. SDS-PAGE techniques combined with Western blotting procedures provide sensitive methods to identify many degradation products from these proteins (Bandman and Zdanis, 1988).

Two of the most difficult proteins to monitor using SDS-PAGE and Western blotting techniques are the large proteins titin and nebulin (Fritz et al., 1989). Several different methods have been used to try to optimize SDS-PAGE techniques to study the variation in titin and nebulin degradation as it relates to meat tenderness (Fritz et al., 1989; Granzier and Wang, 1993). In addition to differences in SDS-PAGE techniques, some controversy exists over whether or not sample preparation (whole muscle homogenates vs. purified myofibrils) influences the interpretation of results that relate the degradation of titin and nebulin to meat tenderness (Fritz and Greaser, 1993; Huff-Lonergan et al., 1995).

The protease μ-calpain has been implicated as being the causative agent for many of the proteolytic changes that occur as meat is aged. μ-Calpain is a low
calcium requiring member of the calpain "family". Calpains are calcium-dependent proteases that are found in nearly every vertebrate tissue that has been tested for their existence (Goll et al., 1992).

In living muscle systems, the calpains have been hypothesized to be instrumental in myofibrillar protein turnover and muscle growth (Goll et al., 1991). In meat that has been held for a period of time after slaughter (postmortem aged), the calpains (especially μ-calpain), through their action on specific myofibrillar proteins, have been hypothesized to enhance the tenderness of whole muscle meat products (Koohmaraie, 1992). Many of the same proteins that are degraded in postmortem tissue are also substrates for the calpains (Olson et al., 1977; Ouali et al., 1990; Koohmaraie et al., 1986). The proteins myosin and actin, which are not substrates for the calpains are also not degraded in postmortem muscle at low temperature (Bandman and Zdanis, 1988).

This project was developed to answer several questions concerning the role of μ-calpain in myofibrillar protein degradation. The objective of the study was several-fold. First, it was desired to determine if the evaluation of the degradation of the large proteins titin and nebulin is affected by the sample preparation method used (purified myofibrils vs. whole muscle homogenates). The second objective was to identify the naturally occurring postmortem degradation products of the myofibrillar proteins titin, nebulin, filamin, desmin, and troponin-T, and to compare these degradation products with those formed when purified myofibrils were incubated with purified μ-calpain under conditions of low temperature and pH. Finally, it was desired to determine the combined effects of selected temperatures, pH values, ionic strengths and calcium levels on the ability of μ-calpain to degrade key myofibrillar proteins.
Dissertation organization

This dissertation is in an alternate style format consisting of an abstract, a general introduction, a general review of literature, four papers prepared for publication, and a concluding summary. The four papers represent the work done by the first author to fulfill requirements for the degree of Doctor of Philosophy. The first two papers were prepared according to the *Journal of Animal Science Style and Form* guide. These papers consist of an Abstract, Introduction, Materials and Methods, Results, Discussion, Implications and Literature Cited sections. The third and fourth papers were prepared for submission to Biochimie and follow the *Instructions to Authors* published by the journal. These papers consist of a Summary, Introduction, Materials and Methods, Results, Discussion, Conclusions and Literature Cited.
GENERAL REVIEW OF LITERATURE

The basic structure of muscle

Muscle is one of the most complex and highly organized tissues found in nature. The details of muscle structure and function play a vital role in determining the quality and palatability attributes of meat and meat products. A knowledge of the structure of muscle is necessary for the understanding of how and why some of these differences in quality (especially tenderness) occur. A short discussion of the structure of muscle follows.

Muscle tissue is classified into three categories: striated, cardiac and smooth. When looked at under the microscope both striated and cardiac muscle exhibit a banding pattern that is transverse to the long axis of the muscle fiber. Smooth muscle does not show this banding pattern. Both smooth and cardiac muscle are classified as involuntary as their function is not normally consciously controlled by the animal. Skeletal muscle is made up of striated muscle. Skeletal muscle is classified as voluntary because it generally can be controlled by the will of the organism (Judge et al., 1989; Pearson and Young, 1989). Because skeletal muscle is the primary tissue that comprises the meat that is consumed, the remainder of the discussion will focus on it.

Skeletal muscle organization  Skeletal muscles can be divided into different “levels” of organization. For the most part, each level is delineated by a sheath of connective tissue. The outer covering of muscle is a thin sheet of connective tissue known as the epimysium. The muscle itself is comprised of a number of structures known as muscle bundles. These bundles are covered by a sheath of connective tissue called the perimysium. Muscle bundles are made up of structures known as muscle fibers. These fibers are long, cylindrical,
multinucleated cells that can be several centimeters long. The diameter of these cells can range from 10 \( \mu \text{m} \) to greater than 100 \( \mu \text{m} \) (Judge et al., 1989). The outer cell membrane of the muscle fiber is known as the sarcolemma. The sarcolemma is in turn, surrounded by a connective tissue sheath known as the endomysium. These different levels of connective tissue (epimysium, perimysium and endomysium) provide a framework for the organization of the entire muscle (Cassens, 1987).

The main functional unit of the muscle fiber is known as the myofibril. The myofibrils are long, cylindrical organelles that average 1-2 \( \mu \text{m} \) in diameter and extend the entire length of the muscle fiber. Within the myofibril is an array of interdigitating thick and thin filaments. These two sets of filaments are aligned parallel to each other. The thick and thin filaments overlap each other in specific regions giving the muscle fiber its typical banding pattern or striated appearance. This striation appears as regions of light and dark bands. The light bands are described as being isotropic when viewed using polarized light and so they are termed \( \text{i-bands} \). The \( \text{i-bands} \) are primarily made up of thin filaments. The dark bands are anisotropic under the same conditions, and so are termed the \( \text{A-bands} \). The \( \text{A-bands} \) are primarily made up of thick filaments. Bisecting the \( \text{i-bands} \) is a dark band known as the Z-line. The region between two Z-lines is termed the sarcomere. Within the sarcomere there is one A-band located between two half \( \text{i-bands} \). This structure is repeated through the myofibril. In resting muscle, the typical length for a sarcomere is 2.5 \( \mu \text{m} \). The A-band is bisected by a narrow band known as the M-line. In the center of the A-band is a slightly lighter region known as the H-zone (Huxley, 1965).
In normal skeletal muscle cells, the thick filaments are approximately 14-16 nm in diameter and are 1.5 μm long. These filaments are primarily made up of approximately 300 myosin molecules. Myosin molecules are rod shaped (tail region) with a globular two headed region on one end. In the thick filament, the myosin molecules are arranged in bundles with the tail regions making up the main shaft of the thick filament. The head regions of the myosin molecules project outward from the main body of the thick filament. The myosin filaments are arranged so that the heads are oriented toward the two ends of the thick filaments, leaving a bare zone in the middle of the filament. Myosin is not the only protein found in the thick filament. The proteins C-protein, myomesin, creatine kinase, 86 kDa protein, X-protein, and H-protein among others are also found in or associated with the thick filament (Pearson and Young, 1989).

The thin filaments of muscle average 6-8 nm in diameter and are approximately 1.0 μm in length. The primary protein that is found in the thin filaments is the protein actin. G-actin (globular form) has a molecular weight of approximately 42,000. The actin molecules themselves are spherical in shape and are arranged in twin strands that are twisted around each other to form the main portion or ‘back-bone” of the thin filament. These actin particles seem to have a “front” and a “back” giving the entire filament directional polarity. The thin filaments are anchored to the Z-line and are attached to the thin filaments on the opposite side of the Z-line in an array of cross connections that give the Z-line its characteristic zigzag pattern (Pearson and Young, 1989).

The thin filaments of muscle also contain the proteins tropomyosin and troponin. Tropomyosin is the second most abundant protein in the thin filament, accounting for approximately 5% of the total protein in the myofibril. Tropomyosin
is made up of two chains that have an approximate weight of 34,000 each. The native tropomyosin molecule has a molecular weight of approximately 68,000 and has a length of 40 nm. In the myofibril, tropomyosin molecules are found as long, thin strands on the actin filaments. The tropomyosin molecules are located near the groove between the paired strands of actin molecules (Murray and Weber, 1974).

Troponin is a complex made up of three subunits, troponin-C (MW 18,000), troponin-I (MW 23,000) and troponin-T (MW 37,000) (Flicker et al., 1982). The three subunits have separate biochemical properties. Troponin-C has the function of binding calcium. Troponin-I can inhibit the interaction between myosin and actin, while troponin-T binds strongly to tropomyosin (Pearson and Young, 1989). The overall shape of the troponin molecule is thought to contain both a globular region as well as a rod-like portion. The total length of the molecule is about 26.5 nm. The rod-like portion comprises around 16.5 nm of the length (Flicker et al., 1982). The entire troponin molecule interacts with approximately one-third of the tropomyosin molecule.

The contraction of muscle The basic principle in contraction involves the shortening of the sarcomere and thus ultimately the muscle by the sliding of the thin filaments past the thick filaments. The force that causes this sliding is generated by the formation and dissolution of cross-bridges between the thick and thin filaments. The myosin heads attach to specific sites on the actin filament and then swivel. This movement draws the thin filaments past the thick filaments before the heads detach and attach again. This cycle of attaching and detaching causes a relative movement of the thin and thick filaments by about 100 angstroms (Murray and Weber, 1974).
Regulation of contraction is accomplished by the actin-troponin-tropomyosin system, as well as by the concentration of ATP and calcium in the myofibril. Phillips et al. (1986) discussed the interaction of this system. In the relaxed state (off-state) the troponin complex binds the tropomyosin and holds it on the outer part of the actin helix thus prohibiting the interaction of myosin and actin. When the concentration of Ca\(^{2+}\) reaches the proper levels the troponin complex binds to the calcium and releases the tropomyosin from its position on the actin strand. The filaments are then in the "on" or active position and the myosin heads are able to bind to actin.

The energy for contraction is provided by the hydrolysis of adenosinetriphosphate (ATP) into two lower energy compounds adenosinediphosphate (ADP) and inorganic phosphate. This hydrolysis reaction takes place on the head of the myosin molecule. The reaction occurs as follows as described by Murray and Weber (1974). ATP has a very strong affinity for myosin and binds to the head very readily. The myosin-ATP complex is then raised to a charged intermediate form that can then bind to actin. Hydrolysis can occur releasing ADP and inorganic phosphate and energy to power contraction. The myosin-actin complex (rigor complex) is maintained until ATP is again bound to myosin and the sequence can be repeated again.

Myofibrillar proteins possibly involved in tenderness

**Titin**  
Titin was first discovered in 1979 (Wang et al., 1979). The protein was described as being extremely large and showed up on 4% polyacrylamide gels as a doublet with an approximate molecular weight of 1 x 10^6. The protein was named titin after the Greek word meaning anything of great size. In this initial
report, an antibody to titin was found to react with the myofibril in specific locations. The antibody reacted strongly at the A-I junction and in the central region of the A-band. It was also noted that weak staining was seen throughout the entire A-band. In addition, the M and Z lines also showed some labeling. It was suggested in this early paper that titin was found in striated muscle (both skeletal and cardiac) and in both vertebrate and invertebrate species.

Maruyama et al. (1976; 1977) had earlier reported the isolation of an elastic and highly insoluble mixture of protein which they named connectin. King and Kurth (1980) showed that this preparation consisted of some protein polypeptides that were larger than myosin. Maruyama et al. (1981) reported that their connectin preparation showed strong immunofluorescence in the A-band and at the Z-line of the myofibril, while the I-bands showed weak staining. These results led them to the conclusion that connectin was located throughout the entire myofibril. It was shown (Maruyama et al., 1981) that this mixture contained the same extremely high molecular weight polypeptide as was found in Wang's original titin preparation.

Titin is the third most abundant protein found in the myofibril (Trinick et al., 1984) making up 8%-10% of the total protein in the myofibril. Titin is third in abundance behind myosin (which makes up approximately 45% of the protein in the myofibril) and actin (which makes up approximately 20% of the protein in the myofibril). While not found to exist in smooth muscle, titin is found in striated muscle, both skeletal and cardiac muscle (Maruyama et al., 1977). Titin is the largest protein yet discovered in nature. The molecular mass of the intact form of titin (T1 also known as α-connectin) has been estimated to be in the neighborhood of 2.8-3 x 10^6 daltons, while its major degradation product T2 (also known as β-connectin) has been estimated to be approximately of 2.6 x 10^6 daltons. Size
variants of the titin molecule have been reported to exist and the variation in size seems to be dependent upon the muscle in which it is found (Wang et al., 1991). The extreme size of titin is probably one of the major reasons that it was not discovered until the late 1970's. Most of the gel systems that were in use prior to that time were insufficient to resolve the protein due to the small pore size of these gels. Clear resolution of titin requires the use of low percentage SDS-PAGE gels (Granzier and Wang, 1993; Huff-Lonergan et al., 1995) as well as very high ratios of acrylamide to bis-acrylamide ranging from 100:1 to 200:1 (Fritz et al., 1989; Huff-Lonergan et al., 1995).

Titin is a highly insoluble protein that is difficult to isolate using normal solvents. In order to extract the intact form of the protein, denaturants such as SDS and/or urea must be used (Wang, 1982; Kimura et al., 1992). T2, on the other hand, can be extracted without using denaturing solvents. Native titin, as this preparation is often called, has been reported to be extracted using solvents such as 0.6 M KCl, in a sodium phosphate buffer at pH 7.0 (Soteriou et al., 1993).

Titin appears to be a “string-like” molecule that has been shown to be very long and extended (Maruyama et al., 1984; Trinick et al., 1984; Wang et al., 1984). Titin is 4-5 nm in diameter and over 1 µm in length (Furst et al., 1988; Nave et al., 1989; Suzuki et al., 1994)

**Localization of titin** Long before titin was first purified in 1979 by Wang et al., the presence of a third set of filaments was suggested by electron microscopy studies. These filaments were described by several names including gap filaments (Sjostrand, 1962; Locker and Leet, 1976), S-filaments (Huxley and Hanson, 1954), connecting filaments (Pringle, 1977) and super thin filaments (McNeill and Hoyle, 1967; Walcott and Ridgeway 1967).
Immunofluorescence has proven to be an invaluable tool in localizing titin in the sarcomere. Many of the polyclonal (as well as monoclonal) antibodies that have been raised against titin bind to the region of the myofibril known as the A-I junction (Fulton and Isaacs, 1991). Monoclonal antibodies have been developed that recognize unique epitopes. These antibodies have shown that titin extends from the M-line at the center of the sarcomere to the Z-line (Furst et al., 1988; Furst et al., 1989; Whiting et al., 1989). Research has shown that the N-terminus of titin is localized at the Z-line and the C-terminus is at or near the M-line (Labeit et al., 1992). This data combined with the proposed length of the titin molecule (> 1 μm) would allow for two titin molecules to span the distance from one Z-line to the next.

**Structure of titin** A portion of the titin molecule has been cloned and sequenced. A large portion of the known sequence of titin is made up of approximately 100 amino acid repeats (Labeit et al., 1990). The majority of these repeats are type I and type II (or motif I and motif II). These same repeats are found in myosin binding proteins such as C-protein, 86 kDa protein and myosin light chain kinase (Fulton and Isaacs, 1991) as well as in the extracellular immunoglobulin and fibronectin families (Labeit et al., 1990). It has been suggested that these myosin binding repeats may help titin perform a ruler-like role in modulating the length of thick filaments in skeletal muscle (Whiting et al., 1989).

Titin has been shown to be a phosphoprotein (Sommerville and Wang, 1988). Titin is phosphorylated mainly on its serine/threonine residues and has approximately 12 moles of phosphate per mole of titin (Sommerville and Wang, 1987; Tanako-Ohmura et al., 1992). Cloning and sequencing studies of the C-terminal region (M-line end) has revealed that titin contains KSP-motifs similar to those seen in neurofilaments. When these regions were studied in vitro, it was
noted that there were high levels of KSP phosphorylating kinases in muscle that is developing, but not in differentiated muscle. It has been suggested that the C-terminal phosphorylation of titin may help to regulate the assembly of M-line proteins during myogenesis to form regular structures (Gautel et al., 1993).

**Character of titin**  
Studies of some of the major degradation products of titin have led to insights into the nature of the titin molecule. Recent reports have localized a 1200 kDa degradation product in the I-band region of the sarcomere that originates from the Z-line end of the long slender titin molecule (Itoh et al., 1988; Kimura et al., 1992; Tanabe et al., 1994). This degradation product has been shown to bind to α-actinin, further evidence that it may interact with the Z-line (Tanabe et al., 1994). This portion of the titin molecule has been estimated to be between 0.34 μm (Tanabe et al., 1994) and 0.36 μm (Suzuki et al., 1994) in resting length myofibrils. In some studies, this fragment has been shown to terminate near the putative N₂ line (Tanabe et al., 1994). Immunoelectron microscopic studies have shown that this region of the titin molecule is highly elastic in nature. Antibodies binding to the titin molecule in the I-band have been shown to change location relative to the Z-line in sarcomeres that have been stretched to different lengths (Itoh et al., 1988). It is possible that this region of the molecule is responsible for the elasticity of the titin molecule that is seen in situ. (Funatsu, et al., 1990).

Investigation of the T2 portion of the titin molecule using antibodies and/or rotary shadowing techniques has led to length estimates of approximately 0.9 μm (Nave et al., 1989; Suzuki et al., 1994). This portion of the molecule is hypothesized to extend from the M-line and contain the C-terminal portion (Nave et al., 1989). This portion of the titin molecule appears to be relatively inextensible.
when it is bound to the thick filaments as shown by studies using antibodies to titin on myofibrils of differing lengths (Itoh et al., 1988). Studies have shown, however, that this portion of the titin molecule is elastic when the myosin filament is removed (Salviati et al., 1990) indicating that firm attachment of the titin molecule suppresses the elastic character of T2. It has been suggested that under conditions of extreme stretch the A-band portion of titin may be recruited, leading to the hypothesis that titin-myosin complexes may act as a “dual stage molecular spring” (Wang et al., 1993). Regardless of the character of T2, it has been proposed that together the 1200 kDa polypeptide and T2 make up the intact titin molecule (Tanabe et al., 1994).

**Role of titin in muscle and meat** In developing muscle cells, titin has been proposed to provide a “scaffold” or a “template” to regulate the length of the thick filament during myofibrillogenesis and organization of the sarcomere (Furst et al., 1989; Fulton and Isaacs, 1991; Trinick, 1992). In adult muscle tissue, titin has been hypothesized to provide an elastic element and to allow the generation of passive tension in living tissue (Wang et al., 1991; Wang et al., 1993). Another proposed role for titin is to aid in maintaining the positioning of the thick and thin filaments within the sarcomere. As the major constituent of the third filament system in the sarcomere coupled with the fact that it is the only element within the sarcomere that spans from the Z-line to the M-line, titin is ideally suited for this role. When one considers that two titin molecules together can span the distance from one Z-line to the next, the role of titin in maintaining sarcomeric integrity is plausible (Robson et al., 1991).

In postmortem tissue, titin may be involved in the development of tenderness due, in part, to some of its proposed roles in living tissue. Degradation of a
structural element that maintains sarcomeric alignment and integrity (such as titin) would seem to aid in disruption of the muscle cell and lead to enhanced tenderness (Robson et al., 1991). Degradation of titin and the conversion of T1 to T2 have been shown to be related in a temporal manner to the loss of elasticity of muscle as it is converted to meat (Takahashi and Saito, 1979). These factors combined with the fact that titin is degraded in postmortem tissue (Lusby et al., 1983; Huff-Lonergan et al., 1995) (as will be discussed in a later section) implicate titin in the postmortem tenderization process.

**Nebulin**  Nebulin is another extremely large protein found to exist in the skeletal muscle sarcomere. Nebulin was first described by Wang et al. (1979) as band 3 due to its migration position on SDS-PAGE gels. The two bands of titin (T1 and the degradation product T2) were designated as bands 1 and 2. Nebulin was initially reported to have a molecular mass of 500 to 600 kDa; however, this figure has been modified and it is now thought to be in the range of 600-900 kDa, depending upon the species and the muscle of origin (Wang and Wright, 1988; Jin and Wang, 1991). Wang and Wright (1988) showed, for example, that nebulin from muscles such as the semitendinosus, the soleus and the sartorius were significantly larger (as determined by SDS-PAGE analysis) than nebulin from the longissimus and the psoas.

Nebulin has been estimated to make up 3-4% of the total myofibrillar protein in mammalian skeletal muscle (Wang, 1982). Nebulin, like titin, has not been found to exist in smooth muscle systems. Interestingly, unlike titin, nebulin has not been found to exist in cardiac muscle (Locker and Wild, 1986; Furst et al., 1988).

Nebulin was so named as it was originally observed by some researchers to be associated with the N2 lines of the sarcomere which are rather "nebulous" in
their appearance (Wang, 1981). These $N_2$ lines are seen in some electron micrographs of skeletal muscle as a dark line that is parallel to the Z-line and runs across the I-band (Franzini-Armstrong, 1970; Locker and Leet, 1976). Some researchers have described the $N_2$-lines as being four lines that appear to be on either side of the Z-line (Locker and Wild, 1984). The exact function of these $N_2$ lines is not known, however, they have been observed to change position as the sarcomere changes in length while maintaining the same proportional distance between the M-line and the Z-line (Locker and Leet, 1976). In fact, the existence of these $N_2$ lines as a naturally occurring entity has been disputed, it has been suggested that the $N_2$ lines may be artifacts of the preparation techniques used for preparing the myofibrils for microscopy (Robson et al., 1991).

Nebulin is an extremely difficult protein to isolate in its native state. The only reported method for isolating intact nebulin is one that utilizes sodium dodecyl sulfate (SDS) (Wang, 1982) to produce a slightly denatured form of the protein. Most of the recent studies dealing with the nature of nebulin have been performed using cloned nebulin fragments (Jin and Wang, 1991; Chen et al., 1993).

**Localization of nebulin**

Nebulin is co-localized with the thin filaments of the skeletal muscle myofibril with the C-terminus at the Z-line end of the filament (Kruger et al., 1991; Wright et al., 1993). As has been done with titin, monoclonal antibodies have been used to locate nebulin more precisely within the sarcomere. Wang and Wright (1988) showed that a monospecific antibody to nebulin bound to six pairs of stripes in the I-band of the skeletal muscle sarcomere. These bands ranged from 0.1 to 1 µm from the Z-line. From this data it was proposed that nebulin could be anchored at the Z-line. More recent studies that
support this hypothesis have shown that radiolabelled α-actinin (a major protein of the Z-line) can bind to nebulin in solid phase binding assays (Nave et al., 1990).

**Molecular character of nebulin**

The amino acid composition of nebulin has been determined. This protein has been shown to have a very high number of positively charged amino acids, and has no homology to any other known protein (Wang and Wright, 1988). Recently, a genetic polymorphism has been found at the porcine nebulin locus (Atac et al., 1995). Nebulin has been shown to be a modular protein that is made up of modules (repeating sequences) of 35 residues. The protein also has been shown to have a superrepeat consisting of seven modules, this pattern of repeats and superrepeats is thought to extend over the length of the molecule (Jin and Wang, 1991; Labeit et al., 1991). Studies using cloned fragments containing these repeats have shown that some two repeat constructs may contain F-actin binding sites (Jin and Wang 1991). One of the two repeat constructs has been shown to slow down the depolymerization rate of F-actin and to speed up the polymerization of G-protein, leading to the hypothesis that nebulin may be part of a “zipper-like composite thin filament” (Chen et al., 1993). It has since been proposed that two nebulin molecules lie along the cleft of the actin helix. These two nebulin molecules are thought to occupy symmetrical positions on the actin filament and may possibly “bridge” the two actin strands in the filament (Pfuhl et al., 1994).

Nebulin was originally thought to be a constituent of the elastic component of muscle because of its presumed association with the N\textsubscript{2}-lines which have been seen to shift their position in relationship to the Z-line in response to stretching of the sarcomere (Wang and Williamson, 1980). It has since been shown, using anti-nebulin antibodies (Wang and Wright, 1988), that epitopes on nebulin do not
change position relative to the Z-line in response to stretching of the sarcomere, and so, in situ, the nebulin molecule is not now thought to be highly elastic when it is associated with the thin filament. Recent studies have indicated that nebulin contributes very little, if at all to the elastic modulus of the thin filament (Yasuda et al., 1995), as the elasticity of the nebulin filament has been shown to be several orders of magnitude lower than the elastic modulus for the thin filament.

**Role of nebulin in muscle and meat**  
Nebulin has been proposed to have several functions in muscle. Because nebulin appears to interact with Z-line proteins (α-actinin), it may be an important structural entity that aids in linking the thin filament to the Z-line (Robson et al., 1991). Nebulin may also play a role in myofibrillogenesis in developing muscle. It is possible that nebulin may act as a template for the developing thin filament (Labeit et al., 1991) and/or act as a stabilizing factor in adult muscle. Several reasons for this hypothesis exist. First nebulin is not found in cardiac muscle (Wang and Wright, 1988). In cardiac tissue, the thin filament lengths are heterogeneous (Robinson and Winegrad, 1977), unlike skeletal muscle which has fairly constant thin filament lengths. In addition, nebulin has been shown to accelerate the polymerization of G-actin. (Chen et al., 1993).

A new role for nebulin has been recently proposed (Root and Wang, 1994). It has been suggested that nebulin might be responsible for regulating the coupling between myosin and actin. Nebulin has been shown to interact with both actin and myosin, and in addition to a regulatory role, may also provide a structural role in regulating the alignment of the thick and thin filaments.

Nebulin may play an important role in the development of meat tenderness and textural properties. Nebulin is degraded quickly in postmortem muscle (Lusby
et al., 1983; Anderson and Parrish, 1989; Huff-Lonergan et al., 1995; Taylor et al., 1995). Due to its proposed roles in anchoring the thin filament to the Z-line and in possibly stabilizing the structure of the thin filaments this hypothesis is plausible.

**Filamin** Filamin is a homodimer that has a molecular mass of approximately 500 kDa. Under the denaturing conditions of SDS-PAGE, filamin from skeletal and cardiac muscle migrates at 245,000 kDa (Price et al., 1994). It is an actin binding protein that has been shown to exist in numerous cell types and has been shown to have the ability to cross-link actin filaments (Price et al., 1994). In the stress fibers of skeletal myoblasts, filamin is seen to be associated with actin filaments (Gomer and Lazarides, 1981). Many different isoforms have been shown to exist (Hock et al., 1990). The amount of filamin in skeletal and cardiac muscle is very low (< 0.1%). In skeletal and cardiac muscle, filamin has been shown to be localized at the periphery of the Z-line and is thought to be associated with intermediate filaments (Price et al., 1994).

The degradation of smooth muscle filamin by calpains has been documented by Davies et al. (1978). Smooth muscle filamin (Mr = 250,000) is cleaved by m-calpain to produce heavy merofilamin (Mr = 240,000) and light merofilamin (Mr = 9,500). This cleavage disrupts the actin-crosslinking ability of filamin, yet the cleaved filamin retains its actin binding ability (Davies et al., 1978). A very similar cleavage pattern resulting in a closely spaced doublet of intact and heavy merofilamin has been shown to occur in myoblasts (Kwak et al., 1993) and was attributed to m-calpain activity. One proposed role of filamin in developing skeletal muscle is to aid in orchestrating the development of the myofibril (Kwak et al., 1993; Price et al., 1994) and its cleavage by calpains may play a role in regulating this action.
Postmortem degradation of filamin could disrupt key linkages that serve to hold myofibrils in lateral register. Degradation of filamin could possibly alter linkages connecting peripheral myofibrils to the sarcolemma by changing interactions between peripheral Z-lines and the sarcolemma via intermediate filament associations.

Desmin  Desmin is a member of the 10 nm diameter intermediate filament group of proteins. Desmin has a molecular mass of 53 kDa and is one of the early proteins to be expressed during myofibrillogenesis (Shaart et al., 1989; Lin et al., 1994). The purified protein has the ability to self-associate and to form filaments when the pH of the solution is lowered to 7.0, or when the ionic strength is increased (Ip et al., 1985). Desmin has been recently shown to be a good substrate for an ADP-ribosyltransferase. The resulting modification inhibits desmin from forming filaments (Huang et al., 1993). In mature myofibrils, desmin has been reported to be located at the periphery of the Z-line (Yagyu et al., 1990). It has been suggested that desmin may play a role in maintaining the alignment of adjacent myofibrils by tying them together at the level of the Z-line (Lazarides, 1982) and may connect myofibrils to other cellular structures, including the sarcolemma (Yagyu et al., 1990). Through these roles, it is possible that desmin may be important in maintaining the structural integrity of muscle cells (Robson et al., 1991). Degradation of desmin by m-calpain has been reported (O'Shea et al., 1978). In both living systems and in postmortem muscle, the degradation of a structural element such as desmin that connects major components of a cell together as well as to the cell membrane, may be important in determining the state of organization of the muscle cell and possibly ultimately the tenderness.
**Troponin-T** Troponin-T ($M_r = 37,000$ on SDS-PAGE gels) is a part of the muscular contraction regulatory complex, troponin, that is important in regulating the Ca$^{2+}$-induced contraction of striated muscle. Troponin-T interacts with tropomyosin at two main positions and covers a large portion of the tropomyosin molecule (Pearlstone and Smillie, 1982) including the overlap between two tropomyosin molecules (White et al., 1987). Troponin-T is susceptible to degradation under conditions such as postmortem aging (Olson et al., 1976; Penny and Dransfield, 1978; Ho et al., 1994). Calpain has been shown to degrade troponin-T in purified myofibrils incubated with the enzyme (Dayton et al., 1975). In addition, the troponin-T fraction of purified troponin has been shown to be degraded by m-calpain (Olson et al., 1977). The major degradation products that have been observed under conditions such as those described above, range in molecular weight from approximately 31,500 to 15,000 daltons (Olson et al., 1977; Penny and Ferguson-Pryce, 1979; Zeece et al., 1986; Ho et al., 1994).

**α–Actinin** α-Actinin has a molecular weight of approximately 200,000 and is made up of two subunits of approximately 100,000 each (Robson et al., 1981). The dimensions of the molecule are approximately $4 \times 50$ Å and it has been suggested that it is the Z-filament of the Z-line (Robson et al., 1981). α–Actinin is thought to cross-link actin filaments across the Z-lines in the myofibrils of striated muscle (Suzuki et al., 1976; Fyrberg et al., 1990). The binding of α-actinin to F-actin appears to be somewhat temperature dependent (Goll et al., 1972; Holmes et al., 1976). The ability of α-actinin to bind F-actin occurs more easily at temperatures in the range of 0-4°C, and is somewhat inhibited at 37°C. It has been shown that when tropomyosin is present at 37°C the binding of α-actinin to F-actin
is almost non-existent (Goll et al., 1972; Holmes et al., 1976). The binding and the interactions between proteins at 37°C seems to be an enigma.

When myofibrils are digested with purified \( \mu \)- and \( m \)-calpain at 25°C it can be seen that \( \alpha \)-actinin is released from the myofibril in an undegraded form. In fact, when purified \( \alpha \)-actinin is incubated with purified \( \mu \)- or \( m \)-calpain it is not apparently degraded (Goll et al., 1991). Hwan and Bandman (1989) have shown, however, that when muscle tissue is incubated at elevated temperatures or for long periods of time at low temperatures, some slight degradation of \( \alpha \)-actinin can be seen to occur.

It has been suggested by Robson et al. (1981) that \( \alpha \)-actinin may play a three-fold role in skeletal muscle. First, it may serve to anchor the thin filaments to the Z-line. Second, it may modify the structure of actin in the thin filaments, and finally, it may help to determine the directionality and regulate the growth of the thin filaments.

**Synemin**  Synemin has been shown to have a molecular weight of 230,000 (Granger and Lazerides, 1980) and is currently classified as an intermediate filament associated protein. Synemin is found in vertebrate skeletal, cardiac and smooth muscle cells (Robson et al., 1991). Synemin and desmin appear to have closely related roles in that they are co-localized in the myofibril at the periphery of the Z-line (Granger and Lazarides, 1979: Robson et al., 1991).

**Vinculin**  Vinculin has a molecular weight of 130,000. Its role in the myofibril is not known at the present time. Some studies suggest that it may play a role in linking actin to the cell membrane (Geiger, 1979). Vinculin may indirectly link the peripheral myofibrils to the costameres that are found at the sarcolemma.
(Johnson and Craig, 1995). Its degradation may affect muscle cell integrity by disrupting linkages of the peripheral myofibrils to the cell membrane.

Zeugmatin  Zeugmatin has a molecular weight of approximately 500,000. When it is examined under SDS-PAGE conditions, it migrates close to nebulin (Maher et al., 1985). Zeugmatin is thought to be at the outer edge of the Z-line. Zeugmatin appears to be especially susceptible to proteolysis, therefore, only very fresh muscle can be used for its isolation and purification (Pearson and Young, 1989).

Rigor mortis

Following the death of the animal, a complex series of physical and chemical changes occur which lead to the muscle being in a stiffened or rigid state termed "rigor mortis". The term literally means "the stiffness of death". This stage is one of the first stages in the conversion of muscle to meat. Events occurring during rigor development and resolution play an important role in determining the perceived tenderness of meat. During the past half-century, much time and effort has been devoted to studying the factors affecting the rate of the onset and the resolution as well as the degree of stiffening obtained throughout the course of the development and decline of rigor mortis.

Immediately after an animal is slaughtered, adenosinetriphosphate (ATP) and creatine phosphate are present in the muscle. The pH of the muscle is near neutrality (pH 6.7-7.2). During normal metabolic processes in the muscle the supply of ATP is continually replenished by oxidative phosphorylation. When the blood supply and its concomitant supply of oxygen is terminated, the muscle goes into an anaerobic state and the level of ATP can be maintained for only a few
hours. During early postmortem times creatine phosphate is used to convert adenosinetriphosphate (ADP) to ATP. Once these reserves are exhausted, the ATP level in the muscle falls. During the postmortem period, anaerobic glycolysis in the system results in the production of lactate from glycogen resulting in a drop of the pH of the muscle. Within a short period of time, the pH can drop from 7.2 to 5.5 (Penny, 1980).

The stiffness that is observed during rigor mortis is caused by the formation of permanent cross-bridges between the actin and myosin filaments. This is essentially the same interaction that occurs in living muscle during contraction, except that when the reserves of ATP are depleted after death there is no longer a constant supply of energy in the form of ATP available to assist in breaking the actomyosin bond at the end of the crossbridge cycle (Judge et al., 1989).

Many physical changes occur during the process of converting muscle to meat. One of the more easily quantified changes is the loss of extensibility when the muscle is subjected to a load. Immediately following death, the muscle is very extensible, it passively stretches under load up to 140% of its resting length (Penny, 1980) and will readily return to its resting length as allowed by the natural elasticity of muscle. During this stage very few irreversible actomyosin cross-bridges have been formed which would prevent this extension. This phase is termed the delay phase of rigor mortis (Bate-Smith and Bendall, 1949). Once the stores of glycogen and creatine phosphate are exhausted, the rephosphorylation of ADP to ATP becomes inadequate to continue to break the actomyosin bonds being formed. The end result is reduced extensibility of the muscle. The stage during which a loss of extensibility is observed in known as the onset phase. This phase begins when the muscle starts to lose its elasticity and lasts until the completion of
rigor mortis. The completion of rigor mortis occurs when all of the creatine phosphate has been used. Characteristically, the muscle is no longer extensible at this point. The time that is required for muscle to pass through these stages of rigor varies from animal to animal and even from muscle to muscle. If muscle that has undergone the effects of rigor mortis is "aged" or held for a certain length of time, the meat will again attain some measure of increased tenderness and pliability (Whitaker, 1959).

Busch et al. (1967) and Goll et al. (1971) suggested that the stages of rigor mortis be defined in terms of the isometric tension that develops in meat (muscle) samples over time postmortem. Isometric tension is that amount of tension that is measured when a muscle strip is held at one end, while the other end is attached to a sensing device. The amount of tension or shortening that develops or declines is measured using a physiograph. Busch et al. (1967) and Goll et al. (1968) proposed that the period of increasing isometric tension be identified with the onset of rigor mortis, while the decrease in isometric tension be identified with the resolution of rigor. Busch and coworkers (1972) showed through a series of experiments that the measurement of the development of isometric tension is a sensitive method for evaluating the onset of rigor mortis. They proposed that the development of postmortem isometric tension patterns is strongly related to the development of changes in muscle length that occur in postmortem muscle during the development of rigor mortis.

Postmortem aging

The practice of holding beef for extended periods of time after death for the purpose of enhancing tenderness is termed postmortem aging. Many studies have been conducted to determine what causes these changes and at what point
optimum tenderization occurs. Studies have recommended periods of time in the neighborhood of 9 days (Paul et al., 1944) or as long as 20 days (Jennings et al., 1978). The period of time for optimum tenderization is so variable because many factors can influence tenderness, one of which is the postmortem storage temperature. In general, by increasing the storage temperature, the rate of tenderizing due to the aging process can be accelerated. This acceleration in the tenderizing process has been observed at temperatures up to 60°C (Davey and Graafhuis, 1976).

**Structural changes**  
Histological examinations of beef muscle structure during aging have shown that structural changes occur during postmortem aging. As early as 1944 (Paul et al., 1944) it was seen that structures that were described as fiber striations became more fragile as postmortem aging time increased. These striations also gradually became lost over a larger area. Changes such as these have been shown to coincide with the differences in tenderness measured either mechanically or by a sensory panel (Paul et al., 1944).

Phase contrast and electron microscopy studies (Schmidt and Parrish, 1971) have also shown that structural changes occur during postmortem aging. In myofibrils that were aged for seven days, myofibrils have been seen to be out of register and not as sharply defined as those myofibrils from unaged samples. In addition, a certain degree of shrinkage could be noted in some of the aged fibers. When the samples were examined using an electron microscope, the aged samples had lost some of their structural integrity. The H-zone of these myofibrils had disappeared and the Z-line was apparently degraded. There were essentially no changes in the M lines or in the thick and thin filaments that could be noted. In
1973, Parrish et al. noted that increased fragmentation of the myofibril at or near the Z-lines was related to an improvement in tenderness.

The change in the structure of the Z-line of the myofibril has been an area of much research in postmortem aging studies for many years. Some of the most recognizable changes that have been seen to occur in meat aged at relatively high temperatures (15°) for 3 days, include lengthening of the A-bands along with the apparent shortening of the I-bands and the complete disappearance of the Z-line (Davey and Gilbert, 1967). Other studies have also shown, in general, that some of the most noticeable changes include apparent disappearance of the Z-lines and/or a weakening of the interaction between the Z-lines and the thin filaments, as well as a weakening of the lateral attachments that hold the myofibrils in place within muscle (Stromer et al., 1967; Davey and Gilbert, 1969; Davey and Dickson, 1970). Others have shown fractures that occur in the A-I junction (Davey and Dickson, 1970; Locker and Wild, 1984; Ouali, 1990), as well as at the N2 line (Ouali, 1990), a line that has been seen in some electron micrographs of skeletal muscle to run parallel to the Z-line and across the I-band (Franzini-Armstrong, 1970). In overstretched muscle, in which there have been observed breaks in the I-band on one side of the Z-line, the N2-line on the intact side of the Z-line has been observed to move toward the Z-line. Locker et al. (1977) surmised that this was due to the association of the N2 line with gap filaments. Often, in samples in which the Z-lines have been shown to be removed or at least were no longer apparent, the myofibrils still retained some of their inherent integrity, indicating that some substance or structural entity still remained in this region that aided in holding the myofibril together (Davey and Gilbert, 1969). Davey and Graafhuis (1976) reported that highly stretched muscle showed a tendency to break at the A-I junction, suggesting
that disruption of the filaments that hold the sarcomere in register, particularly at the A-I junction as well as the Z-line may be important in determining the state of the myofibril during aging and tenderization. Other studies have shown that fragmentation at the interface between the I-band and the Z-line occurs more often in tender than in less tender muscle (Gann and Merkel, 1977).

**Myofibril fragmentation**  Because of the breakage that occurs in the myofibril, there has often been reported to be a greater number of myofibrillar fragments in aged than in unaged meat. This increase in the fragmentation of the myofibril has been used to characterize the aging and amount of tenderization that has taken place. Early attempts to use this information have involved procedures such as counting the number of myofibrils that had one to four sarcomeres. This procedure is based on the fact that as postmortem aging time increases so do the number of shortened myofibrils (Fukasawa et al., 1967). This method, however, is rather tedious and requires a fairly significant amount of skill and experience to master. Another method that is used to determine the amount of fragmentation that has occurred in the myofibril at or near the Z-line, involves using a spectrophotometric method that measures the change in turbidity at 540 nm. As the length of postmortem aging time increases, so does the turbidity of the sample due to the presence of an increased number of fragments (Olson et al., 1976; Culler et al., 1978). This procedure is known as the myofibril fragmentation index (MFI). Based upon this evidence, MacBride and Parrish (1977) have suggested that the term “myofibril fragmentation tenderness” be used to describe the increase in tenderness that occurs parallel with the increase in fragmentation of the myofibril. Indeed, it has been shown that as the degree of fragmentation increases, as measured spectrophotometrically, so does the tenderness of the corresponding
cooked meat, so much so that a significant correlation (correlation coefficient between MFI and panel evaluated tenderness of .75, and between MFI and Warner-Bratzler shear of -.72, keeping in mind that as shear force increases, tenderness decreases) has been observed (Culler et al., 1978), leading to use of the MFI procedure in raw samples to predict tenderness.

**Heat-induced changes**  Some research has been done to examine the changes that occur in meat after cooking. Some of the most notable changes that have been seen to occur in meat cooked to 60°C include the loss of the M-line structure, the beginning of the disruption of the thin filaments, and the start of thick filament coagulation. At 70°C severe disruption of the thin filaments and coagulation of the thick filaments can be noted (Schmidt and Parrish, 1971).

Another structure that has been shown in some studies to survive the cooking process at 60°C are the very fine filaments in muscle tissue that are known as the gap filaments (Locker et al., 1977). It has been hypothesized that the integrity of these filaments (now known to be composed of titin) during cooking may be an important determinant in tenderness of cooked meat (Locker, 1982).

Studies using SDS-PAGE techniques have shown that cooking of meat is associated with the degradation of titin into smaller polypeptides (King et al., 1981; King, 1984; Locker and Wild, 1984). More recent data (Fritz et al., 1992) have shown that as beef is heated at 73°C, there is an increase in the amount of titin degradation products that could be detected using Western blotting techniques. As fresh beef is heated for increasingly longer periods of time at 73°C, there is a progressive increase in the amount of high molecular weight aggregates. When meat samples were injected with a salt (NaCl) and phosphate solution, it was noted that there was little, if any, accumulation of high molecular weight aggregates of
titin. In addition, samples that were heated to lower final internal temperatures (up to 45°C) did not show many degradation products that accumulated. Once heating temperatures were extended above 59°C, however, degradation products became more pronounced. In fact, by the time the internal temperature reached 71°C, intact titin was not readily detected by Western blotting techniques. It was suggested that these differences in the heat-induced changes of titin might be responsible for changes in the perception of tenderness/toughness that occurs as beef is cooked to higher temperatures (Fritz et al., 1992).

The aforementioned studies illustrate that the changes that occur in postmortem muscle are highly specific. In order to gain a better understanding of the processes that take place during postmortem aging, one must first have an understanding of exactly which structural elements or proteins are altered to allow the gross changes in the characteristics of meat to occur. In addition, those elements that are responsible for causing those changes, whether they are enzymatic or physiochemical changes, or a combination of the two need to be studied in detail. The remainder of this review will focus upon the factors thought to be either indicative or responsible for postmortem tenderization.

Postmortem degradation of specific myofibrillar proteins

**Titin** The giant protein titin has been shown to be degraded in postmortem muscle by several researchers (Lusby et al., 1983; Anderson and Parrish, 1989; Paxhia and Parrish, 1988; Fritz and Greaser, 1991; Huff-Lonergan et al., 1995; Taylor et al., 1995). The first major degradation product that was described (and is the most prominent) is termed T2. T2 migrates very closely to the intact form (known as T1) causing the appearance of a doublet on SDS-PAGE gels. T2 has
an apparent molecular weight that has been estimated to be in the neighborhood of 2400 to 2700 kDa, while T1 has an apparent molecular weight of 2800 to 3000 kDa (Kurzban and Wang, 1988; Wang et al., 1991). T2 is thought to originate from the C-terminal end of the titin molecule and extend from the M-line near the center of the sarcomere out past the A-I junction. This degradation product has been estimated to be approximately 0.9 μM in length (Nave et al., 1989; Suzuki et al., 1994). A second, very large degradation product, has been shown to exist. This product has been estimated to have a Mr of 1,200,000, and is therefore referred to as the 1200 kDa polypeptide (Matsuura et al., 1991). The 1200 kDa product is found to exist in the I-band region of the sarcomere and originates from the Z-line end (N-terminus) of the titin molecule (Itoh et al., 1988; Tanabe et al., 1994). This fragment of titin has been estimated to be between 0.34 μm (Tanabe et al., 1994) and 0.36 μm (Suzuki et al., 1994) in length in resting length sarcomeres. Some studies hypothesize that the 1200 kDa polypeptide may terminate in the vicinity of the putative N2 line (Tanabe et al., 1994). Together, the 1200 kDa polypeptide and the T2 portion of titin have been thought to make up the majority of the titin molecule (Tanabe et al., 1994).

Lusby et al. (1983) showed using SDS-PAGE gels, that both titin and nebulin were degraded in postmortem muscle as the time postmortem increased. Fritz and Greaser (1991) examined the postmortem degradation of titin in postmortem bovine psoas major muscle. Using immunofluorescence microscopy they found that while at 45 minutes postmortem most of the myofibrils had two anti-titin bands per sarcomere (less than 1% of the myofibrils exhibited four anti-titin bands per sarcomere), at 48 hours postmortem 65% of the myofibrils had four anti-titin bands per sarcomere. Since the psoas major muscle undergoes most of its
tenderness development very early, within the first few days postmortem, the change that is responsible for this two to four band alteration may also be responsible, in part, for tenderness development.

Paterson et al. (1988) showed that suspending myofibrils in a solution containing 10 mM pyrophosphate resulted in swelling of the myofibrils, at the same time the water holding capacity was increased. This suggested that titin may provide some of the structural restraints that hold the sarcomere together. This observation indicates that titin may provide some of the structural restraints that hold the sarcomere together. It has been hypothesized, based in part, on these results that the extraction of titin allows greater water holding capacity.

Paterson and Parrish (1986) found that both the T1 and T2 bands of titin were present in the less tender rhombiodeus muscle, while only the T2 band was present in the more tender infraspinatus muscle after short periods of aging. Anderson and Parrish (1989) also showed that titin was more rapidly degraded in tender than in less tender steaks from typical slaughter age steers, suggesting that titin may play a role in influencing meat tenderness. This finding was substantiated by Huff-Lonergan et al. (1995). This study (Huff-Lonergan et al., 1995) also implicated the degradation of titin as playing a role in influencing the tenderness of beef from young bulls and old cows, further supporting the hypothesis that titin plays a role in tenderness in general. Additional data from Taylor et al. (1995) also shows that titin is degraded significantly in postmortem muscle.

Various factors such as postmortem pH and temperature have been shown to affect the degradation of titin in situ. In a microscopy study by Orcutt and Dutson (1985) the following combinations of pH and temperature were evaluated for their effect on the degradation of titin (gap filaments) as it could be identified under the
electron microscope: pH 5.5, 37°C; pH 5.5, 0-4°C; pH 7.4, 37°C; pH 7.4, 0-4°C.

Under both temperature conditions at pH 5.5 they noted greater degradation of titin than was seen at pH 7.4 at either temperature. They deduced from this study that pH had a greater effect upon the degradation of titin than did temperature.

Much controversy has been raised in recent years over whether or not titin (and nebulin) play a significant role in postmortem tenderization of beef, or whether it is even significantly degraded during postmortem aging (Fritz et al., 1993). One of the major differences in these studies and those that do show significant titin degradation in relationship to beef tendernessness, such as Huff-Lonergan et al. (1995), is the gel system and the gel sample preparation method that was used. The systems that were used in the studies that did show differences in titin degradation that were related to beef tenderness used gel procedures that allowed for greater separation of the proteins titin and nebulin (Huff-Lonergan et al., 1995). In addition, gel sample procedures, such as the ones used by Fritz et al. (1993) using 100°C solubilization temperatures, have been shown by Granzier and Wang (1993) to result in 50% or more of the titin to be degraded in the sample buffer prior to loading on the gels. The procedures that were used in studies such as Huff-Lonergan et al. (1995) were essentially the same as those used by Granzier and Wang (1993) and have been shown to not have a detrimental effect on the state of titin. This procedure (Huff-Lonergan et al., 1995) used low solubilization temperatures and a slightly different gel sample buffer than was used in Fritz et al., (1993). The study (Fritz et al., 1993) that showed slow degradation and little, if any, correlation between the conversion of T1 to T2 (or the degradation of titin in general) to beef tenderness may have been identifying the degradation product T2 as T1. There may be several reasons for this. First, the separation procedure used
in Fritz et al. (1993) did not show much separation between titin and the next largest protein that was most prominent on the gels, nebulin. This is significant as T1 and T2 would migrate so closely together, that it might be difficult to discern accurately between the two bands. It is possible that in the study (Fritz et al., 1993) the T2 band (which does not degrade noticeably on Coomassie stained SDS-PAGE gels once it is formed) was mistaken for the intact titin band. This cannot be proven, however as in the study by Fritz et al. (1993), the first time point sample that was taken was at two days postmortem. By this point in time, significant degradation of titin may already have occurred, allowing for production of some T2. This T2 could have been mistaken for T1 if that were the case. In addition, this study did not use as a control for the migration of T1, a purified titin standard or a zero time sample. This combination of factors make it difficult to make direct comparisons between Fritz et al. (1993), and others that do show significant degradation of titin over time postmortem (Lusby et al., 1983; Anderson and Parrish; Huff-Lonergan et al., 1995; Taylor et al., 1995).

Nebulin  Several research reports have shown that nebulin is degraded rapidly in postmortem tissue (Lusby et al., 1983; Paterson and Parrish, 1987; Paxiha and Parrish, 1988; Anderson and Parrish, 1989; Fritz and Greaser, 1991; Boles et al., 1992; Huff-Lonergan et al., 1995; Taylor et al., 1995). The possibility that nebulin is involved in the tenderization process was suggested by Anderson and Parrish (1989). In this report, they showed that nebulin bands were less intense or non-existent in myofibrils from steaks that were categorized as “tender” by sensory panel evaluations when compared to myofibrils from “less tender” steaks. A more recent study (Huff-Lonergan et al., 1995) also showed that nebulin was degraded at a faster rate in samples that had lower shear force values and
higher sensory panel scores for tenderness. Degradation of nebulin could be important in postmortem tenderization due to its location in the myofibril and its intimate interaction with the thin filament. Degradation of nebulin could weaken the thin filament linkages at the Z-line and thereby weaken the structure of the muscle cell. Nebulin has also recently been shown to be capable of linking actin and myosin, and is proposed to have a regulatory function in skeletal muscle contraction (Root and Wang, 1994). If this proves to be the case, then it is possible that its degradation may alter actin-myosin interaction in such a way that the alignment of thick and thin filaments is disrupted. This could eventually lead to an increase in postmortem tenderization.

Filamin Filamin, which is thought to be located at the periphery of the Z-disks in skeletal muscle (Price et al., 1994), may exert an influence in tenderness development through its association with the intermediate filaments. Postmortem degradation of filamin could disrupt key linkages that serve to hold myofibrils in lateral register. Degradation of filamin could possibly alter linkages connecting peripheral myofibrils to the sarcolemma by changing interactions between peripheral Z-disks and the sarcolemma via intermediate filament associations. Enhanced degradation of filamin (as evaluated by SDS-PAGE) has been seen in meat samples that had been injected with CaCl₂ (Uytterhaegen et al., 1994), a process that has been shown to stimulate proteolysis and postmortem tenderization (Koohmaraie et al., 1988).

Desmin The intermediate filament protein desmin has been shown to be degraded in postmortem tissue in many studies (Young et al., 1980; Robson et al., 1981; Robson et al., 1984; Koohmaraie et al., 1984a,b; Koohmaraie et al., 1988; Hwan and Bandman, 1989; Whipple and Koohmaraie, 1991). As an example,
Hwan and Bandman (1989) showed that degradation products were detected by a monoclonal antibody to desmin in whole muscle extracts within 4 days of storage at 4°C. This rate of degradation was significantly faster in samples that were held at higher temperatures of 25°C and 37°C. In fact, at the two higher temperatures (25°C and 37°C), the degradation of desmin was noted, but the degradation products were not detected by the monoclonal antibody. In general, this study (Hwan and Bandman, 1989) showed that degradation of desmin took place in about the same time frame as did degradation of troponin-T and titin.

Desmin, through its proposed location around the periphery of the Z-line may possibly play a role in connecting adjacent myofibrils and helping to maintain them in lateral register, as well as to connect myofibrils to other cellular structures, including the sarcolemma (Yagyu et al., 1990). Through these roles, it is possible that desmin may be important in maintaining the structural integrity of muscle cells (Robson et al., 1991). Degradation of a structural element that connects major components of a cell together, as well as to the cell membrane, could affect the development of tenderness.

**Troponin-T and the 30,000-dalton component** Researchers working with the thin filament regulatory protein, troponin, noted the appearance of a 30,000-dalton protein that consistently appeared in their preparations of purified troponin (Dabrowska, et al., 1973). Dabrowska et al., (1973) observed that the appearance of this 30,000-dalton component coincided with the decrease of troponin-B (now known as troponin-T). This same degradation product could be “manufactured” in the early stages of mild digestion of purified troponin-T or myofibrils with trypsin. They hypothesized that the 30,000-dalton component arose naturally from the degradation of troponin-T and was catalyzed by a proteolytic species possibly
active at a neutral pH that was bound to the myofibrils in some manner (Dabrowska, et al., 1973).

The existence of polypeptides appearing in muscle samples that have been held for a period of time (postmortem aged) have been researched for many years. In the early 1970's a band migrating in the range of 30,000 daltons was noted in chicken myofibrils from samples that had been aged for 48 hours. This degradation product was seen to increase in intensity as postmortem aging time increased (Hay et al., 1973; Samejima and Wolfe., 1976). A very similar degradation product was noted in preparations of bovine myofibrils that had been held for a minimum of 24 hours (Penny, 1974).

Olson et al. (1977) also observed the appearance of a 30,000-dalton component in bovine longissimus and semitendinosus muscles that had been postmortem aged at 2°C as well as at 25°C. The appearance of this band was accelerated in the tissue that was aged at 25°C. Along with the appearance of the 30,000-dalton component, the gradual disappearance of troponin-T was noted. In order to more fully characterize the origin of this 30,000-dalton band in beef muscle, purified troponin-T from beef was incubated with the then newly discovered protease CAF (now known as m-calpain). These digestions yielded a 30,000-dalton band and a concomitant decrease in intensity of the troponin-T band.

Since the mid-1970's numerous reports have demonstrated the appearance of a 30,000-dalton component (or bands migrating in this region) and the corresponding decrease in the intensity of troponin-T as postmortem aging time increases (e.g. Olson et al., 1977; Koohmaraie et al., 1984a,b; Ho et al. 1994). A recent study done by Ho et al., (1994) supports the hypothesis that a 30,000-dalton
polypeptide originates from troponin-T. This study showed by using a monoclonal antibody to troponin-T on aged and electrically stimulated beef muscles, that a "family" of polypeptides migrating between approximately 30,000 and 15,000-daltons originated from the degradation of troponin-T. A band that migrated at 30,000-daltons was also electrophoretically purified in this same study, and was shown to be labeled by this same monoclonal antibody to troponin-T.

While most of the available evidence points to troponin-T as the origin of a 30,000-dalton component (Olson et al., 1977; Ho et al., 1994), the exact causative agent of bands migrating in this region has been the subject of some debate. Bands migrating in this region have been shown to be produced in vitro not only by calpains (Olson et al., 1977; Koohmaraie et al., 1986) but also by certain catheptic enzymes such as cathepsin L (Matsukura et al. 1981; Mikami et al., 1987). Other studies on beef homogenates have shown that at above pH 6.0, the addition of Ca\(^{2+}\) ions accelerated the degradation of troponin-T and below pH 6.0 the addition of EDTA accelerated the degradation of troponin-T (Penny and Ferguson-Pryce, 1979). However, the significance of this is not clear as under postmortem conditions, Ca\(^{2+}\) concentrations tend to increase (Jeacocke et al., 1993), favoring the calpain system which has been shown to have significant activity at pH values found in postmortem muscle (Koohmaraie et al., 1986; Kendall et al., 1992).

Olson and Parrish (1977) showed that the intensity of a 30,000-dalton component and the degradation of troponin-T paralleled Warner-Bratzler shear force values (an estimate of meat tenderness) as well as sensory tenderness scores for tenderness. Those samples that were less tender exhibited less degradation of troponin-T and had less intense 30,000-dalton bands, while the more tender samples showed a faster rate of troponin-T degradation and a faster
rate of 30,000-dalton appearance. This relationship was shown to hold for a wide
range in animal age. In a similar study (MacBride and Parrish, 1977), it was seen
that bovine longissimus samples that were significantly more tender after one day
of storage at 2°C exhibited the presence of the 30,000-dalton band, while those that
were designated as tougher did not have the 30,000-dalton band at one day
postmortem. This illustrated that even at one day postmortem, significant
differences in the degradation of myofibrillar proteins can be detected between
tough and tender meat samples.

In the ensuing years, it has been shown that the loss of troponin-T
(and the production of the 30,000-dalton component) is very highly related to the
tenderness of beef. Some reports have estimated that the loss of troponin-T can
account for as much as 60% of the variation in beef tenderness (Penny and
Dransfield, 1979).

The exact contribution of the 30,000-dalton component to tenderness is not
fully understood. It may simply be an indication of the overall proteolysis of
myofibrillar protein in postmortem aged samples. It has also been hypothesized
that troponin-T, which is located periodically along the thin filament could play a
role in maintaining the integrity of the thin filament. Its loss may aid in accelerating
the disruption of the myofibril in this region, and could be one of the factors leading
to fragmentation of the myofibril in the I-band. In this respect, its role in tenderness
may need to be reevaluated (Penny and Dransfield, 1979; Ho et al., 1994;
Uytterhaegen et al., 1994; Huff-Lonergan et al., 1995).
Enzyme systems in muscle/meat tissue

**Cathepsins** The cathepsins that have been the subject of most study in muscle tissue are often referred to as lysosomal enzymes. Many of these enzymes have a pH optimum in the acidic range. The most commonly studied catheptic enzymes in muscle tissue include the cathepsins B, C, D, and L. These cathepsins have been shown to have widely different substrate specificities (Canonico and Bird, 1970). The cathepsins B and C have been shown to degrade myosin and actin (Bandman, 1987). Cathepsin L degrades actin, myosin, α-actinin, troponin and tropomyosin (Okitani et al., 1980). A brief description of some of the more well characterized catheptic enzymes follows.

Cathepsin D is a lysosomal protease that has been found to be present in various organs such as spleen and liver (Okitani et al., 1981). Samarel et al. (1984) examined the multiple forms of cathepsin D in rabbit cardiac muscle. They reported that cathepsin D is initially produced as a 53,000 dalton precursor. Following a limited proteolysis in the lysosome, they showed that an active form of 48,000-daltons was produced. In a study by Schwartz and Bird (1977), the pH optimum for cathepsin D that had been purified from rat liver was 4.0. They showed that cathepsin D could degrade F-actin at pH 5.0 and was inhibited by pepstatin. The rate of hydrolysis of F-actin, however, was only found to be 10% of that reported for myosin. All three of the isoforms of cathepsin D they examined revealed that the same products were produced with each when the cathepsins were incubated with actin and myosin. Robbins et al. (1979) reported a study in which they examined the effect of a partially purified cathepsin D extract from two different sources (bovine muscle and bovine spleen) on bovine myofibrils using SDS-PAGE techniques and scanning electron microscopy. They found that at
temperatures of 25°C or 37°C (pH 5.2-5.3), the structure of the Z-line was disrupted to the point of almost total degradation by forty minutes to one hour of incubation. When they followed the degradation of myosin, they found that myosin heavy chain (approximately 200,000 daltons) was degraded to smaller fragments of approximately 170,000, 150,000, and 80,000 daltons. They found that cathepsin B had relatively little effect on either α-actinin or actin. In 1981, Okitani et al. reported a more detailed study of the properties of cathepsin D and of its action on myofibrils. They found purified cathepsin D from rabbit muscle to be a single polypeptide with a molecular weight of 42,000 daltons. Their cathepsin D preparation optimally hydrolyzed myofibrils at a pH value of 3.0. When they looked specifically at proteins affected by cathepsin D, they found myosin heavy chain to be degraded and a 30,000 dalton polypeptide to be produced at pH 3.8 after a 22 hour incubation at 37°C. These researchers suggested that the proteinase cathepsin D, with its peak proteolytic activity at pH 3.0 and 4.5 may not play as important a role in postmortem proteolysis as other enzymes such as cathepsin L and calpain.

Matsumoto et al. (1983) showed that at pH 3.0, the proteins that were optimally degraded by muscle cathepsin D included troponin I, troponin T, myosin heavy chain, and the slower degradation of tropomyosin and α-actinin. They did not note any degradation of actin or troponin C. When they more closely examined the degradation of myosin heavy chain, they found four major fractions were produced, 155,000, 130,000, 110,000, and 90,000 daltons. Troponin I was broken down into fragments of 13,000 and 11,000 daltons, while troponin-T was hydrolyzed into fragments of 33,000, 20,000, and 11,000 dalton bands. They noted that their results indicated that cathepsin D from muscle was markedly different in
its action toward actin than was the cathepsin D preparation obtained by Schwartz and Bird (1977).

Zeece et al. (1986) reported that purified preparations of cathepsin D revealed two bands with molecular weights of 47,000 daltons and 29,500 daltons when subjected to SDS-PAGE. When they incubated bovine myofibrils with purified cathepsin D at pH 5.5 and 37°C, they found that myosin heavy chain was degraded as was titin. They also noted a slight increase in the degradation of the proteins actin, tropomyosin, troponin-T, troponin I and myosin light chain. When the degree of fragmentation of the myofibrils was examined, they found no significant increase in the amount of fragmentation in the cathepsin D treated myofibrils when compared with untreated myofibrils. When they raised the pH and/or lowered the temperature, they found that cathepsin D was not as active. From their data they concluded that since cathepsin D was active only at such a limited pH and temperature range that it probably does not play a major role in the postmortem tenderization process.

Cathepsin B has been characterized as a thiol protease with a Mr of 27,000. In order to observe the activity of this enzyme, EDTA must be added to the reaction mixture, as the enzyme is inhibited in the presence of heavy metals. In addition, it is also inhibited by leupeptin and iodoacetate (Penny, 1980). Schwartz and Bird (1977) showed that cathepsin B had a pH optimum of 5.2 on native myosin. When cathepsin B is incubated with myosin at a pH of 5.2 and at a temperature of 37°C, the heavy chain portion is broken down to a product with a Mr of 150,000 and to components with molecular weights ranging from 100,000 to 50,000 (Penny, 1980). The action of cathepsin B on F-actin was found to be very similar to that of cathepsin D, producing a major fragment at 35,000 daltons (Schwartz and Bird,
While the pH optimum of both cathepsins B and D appears to be outside the normal pH range of postmortem muscle, it is important to note that the activity of cathepsin B (optimum pH 5.2) is still 50% at pH 5.6 and 20% at pH 6.0. Cathepsin D (optimum pH 4.0) retains 30% of its activity at pH 5.5 (Penny, 1980).

Okitani et al. (1980) reported that the enzyme known as cathepsin L has a molecular weight of 24,000 by estimation with gel filtration. Its activity to degrade myosin was determined to be optimum at a pH of 4.1, however, the enzyme was stable in the pH range of 4.5 to 6.5. They found the most potent inhibitors to be iodoacetate, leupeptin and antipain. Matsukura et al. (1981) reported a study in which they examined the degradation of myofibrillar proteins with cathepsin L. They found that cathepsin L degraded myosin heavy chain, α-actinin, actin, troponin-T and troponin I. They found the most intense degradation to occur around the pH of 4.8. When they looked specifically at the action of cathepsin L on myosin, they found the heavy chains to be degraded, and the light chains to decrease with fragments of 160,000, 92,000, 83,000, and 60,000 being produced. The most optimum pH for the enzyme to degrade myosin heavy chain appeared to be at a pH value of 4.2. Actin was degraded by this enzyme most intensely at a pH of 4.7, with resulting fragments migrating at 40,000, 37,000, and 30,000 daltons. They reported no degradation of tropomyosin and troponin C, but pH 3.7-6.7, troponin-T and troponin I were degraded producing fragments of 30,000 and 13,000 daltons. They also observed the degradation of α-actinin at pH 3.0-3.5 into fragments, the major one occurring at 80,000. From their data, they suggested that cathepsin L may play a role in actin catabolism in living muscle. They also suggested that it may contribute to the tenderizing process that occurs during postmortem aging of meat by possibly causing the breakdown of troponin-T with
the concurrent production of 30,000-dalton component in a similar manner as has been shown with the protease calpain. However, in their study, this activity was shown in the presence of EDTA and not Ca$^{2+}$. Mikami et al. (1987) designed a study in which they specifically analyzed the degradation of myofibrils by cathepsin L, and with lysosomal lysates. In this study they found, unlike Matsukura et al. (1981), that $\alpha$-tropomyosin was degraded after a four hour incubation at 30°C, while $\beta$-tropomyosin was not degraded. They also found actin not to be initially degraded, however, they did see slight degradation at four hours. They found myosin heavy chain to be degraded at pH 5.0 after a four hour incubation period. Troponin-T, troponin I and C-protein were found to be quickly degraded, while titin, nebulin, myosin heavy chain, $\alpha$-actinin and myosin light chains LC$_1$ and LC$_2$ were more slowly degraded. The action of cathepsin L was faster at a pH value of 5.5 than at a pH of 6.0, however, the action at pH 5.5 was slower than at pH 5.0. They noted that after incubation with cathepsin L, several new protein bands appeared on SDS-PAGE gels at 130,000, 120,000, 90,000, 85,000, 80,000, 31,000, and 30,000 daltons. When the incubations were done with only the lysosomal lysate, they found essentially the same degradation pattern that they found with cathepsin L except that there appeared to be a reduced degradation of actin and of the 30,000 dalton component. When they examined electron micrographs of myofibrils incubated with cathepsin L, they found preferential degradation of the Z-lines, with the disappearance of the Z-line after four hours of incubation in the presence of the enzyme. From their results, they concluded that at pH 5.5, cathepsin L may be the most important lysosomal enzyme in postmortem tenderization.

When examining the rate at which cathepsins are active, one must keep in mind several factors. These include the release of the cathepsins from the
lysosomes, the removal of the inhibitors and the temperature and pH of the system under observation. An increase in the temperature and/or lowering of the pH will accelerate the reactions by most of the catheptic enzymes (Penny, 1980).

**Cystatins** The cystatins are a family of protease inhibitors that are specific for cysteine proteases such as cathepsins B, D, and L as well as papain (Barrett, 1987). Also included in this “family” are the kininogens which have the capacity to inhibit the calpains (Ishiguro et al., 1987). There are primarily three families of cystatins. Family 1 is made up of cystatins A and B, and is the only group of the three to be located intracellularly. The major component of family 2 cystatins is cystatin 2. This inhibitor is predominantly extracellular. The third family (family 3) has as its main member the glycoproteins known as the kininogens. Again, this family (family 3) of inhibitors are primarily extracellular (Zeece et al., 1992). In muscle systems cystatins are hypothesized to be one of the main systems responsible for the regulation of cysteine proteinases, particularly cathepsins. The specifics concerning their mechanism of action and their means of control in muscle are unclear at this point in time (Zeece et al., 1992).

The role of cystatins in meat has yet to be elucidated, however, prediction equations for beef tenderness have been developed that have included 24-hour cystatin activity. In a study by Shackleford et al. (1991), an equation that combined 24-hour calpastatin activity, 0-hour μ-calpain activity, and 24-hour cystatin activity was developed. This equation accounted for 63% of the variation in Warner-Bratzler shear-force values measured at fourteen days postmortem. This result and others that are similar have lead some researchers to speculate that cystatins could play a role in postmortem tenderization (Zeece et al., 1992).
Multicatalytic protease  A large intracellular protease complex ($M_r = 700,000$) was discovered in 1983 (Wilk and Orlowski, 1983). This protease is cylindrical in shape and is composed of as many as 24-28 subunits (Orlowski, 1990; Rechsteiner et al., 1993). These subunits are the products of thirteen different genes that are homologous. Several of the subunits have been cloned and sequenced, and it appears that they show little, if any, homology to any other known protease (Orlowski, 1990; Rechsteiner et al., 1993). This enzyme has been characterized as having at least three different catalytic activities with different pH optima. These different proteolytic activities have been identified not by their pH optima alone, but also by their activity on distinct synthetic substrates (Chu-Ping et al., 1994). These have been described as, chymotrypsin-like (pH optimum = 7.0), peptidyl (pH optimum = 8.0), and trypsin-like (pH optimum = 8.5). Because of the presence of several different types of proteolytic activities, the protease complex has been called the multicatalytic protease. However, in the literature, it can be found under several different names including, macropain, proteasome, ingensin and ATP-stimulated protease (Goll, 1991).

The exact action of the multicatalytic protease has not been completely elucidated, but it has been hypothesized that the different catalytic sites act in a coordinated manner to cleave several bonds of a single protein. The mechanism of action may include the movement of the polypeptide intermediates among the different catalytic sites via a channeling system (Dick et al., 1991). Some of the proteins that it has been suggested to be active against include; cyclins, Myc, Fos, and the breakdown of several cytoplasmic proteins with short half-lives (Chu-Ping et al., 1994).
In experiments looking for this protease, the multicatalytic protease has been found to be abundant in cells. This enzyme is thought to be inactive in its “normal” state in cells (McGuire et al., 1989). It has been shown that it can be activated in vitro by compounds including SDS, some polycations, and some unsaturated fatty acids (Goll, 1992). Some studies have indicated that an “activator” protein with an apparent molecular weight of 700,000 may be present in cells. This activator seemed to enhance the activity of the multicatalytic protease in the presence of ATP (Chu-Ping, 1994). The multicatalytic protease has been suggested to play a role in the ubiquitin-system. The ubiquitin system is a system that is involved in tagging proteins for turnover (both normal and abnormal proteins). It has been shown that the multicatalytic protease can degrade ubiquinated proteins in the presence of ATP, but does not require ATP to degrade non-ubiquinated proteins. At this point in time, the multicatalytic protease has not been shown to degrade myofibrillar proteins (Goll, 1992), however, its role in myofibrillar turnover still needs further investigation.

**Calpains and calpastatin** The calpain “system” is a multi-component system composed of several isoforms of the enzyme, calpain, an endogenous inhibitor of the enzyme, named calpastatin, and a possible, yet unidentified activator of the calpains.

The calpains are cysteine proteases that have an absolute requirement for calcium in vitro to initiate full activity. The two most well characterized forms of the enzyme are termed μ-calpain and m-calpain. These isoforms are so named in reference to the difference in the amount of calcium needed by each enzyme for half-maximal activity. In general, μ-calpain requires between 5 to 65 μM Ca^{2+} for half-maximal activity, while m-calpain requires between 300 and 1000 μM Ca^{2+} for
half-maximal activity (Cong et al., 1989; Edmunds et al., 1991, Barrett et al., 1991). These Ca^{2+} requirements vary when specific substrates are used. For example, when physiological substrates such as skeletal muscle myofibrils or purified troponin are used as substrates, it has been shown that μ-calpain requires 65 μM (myofibrils) and 24 μM (troponin) Ca^{2+} for half-maximal activity and m-calpain requires 380 μM (myofibrils) and 580 μM (troponin) Ca^{2+} for half maximal activity. These values are substantially greater than are required for casein, an inexpensive and commonly used substrate. The amount of calcium required for half-maximal activity of μ-calpain when Hammersten casein is the substrate is 2.5 μM Ca^{2+}. For the same substrate, m-calpain requires 390 μM Ca^{2+} (Barrett et al., 1991).

Calpains appear to be ubiquitously found in vertebrate animal cells. While their exact physiological function is not known, they do seem to be specific in both their choice of substrates and in their cleavage of these proteins. For the most part, they cleave their substrates in a limited fashion, leaving large polypeptide fragments. They do not cleave their substrate proteins into their constituent amino acids. Several different classes of substrate proteins for the calpains have been identified and both μ- and m-calpain appear to have similar, if not identical, substrate specificities. Several generalities about substrate cleavage patterns have been drawn in a review of the calpain system by Croall and DeMartino (1991). It appears that the amino acids flanking the calpain cleavage site are not constant. From this it is inferred that other properties of the substrate may affect the susceptibility of the protein to cleavage by calpains. Some possibilities suggested by Croall and DeMartino (1991) include the following: the identity of amino acids at the scissile bond, or at regions distant to the scissile bond, or other features that are unique to the structure of a particular protein or peptide. Some of the proposed
roles of the calpains in vertebrate cells include; activation of enzymes, or at least alteration of the regulation of these enzymes, disassembly and/or remodeling of the cytoskeleton and cleavage of hormone receptors. In the case of certain enzymes, cleavage by calpains can cause them to be constitutively active, and/or alter their regulatory mechanisms. Many of the hormone receptors that are cleaved still retain their binding ability (Goll et al., 1992). In striated muscle, calpains have been shown to cleave many of the myofibrillar proteins including titin (Zeece et al., 1986; Kimura et al., 1992), desmin, troponin-T, troponin-I, tropomyosin and C-protein, but not myosin, actin and troponin-C (Dayton et al., 1975).

**Structure of \( \mu \)- and \( m \)-calpain** Both \( \mu \)- and \( m \)-calpain exist as heterodimers composed of an 80 kDa and a 28 kDa subunit. The 28 kDa subunit is identical in both \( \mu \)- and \( m \)-calpain. In fact, it has been shown to be encoded by a single gene (Suzuki, 1990). The N-terminal region (domain V) of the 28 kDa subunit is rich in Gly. The C-terminal region (domain VI) of this same subunit has four sets of amino acid sequences that predict E-F hand structures, similar to calmodulin, and may predict calcium binding sites (Suzuki, 1990).

The 80 kDa subunits are similar (with approximately 50% sequence homology) and are coded for by different genes. The 80 kDa subunit is composed of four domains, domains I, II, III, and IV. The N-terminal domain, domain I, has not been found to have any sequence homology with any other known protein sequence. The active site of the enzyme is thought to be located in domain II. This domain contains a Cys residue at what is thought to be the active site. This domain also contains a His residue as is found in other cysteine proteases (Suzuki, 1990). This domain, however, does not have a sequence that is closely analogous to other cysteine proteases. This finding has lead to the hypothesis that the evolution
of the calpains stemmed from an ancestral gene that was not the same as the ancestral gene(s) that other cysteine proteases evolved from (Goll, 1991). The third domain, domain III, has not been shown to have any sequence homology to any other known protein. The C-terminal domain, domain IV, has been called "calmodulin-like" because it contains four consecutive helix-loop-helix structures (E-F-hand) that predict calcium binding sites. Although the calpains require calcium for activity, they show no sequence homology to any other known metalloproteases (Suzuki, 1990). Nishimura and Goll (1991) indicate that domain IV of the 80 kDa subunit and domain VI of the 28 kDa subunit (both "calmodulin-like" domains) interact to form the non-covalent binding of the two subunits of the calpain molecule. In the same study, it was shown that fragments of the calpain molecule that contain the active site (all of domain II) did not show any catalytic activity regardless of whether or not calcium was present in the assay. This might possibly indicate that other regions of the molecule are needed to induce conformational changes in the active site to allow it to catalyze protein degradation.

As can be deduced from the above description, the calpains ($\mu$ and m) can be predicted to have eight binding sites for Ca$^{2+}$. Calpains however, based upon the available evidence do not seem to bind this amount of Ca$^{2+}$. Most studies to date seem to indicate that $\mu$-calpain bind two calcium atoms on the 80 kDa subunit, and two on the 30 kDa subunit for a total of four calcium atoms per molecule (Minami et al., 1987; Zimmerman and Schlaepfer, 1988). It appears that m-calpain, while having the same number of predicted calcium binding sites as $\mu$-calpain, binds as many as five or six calcium atoms. Again, as in $\mu$-calpain, the 28 kDa subunit appears to bind only two calcium atoms, while the 80 kDa subunit may
bind three or four calcium atoms (Coolican et al., 1986; Minami et al., 1987; Zimmerman et al., 1988).

**Localization** The most well characterized members of the calpain “family” (μ- and m-calpain) are localized within the cell and under most normal physiological conditions, very little, if any of these enzymes are found to exist outside the confines of the cell (Kumamoto et al., 1992). Both μ- and m-calpain appear to be localized with the plasma membrane and with subcellular organelles (Goll et al., 1992). Within skeletal muscle cells, they are localized with myofibrils, mitochondria, and nuclei. In skeletal muscle myofibrils, the calpains are most densely located at the Z-line, some calpain molecules can be detected at the I-band and the A-band, but the concentration does not appear to be as high in these regions of the myofibril as at the Z-line (Kumamoto et al., 1992). The endogenous inhibitor of the calpains, calpastatin appears to be co-localized with μ-and m-calpain (Goll et al., 1992).

**Autolysis** In the early 1980’s, it was noted that brief incubation of chicken skeletal muscle m-calpain with calcium at low temperatures (0°) reduced the amount of calcium required for half-maximal activity of the enzyme. The lowering of the calcium requirement was quite dramatic, from 400 μM Ca^{2+} required for half maximal activity before incubation with 0.5 mM Ca^{2+} to only 30 μM Ca^{2+} required for half maximal activity (Suzuki et al., 1981). This phenomenon was accompanied by a reduction in mass of both subunits of the enzyme (Suzuki et al., 1981). Later studies with both μ- and m-calpain have shown that, in general, limited autolysis reduces the amount of calcium required for half-maximal activity of m-calpain from 200-1000 μM to 50 to 150 μM concentrations. The calcium requirement for half-maximal activity of μ-calpain is reduced from 3-50 μM (before
autolysis) to 0.6 to 0.8 µM (after autolysis). An interesting effect to note is that the specific activities of both enzymes is largely left unchanged (Edmunds et al., 1991). Both µ and m-calpain undergo a reduction in mass when they are autolyzed. The mass of the 80 kDa subunit of m-calpain is reduced to 78 kDa and the 28 kDa subunit is reduced to 18 kDa. Likewise, the mass of the 80 kDa subunit of µ-calpain is reduced to 76 kDa via a 78 kDa intermediate. The mass of the 28 kDa subunit mass is reduced to 18 kDa as it is in m-calpain (Cong et al., 1989; Hathaway et al., 1982; Suzuki, 1990; Edmunds et al., 1991). It has been shown that brief autolysis of human calpain results in the loss of 19 amino acids from the N-terminus of the 80 kDa subunit of m-calpain, while 27 amino acids are removed from the N-terminus of the 80 kDa subunit of µ-calpain. The 28 kDa subunit, that is common to both enzymes, loses 91 amino acids from its N terminus (Goll et al., 1992).

The physiological significance of this autolysis is not clear. In most cases, the amount of calcium required to initiate autolysis is higher than the calcium required for proteolytic activity. For example, µ-calpain from bovine skeletal muscle has been shown to require 40 to 50 µM Ca^{2+} for half maximal activity for proteolysis, while 190-210 µM Ca^{2+} is required to initiate autolysis. The differences in calcium requirements for proteolytic activity and autolysis for m-calpain are considerably less. The protease m-calpain from bovine skeletal muscle requires 700-740 µM Ca^{2+} for a half maximal rate of hydrolysis of casein and requires 740-780 µM Ca^{2+} for autolysis (Cong et al., 1989). A specific example of how calcium concentration affects the activity of µ-calpain is seen in Cong et al., (1989). They showed that incubation of µ-calpain in the presence of up to 100 µM calcium at 25°C for times as long as one hour resulted in no autolysis,
yet hydrolysis of a casein substrate was observed and reached a maximum at 100 
µM Ca^{2+}. It was shown that almost complete autolysis was achieved within 5 
minutes at calcium concentrations of 200 µM Ca^{2+} and that proteolytic activity 
began to decrease after 3 minutes. The loss of activity after autolysis at 200 µM 
calcium concentrations was accomplished by degradation of the large subunit of 
autolyzed µ-calpain (76 kDa) to a 36 kDa polypeptide.

Interaction with various phospholipids such as phosphatidylinositol, 
phosphatidylserine, and phosphatidylethanolamine (Pontremoli et al., 1985. 
Imajoh et al., 1986) has been shown to lower the Ca^{2+} requirement for autolysis. 
Incubation of µ-calpain with phosphatidylinositol lowers the Ca^{2+} requirement for 
autolysis from 190-210 µM Ca^{2+} in the absence of phosphatidylinositol to 140-150 
µM Ca^{2+} in its presence. Both required calcium concentrations are higher than the 
unautolyzed µ-calpain needs for half maximal activity to hydrolyze casein. On the 
other hand, it has been shown that while m-calpain required 740-780 µM Ca^{2+} for 
half maximal autolysis when no phosphatidylinositol was included in the assay, it 
only required 370-400 µM Ca^{2+} in the presence of this phospholipid. In this case, 
the presence of the phosphatidylinositol lowered the calcium requirement for 
autolysis below that required for half maximal activity to degrade a casein substrate 
(Cong et al., 1989). As stated earlier, the physiological significance of autolysis is 
as of yet unclear.

**pH and temperature optima** The calpains appear to have a pH 
optimum that is near pH 7.5 (Dayton et al., 1976; Yoshimura et al., 1983; Wang 
and Jiang 1991) with a drop in caseinolytic activity below pH 6.5 and above pH 8.0 
(Dayton et al., 1976). Calpain does still retain some activity at pH values in the 
neighborhood of 5.6 (Koohmaraei et al., 1986; Zeece et al., 1986; Wang and
It has been shown that as the pH is lowered to at least 5.8, conditions are more favorable for autolysis (Koohmaraie, 1992).

The temperature optimum for the calpains is approximately 25°C (Dayton et al., 1976) in the presence of excess calcium. Other reports have placed the temperature optimum as high as 30°C (Wang and Jiang, 1991), but most researchers seem to agree that the activity declines rapidly at temperatures higher than 30°C. Most of these studies (which were carried out in the presence of ample calcium for autolysis) infer that the decrease in activity at temperatures above 30°C is due to autolytic inactivation of the enzyme (Dayton et al., 1976). On the other end of the spectrum, calpains are also active at lower temperatures (Koohmaraie et al., 1986; Zeece et al., 1986) although to a lesser degree than at 25°C.

**Ionic strength effects** Few reports have been published describing the effects of ionic strength on the activity of μ-calpain. Most studies report the effects of ionic strength on m-calpain. The caseinolytic activity of m-calpain at pH 7.5 appears to be adversely affected by increased ionic strength. In the presence of KCl, the ability of m-calpain to hydrolyze casein is reduced as the KCl concentration is raised from 75 to 500 mM. Conversely, as KCl concentration is lowered (from 75 to 0 mM KCl at pH 7.5) the activity of m-calpain, as assayed by casein hydrolysis, increases (Tan et al., 1988; Wang and Jiang, 1991). Several explanations for this effect have been proposed. It appears that the ions Na and K do not affect the activity of the enzyme directly, in either the presence or absence of calcium (Wang and Jiang, 1991) so the effect appears to be due to the increased ionic strength of the solution when KCl or NaCl are added. It has been suggested that the effect of added KCl (or NaCl) to the reaction mixture, may in some fashion, alter the substrate in a way that could modify the cleavage sites for calpain and
render them less accessible to the enzyme (Tan et al., 1988). It has also been proposed that a high ionic strength solution may cause conformational changes in the calpain molecule that could lead to an increase in hydrophobicity. This increase in hydrophobicity may allow aggregation of the enzyme. Another effect that has been hypothesized is that the higher ionic strengths may in some manner interfere with the interaction of the calpain enzyme and the calcium it requires for activity (Wang and Jiang, 1991).

The majority of the reports dealing with the effect of ionic strength on calpain activity have used a pH of 7.5 in their assay procedures. When lower pH values are used, ionic strength seems to have a slightly different effect (Kendall et al., 1992). Increasing ionic strength from 32 to 200 mM KCl at a pH of 7.0 seems to increase the caseinolytic activity of m-calpain. Beyond 200 mM KCl, the activity decreases. The same effect has been seen at pH 5.7, increasing the ionic strength up to 100 mM KCl produced a concomitant increase in m-calpain activity. Again, as at higher pH values, the continued increase in ionic strength causes a reduction in calpain activity (Kendall et al., 1992).

**Calpastatin** The endogenous inhibitor of the calpain system is known as calpastatin. Calpastatin is highly specific for the calpains, and is localized along with the calpains at structures including the skeletal muscle myofibrils, mitochondria, and nuclei. The cDNAs for calpastatin from several species have been cloned and sequenced (Maki et al., 1990). The calpastatin that is found in erythrocytes is substantially different from that found in all other cell types. This form of calpastatin has a molecular mass of 48 kDa and migrates under SDS-PAGE conditions as 70 kDa. The type that is found in all other tissues has a molecular mass of between 73 to 78 kDa (depending upon the tissue in which it is
found). This form migrates under SDS-PAGE conditions with an apparent molecular mass of between 107 to 117 kDa (again depending upon the tissue in which it is found). This larger form is made up of four domains and an N-terminal sequence (domain L). Each of these four domains has the ability to inhibit calpain activity. Based on this evidence, it has been hypothesized that one calpastatin molecule can inhibit up to four calpain molecules (Goll et al., 1992).

**Calpain/calpastatin interactions** One of the major factors that must be considered in the search for mechanisms that control calpain activity in vivo, is the interaction between the enzymes μ- and m-calpain and their endogenous inhibitor calpastatin. Calpastatin has been shown to be a competitive inhibitor of the calpains (Maki et al., 1988). Although calpastatin itself has not been shown to bind calcium ions, calcium is required to allow calpastatin to bind to the calpains (Kapprell and Goll, 1989; Maki et al., 1990). This binding appears to be reversible as calcium chelators can cause calpastatin to dissociate from calpain (Melloni et al., 1982; Maki et al., 1990). The amount of calcium required to allow half-maximal binding of calpastatin to calpains is generally lower than that required for half-maximal activity of the unautolyzed and autolyzed forms of m-calpain and for half-maximal activity of autolyzed μ-calpain. The unautolyzed form of the μ-calpain molecule, however, required slightly less calcium for half-maximal activity than for half-maximal binding to calpastatin. Unautolyzed m-calpain requires 150-1000 μM Ca²⁺, autolyzed m-calpain requires 10-40 μM Ca²⁺ and autolyzed μ-calpain requires 10-300 nM Ca²⁺ for half-maximal binding to calpastatin. Unautolyzed μ-calpain requires 35 to 55 μM Ca²⁺ for half-maximal binding to calpastatin (Kapprell and Goll, 1989). On the basis of these figures, Kapprell and Goll (1989)
hypothesized that unautolyzed μ-calpain may not be affected by the presence of calpastatin and could possibly be active.

In recent experiments designed to more precisely characterize the interactions between the calpains and calpastatin, Nishimura and Goll (1991) using autolysis-induced fragments of both μ- and m-calpain, showed that two fragments bound to calpastatin. The first fragment was from the 80 kDa subunit and contained the "calmodulin-like" region of the subunit (domain IV) and a portion of domain III. The other major fragment that was shown to bind calpastatin was from the 28 kDa subunit and also contained the "calmodulin-like" domain, domain VI. Interestingly, the fragments from μ- and m-calpain that contained the active site (domain II) and part of domain I did not bind to calpastatin. This is rather unexpected, as calpastatin has been classified as a competitive inhibitor of the calpains. It was suggested by Nishimura and Goll (1991) that since this fragment neither exhibited activity, nor bound to its competitive inhibitor, calpastatin, the domain I portion of calpain might act as a repressor to the active site of the calpain molecule.

**Role of the calpains under postmortem conditions** The enzymes μ- and m-calpain have been shown to retain at least partial activity in postmortem muscle and under pH and temperature conditions that mimic those seen during postmortem aging (Olson et al., 1977; Koohmaraie et al., 1986; Zeece et al., 1986; Koohmaraie et al., 1987). Under the conditions of pH 5.5-5.8 and 5°C, it has been shown that μ-calpain retains as much as 28% of the caseinolytic activity that it has at pH 7.5 and 25°C (Koohmaraie et al., 1986). This estimate may be low as the assay conditions at pH 5.6 are approaching the isoelectric point of casein (pH 4.6) and casein may have reduced solubility and lower susceptibility to digestion at pH
5.6. \(\mu\)-Calpain can cause many proteolytic changes in myofibrillar proteins at low pH and temperatures. Some of the changes that have been documented at these conditions include the release of \(\alpha\)-actinin, the degradation of desmin, the disappearance of intact troponin-T, and the appearance of polypeptides migrating at 30,000 daltons. \(\mu\)-Calpain is also capable of at least partially removing Z-disks at pH 5.5-5.8 and 5°C (Koohmarai et al., 1986). At higher temperatures (25°C), calpain has been shown to remove Z-disks over a wide range of pH (from pH 5.5 to 7.5) (Dayton et al., 1975; Dayton et al., 1976; Koohmarai et al., 1987). Likewise, m-calpain at reduced temperatures (5°C) and pH (6.5) has been shown to be associated with the degradation of titin, the release of \(\alpha\)-actinin form the myofibril, the degradation of troponin-T, and the appearance of a triplet of bands that migrate at approximately 31,500, 30,000, and 29,000 daltons (Zeece et al., 1986). More recent experiments (Kendall et al., 1992) demonstrate that m-calpain is capable of producing changes in the myofibrillar proteins desmin and troponin-T over a wide range of pH values and ionic strengths.

Studies that have focused on the fate of the major components of the calpain system (\(\mu\)-and m-calpain and calpastatin) in postmortem tissue have shown that while the activity of m-calpain tends to remain constant over a 14-day postmortem aging period, the activities of \(\mu\)-calpain and the inhibitor, calpastatin, also appear to decline rapidly within the first 24 hours postmortem (Koohmarai et al., 1987). Along with these observations is the fact that the myofibril fragmentation index (a measure of the amount of breakage of the myofibril, c.f. MacBride and Parrish, 1977) increases significantly during postmortem aging, especially during the first 24 hours (Koohmarai et al., 1987). Results like those outlined above, when combined with the fact that the calpains will autolyze to the point of inactivity when
given sufficient calcium (Cong et al., 1989; Nishimura and Goll, 1991), have led some researchers to conclude that rapid, early loss of calpain activity may be an indication of early activation of the calpains (Koohmaraie et al., 1987; Koohmaraie et al., 1988).

**The role of calpastatin in postmortem tissue** The calpastatin molecule has been implicated as playing a major role in the development of meat tenderness (Koohmaraie 1992a,b,c). The activity of calpastatin has been shown to decline during postmortem aging (Ducastaing et al., 1985; Koohmaraie et al., 1987). The rate of decline of calpastatin activity is variable, and has been shown to be related to the tenderness of beef as it is aged (Whipple et al., 1990). Correlation coefficients between calpastatin activity at 24 hours postmortem and 14 day shear force values (a mechanical measure of tenderness) have been found to be in the range of 0.39 to 0.69 (Whipple et al., 1990; Shackelford et al., 1991). The hypothesis that has been made that increased calpastatin activity results in decreased calpain activity and thus a decrease in postmortem tenderization (Whipple et al., 1990). These results are somewhat of an enigma, however, as it has been shown that cells contain enough calpastatin to completely inhibit all of the calpain present (Murachi, 1983). An additional complication arises when one considers the fact that in most cases, the calpains require less calcium for the binding of calpastatin than for activity. The only exception to this is the unautolyzed form of μ-calpain which requires slightly less calcium for half-maximal activity than it requires for binding to calpastatin (Kapprell and Goll, 1989). Under these circumstances, it is difficult to envision how most of the forms of calpain can be active under normal conditions. Unautolyzed μ-calpain may be the only form of calpain that may not be affected by the presence of calpastatin and may be active.
(Kapprell and Goll, 1989). Clearly, the relationship between the calpains and calpastatin in muscle cells must be investigated in order to elucidate the regulatory mechanism at work in postmortem tissue that results in variation in meat tenderness.

**Calpain based methods for enhancing meat tenderness** In 1988, Koohmaraie et al., reported a study in which they infused an amount of a 0.3 M calcium chloride solution equal to 10% of the live weight of the animal into lamb carcasses through the carotid artery. They noted that the infused carcasses reached their ultimate shear force values by 24 hours postmortem. Maximum proteolysis as measured by SDS-PAGE techniques was also achieved by 24 hours postmortem. This process is thought to act primarily by fully activating all of the available calpain, both µ-and m-calpain (Koohmaraie, 1992).

Since that initial experiment, numerous studies have been conducted to demonstrate the effectiveness of calcium chloride in accelerating meat tenderness. (e.g. Koohmaraie et al., 1989; Koohmaraie et al., 1990; Wheeler et al., 1991; Moorage et al., 1991). Injection or infusion of calcium chloride at early times postmortem is appealing because of several factors. These include the facts that in most cases the tenderization is complete at 24 hours postmortem, the meat is never over-tenderized, and the process has been shown to be of potential use in hot-boning procedures (Koohmaraie, 1992). This process has been shown to enhance the tenderness of meat from animals that are traditionally less tender such as old cows (Moorage et al., 1991) and those animals that were treated with β-adrenergic agonists (BAA) (Koohmaraie and Shackleford, 1991). Some important drawbacks to the procedure include the potential for off-flavor development (St. Angelo et al.,
1991) and the possibility of enhanced discoloration of the meat under retail storage conditions (Kerth et al., 1995).

Literature cited


SDS-PAGE AND WESTERN BLOTTING COMPARISONS OF PURIFIED MYOFIBRILS AND WHOLE MUSCLE PREPARATIONS FOR EVALUATING TITIN AND NEBULIN IN POSTMORTEM BOVINE MUSCLE\textsuperscript{1,2}

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Abstract

Purified myofibril (MF) and homogenized whole muscle (WM) samples were prepared from A maturity market steers. Samples were removed at 0, 1, 3, 7, 14, and 28 d postmortem. The MF and WM samples from all animals were analyzed by SDS-PAGE (5% gels) and by Western blot analysis using monoclonal antibodies to titin and nebulin. The rate of degradation of the intact forms of titin and nebulin with regard to differences dependent upon sample type (MF vs WM) were examined. The results showed that there was very little difference in the rate of postmortem degradation of the intact form of titin or of intact nebulin with respect to the two types of sample samples examined. Analysis of MF and WM preparations revealed that titin and nebulin were progressively degraded, each at its own rate, with nebulin degrading faster, as postmortem storage time increased. Examination of MF and WM samples showed that the intact form of titin (T1) was absent at the same time postmortem. Intact nebulin also disappeared from both MF and WM preparations at the same time postmortem with respect to sample preparation. Our results indicate either purified MF and WM samples can be used satisfactorily to analyze the rate of degradation of the intact forms of both titin and nebulin.

Key Words: Titin, Nebulin, Postmortem Aging, Beef, SDS-PAGE, Western Blotting

Introduction

The role that the two extremely large proteins of the myofibril, titin (approximately 3,000 kDa) and nebulin (approximately 600 to 900 kDa) may play in the development of meat tenderness has recently been closely examined (Fritz and Greaser, 1993; Huff-Lonergan et al., 1995; Taylor et al., 1995). It has been
suggested that these two proteins are likely to influence meat tenderness (Robson et al., 1991) because of their unique size, structural properties, positions in the myofibril, interactions with other key proteins in the sarcomere and their purported roles in myofibril integrity (Robson, 1995). In muscle samples obtained at death, titin migrates primarily as a single band referred to as T1. At early times postmortem, titin migrates as a doublet, the upper being intact T1 and the lower band, which migrates only slightly faster, being a degradation product of T1, referred to as T2 (Wang et al., 1979). Another high molecular weight degradation product of mammalian titin migrates at approximately 1,200 kDa (Matsuura et al., 1991), and appears with additional time postmortem. Nebulin (600 to 900 kDa), extends from the Z-line to the end of skeletal muscle thin filaments, and is closely associated with or part of the thin filament (Wright et al., 1993; Pfuhl et al., 1994). It has been proposed that nebulin may aid in anchoring the thin filaments to the Z-line and, thus, like titin, may play a significant role in maintaining structural order and integrity in the myofibril (Robson et al., 1991).

Recent studies have differed considerably in the reported rate of postmortem degradation of these proteins, especially titin (c.f. Fritz and Greaser, 1993, with Huff-Lonergan et al., 1995 and Taylor et al., 1995). One possibility for the differences may be in the material examined, namely purified myofibrils (MF) or whole muscle (WM) preparations (Fritz and Greaser, 1993; Huff-Lonergan et al., 1995). Thus the objective of this study was to determine if evaluation of the rate of postmortem degradation of intact titin (designated as T1) and nebulin was significantly influenced by analysis of purified MF versus whole muscle WM homogenates.
Materials and methods

Sources and storage of muscle  Six A maturity market steers were slaughtered at the Iowa State University Meat Laboratory following standard slaughter procedures. The carcasses were not electrically stimulated. A 10-g sample was removed from the region between the 12th and 13th rib of the longissimus muscle at approximately 45 min postmortem (0-day sample). The longissimus thoracis muscle was removed at 24 h postmortem after storage of the carcasses at 4°C. The longissimus muscles were individually vacuum packaged and stored at 2°C. Steaks, .64-cm thick, were removed from the stored muscles at 1, 3, 7, 14, and 28 d postmortem. All samples were used immediately upon removal for preparation of purified MF and WM homogenates.

Myofibril preparation  Myofibrils from at-death and postmortem aged muscle were purified at 2°C, according to the procedure of Huff-Lonergan et al. (1995). Protein concentrations were determined by using the biuret procedure as modified by Robson et al. (1968). The MF samples, suspended in 5 mM Tris-HCl, pH 8.0, were diluted to 3.2 mg/ml and then one vol of each sample was immediately combined with .5 vol of room temperature (25°C) tracking dye solution (3 mM EDTA, 3% [wt/vol] SDS, 30% [vol/vol] glycerol, .003% [wt/vol] pyronin Y, and 30 mM Tris-HCl, pH 8.0) (Wang, 1982) and .1 vol of 2-mercaptoethanol for a final protein concentration of 2 mg/ml. The samples were heated at 50°C for 20 min before loading on polyacrylamide gels.

Whole muscle preparation  The WM samples were prepared by using a modification of the method of Bechtel and Parrish (1983). A .4 g sample of tissue was knife-minced, added to 10 ml of a solution (25°C) containing 2% (wt/vol) SDS, 10 mM sodium phosphate buffer, pH 7.0, and homogenized with a motor-driven
Dounce homogenizer. The sample was then centrifuged at 1500 x g for 15 min at 25°C to remove traces of insoluble components. Protein concentrations of the supernatants were determined by using the bicinchoninic acid (BCA) method (Smith et al., 1985). Samples were diluted to 6.4 mg/ml and then prepared for SDS-PAGE as described for MF. The final concentration of the WM samples was 4 mg/ml.

**Gel system** A 5% polyacrylamide (acrylamide/bisacrylamide = 100:1 [wt/wt]) slab separating gel without a stacking gel was used to examine changes in high molecular weight proteins (approximately 3,000 to 205 kDa). The composition of the gels was as described in Huff-Lonergan et al. (1995). Sixty μg of MF samples and 96 μg of WM samples were loaded onto the gels. Gels (8 cm wide x 7.3 cm tall x 1.5 mm thick) were run on the Bio-Rad Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA). The gels were run at a constant current setting of 3.5 mA/gel for 17 h. Triplicate gels were run. After electrophoresis, gels were either stained for visualization of protein bands, or were transferred by electroelution to a nitrocellulose membrane. For examination of all protein bands, gels were stained a minimum of 12 h in an excess of .1% (wt/vol) Coomassie brilliant blue R-250, 40% (vol/vol) ethanol, and 7% (vol/vol) glacial acetic acid. Gels were destained in an excess of the same solution without the Coomassie brilliant blue R-250.

**Transfer conditions** Gels used for transfer were equilibrated for 15 min at 4°C in a transfer buffer containing 25 mM Tris, 192 mM glycine, 2 mM EDTA, 15% (vol/vol) methanol, and .1% (wt/vol) SDS. The SDS was used in the transfer buffer to aid in mobilization and transfer of very high molecular weight proteins from the gel to the membrane. Samples were blotted onto a nitrocellulose membrane by using a constant voltage setting of 90 V for 150 min.
Western blotting  After transferring, the membranes were blocked for one h at 25°C in blocking solution (80 mM di-sodium hydrogen orthophosphate, anhydrous, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, .1% [vol/vol] polyoxyethylene sorbitan monolaurate [Tween-20], 5% [wt/vol] non-fat dry milk) (Amersham, Arlington Heights, IL). Blots were incubated in solutions containing dilutions of primary antibodies diluted in a solution (PBS-Tween) identical to the blocking solution except with no non-fat dry milk. Primary antibodies used in the Western blotting procedure included, monoclonal anti-nebulin (NB2, Sigma Chemical Co., St Louis, MO) diluted 1:5,000 (antibody:PBS-Tween [vol/vol]) in PBS-Tween, and monoclonal anti-titin (prepared against bovine skeletal muscle titin) cell culture supernatant diluted 1:10 (antibody:PBS-Tween [vol/vol]) in PBS-Tween. Blots were washed three times, ten minutes per wash in PBS-Tween. Bound primary antibodies were labeled with goat-anti-mouse IgG horseradish peroxidase conjugated secondary antibodies (A2554, Sigma Chemical Co., St Louis, MO) diluted 1:5,000 (antibody:PBS-Tween [vol/vol]) in PBS-Tween, for 30 min at 25°C. Blots were rinsed in PBS-Tween three times, ten minutes per wash prior to detection. A chemiluminescent detection system was used as described by the supplier (Amersham) to detect labeled protein bands.

Titin purification  Titin, used for the preparation of monoclonal antibodies, was purified from fresh bovine longissimus muscle MF according to the procedure of Wang (1982). Before final isolation of the purified protein by gel filtration on a 2.6 x 90 cm Sephacryl S-500-HR (Pharmacia Biotech, Piscataway, NJ) column, titin and nebulin were separated by salt fractionation as described by Wang (1982).
Results

Both types of samples (MF and WM) were prepared from all six animals and all samples were examined by both SDS-PAGE and Western blotting with regard to overall qualitative (i.e., no attempt was made to quantify the amount of a given protein band or degradation product, only its presence or absence was evaluated) differences between the two preparations used. Results of SDS-PAGE of MF and WM samples prepared from two animals having similar rates of postmortem degradation are shown in Figure 1. Samples from these two animals were selected because they represent the two extremes observed in our study between MF versus WM samples. Although some differences in the rates of postmortem degradation of titin and nebulin were noted among animals, very little, if any, qualitative differences in the degradation of intact titin and nebulin were noted between the two types (MF and WM) of samples examined for any given animal.

Titin Upon examination of the major titin bands on Coomassie stained 5% gels (Figure 1), few differences were noted between the two types of preparation (compare a with b and c with d in Figure 1). In both the MF and WM samples at 0 d postmortem, T1 was the major form of titin present. At early times postmortem, (e.g. 3 d postmortem) titin appeared as a distinct doublet, with the lower band, T2, more evident at 3 d than at 1 d. In both the MF and WM samples, the T1 form of titin was present for essentially the same period of time postmortem (3 d in the sample shown). We rarely (e.g., animal #2, Figure 1 c) did observe a slightly increased tendency for the doublet pattern of titin to be present at earlier times postmortem in MF samples than in the WM samples from the same animal. However, the T2 band in the MF samples was always faint at 0 d and at early times postmortem (1 d in the samples shown) and at least some of the T1 form was present for the same total
length of time (3 d postmortem) regardless of whether MF or WM samples were examined. The T2 band (large molecular weight degradation product of titin) was the most prominent band at 3 d postmortem in both MF and WM samples, and underwent little additional change as detected by SDS-PAGE, over the remainder of the 28 d postmortem aging period. No obvious difference in the rate of appearance, or in the relative intensity of the T2 band was evident by SDS-PAGE (Figure 1) regardless of whether the MF or WM samples from the same animal were compared. We also found that the degradation product, termed the 1,200 kDa polypeptide (Matsuura et al., 1991), appeared at the same time postmortem (3 d) in both the MF and WM samples.

Western blots of samples from animal #1 shown in Figure 1 probed with a monoclonal titin antibody, are shown in Figure 2. The Western blots showed few, if any, qualitative differences in the rate of postmortem appearance of high molecular weight polypeptides that could be attributed differences in the type of preparation (MF or WM) examined. This observation was found for all six animals in our study (i.e. no differences in the rate of appearance any high molecular weight polypeptides were observed that were dependent upon the type [MF or WM] of sample).

**Nebulin** Essentially no differences attributable to the type of preparation (MF or WM) examined by SDS-PAGE were found in the rate of degradation of intact nebulin (Figure 1). At the time points examined, nebulin was degraded in MF samples at a rate equal to that seen in WM samples with intact nebulin absent by 3 d postmortem in both the samples. Nor were any major qualitative differences in results observed between MF and WM samples (Figure 3) when subjected to the sensitive chemiluminescent Western blotting technique, using a monoclonal
antibody specific for nebulin. Again, intact nebulin, as detected by the antibody, was present over the same time period in both MF and WM samples. Slight differences in the degradation products from nebulin in the two sample types could be noted at three to seven days postmortem, but most of the same bands were detected in both MF and WM samples. Thus, the sample type (i.e. MF or WM) made little difference in determining the apparent overall rate of nebulin degradation.

**Discussion**

Meat represents one of the most structurally complex food products produced. One quality attribute of meat that has been explored for a number of years is tenderness. One of the structural proteins suggested by some (Robson et al., 1991; Huff-Lonergan et al., 1995; Taylor et al., 1995) to play at least a partial role in tenderness development is titin. Interest in the role titin may play in meat tenderness was initiated by observations that a third filament system in the skeletal muscle sarcomere termed gap filaments (Locker and Leet, 1975), was degraded during aging (Davey and Graafhuis, 1976) or by proteolytic treatment *in vitro* (Locker et al., 1977). Since then, it has been shown that gap filaments are composed of titin (Locker et al., 1987). Titin is an elastic protein molecule that spans the distance from the Z-line to the M-line in the sarcomere (Furst et al., 1988). Titin may influence both the structural integrity of the myofibril (Horowits et al., 1986; Horowits and Podolsky, 1987) and the elastic properties of living muscle (Trombitas et al., 1993; Wang et al., 1993). Nebulin, through its interaction with both the Z-line and the thin filaments, could also play a role in maintaining the structural integrity of the myofibril (Robson et al., 1991) and, thus, its degradation may influence the postmortem development of tenderness.
Biochemical techniques have often been employed in an effort to determine changes or differences in structural and regulatory proteins found in muscle and meat that might help explain observed differences in tenderness (e.g., Parrish et al., 1973; Lusby et al., 1983; Fritz et al., 1991). Among the techniques used are SDS-PAGE (e.g. Koohmaraie et al., 1984; Huff-Lonergan et al., 1995) and Western blotting (Bandman and Zdanis, 1988; Fritz et al., 1991; Taylor et al., 1995). Although these techniques are sensitive, there is considerable variation in methods used among the studies. In this study, attention has been focused specifically on the type of sample examined. Purified MF are prepared by using several washing and differential centrifugation steps (Goll et al., 1974). The resulting samples are suspensions of MF almost free of sarcoplasmic and connective tissue proteins. Because MF samples are largely devoid of sarcoplasmic proteins, they provide easier interpretation of SDS-PAGE gels when lower molecular weight (< 200 kDa) proteins are examined. However, a proposed problem the use of MF samples is the potential for a variable degree of protein loss and/or loss of degradation products of myofibrillar proteins that could be soluble in the buffers used during the repeated washing and centrifugation steps (Fritz et al., 1993). The WM samples, on the other hand, contain not only myofibrillar proteins, but also a complex mixture of sarcoplasmic proteins. Thus, WM samples, albeit much faster to prepare, have the distinct disadvantage of containing large amounts of sarcoplasmic proteins that can make SDS-PAGE examination and interpretation of lower molecular weight myofibrillar proteins and degradation products very difficult due to the large number of non-myofibrillar protein bands migrating in the lower molecular weight ranges.
Very few qualitative differences between preparation methods were seen in the rate of appearance of two high molecular weight postmortem-aging-induced products of titin (T2 and 1,200 kDa polypeptide). This indicates that very little, if any, of these two degradation products are lost during the MF preparation procedure. In addition, because the presence of the insoluble intact form of titin (T1) was detected for the same period of time postmortem in both MF and WM preparations, and no difference was obtained between MF and WM samples in the time of disappearance of the intact form of nebulin, it can be concluded that little, if any of these two proteins are lost during the MF preparation procedure. The current study showed that in the cases of titin and nebulin, loss of either intact proteins and/or high molecular weight degradation products in MF preparations were not a significant problem. This study, therefore, demonstrates that either MF or WM preparation procedures can be used to examine the intact forms of titin and nebulin and/or their high molecular weight degradation products.

Implications

Although titin and nebulin are degraded at different times postmortem, little differences in the overall rate of degradation of intact titin and nebulin were detected between purified myofibril and whole muscle samples. Whole muscle samples have the advantage of being easier and quicker to prepare, while myofibril samples may be desirable when cleaner preparations of myofibrillar proteins are needed. Both types of samples provide valid means of evaluating the degradation (time of disappearance) of the intact forms of these two proteins. Either method can be used satisfactorily in studies for comparing the rate of degradation of intact titin and intact nebulin in different postmortem samples.
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Figure 1. Coomassie stained 5% gels of purified myofibrils and whole muscle samples from two bovine samples at 0, 1, 3, 7, 14, and 28 d postmortem. a) Animal #1, purified myofibrils b) Animal #1, whole muscle samples c) Animal #2, purified myofibrils d) Animal #2, whole muscle samples. Abbreviations are as follows: T1 = intact titin, T2 = large (approximately 2,400 kDa) degradation product of intact titin. Myosin = myosin heavy chain. Arrows indicate the tops of the gel well.
Figure 2. Western blots of purified myofibrils (a) and whole muscle samples (b) from a bovine sample (animal #1 in Figure 1) at 0, 1, 3, 7, 14, and 28 d postmortem labeled with a monoclonal antibody to titin. 0-28 refers to d postmortem.
a  Myofibrils
b  Whole Muscle
Figure 3. Western blots of purified myofibrils (a) and whole muscle samples (b) from a bovine sample (animal # 1 in Figure 1) at 0, 1, 3, 7, 14, and 28 d postmortem labeled with a monoclonal antibody to nebulin. 0 - 28 refers to d postmortem.
Myofibrils

Whole Muscle

0 1 3 7 14 28
PROTEOLYSIS OF SPECIFIC MUSCLE STRUCTURAL PROTEINS BY μ-CALPAIN AT LOW PH AND TEMPERATURE IS SIMILAR TO DEGRADATION IN POSTMORTEM BOVINE MUSCLE\textsuperscript{1,2}

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Abstract
Postmortem (PM) and μ-calpain induced degradation of specific skeletal muscle proteins was monitored by using SDS-PAGE and Western blotting techniques. Samples were removed from bovine longissimus thoracis muscle at approximately 45 min PM (at-death = 0 day) for the preparation of at-death myofibrils (MF) and 0-time samples. The LT was excised at 1 day PM, vacuum packaged and stored at 2°C. Samples were removed for Warner-Bratzler shear force analysis and biochemical analysis at 1, 3, 7, 14, 28, and 56 d PM. The protease μ-calpain was purified from bovine skeletal muscle and used to digest at-death MF at pH 5.6, 4°C, 100 μM CaCl₂. Degradation of the proteins titin, nebulin, filamin, desmin, and troponin-T was monitored in the PM-aged and μ-calpain-digested samples by using SDS-PAGE and Western blotting. The PM aged samples that had significantly lower shear force (LSF) values (P<.05) at 1 d PM exhibited faster degradation of these five proteins. In LSF samples, the intact titin band (T1) was absent by 7 d PM and nebulin was absent by 3 d PM. In LSF samples, some filamin was degraded by 3 d PM, but in higher shear force (HSF) samples, degradation was not apparent until 14 days PM. In LSF samples, desmin was degraded more rapidly PM than in HSF samples. Troponin-T was broken down to yield two major polypeptides, of approximately 28 and 30 kDa. These polypeptides appeared earlier PM in LSF samples. Degradation products similar to those observed PM, for all five proteins also were detected in Western blots of μ-calpain digested MF, suggesting the calpain system plays a key role in PM protein degradation.

Key Words: Protein Degradation, Calpain, Beef Tenderness, Postmortem Aging
Introduction

The postmortem (PM) degradation of skeletal muscle proteins and their relationship to meat tenderness and quality have been the subject of considerable research. Several proteins that have been examined include: titin (Lusby et al., 1983; Fritz and Greaser, 1991; Huff-Lonergan et al., 1995a), nebulin (Fritz and Greaser, 1991, Huff-Lonergan et al., 1995a; Taylor et al., 1995), filamin (Uytterhaegen et al., 1992; 1994), desmin (Robson et al., 1981; Whipple et al., 1990; Kooehmaraie et al., 1991), and troponin-T (Olson et al., 1977; Whipple and Kooehmaraie, 1992; Ho et al., 1994). Although most of these proteins have been shown to undergo some PM proteolysis, specific degradation products of these proteins (with the exception of troponin-T) have seldom been carefully identified.

The protease μ-calpain (for review of calpain, see Goll et al., 1992) has been implicated as being the major causative agent for many of the proteolytic changes that occur as meat is aged. Although μ-calpain has been shown to retain at least partial activity under PM conditions of low pH and temperature (Kooehmaraie et al., 1986; Kooehmaraie et al., 1987), many of the specific degradation products resulting from proteolysis under these conditions remain to be characterized.

The objective of this study was two-fold. The first objective was to identify degradation products occurring under normal PM aging conditions, of five myofibrillar and myofibril-associated proteins, namely titin, nebulin, filamin, desmin and troponin-T, in samples differing in Warner-Bratzler shear force values at early times PM. The second objective was to identify the μ-calpain-induced degradation products of the same five proteins from myofibrils (MF) \textit{in vitro} using postmortem-like conditions of pH 5.6 and 4°C and to compare these degradation products to
those products found in naturally aged samples from beef differing in shear force values at one d PM.

Materials and methods

Sources and storage of muscle  Six A maturity market steers were slaughtered at the Iowa State University Meat Laboratory following standard slaughter procedures. The carcasses were not electrically stimulated. A ten-g sample was removed from the region between the 12th and 13th rib of the longissimus thoracis (LT) at approximately 45 min PM (0-day sample). A portion of the LT muscle (.64-cm thick steak and a 2.54-cm thick steak) was removed at 24 h PM after storage of the carcasses at 4°C (one-day sample). The LT muscles were individually vacuum packaged and stored at 2°C. Adjacent .64-cm thick and 2.54-cm thick steaks were removed from the stored LT at 3, 7, 14, 28, and 56 days PM. The .64-cm thick samples were used immediately upon removal for preparation of purified MF. The 2.54-cm steaks were vacuum packaged and frozen at -20°C until they were used for Warner-Bratzler shear force analysis.

Warner-Bratzler shear force determination  Frozen 2.54-cm thick steaks aged 1, 3, 7, 14, 28, and 56 days from all animals were thawed at 2°C for 16 h beforecooking. Steaks were broiled in a General Electric (Chicago Heights, IL) Model CNO2 industrial broiler set at a temperature of 288°C. The surfaces of the steaks were 10.16 cm from the heat source. The steaks were turned when they reached an internal temperature of 24°C and were removed from the heat at an internal temperature of 65°C. Six 1.27-cm diameter cores were removed parallel to the muscle fiber direction from the central, medial and lateral portions of the steaks (two cores per location). Cores were sheared through the center, perpendicular to the fiber direction, with a Warner-Bratzler shear device attached to an Instron
Universal Testing Device (Model 4502) controlled with a Model 4500 computer assist module (Instron, Canton, MA). Peak shear force values were recorded as kg/1.27-cm diameter core.

**Myofibrill preparation** MF from at-death and aged muscle were prepared at 2°C according to the procedure of Huff-Lonergan et al. (1995a). Protein concentrations were determined by using the biuret procedure as modified by Robson et al. (1968). MF suspended in 5 mM Tris-HCl, pH 8.0, were diluted to 3.2 mg/ml. One volume of each sample was immediately combined with .5 volumes of 25°C tracking dye (3 mM EDTA, 3% [wt/vol] SDS, 30% [vol/vol] glycerol, .003% [wt/vol] pyronin Y, and 30 mM Tris-HCl, pH 8.0) (Wang, 1982) and .1 volume of β-mercaptoethanol (MCE) for a final protein concentration of 2 mg/ml. Samples were heated at 50°C for 20 min before electrophoresis.

**Purification of μ-calpain** The μ-calpain used in this study was purified from 4.5 kg of bovine semimembranosus muscle obtained 45 min PM according to the methods modified from Edmunds et al. (1991). The muscle was ground and homogenized in six volumes (wt/vol; weight in grams of the original ground muscle sample) of 5 mM EDTA, .1% (vol/vol) MCE, 20 mM Tris-HCl, pH 7.5 (TEM) containing 2.5 μM trans-Epoxysuccinyl-L-leucylamido-[4-guanidino]butane (E-64), 100 mg/ml ovomucoid trypsin inhibitor, and 2 mM phenylmethylsulfonylfluoride (PMSF). The homogenate was centrifuged at 17,700 x g for 30 min. After filtration of the supernatant and adjustment of the pH to 7.5 with solid Tris, proteins were salted out between 0 and 45% ammonium sulfate saturation. Proteins were pelleted at 17,700 x g for 30 min, resuspended in TEM and dialyzed against TEM. The supernatant was loaded onto a 5 cm x 50 cm QA-52 (Whatman, Hillsboro, OR) column previously equilibrated in TEM. After washing with TEM to remove
unbound proteins, the column was eluted with a linear gradient of 0 to 500 mM KCl in TEM, pH 7.5. Crude \( \mu \)-calpain was eluted between 115 mM KCl and 180 mM KCl. Fractions containing \( \mu \)-calpain activity were pooled and loaded onto a 2.6 cm x 37 cm phenyl-Sepharose (Pharmacia Biotech Inc., Piscataway, NJ) column that had been previously equilibrated in 125 mM KCl, TEM, pH 7.5. After washing with the equilibration buffer, \( \mu \)-calpain was eluted with 5 mM EDTA, .1% (vol/vol) MCE, and 20 mM Tris, pH 7.5. Pooled \( \mu \)-calpain from the phenyl-Sepharose column was adjusted to .8 M ammonium sulfate and was loaded onto a 2.6 cm x 28 cm butyl-Sepharose (Pharmacia Biotech Inc., Piscataway, NJ) column that had been previously equilibrated in .8 M ammonium sulfate, 5 mM EDTA, .1% (vol/vol) MCE, and 20 mM Tris, pH 7.5. After washing, the column was eluted with a linear gradient of .8 M ammonium sulfate to 0 mM ammonium sulfate in TEM. The pooled \( \mu \)-calpain-containing fractions were dialyzed against TEM and loaded onto a 1.6 cm x 50 cm DEAE-TSK (Supelco Inc., Bellefonte, PA) column that had been previously equilibrated in 1 mM EDTA, .1% (vol/vol) MCE, 20 mM MES-Tris (MES=2-[N-Morpholino]ethanesulfonic acid), pH 6.5. The \( \mu \)-calpain was eluted from this final column with a linear 0 to 150 mM KCl gradient in the equilibration buffer.

**Calpain activity assay** The activity of \( \mu \)-calpain was monitored during the purification procedure by measuring the release of trichloroacetic acid-soluble polypeptides resulting from the digestion of casein by the \( \mu \)-calpain (Koohmaraie, 1990).

**Digestion procedure** At-death MF from bovine longissimus muscle were prepared according to Huff-Lonergan et. al. (1995a) and stored in 50% (vol/vol) glycerol at -20°C until used. For each assay, four ml of glycerinated MF were spun
at 3100 x g for six min at 4°C. Pellets were washed with 2 ml of 5 mM Tris-HCl, pH 8.0 and then centrifuged at 3,100 x g for six min at 4°C. The supernatants were removed and the myofibril pellets were washed twice in 2 ml of a 4°C buffer containing 165 mM NaCl, 50 mM MES-Tris pH 5.6 (pH was adjusted at 4°C with cold 2 M Tris). Following each wash, the samples were centrifuged at 1,100 x g for six min. After the final spin, the pellets were resuspended in two ml of the same buffer, the pH and the conductivity of the samples was monitored, and adjusted (if necessary) to pH 5.6 and 165 mM NaCl. Protein concentrations were measured by using the biuret procedure as modified by Robson et al. (1968). Concentrations were adjusted with buffer (165 mM NaCl, 50 mM MES-Tris pH 5.6) to 4 mg of protein per ml, 100 μM CaCl₂, and 15 mM MCE. After the samples were allowed to equilibrate for five min in a 4°C circulating water bath, μ-calpain was added at a ratio of 1:800 (wt/wt; μ-calpain:myofibrillar protein). Control samples were the same, except 20 mM EDTA was added to the myofibril/CaCl₂/MCE mixture prior to the addition of μ-calpain (calpain control) or no μ-calpain was added (buffer control). Final reaction volumes were 2.5 ml. Samples (.4 ml) were removed after 0, 2, 15, 60, and 120 min of digestion, and added to an aliquot of 200 mM EDTA to bring the final concentration of EDTA to 20 mM to stop the reaction. Samples were centrifuged at 12,000 x g for 15 min at 4°C. The supernatant was removed, its volume measured, and reserved. An amount of 5 mM Tris-HCl, pH 8.0 equal to the amount of supernatant removed was added to resuspend the respective pellets. After thorough resuspension of the MF, pyronin Y tracking dye (3 mM EDTA, 3% [wt/vol] SDS, 30% [vol/vol] glycerol, .003% [wt/vol] pyronin Y, 120 mM DL-dithiothreitol [DTT] and 30 mM Tris-HCl, pH 8.0) (Wang, 1982) was added. The final protein concentration of the samples in tracking dye was 2 mg/ml. The
reserved supernatant was added to 2x concentrated pyronin Y tracking dye at a ratio of 1:1 (vol/vol). Samples were immediately heated at 50°C for 20 min and loaded onto gels. All digestions were done in triplicate.

**Gel system** To observe changes in both high and low molecular weight proteins two SDS-PAGE systems were used. Gradient gels (3.2 to 12%), without stacking gels, were used to examine changes in high molecular weight proteins (3,000 to 100 kDa). The gels were made using acrylamide (acrylamide:N,N'-bis-methylene acrylamide = 100:1 [wt/wt]), and 2 mM EDTA, .1% (wt/vol) SDS, .67% (vol/vol) N,N,N',N'-tetramethylethylenediamine (TEMED), .1% (wt/vol) ammonium persulfate (APS), and .375 M Tris-HCl, pH 8.0. Glycerol was added to the 12% solution to make a final concentration of glycerol 15% (vol/vol) to facilitate formation of the gradient. An 18% acrylamide gel system was used to identify smaller polypeptides (205-9 kDa). These gels were made using acrylamide (acrylamide:N,N'-bis-methylene acrylamide = 100:1 [wt/wt]), .1% (wt/vol) SDS, .67% TEMED, .1% (wt/vol) APS, and .375 M Tris-HCl, pH 8.8. A 5% stacking gel was used over the 18% gel and contained acrylamide (acrylamide:N,N'-bis-methylene acrylamide = 100:1), and .1% (wt/vol) SDS, .67% (vol/vol) TEMED, .1% (wt/vol) APS, and .375 M Tris-HCl, pH 6.8. Gels (8 cm wide x 9 cm tall x 1.5 mm thick) were run on Hoefer SE260 Mighty Small II units (Hoefer Scientific Instruments, San Francisco, CA). The running buffer used in both the upper and lower chambers of the slab gel unit contained 25 mM Tris, 192 mM glycine, 2 mM EDTA and .1% (wt/vol) SDS. Twenty µg of myofibril samples and of pellets obtained from the digested samples in tracking dye were loaded onto the gels. Twenty µl of the supernatant samples in tracking dye were loaded onto the gels. The 3.2-12% gels were run at a constant current setting of 6 mA/gel for
approximately 18 h at 25°C and the 18% gels were run at a constant voltage setting of 40 volts for 17 h at 25°C. Following electrophoresis, gels were either stained for visualization of protein bands, or were transferred by electroelution to PVDF membranes. Gels used for examination of all protein bands were stained for a minimum of 12 h in an excess of a solution containing .1% (wt/vol) Coomassie brilliant blue R-250, 40% (vol/vol) ethanol, and 7% glacial acetic acid. Gels were destained in an excess of the same solution without the Coomassie brilliant blue R-250.

**Transfer conditions** After running, gels used for transfer were equilibrated for 15 min at 4°C in either 25 mM Tris, 192 mM glycine, 2 mM EDTA, 15% (vol/vol) methanol, and .1% (wt/vol) SDS (3.2 to 12% gels), or in the same solution minus the SDS (18% gels). The SDS was used in the transfer buffer for the 3 to 12% gels to aid in mobilization of very high molecular weight proteins from the gel. Samples were blotted onto PVDF membranes using a Hoefer TE22 Mighty Small Transphor electrophoresis unit at a constant voltage setting of 90 V for 75 min (18% gels for transfer of desmin and troponin-T and 3.2 to 12% gels for transfer of nebulin and filamin) or at 90 V for 5 h to transfer titin (3.2 to 12% gels). The temperature of the transfer buffer was maintained between 4°C and 10°C by using a refrigerated circulating water bath.

**Western blotting** After transferring, the membranes were blocked for one hour at 25°C in blocking solution (80 mM di-sodium hydrogen orthophosphate, anhydrous, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, .1% [vol/vol] polyoxyethylene sorbitan monolaurate [Tween-20], and 5% [wt/vol] non-fat dry milk) prepared as described by the supplier of the chemiluminescent detection system (Amersham, Arlington Heights, IL). Primary antibodies used in
the Western blotting procedure included: polyclonal anti-desmin (prepared against porcine skeletal muscle desmin), diluted 1:15,000 in blocking solution; polyclonal anti-filamin (prepared against avian smooth muscle filamin) diluted 1:20,000 in blocking solution; monoclonal anti-nebulin (NB2, Sigma Chemical Co., St Louis, MO) diluted 1:5000 in PBS-Tween (same solution as for the blocking solution except the non-fat dry milk was not added); monoclonal anti-troponin-T (JLT-12, Sigma Chemical Co.) diluted 1:20,000 in PBS-Tween; monoclonal anti-titin (4C7, prepared against bovine skeletal muscle titin) cell culture supernatant diluted 1:10 in PBS-Tween. Incubation times for the blots in primary antibody are given in the figure legends. Blots were washed three times, ten min per wash in PBS-Tween (for blots labeled with monoclonal antibodies) or blocking solution (for blots labeled with polyclonal antibodies). Bound primary antibodies were labeled with either goat-anti-rabbit (used for blots labeled with polyclonal primary antibodies) IgG horseradish peroxidase conjugated secondary antibodies (A9169, Sigma Chemical Co., St Louis, MO), diluted 1:5000 in blocking solution, or with goat-anti-mouse (used for blots labeled with monoclonal primary antibodies) IgG horseradish peroxidase conjugated secondary antibodies (A2554, Sigma Chemical Co., St Louis, MO) diluted 1:5000 in PBS-Tween, for 30 min at 25°C. Blots were rinsed in PBS-Tween three times, ten min per wash, before detection. A chemiluminescent system (Amersham) was used to detect labeled protein bands as described by the supplier.

**Titin purification**  
Titin, used for the preparation of monoclonal antibodies, was purified from fresh bovine longissimus muscle MF according to the procedure of Wang (1982). Before final isolation of the purified protein by gel filtration on a
2.6 x 90 cm Sephacryl S-500-HR (Pharmacia Biotech, Piscataway, NJ) column, titin and nebulin were separated by salt fractionation as described by Wang (1982).

Results

Gels and Western blots shown of the naturally aged myofibril (MF) samples are representative of samples that differed significantly (P<.05) in their Warner-Bratzler shear force values at one day PM. The lower shear force (LSF) samples had mean shear force values between 2.6 and 2.9 kg/1.27-cm diameter core, while the higher shear force (HSF) samples had mean shear force values between 5.1 and 6.1 kg/1.27 cm-diameter core (SEM = .566).

Titin and nebulin General changes in the large proteins (Mr > myosin heavy chain) can be seen in the 3.2-12% gradient gels in Figure 1. The naturally aged MF samples from steaks with significantly (P<.05) lower shear force values (Figure 1, a) exhibited earlier disappearance of the intact forms of titin (T1) and nebulin than did the MF samples from steaks with significantly (P<.05) higher shear force values (Figure 1, b). In both the higher and lower shear force samples, intact nebulin was degraded faster than intact titin (Figure 1, a and b). In the MF from the LSF samples (Figure 1, a), intact titin could be detected through 3 days PM, while intact nebulin was not detected beyond one day PM. In the MF from the HSF samples (Figure 1, b) the same temporal relationship between intact titin and nebulin was noted. The MF from the HSF samples possessed intact titin through 7 days of PM aging. Nebulin, on the other hand, was not seen in its intact form beyond 3 days PM in the MF from the HSF samples in this study. This phenomenon has also been observed in other studies (e.g. Huff-Lonergan et al., 1995a; Taylor et al., 1995).
When the degradation patterns from the naturally aged samples were compared to the gels of the μ-calpain digested MF samples (Figure 1, c), it could be seen that after incubation at 4°C, pH 5.6, 100 μM CaCl₂ in the presence of μ-calpain, many of the same changes occurred. Most notably, nebulin was degraded quickly, the band corresponding to intact nebulin was degraded before intact titin (T1). Intact titin was substantially degraded after 120 min of incubation to predominantly the T2 form. In contrast, the control sample showed no degradation at 120 min, indicating the changes were due to μ-calpain and not to the incubation conditions of buffer and temperature.

Examination of Western blots provided more detailed information on the degradation of these two large structural proteins, titin and nebulin. Figure 2 shows Western blots probed with a monoclonal antibody to titin. Again, as was seen in the gels, the MF from LSF (Figure 2, a) samples exhibited earlier degradation of titin than did the MF from the HSF samples (Figure 2, b). T1 disappeared from the blots earlier in the MF from the LSF samples than in the MF from the HSF samples. In addition, the monoclonal antibody detected a titin degradation product that migrated at approximately 1200 kDa. In the MF from the LSF samples (Figure 2, a), the 1200 kDa polypeptide was detected by one day PM, while in the MF from the HSF samples (Figure 2, b), it was not detected until 3 days PM. As the PM aging time increased through 7 days PM in the MF from the LSF samples (Figure 2, a) and 14 days PM in the MF from the HSF samples (Figure 2, b), this 1200 kDa band also decreased. The 1200 kDa polypeptide was not detected by the antibody at or beyond 28 days PM in either the MF from the LSF samples or the MF from the HSF samples, indicating further degradation of this polypeptide. Degradation of T2 that could not be detected in the gels was detected by the Western blotting procedure.
By 7 days PM in the MF from the LSF samples (Figure 2, a) and by 28 days PM in the MF from the HSF samples (Figure 2, b), the titin antibody failed to recognize the T2 band of titin, even though this band could still be seen through 56 days PM on Coomassie stained gels.

The blots of the purified MF that were digested with μ-calpain at pH 5.6, 4°C 100 μM CaCl₂ (Figure 2, c) revealed the disappearance of the T1 band by 60 min of incubation. A polypeptide migrating at 1200 kDa also increased over the incubation period, up to 60 min. By 120 min, however, the band was not strongly detected by this antibody. Additionally, as in the naturally aged samples, the T2 band of titin was not recognized by the monoclonal antibody after prolonged times of incubation (120 min). As seen in the gel sample of the digests, the 120 minute control did not appear to be significantly different from the zero time sample, indicating little if any affect of the buffer or temperature on the proteins examined.

Failure of the monoclonal antibody to recognize either the 1200 kDa polypeptide or T2 after extended times PM and after prolonged digestion times indicates that proteolysis of these two polypeptides occurs, even though this degradation is not seen on Coomassie stained gels.

Western blots probed with a monoclonal antibody to nebulin (Figure 3) showed the same trend as was observed in the gels. Intact nebulin was not recognized by the antibody beyond one day PM in the MF from the LSF samples (Figure 3, a). Intact nebulin was detected through 3 days PM in the MF samples from HSF samples (Figure 3, b). The MF that were digested with μ-calpain at pH 5.6, 4°C (Figure 3, c) showed rapid degradation of intact nebulin. After 15 min of incubation, intact nebulin was not detected by the antibody.
Upon further examination of Figures 2 and 3, it can be seen that in both the MF samples from the naturally aged LSF and HSF samples as well as in the μ-calpain digested samples, the degradation of nebulin preceded the degradation of titin. This indicates that nebulin is slightly more susceptible to degradation by endogenous proteases (including μ-calpain) than is titin.

When the naturally aged MF from the LSF samples and MF from the HSF and the μ-calpain digested samples were run on 18% gels and transferred to PVDF for Western blotting, neither the monoclonal titin antibody, nor the monoclonal nebulin antibody were able to detect any degradation products (results not shown). This suggests that the lower molecular weight degradation products were rendered unrecognizable to the antibody, or were small enough that they were not resolved on the 18% gels used in this study. No titin or nebulin degradation products that could be recognized by the particular monoclonal antibodies used in this study were detected in the supernatant of the digested samples run on either the 3.2-12% (figures 2,c and 3, c) or 18% gels (not shown). This indicates that it is highly unlikely that any degradation products recognized by these two antibodies were lost in the MF preparations of the naturally aged samples.

Filamin Blots that were probed with a polyclonal antibody to filamin are shown in Figure 4. MF samples from the LSF steaks showed the appearance of an approximately 240 kDa product in addition to intact filamin by 3 days PM (Figure 4, a). Appearance of this product indicates degradation or disruption of intact filamin. In contrast, the approximately 240 kDa product was not apparent in the MF from the HSF samples until 14 days PM (Figure 4, b).

When the myofibril samples that were digested with μ-calpain at pH 5.6, 4°C were examined by Western blotting, it was seen that the polyclonal antibody
recognized the presence of a degradation product at 60 min of incubation (Figure 4, c). Again, as in the naturally aged MF sample, the degradation product that was recognized migrated very closely to the intact form, giving the appearance of a doublet. From the results in Figure 4 it can be seen that μ-calpain is capable of producing the same or nearly the same degradation pattern of filamin as was seen in the naturally aged samples.

**Desmin** Blots of MF from the LSF samples that were probed with a polyclonal antibody to desmin revealed three degradation products (Figure 5, a). The first products to appear were detected at 3 days PM and migrated at approximately 45 kDa and 38 kDa. The 45 kDa polypeptide appeared to be transient and was not detected beyond 14 days PM in the MF from the LSF samples, indicating that it was further degraded. The 38 kDa polypeptide, after it appeared at 3 days PM was present throughout the 56 day aging period. Also appearing in the MF from the LSF sample was a 35 kDa polypeptide. This polypeptide was not seen until 56 days PM and was very faint.

Blots of the MF from the HSF samples showed slower degradation of desmin (Figure 5, b). The 45 kDa polypeptide was not apparent until 7 days PM and remained through 28 days PM. The 38 kDa polypeptide appeared at 14 days PM, and like in the MF from the LSF samples, remained throughout the rest of the aging period (56 days). Unlike in the MF from the LSF samples, the presence of a 35 kDa polypeptide was not detected in the MF from the HSF samples.

Blots of MF that had been incubated with purified μ-calpain showed the presence of a 38 kDa and a 35 kDa polypeptide when probed with the polyclonal desmin antibody (Figure 5, c). In these blots, the 38 kDa polypeptide appeared first (after 2 min of incubation) and remained throughout the digestion time (120 min).
The 35 kDa polypeptide appeared later (after 15 min of incubation) and it, like the 38 kDa polypeptide, remained through the 120 minute incubation. The 45 kDa polypeptide was not seen to appear in the μ-calpain digested MF samples. This could be due to degradation of the 45 kDa polypeptide prior to the sampling time. μ-Calpain, in the amount added to the purified MF, was able to fully degrade the intact desmin within 60 min of incubation. The naturally aged MF, on the other hand, continued to show intact desmin throughout the aging period. Either there was less active μ-calpain available in relation to the total amount of substrate protein, or the conditions were not as favorable for μ-calpain-induced degradation of desmin in the PM muscle samples as they were in the purified MF. In either case, it is possible that the absence of the 45 kDa polypeptide in the digested samples is due to its transient nature. It may have been formed and subsequently degraded before a sample was taken for analysis.

Troponin-T The degradation of troponin-T and the appearance of bands migrating at approximately 30 kDa has often been reported (MacBride and Parrish, 1977; Olson et al., 1977; Wheeler and Koohmaraie, 1994). Blots of MF from the LSF and HSF samples in this study that were probed with a monoclonal antibody to troponin-T also showed a difference in the degradation of this thin filament regulatory protein (Figure 6, a and b). There were two distinct degradation products migrating at approximately 30 kDa and 28 kDa that were labeled by the troponin-T monoclonal antibody. In the MF from the LSF samples (Figure 6, a) both the 30 kDa and the 28 kDa polypeptides appeared by 3 days PM. In addition, intact troponin-T was present on the blots through 14 days PM. The MF from the HSF samples (Figure 6, b) showed a marked increase in the amount of time before both the 30 kDa and 28 kDa polypeptides were recognized by the monoclonal
antibody. The 30 kDa polypeptide was not prominently recognized by the troponin-T monoclonal antibody until 7 days PM. The 28 kDa polypeptide was not recognized until 14 days PM. Intact troponin-T was present on the blots of MF from HSF samples through 28 days PM. The retardation of the appearance of the 30 kDa and 28 kDa polypeptides and the increased amount of the time that intact troponin-T could be detected on the blots of the MF from the HSF samples are reflective of the reduced tenderness as measured by Warner-Bratzler shear force values.

The purified MF that were digested with purified μ-calpain also showed the development of a 30 kDa polypeptide and a 28 kDa polypeptide (Figure 6, c). These degradation products appeared within 60 min of incubation with the μ-calpain. The presence of these polypeptides indicate that μ-calpain is capable of producing these degradation products under PM conditions of pH 5.6 and 4°C.

Discussion

Titin Titin, an extraordinarily large protein (approximately 3000 kDa), has been shown to span the distance from the Z-line to nearly the M-line (Furst et al., 1988), fully half of the skeletal muscle sarcomere. One possible role that titin may serve is to aid in maintaining the structural integrity of the myofibril. Titin has been proposed to be a structural entity that keeps the thick filaments in register in the middle of the sarcomere. Titin, based on the fact that its I-band portion is elastic in nature, may also play a role in maintaining elasticity of skeletal muscle cells (Wang et al., 1991, Wang et al., 1993). Due to the afore mentioned roles of titin in living cells, the possibility exists that its degradation may lead to weakening of the lateral
structure of the myofibrillar sarcomere. This weakening, in conjunction with other physiochemical forces, could lead to enhanced tenderness.

The degradation of intact titin (T1) has been observed in several studies (Lusby et al., 1983; Zeece et al., 1986; Astier et al., 1993). A major degradation product that migrates only slightly faster under SDS-PAGE conditions than T1 is termed T2 (approximately 2400 kDa) (Kurzban and Wang, 1988). Another degradation product that has been observed migrates at 1200 kDa under SDS-PAGE conditions (Matsuura et al., 1991; Takahashi et al., 1992). This polypeptide has been shown to contain the portion of titin that extends from near the Z-line to near the putative N₂ line in the l-band (Kimura et al., 1992; Kawamura et al., 1995).

A polypeptide migrating in the region of 1200 kDa was observed in the current study. This polypeptide was recognized by a monoclonal antibody prepared against intact titin (Figure 2). The 1200 kDa polypeptide was observed to appear faster in LSF samples than in HSF samples. The same monoclonal antibody that recognized T1 and the 1200 kDa polypeptide also initially recognized the other large degradation product, T2. This antibody, which labels at the A-I junction as revealed by immunofluorescence microscopy (results not shown), may recognize a repeated epitope that is common to both the 1200 kDa and the T2 portion of the molecule. Reports of monoclonal titin antibodies that recognize multiple epitopes are not uncommon (Itoh et al., 1988; Furst et al., 1989). The T2 polypeptide was also apparently degraded or altered. After prolonged periods of PM storage or µ-calpain digestion, the antibody ceased to recognize T2. The degradation of T2 also appeared to be related to the rate of tenderization, as it appeared to be degraded faster in LSF samples.
Nebulin  Nebulin, another extremely large (600-800 kDa) (Jin and Wang, 1991) relatively inextensible protein that is found only in skeletal muscle, spans the distance from the Z-line to near the end of the thin filament. Nebulin, which is thought to be intimately associated with the thin filament (Pfuhl et al., 1994), has been proposed to aid in anchoring the thin filament to the Z-line (Wang and Wright, 1988; Komiyama et al., 1992). Degradation of nebulin could weaken the thin filament linkages at the Z-line and thereby weaken the structure of the muscle cell. Nebulin has also recently been shown to be capable of linking actin and myosin and is proposed to have a regulatory function in skeletal muscle contraction (Root and Wang, 1994). If this proves to be the case, then it is possible that its degradation may alter actin-myosin interactions in such a way that the alignment of thick and thin filaments is disrupted. This could eventually lead to an increase in PM tenderization. Nebulin degradation, as seen in this study (Figure 3) and others (Huff-Lonergan et al., 1995a; Taylor et al., 1995) does seem to be related to PM tenderization.

Filamin  Filamin is a large (Mr=245,000 in skeletal and cardiac muscle) actin binding protein that has been shown to exist in numerous cell types (Price et al., 1994). Many different isoforms have been shown to exist (Hock et al., 1990). the amount of filamin in skeletal and cardiac muscle is very low (<.1%). In skeletal and cardiac muscle, filamin has been shown to be localized at the periphery of the Z-disk and is thought to be associated with intermediate filaments (Price et al., 1994). Postmortem degradation of filamin could disrupt key linkages that serve to hold MF in lateral register. Degradation of filamin could possibly alter linkages connecting peripheral MF to the sarcolemma by changing interactions between peripheral Z-disks and the sarcolemma via intermediate filament associations.
This study showed that some filamin was degraded to form an approximately 240 kDa degradation product in both naturally aged MF and in μ-calpain digested MF (Figure 4). This same doublet formation (composed of intact and degraded filamin) has been seen in cultured embryonic skeletal muscle cells and has been attributed to calpain activity (Kwak et al., 1993). Other reports (Uytterhaegen et al., 1994) have seen increased degradation of filamin in samples injected with CaCl₂, a process that has been shown to stimulate proteolysis and PM tenderization (Koohmaraie et al., 1988b). The fact that the current study shows that the degradation of filamin in the naturally aged MF occurs at different rates in samples with different shear force values indicates that its degradation is either directly involved in tenderization, through disruption of key linkages, or is an indicator of PM proteolysis. Further studies that employ sensitive detection methods (e.g. Western blotting, immunofluorescence) are needed to determine the role of filamin in skeletal muscle systems and PM tenderization.

Desmin Desmin, a member of the 10 nm diameter intermediate filament group (O'Shea et al., 1981) that is localized at the periphery of the Z-disk in skeletal muscle (Richardson et al., 1981), has been suggested to play a role in PM tenderization (Hwan and Bandman, 1989; Whipple and Koohmaraie, 1991). Desmin, as a component of intermediate filaments, has been shown to surround the Z-lines of MF and to connect adjacent MF at the level of the Z-line, as well as to connect MF to other cellular structures, including the sarcolemma (Yagyu et al., 1990). Through these roles, it is possible that desmin may be important in maintaining the structural integrity of muscle cells (Robson et al., 1991). Degradation of a structural element that connects major components of a cell
together, as well as to the cell membrane, could affect the development of
tenderness.

This study, in agreement with others, found that desmin is degraded during
PM storage (Koohmaraie et al., 1984a,b; Koohmaraie et al., 1988a; Hwan and
Bandman, 1989), and that it was degraded more rapidly in samples with lower
shear force values than in samples with higher shear force values. The major
degradation product that was seen in both the LSF and HSF samples was a
polypeptide of approximately 38 kDa (Figure 5). This degradation product was
seen to appear also in μ-calpain digested MF in this study. The fact that the 38 kDa
polypeptides produced in both MF from naturally aged samples and in μ-calpain
digested purified MF, were detected by the same antibody suggests that these
could be the same polypeptide. μ-Calpain may be, at least in part responsible for
the production of the 38 kDa polypeptide under normal PM conditions. The fact
that this polypeptide appeared at earlier times PM in LSF samples suggests that its
generation may be linked to PM tenderization processes.

**Troponin-T** For many years, it has been recognized that the degradation of
troponin-T and the appearance of polypeptides migrating at approximately 30 kDa
are strongly related to the tenderness of beef (MacBride and Parrish, 1977; Olson
and Parrish, 1977; Penny and Dransfield, 1979). It has been shown that purified
bovine troponin-T can be degraded by calpain *in vitro* to produce polypeptides in
the 30 kDa region (Olson et al., 1977). In addition, polypeptides in the 30 kDa
region found in aged bovine muscle have been shown to be products of troponin-T
by using Western blotting techniques (Ho et al., 1994). In the current study,
increasing PM aging time was also shown to be associated with an appearance of
2 major polypeptides that were labeled with a monoclonal antibody to troponin-T, a
30 kDa polypeptide and a 28 kDa polypeptide. In addition, the increasing postmortem aging time was also associated with a loss of troponin-T, as has been seen in numerous studies (Olson et al., 1977; Koohmaraie et al., 1984; Ho et al., 1994). The antibody used in this study appears to label two very closely spaced bands corresponding to intact troponin-T. This is possibly due to isoforms of troponin-T that exist in skeletal muscle (Clarke et al., 1976; Briggs et al., 1990; Malhotra, 1994). Both the appearance of the 30 and 28 kDa bands and the disappearance of the intact troponin-T band in this study appear to be very strongly related to the shear force (LSF vs. HSF) classification of the samples. Samples with significantly higher shear force values showed a delayed appearance of both the 28 and 30 kDa bands as well as delayed degradation of intact troponin-T (Figure 6), indicating slower degradation of troponin-T in samples with higher shear force values. Under conditions of pH and temperature (pH 5.6 and 4°C) that commonly occur in postmortem aged samples, it can be seen (Figure 6) that the protease μ-calpain is capable of catalyzing the development of polypeptides migrating at 28 and 30 kDa. This, coupled with the fact that the degradation products in the naturally aged MF and μ-calpain digests are labeled with the same highly specific monoclonal antibody suggests that these could be the same polypeptides and supports the hypothesis that μ-calpain is at least partly responsible for the PM degradation of troponin-T and the concomitant production of the 28 and 30 kDa polypeptides.

Degradation of troponin-T may simply be an indicator of overall PM proteolysis. However, because troponin-T is an integral part of skeletal muscle thin filaments (Greaser and Gergely, 1971; Flicker et al., 1982) its role in PM tenderization may warrant more careful examination as has been suggested
previously (Ho et al., 1994; Uytterhaegen et al., 1994; Huff-Lonergan et al., 1995). Several reasons for this possibility exist. Troponin-T makes up the elongated portion of the troponin complex and through its interaction with tropomyosin aids in regulating the thin filament during skeletal muscle contraction. It is possible that degradation of troponin-T and disruption of its interactions with other thin filament proteins may aid in the disruption of the thin filament in the I-band, leading to fragmentation of the myofibril. Additionally, since troponin-T is part of the regulatory complex that aids in mediating actin-myosin interactions, it could be possible that its degradation may lead to changes involving thick and thin filament interactions. Regardless of whether or not it aids in disruption of the thin filament and/or the I-band, alters thick and thin filament interactions, or simply reflects overall protein degradation, degradation of troponin-T into 30 and 28 kDa polypeptides appears to be a good indicator of beef tenderness.

The degradation of all five of the proteins examined in this study appeared to occur more quickly in LSF samples than in HSF samples. In the LSF samples, major changes took place between 1 to 3 days PM while in HSF samples, the major changes took place much later and over a longer period of time, between 3 and 14 days PM.

It appears that PM tenderization does not depend upon the degradation of one single protein, rather tenderization is probably linked to structural changes occurring in several key regions of the muscle cell. Just as the overall integrity and function of muscle cells does not depend upon a single protein, but on the coordinated action of several proteins, the structural weakening of those muscle cells must also not depend upon the degradation a single cytoskeletal protein either. The proteins examined in this study are located in different regions of the
muscle cell, and most have been implicated as being important in maintaining the structure and function of the muscle cell in some manner. In addition, these proteins are located at regions that appear to be affected during PM aging, including areas near the Z-line and in the I-band. Degradation of proteins such as desmin and filamin, located at the periphery of the Z-line, may disrupt the lateral register of the MF themselves as well as their attachments to the cell membrane. Degradation of the proteins within the myofibril that are associated with the thick and thin filaments may allow lateral movement to occur within the sarcomere of PM aged samples. Titin, nebulin and troponin-T, through their ability to directly interact with, or modulate the interaction between, proteins of the thick and thin filaments and/or the Z-line have the opportunity to play key roles. Disruption of these proteins, especially titin and nebulin, may allow for further physiochemical changes that lead to myofibril fragmentation and ultimately tenderization. The fact that the proteins in this study were degraded more quickly in LSF than in HSF samples indicates that degradation of these proteins may be related in some manner to PM tenderization.

As seen in this study, μ-calpain has the ability under PM conditions of relatively low pH and temperature, to degrade titin, nebulin, filamin, desmin and troponin-T into many of the same degradation products as were produced in naturally aged MF. This further implicates μ-calpain as being a catalyst for the changes occurring in PM muscle. Further research is needed to determine the factors that govern the interactions between μ-calpain and its substrates within the skeletal muscle cell.
Implications

This study supports the hypothesis that the protease \( \mu \)-calpain is responsible for many of the significant changes that occur in postmortem aged beef. \( \mu \)-Calpain, under conditions of low pH and temperature, produces many similar polypeptides in purified MF as can be seen to develop in postmortem aged tissue. The appearance of several proteolytic products of the proteins titin, nebulin, filamin, desmin, and troponin-T in naturally aged beef seem to be related to postmortem tenderization. The fact that the afore mentioned proteins are degraded in a similar time frame, suggests that disruption of several key proteins is important in postmortem tenderization. Further studies on processes controlling the degradation of key myofibrilar proteins by \( \mu \)-calpain could lead to the development of more efficient methods for producing consistently tender beef.
Literature cited


Figure 1. Coomassie stained 3.2-12% gradient SDS-PAGE gels of myofibrils

a) Naturally aged myofibrils isolated at 0, 1, 3, 7, 14, 28, and 56 days postmortem from a low shear force (LSF) sample. b) Naturally aged myofibrils isolated at 0, 1, 3, 7, 14, 28, and 56 days postmortem from a high shear force (HSF) sample. 0-56 refers to days postmortem. c) Pellets and supernatants from purified at-death myofibrils incubated at 4°C, pH 5.6 with μ-calpain and sampled after 0, 2, 15, 60, and 120 minutes of incubation. 0-120 refers to minutes incubation with μ-calpain. C = digest buffer control sample (without μ-calpain) after 120 minutes of incubation at 4°C, pH 5.6. Other abbreviations are as follows: T1 = intact titin, T2 = Large (2400 kDa) degradation product of titin, MHC = myosin heavy chain. Open triangle designates the position of T1. Closed arrowhead designates the position of T2. Small arrow designates the position of nebulin.
Figure 2. Western blots of myofibril samples run on 3.2-12% gradient gels and transferred to PVDF membrane. The blots were incubated with monoclonal titin antibody 4C7 (diluted 1:10 in PBS-Tween) overnight at 4°C. a) Naturally aged myofibrils isolated at 0, 1, 3, 7, 14, 28, and 56 days postmortem from a low shear force (LSF) sample b) Naturally aged myofibrils isolated at 0, 1, 3, 7, 14, 28, and 56 days postmortem from a high shear force (HSF) sample 0 - 56 refers to days postmortem. c) Pellets and supernatants from purified at-death myofibrils incubated at 4°C, pH 5.6 with μ-calpain and sampled after 0, 2, 15, 60, and 120 minutes of incubation. 0 -120 refers to minutes of incubation with purified μ-calpain. C= digest buffer control sample after 120 minutes of incubation at 4°C, pH 5.6, and 100 μM CaCl₂. Open triangle designates the position of T1. Closed arrowhead designates the position of T2. Abbreviations are as follows: T1 = intact titin, T2 = Large (2400 kDa) degradation product of titin, 1200 K = 1200 kDa degradation product of titin, MHC = myosin heavy chain,
a

T1
T2
1200 K-

b

T1
T2
1200 K-

μ-Calpain Digested Myofibrils
pH 5.6  4°C

Pellet  Supernatant
0 2 15 60 120 C 0 2 15 60 120 C
Figure 3. Western blots of myofibril samples run on 3.2-12% gradient gels and transferred to PVDF membrane. The blots were incubated with monoclonal nebulin antibody NB2 (diluted 1:5000) overnight at 4°C. a) Naturally aged myofibrils isolated at 0, 1, 3, 7, 14, 28, and 56 days postmortem from a low shear force (LSF) sample b) Naturally aged myofibrils isolated at 0, 1, 3, 7, 14, 28, and 56 days postmortem from a high shear force (HSF) sample. 0 - 56 refers to days of postmortem aging. c) Pellets and supernatants from purified at-death myofibrils incubated at 4°C, pH 5.6 with μ-calpain and sampled after 0, 2, 15, 60, and 120 minutes of incubation. 0 - 120 refers to minutes of incubation with purified μ-calpain. C= digest control sample after 120 minutes of incubation at 4°C, pH 5.6 and 100 μM CaCl₂.
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Nebulin

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C

μ-Calpain Digested Myofibrils

Pellet | Supernatant
---     | ---
0      | 2 15 60 120 C
2      | 15 60 120 C

Figure 4. Western blots of myofibril samples run on 3.2-12% gradient gels and transferred to PVDF membrane. The blots were incubated with polyclonal filamin antibody (diluted 1:20,000 in blocking solution) for two hours at room temperature. a) Naturally aged myofibrils isolated at 0, 1, 3, 7, 14, 28, and 56 days postmortem from a low shear force (LSF) sample b) Naturally aged myofibrils isolated at 0, 1, 3, 7, 14, 28, and 56 days postmortem from a high shear force (HSF) sample. 0 - 56 refers to days of postmortem aging. c) Pellets and supernatants from purified at-death myofibrils incubated at 4°C, pH 5.6 with μ-calpain and sampled after 0, 2, 15, 60, and 120 minutes. 0 - 120 refers to minutes of incubation with μ-calpain. C= digest buffer control sample after 120 minutes of incubation at 4°C, pH 5.6 and 100 μM CaCl₂. Closed triangle designates the position of intact filamin. Open triangle designates the position of the large degradation product of filamin.
Filamin

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\( \mu \)-Calpain Digested Myofibrils

\( \text{pH} 5.6 \quad 4^\circ C \)

Pellet          Supernatant

 Filamin

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Figure 5. Western blots of myofibril samples run on 18% gels and transferred to PVDF membrane. The blots were incubated with a polyclonal antibody to desmin (diluted 1:15,000 in blocking solution) for one hour at room temperature (25°C). a) Naturally aged myofibrils isolated at 0, 1, 3, 7, 14, 28, and 56 days postmortem from a low shear force (LSF) sample. b) Naturally aged myofibrils isolated at 0, 1, 3, 7, 14, 28, and 56 days postmortem from a high shear force (HSF) sample. 0 - 56 refers to days of postmortem aging. c) Pellets and supernatants from purified at-death myofibrils incubated at 4°C, pH 5.6 with μ-calpain and sampled after 0, 2, 15, 60, and 120 minutes of incubation. 0 - 120 refers to minutes of incubation with purified μ-calpain. C= digest buffer control sample after 120 minutes of incubation at 4°C, pH 5.6 and 100 μM CaCl₂.
a) LSF

Desmin

38 K-
35 K-

b) HSF

Desmin

38 K-

38 K-

35 K-

35 K-

C) μ-Calpain Digested Myofibrils
pH 5.6  4°C

Pellet  Supernatant

0  2  15  60  120  C  0  2  15  60  120  C

Desmin

38 K-

35 K-

38 K-

35 K-
Figure 6. Western blots of myofibril samples run on 18% gels and transferred to PVDF membrane. The blots were incubated with monoclonal troponin-T antibody JLT-12 (diluted 1:20,000 in PBS-Tween) for one hour at room temperature (25°C). a) Naturally aged myofibrils isolated at 0, 1, 3, 7, 14, 28, and 56 days postmortem from a low shear force (LSF) sample. b) Naturally aged myofibrils isolated at 0, 1, 3, 7, 14, 28, and 56 days postmortem from a high shear force (HSF) sample. 0 - 56 refers to days of postmortem aging. c) Pellets and supernatants from purified at-death myofibrils incubated at 4°C, pH 5.6 with μ-calpain and sampled after 0, 2, 15, 60, and 120 minutes of incubation. 0 - 120 refers to minutes of digestion with purified μ-calpain. C = digest buffer control sample after 120 minutes of incubation at 4°C, pH 5.6 and 100 μM CaCl₂. Arrows indicate the positions of two isoforms of intact troponin-T.
**Troponin-T**

30 K- 28 K-

- LSF
- HSF

- Troponin-T
  - 30 K-
  - 28 K-

**μ-Calpain Digested Myofibrils**

pH 5.6 4°C

- Pellet
- Supernatant

- Troponin-T
  - 30 K-
  - 28 K-
EFFECTS OF THE PROTEASE \( \mu \)-CALPAIN ON PURIFIED MYOFIBRILS
AT LOW TEMPERATURE UNDER DIFFERENT CONDITIONS OF PH
AND IONIC STRENGTH

A paper to be submitted to Biochimie

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; E-64, trans-Epoxysuccinyl-
L-leucylamido-(4-guanidino)butane; Tris, tris[hydroxymethyl]aminomethane; MCE,
\( \beta \)-mercaptoethanol; PMSF, Phenylmethylsulfonylfluoride; KCl, potassium chloride;
MES, 2-[N-Morpholino] ethanesulfonic acid; TCA, trichloroacetic acid; HCl,
hydrochloric acid; CaCl\(_2\), calcium chloride; SDS, sodium dodecyl sulfate; SDS-
PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DTT, DL-
Dithiothreitol; TEMED, N\'N\'N\'N\'-tetramethylethylenediamine; APS, ammonium
Summary

The protease \( \mu \)-calpain has been implicated in the degradation of key myofibrillar proteins in many situations including; myofibrillar protein turnover and muscle growth. Temperature, pH and ionic strength are known to affect calpain activity, however, the combination of these factors on the proteolytic activity of \( \mu \)-calpain on myofibrillar proteins and the specific degradation products produced has not been fully documented. In this study, the degradation of the myofibrillar proteins titin, nebulin, filamin, desmin and troponin-T by \( \mu \)-calpain under varied pH and ionic strength conditions at low temperature (4°C) was monitored using SDS-PAGE and highly sensitive chemiluminescent Western blotting techniques. \( \mu \)-Calpain, purified from bovine skeletal muscle, was used to digest purified bovine skeletal muscle myofibrils in the presence of 100 \( \mu \)M CaCl\(_2\), under the following conditions at 4°C: 1) pH 7.0, 165 mM NaCl, 2) pH 7.0, 330 mM NaCl, 3) pH 5.6, 165 mM NaCl, and 4) pH 5.6, 330 mM NaCl. \( \mu \)-Calpain degraded the proteins titin, nebulin and desmin faster than it degraded filamin and troponin-T under all four conditions. The activity of \( \mu \)-calpain on all five proteins was greatest at pH 7.0, 165 mM NaCl and was lowest at pH 7.0, 330 mM NaCl. The proteins troponin-T and filamin were not noticeably degraded under the conditions of pH 7.0, 330 mM NaCl. This study demonstrates that \( \mu \)-calpain has the ability at low temperatures to degrade the proteins titin, nebulin, filamin, desmin and troponin-T under conditions that mimic those found in postmortem tissue. Neither low pH, nor the combination
of low pH and high ionic strength were effective in inhibiting μ-calpain from degrading any of the proteins examined.

key words: μ-calpain / myofibrillar proteins / pH / ionic strength

Introduction

The calpain enzyme system has been widely studied in muscle systems by muscle biologists for many years [1-6]. Calpains were first characterized in mammalian skeletal muscle by Dayton et al. [7] and this family of calcium-dependent cysteine proteinases are found in almost every vertebrate tissue that has been examined for their existence [6, 8]. The calpains have been shown to degrade myofibrillar proteins including; titin [9-11], nebulin [11], filamin [11, 12], C-protein [7], desmin [11, 13], troponin-I [7], tropomyosin [7] and troponin-T [2, 7, 11, 14] to name a few. Meanwhile, they have not been shown to degrade the two most abundant myofibrillar proteins, actin and myosin [7]. Additionally, while one of the most observable microscopic changes involves removal of the Z-lines, calpains have been shown to release, but not to degrade, one of the predominant Z-line proteins, α-actinin [15].

In living muscle systems, the calpains have been hypothesized to be instrumental in myofibrillar protein turnover and muscle growth [7, 16]. Many of the same proteins that are substrates for the calpains are also degraded in postmortem tissue [3, 4, 11, 17, 18]. The proteins actin and myosin, which are not substrates for calpain, are also not degraded in postmortem muscle [19].

Postmortem tissue provides a set of conditions that are in many respects vastly different from those that are found in living tissue. During the first few hours
after death, the temperature of muscle declines from 37°C to temperatures as low as 2-4°C and the pH declines from near 7.0 to approximately 5.6. At the same time, the ionic strength of the tissue increases from an equivalent of approximately 165 mM NaCl to nearly twice that value [20, 21]. Calcium is also released from its intracellular stores [22] allowing for as much as 100-fold increases or more in free calcium concentration in the muscle tissue [17]. While the increase in Ca^{2+} concentration would favor the activity of μ-calpain (requiring approximately 65 μM Ca^{2+} for half-maximal activity on myofibrils, [14]) it has been argued that the decreased temperature and pH would inhibit its action in postmortem muscle given that the pH optimum is near neutrality, and the temperature optimum is at 25°C [23]. The rise in ionic strength of postmortem muscle would also seem to not favor calpain activity as m-calpain has been shown to be somewhat inhibited by increases in ionic strength [24, 25, 26]. While μ-calpain has been shown to have some action on skeletal muscle myofibrils under conditions of pH 5.5-5.8 and 5°C [4], the effect of ionic strength has not been as carefully examined in combination with low pH and temperature, nor have the degradation products of key myofibrillar proteins been carefully documented.

The objective of this study was to examine the combined effects of pH and ionic strength on the ability of highly purified μ-calpain to degrade specific myofibrillar proteins at low temperature and pH and to identify μ-calpain induced degradation products of key myofibrillar proteins. The pH and ionic strength ranges were chosen to encompass those potentially found in both living and postmortem muscle.
Materials and methods

Purification of μ-calpain The μ-calpain used in this study was purified from 4.5 kg of bovine semimembranosus muscle obtained 45 minutes postmortem according to the method of Edmunds et al. [5] with modifications. The procedure was as follows. The muscle was ground and homogenized in 6 volumes of 5 mM EDTA, 0.1% (volume/volume) MCE, 20 mM Tris, pH 7.5 (TEM) containing 2.5 μM E-64, 100 mg/ml ovomucoid trypsin inhibitor and 2 mM PMSF. The homogenate was centrifuged at 17,700 x g for 30 minutes. After filtration of the supernatant and adjustment of the pH to 7.5 with solid Tris, proteins were salted out between 0 and 45% saturated ammonium sulfate. Proteins were pelleted at 17,700 x g for 30 minutes, resuspended in TEM and dialyzed. The supernatant was loaded onto a 5 cm x 50 cm QA-52 (Whatman, Hillsboro, OR) column. After washing to remove unbound proteins, the column was eluted with a linear gradient of 0 to 500 mM KCl in TEM, pH 7.5. Crude μ-calpain was eluted between 115 mM KCl and 180 mM KCl. Fractions containing μ-calpain activity were pooled and were loaded onto a 2.6 cm x 37 cm phenyl-Sepharose (Pharmacia Biotech Inc., Piscataway, NJ) column that had been previously equilibrated in 125 mM KCl, TEM, pH 7.5. After washing with the equilibration buffer, μ-calpain was eluted with 20 mM Tris, 5 mM EDTA and .1% (volume/volume) MCE, pH 7.5. Pooled μ-calpain from the phenyl-Sepharose column was adjusted to 0.8 M ammonium sulfate and was loaded onto a 2.6 cm x 28 cm butyl-Sepharose (Pharmacia Biotech Inc., Piscataway, NJ) column that had been previously equilibrated in 0.8 M ammonium sulfate, 20 mM Tris, 5 mM EDTA and 0.1% (volume/volume) MCE, pH 7.5. After washing, the column was eluted with a linear gradient of 0.8 M ammonium sulfate to 0 mM ammonium sulfate in TEM. The sample was dialyzed and loaded onto a 1.6 cm x
50 cm DEAE-TSK (Supelco Inc., Bellefonte, PA) column equilibrated in 1 mM EDTA, 0.1% (volume/volume) MCE, 20 mM Tris-MES, pH 6.5. The \( \mu \)-calpain was eluted from this final column with a linear 0 to 150 mM KCl gradient.

**Calpain activity assay** The activity of \( \mu \)-calpain was monitored during the purification procedure by measuring the release of TCA-soluble polypeptides resulting from the digestion of casein by the \( \mu \)-calpain [27].

**Digestion procedure** Myofibrils from at death bovine longissimus muscle were prepared according to the procedure of Huff-Lonergan et al. [27] and were stored in 50% (volume/volume) glycerol at -20°C until use. For each assay, 4 ml of glycerinated myofibrils were spun at 3100 \( \times \) g for 6 minutes at 4°C. Pellets were washed with 2 ml 5 mM Tris-HCl, pH 8.0 and then spun at 3100 \( \times \) g for 6 minutes at 4°C. The supernatant was removed and the myofibril pellets were washed twice in 2 ml of one of the following 4°C buffers; 165 mM NaCl in 50 mM MES-Tris pH 5.6, 330 mM NaCl in 50 mM MES-Tris pH 5.6, 165 mM NaCl in 50 mM HEPES-Tris, pH 7.0, or 330 mM NaCl in 50 mM HEPES-Tris, pH 7.0. Following each wash, the samples were spun at 1100 \( \times \) g for 6 minutes. After the final spin, the pellets were resuspended in 2 ml of the respective buffer, the pH and the conductivity of the samples was monitored and adjusted (if necessary) to meet the desired pH and conductivity parameters. Protein concentrations were measured using the biuret procedure as modified by Robson et al. [28]. Concentrations were adjusted using the desired buffer to 4 mg of protein per ml, 100 \( \mu \)M CaCl\(_2\), and 15 mM MCE. After the samples were allowed to equilibrate in a 4°C circulating water bath, \( \mu \)-calpain was added at a ratio of 1:800 (\( \mu \)-calpain:myofibrillar protein, weight/weight). Control samples were the same, except 20 mM EDTA was added to the myofibril/CaCl\(_2\)/MCE mixture prior to the addition of \( \mu \)-calpain (calpain control) or
no μ-calpain was added (buffer control). Final reaction volumes were 2.5 ml. Samples (.4 ml) were removed after 0, 2, 15, 60, and 120 minutes of digestion and added to an aliquot of 200 mM EDTA to bring the final concentration of EDTA to 20 mM to stop the reaction. Samples were spun at 12,000 x g for 15 minutes at 4°C. The supernatant was removed, measured and reserved. An amount of 5 mM Tris-HCl, pH 8.0 equal to the amount of supernatant removed was added to resuspend the pellets. After thorough mixing, pyronin Y tracking dye [3 mM EDTA, 3% (weight/volume) SDS, 30% (volume/volume) glycerol, 0.003% (weight/volume) pyronin Y, 120 mM DTT and 30 mM Tris-HCl, pH 8.0] [30] was added. The final concentration of the samples in tracking dye was 2 mg/ml. The reserved supernatant was added to 2x pyronin Y tracking dye at a ratio of 1:1. Samples were immediately heated at 50°C for 20 minutes and loaded onto gels. All digestions were done in triplicate.

**Gel system** To observe changes in both high and low molecular weight proteins two gel systems were used. Stackless 3.2-12% gradient gels were used to examine changes in high molecular weight proteins (3000-100 kDa). The gels were made using a 30% stock solution of acrylamide (acrylamide:N,N'-bis-methylene acrylamide = 100:1), and 2 mM EDTA, 0.1% (weight/volume) SDS, .67% (volume/volume) TEMED, 0.1% (weight/volume) APS, and 0.375 M Tris-HCl, pH 8.0. Fifteen percent (volume/volume) glycerol was added to the 12% gel solution to stabilize the gradient. An 18% gel system was used to identify smaller polypeptides (205-9 kDa). These gels were made using a 30% stock solution of acrylamide (acrylamide:N,N'-bis-methylene acrylamide = 100:1), 0.1% (weight/volume) SDS, 0.67% TEMED, 0.1% (weight/volume) APS, and 0.375 M Tris-HCl, pH 8.8. A 5% stacking gel was used on the 18% gels containing
acrylamide (acrylamide: N,N'-bis-methylene acrylamide = 100:1), and 0.1% (weight/volume) SDS, 0.67% (volume/volume) TEMED, 0.1% (weight/volume) APS, and 0.375 M Tris-HCl, pH 6.8. Gels (8 cm wide x 9 cm tall x 1.5 mm thick) were run on the Hoefer SE260 Mighty Small II (Hoefer Scientific Instruments, San Francisco, CA). The running buffer used in both the upper and lower chambers of the slab gel unit was 25 mM Tris, 192 mM glycine, 2 mM EDTA and 0.1% (weight/volume) SDS. Twenty µg of pellets from the digested samples were loaded onto the gels. Twenty µl of the supernatant samples in tracking dye were loaded onto the gels. The 3.2-12% gels were run at a constant current setting of 6 mA/gel for approximately 18 hours at room temperature and the 18% gels were run at a constant voltage setting of 40 volts for 17 hours at room temperature. Following electrophoresis, gels were either stained for visualization of protein bands, or were transferred by electroelution to PVDF membranes. Gels for examination of all protein bands were stained a minimum of 12 hours in an excess of a solution containing 0.1% (weight/volume) of Coomassie brilliant blue R-250, 40% (volume/volume) ethanol and 7% glacial acetic acid. Gels were destained in an excess of the same solution without the Coomassie brilliant blue R-250.

**Transfer conditions**  
After running, gels for transfer were equilibrated in either 25 mM Tris, 192 mM glycine, 2 mM EDTA, 15% (volume/volume) methanol and 0.1% (weight/volume) SDS (3.2-12% gels) or in the same solution minus the SDS (18% gels). Gels were equilibrated for 15 minutes in the buffer at 4°C. SDS was used in the transfer buffer for the 3-12% gels to aid in mobilization of very high molecular weight proteins from the gel. Samples were blotted onto PVDF membranes using a Hoefer TE22 Mighty Small Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) at a constant voltage setting of
90 V for 1 hour and 15 minutes or at 90 V for 5 hours to transfer titin. The temperature of the transfer buffer was maintained between 4°C and 10°C by using a refrigerated circulating bath.

**Western blotting** After transferring, the membranes were blocked for one hour at room temperature (25°C) in blocking solution [80 mM di-sodium hydrogen orthophosphate, anhydrous, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, 0.1% (volume/volume) Tween-20, and 5% (weight/volume) non-fat dry milk]. Primary antibodies used in the Western blotting procedure included; polyclonal anti-desmin (prepared against porcine skeletal muscle desmin), diluted 1:15,000 in blocking solution, polyclonal anti-filamin (prepared against avian smooth muscle filamin) diluted 1:20,000 in blocking solution, monoclonal anti-nebulin (NB2, Sigma Chemical Co., St Louis, MO) diluted 1:5000 in PBS-Tween [PBS-Tween = 80 mM di-sodium hydrogen orthophosphate, anhydrous, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, 0.1% (volume/volume) Tween-20], monoclonal anti-troponin-T (JLT-12, Sigma Chemical Co., St Louis, MO) diluted 1:20,000 in PBS-Tween, monoclonal anti-titin (4C7, prepared against bovine skeletal muscle titin) cell culture supernatant diluted 1:10 in PBS-Tween. Incubation times for the blots in primary antibody are indicated in the figure legends. Blots were washed three times, ten minutes per wash in PBS-Tween (blots labeled with monoclonal antibodies) or blocking solution (blots labeled with polyclonal antibodies). Bound primary antibodies were labeled with either goat-anti-rabbit (used for blots labeled with polyclonal primary antibodies) IgG horseradish peroxidase conjugated secondary antibodies (A9169, Sigma Chemical Co., St Louis, MO), diluted 1:5000 in blocking solution, or goat-anti-mouse (used for blots labeled with monoclonal primary antibodies) IgG.
horseradish peroxidase conjugated secondary antibodies (A2554, Sigma Chemical Co., St Louis, MO) diluted 1:5000 in PBS-Tween, for 30 minutes at room temperature. Blots were rinsed in PBS-Tween three times, ten minutes per wash prior to detection. A chemiluminescent detection system (Amersham, Arlington Heights, IL) was used to detect labeled protein bands.

**Titin purification**  Titin, for the preparation of monoclonal antibodies, was purified from fresh bovine longissimus muscle myofibrils according to the procedure of Wang [30]. Prior to final isolation of the purified protein by gel filtration using a 2.6 x 90 cm Sephacryl S-500-HR (Pharmacia Biotech, Piscataway, NJ) column, titin and nebulin were separated by salt fractionation. Titin complexed with SDS was selectively precipitated by bringing the NaCl concentration of the SDS-solubilized myofibril proteins to a final concentration of between 0.64 and 0.7 M. The precipitated titin was resuspended using 20 mM EDTA, 80 mM DTT, 20% SDS, 4 mM PMSF, 200 mM Tris-HCl, pH 8.0 [30] before the gel filtration step.

**Results**

**SDS-PAGE**  The μ-calpain that was purified for use in this study was a highly homogeneous preparation as can be seen in Figure 1. This figure shows the column fractions from the final step in the purification procedure that had high activity. These fractions were pooled (Figure 1, lane P) and used for the digestions in this study.

Figure 2 represents the Coomassie stained 3.2-12% gradient gels of the μ-calpain digest. Under conditions of relatively low ionic strength (165 mM NaCl) and pH 7.0 (Figure 2, a) μ-calpain was seen to have a very rapid effect on the two large structural proteins titin and nebulin. As can be seen in the pellet fraction, the intact form of titin, T1 was degraded to primarily the T2 form within 15 minutes.
Likewise, nebulin was also quickly degraded with very little intact nebulin remaining in the pellet by two minutes. Other protein bands were seen to be degraded under these same conditions of pH and temperature. A protein band migrating just faster than myosin heavy chain (band e, Figure 2, a) was degraded by 60 minutes. In addition, at 60 minutes in the pellet fraction a band (band f, Figure 2, a) was seen to appear and remain through 120 minutes of incubation.

In the supernatant, a high molecular weight polypeptide appeared after 15 minutes of incubation. This polypeptide was replaced by 60 minutes with a slightly faster migrating polypeptide (band g, Figure 2) that remained through 120 minutes. Also appearing in the supernatant at 15 minutes and remaining through 120 minutes was a polypeptide that migrated faster than myosin heavy chain (band h, Figure 2). A band (band i, Figure 2) migrating with the approximate molecular mass of α-actinin was seen in the supernatant at 15 minutes and to increase in intensity through 120 minutes. None of the antibodies used in this study identified bands e-i.

Under higher ionic strength conditions (330 mM NaCl) at pH 7.0 (Figure 2, b), the T1 band was degraded to T2 by 120 minutes. Nebulin was degraded more quickly than T1 under the conditions of pH 7.0, 165 mM NaCl. Intact nebulin was degraded by 60 minutes. There was a general increase in the solubility of proteins (Figure 2, b) when compared to the other treatments (Figure 2, a, c, d), but no significant differences were seen in the digested treatments when compared to the controls.

μ-Calpain was able to catalyze the degradation of several myofibrillar proteins at the lower pH of 5.6. At pH 5.6, 165 mM NaCl (Figure 2, c), T1 was partially degraded by 120 minutes postmortem. Intact nebulin was degraded by 60
minutes. A band migrating with the approximate molecular mass of α-actinin (Figure 2, c, band i) could be detected in the supernatant of the 60 minute digestion period. The μ-calpain digest done at pH 5.6, 330 mM NaCl (Figure 2, d) resulted in T1 being mostly degraded by 60 minutes. Intact nebulin was largely degraded by 2 minutes and was not apparent on the gels by 15 minutes.

**Western blots**

**Titin** The Western blots probed with a monoclonal antibody to titin (4C7) (Figure 3) revealed that μ-calpain was able to degrade titin fastest at the conditions of lower ionic strength (165 mM NaCl) and pH 7.0 (Figure 3, a) Within 15 minutes, both T1 and T2 were degraded to the point that the monoclonal antibody no longer was able to detect T1 and only weakly recognized T2. Within 60 minutes, neither band (T1 or T2) was detected by the antibody. Under conditions of pH 7.0, 330 mM NaCl (Figure 3, b), μ-calpain appeared to degrade titin at a much slower rate. The antibody was able to recognize T1 through 60 minutes of digestion, and recognized both T2 and 1200 kDa band in the digestion at 120 minutes. The two digestions that were done at the lower pH of 5.6 (165 and 330 mM NaCl; Figure 3, c and d) showed a rate of titin degradation that was intermediate to the two digestions done at pH 7.0. Both digestions done at pH 5.6 showed that intact titin (T1) was recognized by the monoclonal antibody through 15 minutes. T2, the highest molecular weight degradation product of titin was recognized by the antibody through 60 minutes, in both digestions done at pH 5.6, irrespective of the ionic strength conditions. Likewise, a 1200 kDa band was detected through 60 minutes in both pH 5.6 conditions. Neither T2 nor the 1200 kDa polypeptide were recognized at 120 minutes. No form of titin in the supernatant was recognized by the monoclonal antibody. There were no titin
degradation products smaller than the 1200 kDa polypeptide that could be identified by the monoclonal antibody, 4C7, in any of the samples on either on the 3.2-12% gradient gels (Figure 3) or on 18 % gels (results not shown) under any digestion conditions.

**Nebulin** Western blots probed with a monoclonal antibody to nebulin (NB2) (Figure 4) showed that nebulin was degraded by μ-calpain more quickly under all conditions examined than was titin. Within 15 minutes, at pH 7.0, 165 mM NaCl (Figure 4, a), pH 5.6, 165 mM NaCl (Figure 4, c), and pH 5.6, 330, mM NaCl (Figure 4, d), the intact nebulin band that was recognized by the antibody was absent from the blots. The slowest rate of degradation of nebulin by μ-calpain took place under the conditions of pH 7.0, 330 mM NaCl (Figure 4, b). Under these digestions conditions, the antibody, NB2, strongly recognized the intact nebulin band through 15 minutes of digestion. Even after 60 minutes of incubation with μ-calpain a slight amount of intact nebulin could be detected.

No nebulin degradation products were detected by the antibody, either in the pellets or in the supernatants on either 3.2-12% gradient gels (Figure 4, a-d) or 18% gels (results not shown).

**Filamin** The protease μ-calpain degraded some filamin into an approximately 240 kDa product within 15 minutes of incubation at pH 7.0, 165 mM NaCl (Figure 5, a). Within 60 minutes, filamin was completely degraded under those same conditions. At pH 7.0, 330 mM NaCl (Figure 5, b), degradation of filamin appeared to be negligible. Under the conditions of pH 5.6, 165 mM NaCl (Figure 5, c) and pH 5.6, 330 mM NaCl (Figure 5, d), some filamin was degraded to an approximately 240 kDa product by 60 minutes. The doublet remained, with very little evidence of further degradation at 120 minutes.
Desmin  Intact desmin was rapidly degraded by μ-calpain under the conditions of pH 7.0, 165 mM NaCl (Figure 6, a). After 2 minutes, degradation products with approximate molecular masses of 45 and 38 kDa were apparent. Within 15 minutes intact desmin could not be detected, nor could any of its degradation products. Under conditions of pH 7.0 and 330 mM NaCl (Figure 6, b), desmin showed little degradation until 60 minutes. At that time, a 38 kDa degradation product was detected. This degradation product remained through the entire 120 minutes. In addition, while the band corresponding to intact desmin decreased in intensity by 120 minutes, it never completely disappeared from the blots as it did in the other digestion conditions. The two digestions that were done at pH 5.6 (Figure 6, c and d) both showed more extensive degradation of desmin than was seen at pH 7.0, 330 mM NaCl (Figure 6, b). However, the degradation was not as extensive as that seen at pH 7.0, 165 mM NaCl (Figure 6, a). Under the conditions of pH 5.6, 165 mM NaCl (Figure 6, c), a degradation product migrating at 38 kDa was detected after 2 minutes of digestion. A product migrating at 35 kDa was detected by 15 minutes of digestion. After their appearance, both the 38 kDa and 35 kDa polypeptides remained throughout the 120 minute digestion. μ-Calpain was able to completely digest intact desmin within 60 minutes at pH 5.6, 165 mM NaCl (Figure 6, c). When the ionic strength was doubled as in the 330 mM NaCl, pH 5.6 digestion (Figure 6, d), μ-calpain was still able to degrade desmin in a manner that was similar to that seen under the conditions of pH 5.6, 165 mM NaCl (Figure 6, c), with the exception that a transient 45 kDa degradation product was detected after 2 and 15 minutes of incubation.

Troponin-T  The pH 7.0, 165 mM NaCl digestion condition showed the most rapid degradation of troponin-T by μ-calpain of any of the conditions
examined in this study (Figure 7, a). Within 15 minutes of incubation, a 28 kDa degradation product was identified. In addition, a band migrating at approximately 25 kDa was faintly present by 15 minutes, and was a prominent band by 60 minutes of incubation with μ-calpain. Within 60 minutes of incubation, the intact form of troponin-T could not be detected. When the conditions of pH 7.0, 330 mM NaCl (Figure 7, b), were examined, it was noted that very little, if any, of the troponin-T was degraded over the entire 120 minute incubation period. Again, as was seen in the majority of the other proteins in this study, under the two pH 5.6 digestion conditions (Figure 7, c and d), the degradation of troponin-T by μ-calpain was intermediate to the two pH 7.0 conditions. In both the pH 5.6, 165 mM NaCl (Figure 7, c) and the pH 5.6, 330 mM NaCl (Figure 7, d) digestion, two main degradation products that migrated at approximately 30 and 28 kDa were observed to appear after 60 minutes. Under both pH 5.6 conditions (Figure 7, c and d), these two degradation products remained through the 120 minute digestion period.

It was also noted that the intact form of troponin-T was slightly more soluble in the 330 mM NaCl digestion conditions (Figure 7, b and d) than in the 165 mM NaCl conditions (Figure 7, a and d). In both the pH 7.0, 330 mM NaCl (Figure 7, b) and the pH 5.6, 330 mM NaCl (Figure 7, d) conditions, the monoclonal troponin-T antibody detected the intact form, but no degradation products in the supernatant fractions.

Discussion

Titin and nebulin Titin is known to be degraded in postmortem muscle in which the pH and ionic strength conditions are vastly different from the conditions typically encountered in living muscle systems. Titin has also been found to be
altered in damaged cardiac muscle tissue [31]. μ-calpain has been shown, under certain circumstances, to have the capability to degrade intact titin (T1) to produce the large proteolytic fragment T2 [9, 10]. Given the wide range of pH and ionic strength conditions examined in the current study, it is apparent that μ-calpain is also capable of causing many of the same degradative changes in titin that are seen in muscle/meat systems, the most well characterized of which is the disappearance of T1 and the production of T2 [28, 32, 33]. Another significant polypeptide that is seen to appear in damaged muscle or in postmortem aged beef is a degradation product of titin with an approximate molecular weight of 1200 [34, 35]. In all of the conditions examined in this study, the protease μ-calpain was able to catalyze the degradation of intact titin (T1). At pH 7.0, 165 mM NaCl, pH 5.6, 165 mM NaCl and pH 5.6, 330 mM NaCl (Figure 3, a, c, d), μ-calpain degraded T2 and a titin degradation product migrating at approximately 1200 kDa.

Recent reports have localized a 1200 kDa degradation product in the I-band region of the sarcomere that originates from the Z-line end of the long, slender titin molecule [36, 37]. This portion of the titin molecule has been estimated to be between 0.34 μm [37] and 0.36 μm [38] long in resting length myofibrils. In some studies, this fragment has been shown to terminate near the putative N2 line [37]. T2 has been estimated to be approximately 0.9 μm in length and extends from at or near the M-line [38, 39]. It has been hypothesized that together these two fragments make up the titin molecule [37]. The fact that the monoclonal antibody used in this study recognized both the approximately 1200 kDa fragment as well as T2 indicates that these two fragments may have similar epitopes. In several studies using monoclonal antibodies against titin, titin has been shown to have repeating epitopes that are found in both the I-band region and the A-band region of the
myofibril. [36, 40]. Given that a large part of the structure of titin is made up of approximately 100 amino acid repeats [41, 42, 43], this is not surprising. In fact, Maruyama et al. [43] reported cloning a portion of the C-terminal region after screening a cDNA library with an antibody to the N-terminal 1200 kDa polypeptide. Additionally, α-actinin, one of the major proteins of the Z-line, has been shown to bind to the T2 portion of titin [44] as well as to a 1200 kDa polypeptide [37]. While the binding of α-actinin to T2 would seem to have very little functional significance (as T2 does not extend to the Z-line) it may indicate that these two titin fragments possess similar amino acid sequences and likewise similar epitopes that may be recognized by the same antibody.

Nebulin was degraded very quickly under all of the conditions examined in this study (Figure 4). The anti-nebulin antibody, NB2, used in the current study labels an epitope that is approximately 0.22 μm from the Z-line [45], close to the region of the myofibril where the N2 line is often observed in the I-band [45, 46]. Myofibrils have been shown to fragment at or near the Z-line in the I-band in postmortem aged tissue [33, 47], and in myofibrils incubated with calpain, the 400 Å periodicity of the I-band has been seen to be lost in addition to alterations at the Z-line [1]. Disruption of nebulin at the region of the N2 line in the I-band, combined with degradation of titin at or near this same region (as suggested by the loss of the 1200 kDa epitope), may contribute to the fragmentation of the myofibril in the I-band region.

Filamin Filamin exists as a homodimer that has a molecular mass of approximately 500 kDa. Under the denaturing conditions of SDS-PAGE techniques, the skeletal muscle isoform of filamin migrates at 245,000 daltons [48]. In the stress fibers of skeletal myoblasts, filamin is seen to be associated with actin
Filamin is characterized as an actin binding protein that has the ability to cross-link actin filaments. Filamin has also been proposed to be associated with intermediate filaments and is thought to be localized around the periphery of the Z-disks in skeletal muscle myofibrils. To date, the exact function of filamin in skeletal muscle is not precisely known. The content of filamin in skeletal muscle is very low, estimates have been made that place the concentration of filamin in this tissue in the range of 0.8 +/- 0.1 mg of filamin per gram of total protein. The degradation of smooth muscle filamin by calpains has been documented by Davies et al., Smooth muscle filamin (Mr 250,000) is cleaved by μ-calpain to produce heavy merofilamin (Mr 240,000) and light merofilamin (Mr 9500). This cleavage disrupts the actin-crosslinking ability of filamin, yet the cleaved filamin retains its actin binding ability. A very similar cleavage pattern resulting in a closely spaced doublet composed of intact and heavy merofilamin has been shown to occur in myoblasts and was attributed to μ-calpain activity. One proposed role of filamin in developing skeletal muscle is to aid in orchestrating the development of the myofibril and its cleavage by calpains may play a role in regulating this action.

The degradation of filamin, as seen in this study, was most rapid under the conditions of pH and ionic strengths most closely approximating those found in cells under normal physiological conditions (relatively low ionic strength, 165 mM NaCl, and a pH of near neutrality, pH 7.0). The fact that at pH 7.0 and high ionic strength conditions (330 mM NaCl) did not result in any detectable degradation of filamin is puzzling, but may suggest some conformational change in filamin under these conditions, making cleavage sites less available, or possibly some alteration in the conformation of the enzyme that may affect the activity of the
μ-calpain itself. Under the conditions of lower pH (pH 5.6), the ionic strength of the buffer did not appear to have a major effect upon the degradation of filamin. Both digestions that were done at pH 5.6 occurred at a rate that was slower than that seen at pH 7.0, 165 mM NaCl. The slower degradation at pH 5.6, 165 mM NaCl when compared to the pH 7.0, 165 mM NaCl conditions may be indicative of the suboptimal conditions for calpain activity that are reported for the enzyme at lower pH values [1]. Why μ-calpain was able to digest filamin at pH 5.6, 330 mM NaCl and not at pH 7.0, 330 mM NaCl is an enigma, but again may be due to differences in the conformational state of the substrate, the enzyme, or both under these conditions.

**Desmin** Desmin is a member of the 10 nm diameter intermediate filament group of proteins. Desmin has a molecular mass of 53 kDa and is one of the early proteins to be expressed during myofibrillogenesis [54, 55]. The purified protein has the ability to self-associate and to form filaments when the pH of the solution is lowered to 7, or when the ionic strength is increased [56]. Desmin has been recently shown to be a good substrate for an ADP-ribosyltransferase. The resulting modification inhibits desmin from forming filaments [57]. In mature myofibrils desmin has been reported to be located at the periphery of the Z-line [58]. It has been suggested that desmin may play a role in maintaining the alignment of adjacent myofibrils by tying them together at the level of the Z-line [59].

Degradation of desmin by μ-calpain has been reported [13]. In both living systems and in postmortem muscle the degradation of desmin by the calpains may be important in determining the state of organization of the muscle cell.

In the current study, the degradation of desmin was most rapid at pH 7.0 and 165 mM NaCl with intact desmin being completely degraded within 15 minutes of
digestion. On the other hand, the degradation of desmin was the slowest at pH 7.0 and 330 mM NaCl, again suggesting some alteration in the substrate, desmin or in the enzyme itself. The two lower pH conditions (pH 5.6 and either 165 or 330 mM NaCl) appeared to proceed at a rate that was intermediate to the two pH 7.0 conditions, however, the digestion pH 5.6, 330 mM NaCl appeared to proceed at a slightly slower rate than did the pH 5.6, 165 mM NaCl. Under these conditions a transient 45 kDa polypeptide was observed to remain through 15 minutes and was not detected by the antibody beyond that time point. This degradation product was not seen to appear in the samples that were digested at pH 5.6, 165 mM NaCl, possibly due to more rapid degradation resulting in its disappearance before the sampling period. It is also possible that under the conditions chosen, desmin, which seems to be very sensitive to changes in both pH and ionic strength conditions, may undergo slight changes in conformation that may render certain cleavage sites more or less susceptible to calpain action. This may then result in the slight differences in the degradation patterns seen in the different conditions used in this study.

Troponin-T  Troponin-T (Mr 37,000 on SDS-PAGE gels) is a part of the regulatory protein complex, troponin, that is important in regulating the Ca\(^{2+}\)-induced contraction of striated muscle. Troponin-T interacts with tropomyosin at two main positions and covers a large portion of the molecule [60] including the overlap between two tropomyosin molecules [61]. Troponin-T has been shown to be susceptible to degradation under conditions such as postmortem aging [2, 62, 63]. Calpain has been shown to degrade troponin-T in purified myofibrils incubated with the enzyme [7, 11]. In addition, the troponin-T fraction of purified troponin has been shown to be degraded by \( \mu \)-calpain [2]. The major degradation
products that have been observed under conditions such as those described above range in molecular weight from approximately 31,500 to 15,000 daltons [2, 9, 63, 64].

In the current study, \( \mu \)-calpain was shown to degrade troponin-T to produce polypeptides ranging from approximately 30,000 daltons to approximately 25,000 daltons under three of the four conditions examined. Again, as was seen in the other proteins examined in this study, \( \mu \)-calpain degraded troponin-T the fastest under the conditions of pH 7.0 and 165 mM NaCl, and somewhat slower under the conditions of pH 5.6, 165 mM NaCl and pH 5.6, 330 mM NaCl. \( \mu \)-Calpain did not appear to degrade troponin-T under the conditions of pH 7.0, 330 mM NaCl. This phenomenon has been seen in other reports using m-calpain. Kendall et al. [26] reported that while m-calpain degraded troponin-T at lower ionic strengths at pH 7.0, the enzyme failed to degrade troponin-T under the conditions of pH 7.0, 300 mM KCl and 400 mM KCl or under the conditions of pH 6.2, 300 mM KCl and 400 mM KCl.

Conclusions

The proteins examined in this study appeared to differ in their susceptibility to proteolysis by \( \mu \)-calpain. Under the four conditions used, the proteins titin, nebulin and desmin appeared to be the most susceptible to \( \mu \)-calpain digestion. The proteins filamin and troponin-T, were degraded more slowly than titin, nebulin and desmin under the conditions of pH 7.0, 165 mM NaCl; pH 5.6, 165 mM NaCl; pH 5.6, 330 mM NaCl. Filamin and troponin-T were not noticeably degraded at pH 7.0, 330 mM NaCl, while the intact forms of the proteins titin, nebulin and desmin were degraded under these conditions. This difference in susceptibility, especially,
at the higher pH and ionic strength conditions could arise from pH/ionic strength induced conformational changes in the proteins. These changes may alter the susceptibility of these proteins to calpain digestion by rendering specific cleavage sites inaccessible.

The effect of the pH/ionic strength conditions on the calpain molecule cannot be ignored. The calpain enzyme has been shown to have reduced activity at lower pH values and higher ionic strengths [1, 24, 25, 26]. It is possible that under the pH/ionic strength conditions of pH 7.0, 330 mM NaCl conformational changes in the calpain molecule itself could occur that may allow for an increase in its hydrophobicity and lead to some aggregation of the enzyme as has been suggested by other reports [25]. It has also been suggested [25] that higher ionic strengths at pH values of near neutrality may interfere with the enzyme and its interaction with its needed calcium, thereby reducing its activity under these conditions.

The reduced activity of \( \mu \)-calpain under the conditions of pH 5.6, 165 mM NaCl is not an unexpected phenomenon. Several reports show reduced activity of calpain under acidic conditions [1, 25, 26, 65]. pH values as low as 5.8 have been shown to increase the rate of autolysis of \( \mu \)-calpain [65]. This increased rate of autolysis may account for some of the reduction in activity at low pH values. The fact that \( \mu \)-calpain retains greater activity under the conditions of pH 5.6, 330 mM NaCl than it had under the conditions of pH 7.0, 330 mM NaCl on all five of the proteins examined, may indicate a synergistic relationship between pH and ionic strength and their effect on calpain activity. It is possible that conformational changes of the enzyme induced by the lowered pH outweigh the effects of
increased ionic strength under the conditions examined, or it may reflect some change in the substrate proteins.

On the basis of these results, the low pH and relatively higher ionic strengths found in postmortem muscle would not appear to inhibit the μ-calpain-induced degradation of the five proteins studied. This study further implicates μ-calpain in postmortem protein degradation.

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Figure 1. Coomassie stained SDS-PAGE gel of DEAE-TSK column fractions of purified bovine \( \mu \)-calpain. A sample of the pooled fractions used in the digestions was loaded on the far right lane. 1-10 refers to active column fractions from the final column in the purification scheme. P = pooled \( \mu \) calpain samples. 80 K = 80 kDa subunit of \( \mu \)-calpain, 28 K = 28 kDa subunit of \( \mu \)-calpain.
Figure 2. Coomassie-stained 3.2-12% gradient gels of pellets and supernatants from the $\mu$-calpain digests. 0-120 refers to minutes of digestion with $\mu$-calpain. Closed arrowhead indicates the position of intact titin (T1). Open triangle indicates the position of a large degradation product of titin (T2). a) Digest done at pH 7.0, 165 mM NaCl, 100 $\mu$M CaCl$_2$. C1 = 120 minute enzyme control, C2 = 120 minute buffer control, T1 = intact titin, T2 = High molecular weight degradation product of titin, MHC = myosin heavy chain, e-i = unknown bands altered by $\mu$-calpain degradation. b) Digest done at pH 7.0, 330 mM NaCl, 100 $\mu$M CaCl$_2$, C = 120 minute buffer control, T1 = intact titin, T2 = high molecular weight degradation product of titin, MHC = myosin heavy chain. c) Digest done at pH 5.6, 165 mM NaCl, 100 $\mu$M CaCl$_2$. C = 120 minute buffer control, T1 = intact titin, T2 = high molecular weight degradation product of titin, MHC = myosin heavy chain, i = band altered by $\mu$-calpain digestion. d) Digest done at pH 5.6, 330 mM NaCl, C = 120 minute buffer control, T1 = intact, T2 = high molecular weight degradation product. MHC = myosin heavy chain.
a pH 7.0, 165 mM NaCl

b pH 7.0, 330 mM NaCl

c pH 5.6, 165 mM NaCl

d pH 5.6, 330 mM NaCl
Figure 3. Western blots of 3.2-12% SDS-PAGE gradient gels of pellets and supernatants from the μ-calpain digests. Blots were incubated overnight at 4°C with a monoclonal antibody, 4C7, to titin. C = digest buffer control, T1 = intact titin, T2 = High molecular weight degradation product of titin, 1200 K = 1200 kDa degradation product of titin. a) Digest done at pH 7.0, 165 mM NaCl, 100 μM CaCl$_2$. b) Digest done at pH 7.0, 330 mM NaCl, 100 μM CaCl$_2$. c) Digest done at pH 5.6, 165 mM NaCl, 100 μM CaCl$_2$. d) Digest done at pH 5.6, 330 mM NaCl, 100 μM CaCl$_2$. Closed arrowhead designates the position of T1. Open triangle designates the position of T2.
a  pH 7.0, 165 mM NaCl

b  pH 7.0, 330 mM NaCl

c  pH 5.6, 165 mM NaCl

d  pH 5.6, 330 mM NaCl
Figure 4. Western blots of 3.2-12% SDS-PAGE gradient gels of pellets and supernatants from the μ-calpain digests. Blots were incubated overnight at 4°C with a monoclonal antibody, NB2, to nebulin. C = calpain control. a) Digest done at pH 7.0, 165 mM NaCl, 100 µM CaCl$_2$.
   b) Digest done at pH 7.0, 330 mM NaCl, 100 µM CaCl$_2$.
   c) Digest done at pH 5.6, 165 mM NaCl, 100 µM CaCl$_2$.
   d) Digest done at pH 5.6, 330 mM NaCl, 100 µM CaCl$_2$. 
Figure 5. Western blots of 3.2-12% SDS-PAGE gradient gels of pellets and supernatants from the μ-calpain digests. Blots were incubated at room temperature with a polyclonal antibody to filamin. C = calpain control. a) Digest done at pH 7.0, 165 mM NaCl, 100 μM CaCl₂. Open triangle designates the position of intact filamin. Small arrow designates the position of the large degradation product of filamin. b) Digest done at pH 7.0, 330 mM NaCl, 100 μM CaCl₂. c) Digest done at pH 5.6, 165 mM NaCl, 100 μM CaCl₂. Open triangle designates the position of intact filamin. Small arrow designates the position of the large degradation product of filamin. d) Digest done at pH 5.6, 330 mM NaCl, 100 μM CaCl₂. Open triangle designates the position of intact filamin. Small arrow designates the position of the large degradation product of filamin.
a  pH 7.0, 165 mM NaCl

Pellet  |  Supernatant
---|---
0 2 15 60 120 C 0 2 15 60 120 C

Filamin - △ - r + -

b  pH 7.0, 330 mM NaCl

Pellet  |  Supernatant
---|---
0 2 15 60 120 C 0 2 15 60 120 C

Filamin - - - - - - - - - -

c  pH 5.6, 165 mM NaCl

Pellet  |  Supernatant
---|---
0 2 15 60 120 C 0 2 15 60 120 C

Filamin - △ - r + -

d  pH 5.6, 330 mM NaCl

Pellet  |  Supernatant
---|---
0 2 15 60 120 C 0 2 15 60 120 C

Filamin - - - - - - - - - -

Figure 6. Western blots of 18% SDS-PAGE gels of pellets and supernatants from \( \mu \)-calpain digests.

Blots were incubated for one hour at room temperature with a polyclonal antibody to desmin. 

\( C = \) calpain control, \( 45 \, K = 45,000 \) dalton degradation product of desmin, \( 38 \, K = 38,000 \) dalton degradation product of desmin, \( 35K = 35,000 \) dalton degradation product of desmin. 

a) Digest done at pH 7.0, 165 mM NaCl, 100 \( \mu \)M CaCl\(_2\).  

b) Digest done at pH 7.0, 330 mM NaCl, 100 \( \mu \)M CaCl\(_2\).  

c) Digest done at pH 5.6, 165 mM NaCl, 100 \( \mu \)M CaCl\(_2\).  

d) Digest done at pH 5.6, 330 mM NaCl, 100 \( \mu \)M CaCl\(_2\).
a  pH 7.0, 165 mM NaCl

b  pH 7.0, 330 mM NaCl

c  pH 5.6, 165 mM NaCl

d  pH 5.6, 330 mM NaCl

Desmin
45 K-
38 K-
36 K-

Desmin
38 K-
35 K-

Desmin
45 K-
38 K-
35 K-

Desmin
38 K-
35 K-

Supernatant

Pellet

0  2  15  60  120  C  0  2  15  60  120  C

0  2  15  60  120  C  0  2  15  60  120  C

0  2  15  60  120  C  0  2  15  60  120  C

0  2  15  60  120  C  0  2  15  60  120  C
Figure 7. Western blots of 18% SDS-PAGE gels of pellets and supernatants from μ-calpain digests.

Blots were incubated for one hour at room temperature with a monoclonal antibody, JLT-12, to troponin-T. C = calpain control, 28 K = 28,000 dalton degradation product of troponin T, 30 K = 30,000 dalton degradation product of troponin-T. Arrowheads designate the positions of two isoforms of intact troponin-T.  
a) Digest done at pH 7.0, 165 mM NaCl, 100 μM CaCl₂.  
b) Digest done at pH 7.0, 330 mM NaCl, 100 μM CaCl₂.  
c) Digest done at pH 5.6, 165 mM NaCl, 100 μM CaCl₂.  
d) Digest done at pH 5.6, 330 mM NaCl, 100 μM CaCl₂.
EFFECTS OF THE PROTEASE \( \mu \)-CALPAIN ON PURIFIED MYOFIBRILS 
AT PHYSIOLOGICAL TEMPERATURE UNDER SELECTED 
CONDITIONS OF CALCIUM CONCENTRATION, PH, AND IONIC 
STRENGTH

A paper to be submitted to Biochimie

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; E-64, trans-Epoxysuccinyl-
\( L \)-leucylamido-(4-guanidino)butane; Tris, tris[hydroxymethyl]aminomethane; MCE, 
\( \beta \)-mercaptoethanol; PMSF, Phenylmethylsulfonylfluoride; KCl, potassium chloride; 
MES, 2-[N-Morpholino] ethanesulfonic acid; TCA, trichloroacetic acid; HCl, 
hydrochloric acid; CaCl\(_2\), calcium chloride; SDS, sodium dodecyl sulfate; SDS-
PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DTT, \( DL \)- 
Dithiothreitol; TEMED, \( N'N'N'N' \)-tetramethylethylenediamine; APS, ammonium 
persulfate; PVDF, polyvinylidene difluoride; Tween -20, polyoxyethylene sorbitan 
monolaurate.
Summary

The protease μ-calpain requires between 2.5 μM Ca²⁺ to 65 μM Ca²⁺ for half maximal activity. Autolysis of μ-calpain has been shown to require 190-210 μM Ca²⁺. Calpains exhibit maximal activity without autolysis when calcium levels are near 100 μM. In this study, the degradation of the myofibrillar proteins titin, nebulin, filamin, desmin, and troponin-T by μ-calpain was examined under varied pH and ionic strength conditions at 37°C and at 100 and 200 μM Ca²⁺ levels using SDS-PAGE and highly sensitive chemiluminescent Western blotting techniques. Myofibril samples incubated at 37°C, 100 μM Ca²⁺, pH 7.0 and either 165 mM NaCl or 330 mM NaCl exhibited very little degradation of myofibrillar protein. When the samples were incubated at 37°C, 100 μM Ca²⁺, pH 5.6 and either 165 mM NaCl or 330 mM NaCl the degradation was evident. Myofibrils incubated at 200 μM Ca²⁺, pH 7.0, 165 mM NaCl or 330 mM NaCl had dramatically different results from those seen at 100 μM Ca²⁺, pH 7.0, 165 mM NaCl or 330 mM NaCl. Within 2 to 15 minutes at 200 μM Ca²⁺ most of the proteins examined showed significant degradation in both conditions at pH 7.0, while none of the proteins showed significant degradation when the digestions were done at 100 μM Ca²⁺, pH 7.0 and either 165 or 330 mM NaCl. The amount of myosin that was released from the myofibril was greatly increased in the supernatant of the 200 μM Ca²⁺, pH 7.0, 330 mM NaCl digestion by 60 minutes when compared to 0 minute and 60 minute controls. This study demonstrates that the combination of physiological temperature and pH may have a dramatic effect on the calcium requirements needed by μ-calpain to degrade myofibrillar proteins.

key words: μ-calpain / myofibrillar proteins / pH / ionic strength / temperature
Introduction

The calpains are cysteine proteinases that have an absolute requirement for calcium in vitro to initiate full activity. The two most well characterized forms of the enzyme are \( \mu \)-calpain and m-calpain. Both isozymes are heterodimers made up of an 80 and a 28 kDa subunit [1]. The nomenclature for these two isozymes reflects the difference in the amount of calcium each requires for half-maximal activity. Estimates of \( \text{Ca}^{2+} \) requirements for half-maximal activity of \( \mu \)-calpain range form 2.5 \( \mu \text{M Ca}^{2+} \) to 65 \( \mu \text{M Ca}^{2+} \) (depending upon the substrate) and for m-calpain, the range is between 300 \( \mu \text{M Ca}^{2+} \) and 1000 \( \mu \text{M Ca}^{2+} \) [2-4]. The majority of these estimates have been made under the conditions of 25°C and pH 7.5, conditions which have been shown to be most favorable for calpain activity [5,6].

When the calpains are briefly incubated with specific amounts of calcium they undergo autolysis. This autolysis is characterized by a dramatic lowering of the amount of calcium required for half-maximal activity [7] without altering the specific activity of the enzymes. The calcium requirements are lowered from 2.5-65 \( \mu \text{M Ca}^{2+} \) (before autolysis) to 0.6-0.8 \( \mu \text{M Ca}^{2+} \) (after autolysis) for \( \mu \)-calpain, and from 300-1000 \( \mu \text{M Ca}^{2+} \) (before autolysis) to 3-50 \( \mu \text{M Ca}^{2+} \) (after autolysis) for m-calpain [3]. The autolysis of both of these forms of the enzyme results in a reduction of the masses of both the 80 kDa and the 30 kDa subunits of both \( \mu \)-and m-calpain. The physiological significance of this autolysis is not clear. In most cases, the amount of calcium required to initiate autolysis is higher than the calcium required for half-maximal activity for proteolysis. Incubation of \( \mu \)-calpain in the presence of 100 \( \mu \text{M Ca}^{2+} \) at 25°C has been shown to result in maximal activity of \( \mu \)-calpain on a casein substrate [2]. Autolysis of \( \mu \)-calpain has been shown to require 190-210 \( \mu \text{M Ca}^{2+} \) [2], levels that are clearly higher than those needed for
activity. Incubation with phospholipids such as phosphatidylinositol, has been shown to reduce the level of Ca\(^{2+}\) required for autolysis [8,9]. However, the calcium requirements are still usually higher than those needed for half maximal activity against casein [2]. It has also been shown that as the pH is lowered to at least 5.8, conditions are more favorable for autolysis [10].

The calpain enzyme system in muscle systems has been hypothesized to play a key role in myofibrillar protein turnover and in muscle growth [11]. Indeed, the calpains have been shown to be very selective in the proteins that they degrade, targeting proteins such as titin [12,13], nebulin [E Huff-Lonergan, S Sernett, FC Parrish, Jr., RM Robson, unpublished observations], filamin [14], C-protein [15], desmin [16], troponin-I [15], tropomyosin [15], and troponin-T [15,17,18], while not degrading the two most abundant proteins in the myofibril, actin and myosin [19].

The objective of this study was to examine the combined effects of pH, ionic strength and calcium level at physiological temperature (37\(^\circ\)C) on muscle proteins in order to gain a better understanding of the events controlling \(\mu\)-calpain orchestrated degradation of selected skeletal muscle proteins.

**Materials and methods**

**Purification of \(\mu\)-calpain** The \(\mu\)-calpain used in this study was purified from 4.5 kg of bovine semimembranosus muscle obtained 45 minutes postmortem according to the method of Edmunds et al. [3] with modifications. The procedure was as follows. The muscle was ground and homogenized in 6 volumes of 5 mM EDTA, 0.1% (volume/volume) MCE, 20 mM Tris, pH 7.5 (TEM) containing 2.5 \(\mu\)M (E-64), 100 mg/ml ovomucoid trypsin inhibitor and 2 mM (PMSF). The homogenate
was centrifuged at 17,700 x g for 30 minutes. After filtration of the supernatant and adjustment of the pH to 7.5 with solid Tris, proteins were salted out between 0 and 45% ammonium sulfate. Proteins were pelleted at 17,700 x g for 30 minutes, resuspended in TEM and dialyzed. The supernatant was loaded onto a 5 cm x 50 cm QA-52 (Whatman, Hillsboro, OR) column. After washing to remove unbound proteins, the column was eluted with a linear gradient of 0 to 500 mM KCl in TEM, pH 7.5. Crude μ-calpain was eluted between 115 mM KCl and 180 mM KCl. Fractions containing μ-calpain activity were pooled and were loaded onto a 2.6 cm x 37 cm phenyl-Sepharose (Pharmacia Biotech Inc., Piscataway, NJ) column that had been previously equilibrated in 125 mM KCl, TEM, pH 7.5. After washing with the equilibration buffer, μ-calpain was eluted with 20 mM Tris, 5 mM EDTA and 0.1% (volume/volume) MCE, pH 7.5. Pooled μ-calpain from the phenyl-Sepharose column was adjusted to 0.8 M ammonium sulfate and was loaded onto a 2.6 cm x 28 cm butyl-Sepharose (Pharmacia Biotech Inc., Piscataway, NJ) column that had been previously equilibrated in 0.8 M ammonium sulfate, 20 mM Tris, 5 mM EDTA and 0.1% (volume/volume) MCE, pH 7.5. After washing, the column was eluted with a linear gradient of 0.8 M ammonium sulfate to 0 mM ammonium sulfate in TEM. The sample was dialyzed and loaded onto a 1.6 cm x 50 cm DEAE-TSK (Supelco Inc., Bellefonte, PA) column equilibrated in 1 mM EDTA, .1% (volume/volume) MCE, 20 mM Tris-MES, pH 6.5. The μ-calpain was eluted from this final column with a linear 0 to 150 mM KCl gradient.

Calpain activity assay. The activity of μ-calpain was monitored during the purification procedure by measuring the release of trichloroacetic acid (TCA) soluble polypeptides resulting from the digestion of casein by the μ-calpain [20].
**Digestion procedure**  At-death myofibrils from bovine longissimus muscle were prepared according to the procedure of Huff-Lonergan et al. [21] and were stored in 50% (volume/volume) glycerol at -20°C until use. For each assay, 4 ml of glycerinated myofibrils were spun at 3100 x g for 6 minutes at 4°C. Pellets were washed with 2 ml 5 mM Tris-HCl, pH 8.0 and then spun at 3100 x g for 6 minutes at 4°C. The supernatant was removed and the myofibril pellets were washed twice in 2 ml of one of the following 4°C buffers; 165 mM NaCl in 50 mM MES-Tris pH 5.6, 330 mM NaCl in 50 mM MES-Tris, pH 5.6, 165 mM NaCl in 50 mM HEPES-Tris, pH 7.0, or 330 mM NaCl in 50 mM HEPES-Tris, pH 7.0. Following each wash, the samples were spun at 1100 x g for 6 minutes. After the final spin, the pellets were resuspended in 2 ml of the respective buffer, brought to 37°C in a circulating water bath and the pH and the conductivity of the samples was monitored and adjusted (if necessary) to meet the desired pH and conductivity parameters. Protein concentrations were measured using the Biuret procedure as modified by Robson et al. [22]. Concentrations were adjusted (using the desired buffer) to 4 mg of protein per ml, 100 µM CaCl₂ or 200 µM CaCl₂, and 15 mM MCE. µ-Calpain was added at a ratio of 1:800 (µ-calpain/myofibrillar protein, weight/weight). Control samples were the same, except 20 mM EDTA was added to the myofibril/CaCl₂/MCE mixture prior to the addition of µ-calpain (enzyme control) or no µ-calpain was added (buffer control). Final reaction volumes were 2.5 ml. Samples (0.4 ml) were removed after 0, 2, 15, and 60 minutes of digestion and added to an aliquot of 200 mM EDTA to bring the final concentration of EDTA to 20 mM to stop the reaction. Samples were spun at 12,000 x g for 15 minutes at room temperature (25°C). The supernatant was removed, measured and reserved. An amount of 5 mM Tris-HCl, pH 8.0 equal to the amount of supernatant removed was
added to resuspend the pellets. After thorough mixing, pyronin Y tracking dye [3 mM EDTA, 3% (weight/volume) SDS, 30% (volume/volume) glycerol, 0.003% (weight/volume) pyronin Y, 120 mM DTT and 30 mM Tris-HCl, pH 8.0] [23] was added. The final concentration of the samples in tracking dye was 2 mg/ml. The reserved supernatant was added to 2x pyronin Y tracking dye at a ratio of 1:1. Samples were immediately heated at 50°C for 20 minutes and loaded onto gels. All digestions were done in triplicate.

**Gel system** To observe changes in both high and low molecular weight proteins two gel systems were used. Stackless 3.2-12% gradient gels were used to examine changes in high molecular weight proteins (3000-100 kDa). The gels were made using a 30% stock solution of acrylamide (acrylamide:N,N'-bis-methylene acrylamide = 100:1), and 2 mM EDTA, .1% (weight/volume) SDS, 0.67% (volume/volume) TEMED, 0.1% (weight/volume) APS, and .375 M Tris-HCl, pH 8.0. Fifteen percent (volume/volume) glycerol was added to the 12% gel solution to stabilize the gradient. An 18% gel system was used to identify smaller polypeptides (205-9 kDa). These gels were made using a 30% stock solution of acrylamide (acrylamide:N,N'-bis-methylene acrylamide = 100:1), 0.1% (weight/volume) SDS, 0.67% TEMED, 0.1% (weight/volume) APS, and 0.375 M Tris-HCl, pH 8.8. A 5% stacking gel was used on the 18% gels containing acrylamide (acrylamide:N,N'-bis-methylene acrylamide = 100:1), and 0.1% (weight/volume) SDS, 0.67% (volume/volume) TEMED, 0.1% (weight/volume) APS, and 0.375 M Tris-HCl, pH 6.8. Gels (8 cm wide x 9 cm tall x 1.5 mm thick) were run on the Hoefer SE260 Mighty Small II (Hoefer Scientific Instruments, San Francisco, CA). The running buffer used in both the upper and lower chambers of the slab gel unit was 25 mM Tris, 192 mM glycine, 2 mM EDTA and 0.1%
Twenty μg of pellets from the digested samples were loaded onto the gels. Twenty μl of the supernatant samples in tracking dye were loaded onto the gels. The 3.2-12% gels were run at a constant current setting of 6 mA/gel for approximately 18 hours at room temperature and the 18% gels were run at a constant voltage setting of 40 volts for 17 hours at room temperature. Following electrophoresis, gels were either stained for visualization of protein bands, or were transferred by electroelution to PVDF membranes. Gels for examination of all protein bands were stained a minimum of 12 hours in an excess of a solution containing 0.1% (weight/volume) of Coomassie brilliant blue R-250, 40% (volume/volume) ethanol and 7% glacial acetic acid. Gels were destained in an excess of the same solution without the Coomassie brilliant blue R-250.

**Transfer conditions** After running, gels for transfer were equilibrated in either 25 mM Tris, 192 mM glycine, 2 mM EDTA, 15% (volume/volume) methanol and .1% (weight/volume) SDS (3.2-12% gels) or in the same solution minus the SDS (18% gels). Gels were equilibrated for 15 minutes in the buffer at 4°C. SDS was used in the transfer buffer for the 3-12% gels to aid in mobilization of very high molecular weight proteins from the gel. Samples were blotted onto PVDF membranes using a Hoefer TE22 Mighty Small Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) at a constant voltage setting of 90 V for 1 hour and 15 minutes (nebulin, filamin, desmin, troponin-T) or at 90 V for 5 hours (titin). The temperature of the transfer buffer was maintained between 4°C and 10°C by using a refrigerated circulating bath.

**Western blotting** After transferring, the membranes were blocked for one hour at room temperature (25°C) in blocking solution [80 mM di-sodium hydrogen orthophosphate, anhydrous, 20 mM sodium dihydrogen orthophosphate, 100 mM
sodium chloride, .1% (volume/volume) Tween-20, and 5% (weight/volume) non-fat dry milk]. Primary antibodies used in the Western blotting procedure included; polyclonal anti-desmin (prepared against porcine skeletal muscle desmin), diluted 1:15,000 in blocking solution, polyclonal anti-filamin (prepared against avian smooth muscle filamin) diluted 1:20,000 in blocking solution, monoclonal anti-nebulin (NB2, Sigma Chemical Co., St Louis, MO) diluted 1:5000 in PBS-Tween [PBS-Tween = 80 mM di-sodium hydrogen orthophosphate, anhydrous, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, 0.1% (volume/volume) Tween-20], monoclonal anti-troponin-T (JLT-12, Sigma Chemical Co., St Louis, MO) diluted 1:20,000 in PBS-Tween, monoclonal anti-titin (4C7, prepared against bovine skeletal muscle titin) cell culture supernatant diluted 1:10 in PBS-Tween. Incubation times for the blots in primary antibody are indicated in the figure legends. Blots were washed three times, ten minutes per wash in PBS-Tween (blots labeled with monoclonal antibodies) or blocking solution (blots labeled with polyclonal antibodies). Bound primary antibodies were labeled with either goat-anti-rabbit (used for blots labeled with polyclonal primary antibodies) IgG horseradish peroxidase conjugated secondary antibodies (A9169, Sigma Chemical Co., St Louis, MO), diluted 1:5000 in blocking solution, or goat-anti-mouse (used for blots labeled with monoclonal primary antibodies) IgG horseradish peroxidase conjugated secondary antibodies (A2554, Sigma Chemical Co., St Louis, MO) diluted 1:5000 in PBS-Tween, for 30 minutes at room temperature. Blots were rinsed in PBS-Tween three times, ten minutes per wash prior to detection. A chemiluminescence detection system (Amersham, Arlington Heights, IL) was used to detect labeled protein bands.
Titin purification  Titin, for the preparation of monoclonal antibodies, was purified from fresh bovine longissimus muscle myofibrils according to the procedure of Wang [23]. Prior to final isolation of the purified protein by gel filtration using a 2.6 x 90 cm Sephacryl S-500-HR (Pharmacia Biotech, Piscataway, NJ) gel filtration column, titin and nebulin were separated by salt fractionation. Titin complexed with SDS was selectively precipitated by bringing the NaCl concentration of the SDS-solubilized myofibril proteins to a final concentration of between 0.64 and 0.7 M. The precipitated titin was resuspended using 20 mM EDTA, 80 mM DTT, 20% SDS, 4 mM PMSF, 200 mM Tris-HCl, pH 8.0 [25] before the gel filtration step.

Results

100 µM calcium digestions  Myofibril samples that were incubated at 37°C, 100 µM Ca²⁺, pH 7.0 and either 165 mM NaCl or 330 mM NaCl exhibited very little degradation of myofibrillar protein as detected by both SDS-PAGE (Figure 1, a, b) and Western blots (Figures 2-6, a, b). When the samples were incubated at 37°C, 100 µM Ca²⁺, pH 5.6 and either 165 mM NaCl or 330 mM NaCl the degradation was evident (Figures 1-6, c, d).

Titin  The Coomassie stained 3.2-12% gels (Figure 1) and Western blots (Figure 2) showed very little, if any, change in the titin band over the 60 minute incubation period in the myofibril samples incubated with µ-calpain at 100 µM Ca²⁺, pH 7.0 and either 165 mM NaCl or 330 mM NaCl (Figure 1, a and b, and Figure 2, a and b). However when the pH was lowered to 5.6 (Figure 1, c and d, and Figure 2, c and d), the degradation of titin was rapid. Within 15 minutes at both 100 µM Ca²⁺, pH 5.6, 165 mM NaCl (Figure 1, c, and Figure 2, c) and 330 mM
NaCl (Figure 1, d, and Figure 2, d) the intact form of titin was largely degraded. Under the conditions of 100 µM Ca\(^{2+}\), pH 5.6, 165 mM NaCl (Figure 2, c), proteolysis by µ-calpain of the high molecular weight titin degradation product T2 and the titin degradation product migrating at approximately 1200 kDa was also evident by 15 minutes postmortem on Western blots (Figure 2, c). The band corresponding to T2 and the band migrating at approximately 1200 kDa were also degraded, but at a much slower rate in the samples incubated with µ-calpain at 100 µM Ca\(^{2+}\), pH 5.6, 330 mM NaCl (Figure 2, d) than at pH 5.6, 165 mM NaCl (Figure 2, c). At 15 minutes T2 and the 1200 kDa band were both strongly recognized by the monoclonal antibody (Figure 2, d). Within 60 minutes, the 1200 kDa band was still recognized, (although not as strongly as at 15 minutes) but the T2 band was only weakly recognized.

There were no bands in the pellets smaller than the 1200 kDa band that were recognized by this monoclonal antibody on either the 3.2-12% gradient gels (Figure 2) or on 18% gels (results not shown) by the monoclonal antibody used.

When the supernatants from the digestions were examined on both 3.2-12% gradient gels (Figure 2) or 18% gels (results not shown) there were no bands recognized by monoclonal antibody (4C7) that were soluble under the conditions used.

**Nebulin** Again, as was the case for titin, there was no observable degradation of nebulin by µ-calpain under the conditions of 100 µM Ca\(^{2+}\), pH 7.0 and either 165 mM NaCl or 330 mM NaCl as seen in both Coomassie stained gels (Figure 1, a and b) Western blots (Figure 3, a and b). When the pH of the incubation was lowered to 5.6 with all other conditions remaining the same, the degradation of nebulin was evident (Figure 1, c and d and Figure 3, c and d).
Within 15 minutes intact nebulin could not be discerned on the SDS-PAGE gels (Figure 1, c and d), and the monoclonal antibody, NB2, was unable to recognize intact nebulin (Figure 3, c and d). The antibody failed to recognize degradation products on Western blots of the 3.2-12% gradient gels (Figure 3, c) of the 100 μM Ca^{2+}, pH 5.6, 165 mM NaCl digest. However, slight traces of a degradation product were detected at 2 minutes on the Western blots of 3.2-12% gradient gels of the 100 μM Ca^{2+}, pH 5.6, 330 mM NaCl digest (Figure 3, d) indicating that the protein may have been degraded slightly more slowly under the high ionic strength conditions. The antibody did not recognize any degradation products on the Western blots of 18% gels (results not shown). The antibody also did not recognize any degradation products in the supernatant on either 3.2-12% gels (Figure 3) or 18% gels (not shown) under any of the conditions examined.

**Filamin**  The degradation of filamin was not detected on Western blots of the digests done under the conditions of 100 μM Ca^{2+}, pH 7.0, 165 mM NaCl or 330 mM NaCl (Figure 4, a and b). There did appear to be a slight amount of filamin detected in the supernatant in the 100 μM Ca^{2+}, pH 7.0, 330 mM NaCl (Figure 4, b) digest indicating a tendency toward enhanced solubility of the protein under those conditions. When the myofibrils were digested under the conditions of 100 μM Ca^{2+}, pH 5.6, 165 mM NaCl (Figure 4, c), filamin appeared as a doublet composed of the intact form (upper band) and a degraded form (lower band) within 15 minutes of incubation with the upper band (intact form) being the most prominent. By 60 minutes further degradation was apparent as the lower band (degraded form) of the doublet was more prominent. When the ionic strength was doubled (making the conditions 100 μM Ca^{2+}, pH 5.6, 330 mM NaCl), very little degradation was detected by the polyclonal antibody (Figure 4, d). At 60 minutes
of incubation, a very faint lower band was observed. This indicated much slower degradation of filamin under these conditions.

**Desmin**  Like the other proteins in this study, desmin was not noticeably degraded by μ-calpain under the conditions of 100 μM Ca^{2+}, pH 7.0 and either 165 mM NaCl or 330 mM NaCl (Figure 5, a and b). Intact desmin was degraded completely within 15 minutes of digestion under the conditions of 100 μM Ca^{2+}, pH 5.6, 165 mM NaCl (Figure 5, c). The degradation of desmin by μ-calpain proceeded more slowly when the ionic strength of the solution was doubled (100 μM Ca^{2+}, pH 5.6, 330 mM NaCl, Figure 5, d). After 15 minutes of digestion, two bands were identified by the polyclonal antibody to desmin, a band migrating at approximately 45,000 and a more strongly labeled band migrating at 38,000 (Figure 5, d). By the end of the 60 minute incubation period, there was still a slight amount of intact desmin remaining.

**Troponin-T**  Little change in troponin-T was noted in the μ-calpain digestions done at 100 μM Ca^{2+}, pH 7.0 and either 165 mM NaCl (Figure 6, a) or 330 mM NaCl (Figure 6, b). Some troponin-T was noted in the supernatant in the 100 μM Ca^{2+}, pH 7.0, 165 mM NaCl digestion (Figure 6, a), however troponin-T appeared to be more soluble under the conditions of 100 μM Ca^{2+}, pH 7.0, 330 mM NaCl (Figure 6, b). Under the conditions of 100 μM Ca^{2+}, pH 5.6, 165 mM NaCl (Figure 6, c) the degradation of troponin-T by μ-calpain was evident. Within 15 minutes of digestion, a degradation product migrating at 30,000 daltons was detected. By 60 minutes, three bands migrating at approximately 30,000, 28,000, and 25,000 were recognized by the monoclonal antibody to troponin-T. When the digestions that were done at 100 μM Ca^{2+}, pH 5.6, 330 mM NaCl (Figure 6, d) were examined, it was noted that there were no degradation products of troponin-T that
were recognized by the antibody. However, significantly more intact troponin-T was evident in the supernatant under the conditions of 100 μM Ca²⁺, pH 5.6, 330 mM NaCl (Figure 6, d) than under the conditions of 100 μM Ca²⁺, pH 5.6, 165 mM NaCl (Figure 6, c) indicating that the protein was more soluble under the high ionic strength conditions.

200 μM calcium digestions When the myofibrils were incubated at 200 μM Ca²⁺, pH 7.0, 165 mM NaCl or 330 mM NaCl (Figure 7, a and b) the results were dramatically different from those seen at 100 μM Ca²⁺, pH 7.0, 165 mM NaCl or 330 mM NaCl (Figure 1, a and b). Within 2 to 15 minutes at 200 μM Ca²⁺, most of the proteins examined showed significant degradation in both conditions that were done at pH 7.0 (Figures 7-12, a, b), while none of the proteins showed significant degradation when the digestions were done at 100 μM Ca²⁺, pH 7.0 and either 165 or 330 mM NaCl (Figures 1-6, a, b). In addition, the amount of myosin that was released from the myofibril was greatly increased in the supernatant of the 200 μM Ca²⁺, pH 7.0, 330 mM NaCl digestion (Figure 7, b), especially by 60 minutes when compared to both the 0 minute and 60 minute controls. This phenomenon was not seen under any other digestion conditions.

Titin Upon examination of the Coomassie stained gels of the samples digested at 200 μM Ca²⁺, pH 7.0, 165 mM NaCl (Figure 7, a) it was noted that a high molecular weight degradation product was present in the supernatant from 2 minutes until the end of the digestion at 60 minutes. This degradation product was slightly larger than intact nebulin and migrated at approximately 1000 kDa. Because of its size, this degradation product was probably from titin, although the monoclonal titin antibody used in this study did not recognize it. A degradation
product migrating in the same position was seen to occur in all of the digestions
done at 200 μM Ca$^{2+}$ regardless of the pH or ionic strength of the solution.

The Western blots using the monoclonal antibody to titin (Figure 8) showed
after 2 minutes at 200 μM Ca$^{2+}$, pH 7.0, 165 mM NaCl (Figure 8, a) in the presence
of μ-calpain, the antibody failed to recognize any form of titin, indicating
considerable degradation occurred at a very short time under these conditions.
When the ionic strength was raised by increasing the NaCl concentration to 330
mM the degradation of titin at 200 μM Ca$^{2+}$ and pH 7.0 was slightly slower (Figure
8, b). Within 15 minutes, the antibody no longer recognized T1 or T2, yet it did still
recognize the band migrating at 1200 kDa. When the Coomassie-stained SDS-
PAGE gel of the 200 μM Ca$^{2+}$, pH 7.0, 330 mM NaCl digest was examined (Figure
7, b), it was seen that a band that migrated at the same position as T2 was present
in the supernatant. The monoclonal titin antibody however, did not recognize this
band. It is possible that the band is a slightly truncated form of T2, with only a small
portion (probably the portion containing the epitope for the antibody) missing.

When the digestions done a 200 μM Ca$^{2+}$ and pH 5.6 (Figure 8, c and d)
were considered, it was again seen that the degradation of titin proceeded faster in
the conditions done in 165 mM NaCl (Figure 8, c) than in 330 mM NaCl (Figure 8,
d). Within 2 minutes at 200 μM Ca$^{2+}$, pH 5.6, 165 mM NaCl (Figure 8, c), the
antibody again failed to recognize any form of titin, while at pH 5.6, 330 mM NaCl
(Figure 8, d) the antibody still recognized T1, T2 and a band migrating at 1200 kDa
at 2 minutes. By 60 minutes of digestion at pH 5.6, 330 mM NaCl (Figure 8, d), the
antibody still faintly recognized the T2 band and somewhat more strongly the 1200
kDa band.
**Nebulin** Nebulin was degraded very quickly regardless of the pH or the ionic strength when the Ca\(^{2+}\) concentration was raised to 200 \(\mu\)M (Figures 7 and 9). Under all four conditions (Figure 9 a, b, c, and d), intact nebulin was degraded by 2 minutes of digestion. This was in contrast to the digestion done at 100 \(\mu\)M Ca\(^{2+}\) in which the pH 7.0 (Figure 3, a and b) samples showed little, if any, degradation of nebulin, and the pH 5.6 digestions did not show any degradation until after 2 minutes (Figure 3, c and d).

**Filamin** Filamin was quickly degraded under the conditions of 200 \(\mu\)M Ca\(^{2+}\) and pH 7.0, 165 mM NaCl (Figure 10, a), pH 5.6, 165 mM NaCl (Figure 10, c). Under the low ionic strength condition at pH 7.0 (Figure 10, a), the antibody did not recognize any form of filamin after 2 minutes of incubation with \(\mu\)-calpain. At 165 mM NaCl and a pH of 5.6 (Figure 10, c), a doublet composed of intact filamin and a high molecular weight degradation product was detected by the polyclonal antibody. By 15 minutes of incubation, the antibody no longer recognized either the intact form or the lower band of the doublet.

Under the two higher ionic strength conditions (Figure 10, b and d), filamin was degraded noticeably more slowly. When the digestion conditions were 200 \(\mu\)M Ca\(^{2+}\) and pH 7.0, 330 mM NaCl (Figure 10, b) and pH 5.6, 330 mM NaCl (Figure 10, d), filamin was degraded more slowly. At two minutes, the polyclonal antibody recognized a doublet composed of intact filamin and degraded filamin. This doublet remained throughout the digestion period in both of the higher ionic strength conditions at 200 \(\mu\)M Ca\(^{2+}\) (Figure 10, c and d), indicating that filamin was less susceptible to degradation under the conditions of high ionic strength. Filamin appeared to be the most soluble under the conditions of pH 7.0, 330 mM NaCl (Figure 10, b).
These results at 200 μM Ca\(^{2+}\) (Figure 10) are in sharp contrast to those seen at 100 μM Ca\(^{2+}\) (Figure 4) in which only the pH 5.6, 165 mM NaCl digestion condition (Figure 4, c) showed a significant rate of degradation. Even under those conditions (pH 5.6, 165 mM NaCl, 100 μM Ca\(^{2+}\) ), the rate of degradation was considerably slower when compared to the pH 5.6, 165 mM NaCl done at 200 μM Ca\(^{2+}\) (Figure 10, c).

**Desmin**  At 200 μM Ca\(^{2+}\), the degradation of desmin proceeded at a rapid rate (Figure 11), especially when compared to the digestions done at 100 μM Ca\(^{2+}\) (Figure 5). In all four of the conditions tested at the 200 μM calcium level, the degradation of the intact form of desmin was complete within 2 minutes (Figure 11).

**Troponin-T**  When the calcium concentration was raised to 200 μM, the degradation of troponin-T was enhanced (Figure 12), but the difference in rate of digestion between 200 and 100 μM Ca\(^{2+}\) conditions was not as great as for the other four proteins in the study. At 100 μM Ca\(^{2+}\) the only condition that showed a significant rate of degradation of troponin-T was at pH 5.6, 165 mM NaCl (Figure 6, c). At 200 μM Ca\(^{2+}\) degradation was seen to occur at pH 7.0, 165 mM NaCl (Figure 12, a), pH 7.0, 330 mM NaCl (Figure 12, b), and pH 5.6, 165 mM NaCl (Figure 12, c). No degradation products were detected under the conditions of 200 μM Ca\(^{2+}\), pH 5.6, 330 mM NaCl (Figure 12, d).

When the digestion conditions were pH 7.0, 165 mM NaCl (Figure 12) the only degradation product seen after 15 minutes and at one hour was a band that migrated at approximately 28,000 daltons (Figure 12, a). When the pH was lowered to 5.6 (pH 5.6, 165 mM NaCl; Figure 12, c) three bands were seen by 15 minutes and remained throughout the 60 minute digestion period. These bands migrated at approximately 30,000, 28,000, and 25,000 daltons. Under the
conditions of 200 μM Ca²⁺, pH 7.0, 330 mM NaCl (Figure 12, b) a light band migrating at approximately 28,000 was detected at 15 minutes and at 60 minutes. Under these same conditions, the thin filament-associated protein troponin-T appeared to increase in the supernatant through 60 minutes of incubation. There was significantly more troponin-T in the 60 minute digested sample (pH 7.0, 330 mM NaCl Figure 12, b, lane 60) than in the 60 minute buffer control sample (Figure 12, b, lane C).

In the samples that were digested at 200 μM Ca²⁺, pH 7.0, 330 mM NaCl there appeared to be a dramatic increase the release of actin into the supernatant (Figure 14, b) when compared both with the other digestions done at 200 μM Ca²⁺ (Figure 14, a, c, and d), as well as with those done at 100 μM Ca²⁺ (Figure 13). This appeared to be accompanied by an increase in the amount of troponin-T released as well (Figure 12, b), possibly indicating structural damage to the myofibril resulting in release of the thin filament or thin filament proteins. Release of myosin heavy chain under these same conditions was also seen in these same samples (pH 7.0, 330 mM NaCl, Figures 7, b and 14, b). The release of these proteins cannot be explained by a general increase in solubilization as neither the buffer control nor the enzyme control (incubated for one hour under the same conditions) showed this response.

Discussion

Susceptibility of proteins Of the five proteins examined in this study, nebulin appeared to be the most sensitive to proteolysis by μ-calpain when all of the conditions were taken into consideration. At the 200 μM calcium level, desmin appeared to be equally as susceptible as nebulin, at the time points examined.
Under all conditions of pH and ionic strength at 200 μM Ca\(^{2+}\), the intact forms and/or degradation products of both nebulin and desmin were not detectable at the earliest sampling time point, indicating extensive degradation of these two proteins had occurred within 2 minutes.

The protein troponin-T appeared to be the least sensitive to proteolysis by μ-calpain. Under all conditions tested, a significant amount of intact troponin-T remained throughout the 60 minute incubation. In the high ionic strength digestions, there appeared to be very little degradation of troponin-T at both the 100 μM and 200 μM Ca\(^{2+}\) concentrations. Under the conditions of 200 μM Ca\(^{2+}\), pH 7.0, 330 mM NaCl (Figure 12) a slight amount of a degradation product migrating at approximately 28,000 was detected, however, the amount was very small in comparison to the amount of intact troponin-T that remained. This effect of ionic strength on the susceptibility of troponin-T to calpain degradation has been seen in other reports. Kendall et al. [18] showed that at 25°C and KCl levels of 300 mM and above, m-calpain did not noticeably degrade intact troponin-T as seen on Coomassie stained SDS-PAGE gels. Under the conditions of 4°C, μ-calpain also has been shown to not degrade troponin-T at NaCl levels of 330 mM [E Huff-Lonergan, S Sernett, FC Parrish, Jr., RM Robson, unpublished results]. The results of the current study, combined with the afore mentioned reports [18] may suggest that higher ionic strength affects the conformation of the troponin-T molecule (or the entire troponin complex) in such a manner as it could make the calpain sensitive sites unavailable for cleavage.

It can be seen in Figures 7 and 14 that the combination of the higher Ca\(^{2+}\) concentration, pH, and ionic strength in the presence of μ-calpain caused the release of myosin, actin (Figure 14) and troponin-T (Figure 12) from the myofibril
indicating that some structural constraint was removed that once held these proteins in place within the myofibril. While many proteins are more soluble at higher ionic strengths, the release of these proteins cannot be solely ascribed to increased solubility alone, as the controls (both buffer and calpain controls) that were incubated under the same conditions for the same amount of time did not show this affect (Figures 7, b, lane C; 12 b, lane C; and 14, b, lane C). This affect was also not seen under conditions of relative inactivity of μ-calpain (100 μM Ca²⁺, pH 7.0, 330 mM NaCl, Figures 1, 6, and 13) in which the ionic strength, pH and temperature were the same as when the proteins myosin, actin and troponin-T were released.

**Effects of ionic strength and pH**  Ionic strength appeared to have an effect upon the ability of μ-calpain to degrade the majority of the proteins examined in this study. When the calcium levels were in the range of 100 μM, a doubling of the ionic strength (from 165 mM NaCl to 330 mM NaCl) significantly decreased the degradation at pH 5.6 (there was no evidence of proteolysis at 100 μM Ca²⁺ and pH 7.0) of four of the five proteins in the study, with nebulin being the exception. The same decrease in proteolysis at the higher ionic strength at both pH 7.0 and 5.6 was seen when the calcium levels were raised to 200 μM (Figures 7-12) for the proteins titin, filamin and troponin-T. The proteins nebulin and desmin appeared to be very sensitive to degradation by μ-calpain under all of the conditions examined, with the exception of 100 μM Ca²⁺, pH 7.0. The difference in susceptibility exhibited by the proteins in this study may arise from differences in conformational changes induced by the pH/ionic strength conditions. These changes may render specific cleavage sites unavailable and thereby affect the susceptibility of these proteins to proteolysis by μ-calpain.
The ionic strength of the solution may have an effect on the conformation of the \(\mu\)-calpain enzyme. The reduction in activity of calpains under conditions of higher ionic strength has been seen in other studies [18,24,25]. Possible explanations for this reduction in activity include the fact that the higher ionic strengths may interfere in some manner with the interaction between the enzyme and the calcium ion. It has also been suggested that higher ionic strengths may increase the hydrophobicity of the enzyme and allow for aggregation of \(\mu\)-calpain [25] or the substrate proteins.

**Effects of calcium level and pH** This study demonstrates that the combination of physiological temperature and pH may have a dramatic effect on the calcium requirements needed by \(\mu\)-calpain to degrade myofibrillar proteins. When conditions were at or near physiological levels of pH and temperature (pH 7.0 and 37°C) the calpains were not shown to have any discernible activity when calcium levels were in the range of 100 \(\mu\)M. \(\text{Ca}^{2+}\) concentrations of 100 \(\mu\)M have been shown at 25°C to provide maximum hydrolysis of a casein substrate under the conditions of 25°C and pH 7.5 without causing autolysis [2]. Indeed, under the same conditions of pH and ionic strength but lower temperatures (4°C), 100 \(\mu\)M \(\text{Ca}^{2+}\) has been shown to be sufficient to activate proteolysis of the same five proteins that were examined in this study [E Huff-Lonergan, S Sernett, FC Parrish, Jr., RM Robson, unpublished results]. It was expected therefore that in the presence of 100 \(\mu\)M \(\text{Ca}^{2+}\) and at a temperature of 37°C and pH 7.0, proteolysis would proceed if not at the same rate, maybe faster. One hypothesis that might be raised upon initial examination of the data at 37°C, pH 7.0 and 100 \(\mu\)M \(\text{Ca}^{2+}\), is that rapid autolysis leading to inactivation of the enzyme is occurring prior to hydrolysis of the substrate myofibrillar proteins at the higher temperature. However other
reports [2,26] have shown that autolysis of \( \mu \)-calpain requires \( \text{Ca}^{2+} \) levels in the neighborhood of 200 \( \mu \text{M} \). The current study also tends to disprove the inactivation hypothesis as when \( \text{Ca}^{2+} \) levels were raised to 200 \( \mu \text{M} \), the degradation of most of the proteins occurred rapidly, indicating that at 100 \( \mu \text{M} \text{Ca}^{2+} \) the calpain was not autolyzed to the point of inactivity, it was simply not active on the myofibrillar proteins in the study at 100 \( \mu \text{M} \text{Ca}^{2+} \). These results suggest that under conditions of physiological temperature and pH 7.0, another mechanism may aid in modulating the activity of the calpain enzyme.

When the pH of the solution was lowered to 5.6 and the \( \text{Ca}^{2+} \) level remained at 100 \( \mu \text{M} \), the enzyme exhibited significant activity on all five of the proteins examined under the low ionic strength conditions (Figures 1-6). This may suggest that pH-induced conformational changes in the protease itself may play a role in activating the enzyme. Indeed, \( \mu \)-calpain has been shown to exhibit both a lowered requirement of \( \text{Ca}^{2+} \) for autolysis as well as an increased \( V_{\text{max}} \) for proteolytic activity in the presence of acidic phospholipids (e.g. phosphatidylinositol) with those phospholipids containing a higher number of negatively charged phosphate groups exhibiting greater effects [27,28]. This may illustrate a response to a localized reduction in pH leading to greater sensitivity to calcium under the conditions tested.

The fact that at pH 7.0, 37°C, 200 \( \mu \text{M} \text{Ca}^{2+} \) caused rapid activation of \( \mu \)-calpain (as evidenced by extensive degradation of the majority of the proteins at pH 7.0, 165 mM NaCl) while 100 \( \mu \text{M} \text{Ca}^{2+} \) did not, may indicate that under the conditions of 37°C and pH 7.0, the enzyme requires a higher concentration of \( \text{Ca}^{2+} \) to be active. Under the same pH and ionic strength conditions (pH 7.0, 165 mM NaCl, [E Huff-Lonergan, S Sernett, FC Parrish, Jr., RM Robson, unpublished...
results]) at 4°C, 100 μM Ca²⁺ was sufficient to allow μ-calpain to catalyze the degradation of the same proteins examined in the current study. Since the level of calcium (200 μM) that did allow μ-calpain to degrade the proteins was in the range that has been shown to initiate autolysis [2] it may be possible that a dramatic change in conformation is needed at 37°C to allow μ-calpain to be active against myofibrillar proteins. The possibility that autolysis of the enzyme plays a role in initiating activity at physiological temperatures cannot be easily ruled out. It has been shown that μ-calpain more rapidly undergoes autolysis at lower pH values (pH 5.8) [10]. The fact that at 100 μM Ca²⁺ and pH 5.6, μ-calpain did exhibit significant activity towards myofibrillar proteins while at pH 7.0 and 100 μM Ca²⁺ it did not, may indicate that autolysis or some other conformational change is needed to activate the enzyme at 37°C.

This study does not exclude the possibility that there is a dramatic increase in the amount of calcium chelated by calcium binding myofibrillar proteins at 37°C, pH 7.0, thus drastically reducing the level of free calcium in solution. However, the increase in amount of calcium chelated at 37°C would seem to be very high in order to reduce the level of calcium below that needed to initiate the activity of this low calcium requiring form of the enzyme.

As the pH and temperature levels used (37°C, and pH 7.0) are close to physiological levels, these results may point to combinations of specific parameters (e.g. temperature, pH and calcium level) that may play a more critical role in controlling calpain activity against myofibrillar proteins in vivo than have been suggested in in vitro studies done at lower temperatures
Conclusions

The fact that many factors affect the observed activity of \( \mu \)-calpain on myofibrillar proteins under conditions of physiological temperature (37°C) was demonstrated by this study. In addition to ionic strength, which has been shown to reduce the activity of \( \mu \)-calpain against the myofibrillar proteins, the other factors of pH and Ca\(^{2+}\) concentration exhibit unique effects on protein degradation by \( \mu \)-calpain at this temperature. This study may indicate that \( \mu \)-calpain exhibits a unique conformation or stability at the conditions of pH 7.0, 100 \( \mu \)M Ca\(^{2+}\) and 37°C that suppresses the activity of the enzyme under this set of conditions. While the effect of these conditions on the structure of the substrate proteins is not known, this study indicates the need to further investigate the physiological parameters of pH and temperature in more studies to more carefully elucidate the mechanism of calpain control in muscle cells.

Acknowledgments

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Figure 1. Coomassie stained 3.2-12% SDS-PAGE gradient gels of the pellets and supernatants from the $\mu$-calpain digested myofibrils digested in the presence of 100 $\mu$M CaCl$_2$. 0, 2, 15, 60 = minutes of incubation, T1 = intact form of titin (closed arrowhead), T2 = high molecular weight degradation product of titin (open triangle), MHC = myosin heavy chain, C = 60 minute buffer control. a) Digestion done at pH 7.0, 165 mM NaCl. b) Digestion done at pH 7.0, 330 mM NaCl. c) Digestion done at pH 5.6, 165 mM NaCl. d) Digestion done at pH 5.6, 330 mM NaCl.
Figure 2. Western blots of 3.2-12% gradient gels of the pellets and supernatants from the \( \mu \)-calpain digested myofibrils digested in the presence of 100 \( \mu \)M CaCl\(_2\). Blots were incubated overnight at 4°C with a monoclonal antibody (4C7) to titin. 0, 2, 15, 60 = minutes of incubation with \( \mu \)-calpain. C = 60 minute buffer control. Closed arrowhead designates the position of T1. Open triangle designates the position of T2. a) Digest done at pH 7.0, 165 mM NaCl, 100 \( \mu \)M CaCl\(_2\). b) Digest done at pH 7.0, 330 mM NaCl, 100 \( \mu \)M CaCl\(_2\). c) Digest done at pH 5.6, 165 mM NaCl, 100 \( \mu \)M CaCl\(_2\). d) Digest done at pH 5.6, 330 mM NaCl, 100 \( \mu \)M CaCl\(_2\).
Figure 3. Western blots of 3.2-12% SDS-PAGE gradient gels of the pellets and supernatants from the \( \mu \)-calpain digested myofibrils digested in the presence of 100 \( \mu \)M CaCl\(_2\). Blots were incubated overnight at 4°C with a monoclonal antibody to nebulin (NB2). 0, 2, 15, 60 = minutes of incubation, C = 60 minute buffer control. a) Digest done at pH 7.0, 165 mM NaCl, 100 \( \mu \)M CaCl\(_2\). b) Digest done at pH 7.0, 330 mM NaCl, 100 \( \mu \)M CaCl\(_2\). c) Digest done at pH 5.6, 165 mM NaCl, 100 \( \mu \)M CaCl\(_2\). d) Digest done at pH 5.6, 330 mM NaCl, 100 \( \mu \)M CaCl\(_2\).
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**Figure 4.** Western blots of 3.2-12% SDS-PAGE gradient gels of the pellets and supernatants from the \( \mu \)-calpain digests digested myofibrils digested in the presence of 100 \( \mu \)M \( \text{CaCl}_2 \). Blots were incubated at room temperature for 2 hours with a polyclonal antibody to filamin. 0 - 60 = minutes of incubation with purified \( \mu \)-calpain, C = 60 minute buffer control. a) Digest done at pH 7.0, 165 mM NaCl, 100 \( \mu \)M CaCl\(_2\). b) Digest done at pH 7.0, 330 mM NaCl, 100 \( \mu \)M CaCl\(_2\). c) Digest done at pH 5.6, 165 mM NaCl, 100 \( \mu \)M CaCl\(_2\). Closed arrowhead designates the position of intact filamin. Small arrow designates the position of the large degradation product of filamin. d) Digest done at pH 5.6, 330 mM NaCl, 100 \( \mu \)M CaCl\(_2\).
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Filamin: 
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Figure 5. Western blots of 18% SDS-PAGE gels of the pellets and supernatants from the \( \mu \)-calpain digests digested myofibrils digested in the presence of 100 \( \mu \)M CaCl\(_2\). Blots were incubated at room temperature for one hour with a polyclonal antibody to desmin. 0, 2, 15, 60 = minutes of incubation, 42 K = 42,000 dalton degradation product of desmin, 38 K = 38,000 dalton degradation product of desmin, C = 60 minute buffer control. a) Digest done at pH 7.0, 165 mM NaCl, 100 \( \mu \)M CaCl\(_2\). b) Digest done at pH 7.0, 330 mM NaCl, 100 \( \mu \)M CaCl\(_2\). c) Digest done at pH 5.6, 165 mM NaCl, 100 \( \mu \)M CaCl\(_2\). d) Digest done at pH 5.6, 330 mM NaCl, 100 \( \mu \)M CaCl\(_2\).
a pH 7.0, 165 mM NaCl

Desmin

---

b pH 7.0, 330 mM NaCl

Desmin

---

c pH 5.6, 165 mM NaCl

---

d pH 5.6, 330 mM NaCl

Desmin

45 K-

38 K-
Figure 6. Western blots of 18% SDS-PAGE gels of the pellets and supernatants from the μ-calpain digested myofibrils digested in the presence of 100 μM CaCl₂. Blots were incubated at room temperature for one hour with a monoclonal antibody to troponin-T. 0, 2, 15, 60 = minutes of incubation, 30 K = 30,000 dalton degradation product of troponin-T, 28 K = 28,000 dalton degradation product of troponin-T, C = 60 minute buffer control. a) Digest done at pH 7.0, 165 mM NaCl, 100 μM CaCl₂. b) Digest done at pH 7.0, 330 mM NaCl, 100 μM CaCl₂. c) Digest done at pH 5.6, 165 mM NaCl, 100 μM CaCl₂. d) Digest done at pH 5.6, 330 mM NaCl, 100 μM CaCl₂. Arrowheads indicate the positions of two isoforms of intact troponin-T.
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Troponin-T levels are shown for each condition.

- **Troponin-T**
  - 30 K-
  - 28 K-
Figure 7. Coomassie stained 3.2-12% SDS-PAGE gradient gels of the pellets and supernatants from the $\mu$-calpain digested myofibrils digested in the presence of 200 $\mu$M CaCl$_2$. 0, 2, 15, 60 = minutes of incubation, T1 = intact form of titin (closed arrowhead), T2 = high molecular weight degradation product of titin (open triangle), MHC = myosin heavy chain. a) Digestion done at pH 7.0, 165 mM NaCl, C = 60 minute buffer control. b) Digestion done at pH 7.0, 330 mM NaCl, C1 = 60 minute enzyme control, C2 = 60 minute buffer control. c) Digestion done at pH 5.6, 165 mm NaCl, C = 60 minute buffer control. d) Digestion done at pH 5.6, 330 mM NaCl, C = 60 minute buffer control.
a  pH 7.0, 165 mM NaCl
Pellet  Supernatant

MHC

T1  T2

Nebulin

b  pH 7.0, 330 mM NaCl
Pellet  Supernatant

MHC

T1  T2

Nebulin

200 μM

c  pH 5.6, 165 mM NaCl
Pellet  Supernatant

MHC

T1  T2

Nebulin

d  pH 5.6, 330 mM NaCl
Pellet  Supernatant

MHC

T1  T2

Nebulin
Figure 8. Western blots of 3.2-12% SDS-PAGE gradient gels of the pellets and supernatants from the μ-calpain digested myofibrils digested in the presence of 200 μM CaCl₂. Blots were incubated overnight at 4°C with a monoclonal antibody to titin (4C7). 0, 2, 15, 60 = minutes of incubation, T1 = intact form of titin, T2 = high molecular weight degradation product of titin, 1200 K = titin degradation product migrating at approximately 1,200,000 C = 60 minute buffer control. Closed arrowhead designates the position of T1. Open triangle designates the position of T2. a) Digest done at pH 7.0, 165 mM NaCl, 200 μM CaCl₂. b) Digest done at pH 7.0, 330 mM NaCl, 200 μM CaCl₂. c) Digest done at pH 5.6, 165 mM NaCl, 200 μM CaCl₂. d) Digest done at pH 5.6, 330 mM NaCl, 200 μM CaCl₂.
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### Diagrams

#### Diagram 1

- **Condition:** pH 7.0, 165 mM NaCl
- **Pellet:** 0 2 15 60 C
- **Supernatant:** 0 2 15 60 C

#### Diagram 2

- **Condition:** pH 7.0, 330 mM NaCl
- **Pellet:** 0 2 15 60 C
- **Supernatant:** 0 2 15 60 C

#### Diagram 3

- **Condition:** pH 5.6, 165 mM NaCl
- **Pellet:** 0 2 15 60 C
- **Supernatant:** 0 2 15 60 C

#### Diagram 4

- **Condition:** pH 5.6, 330 mM NaCl
- **Pellet:** 0 2 15 60 C
- **Supernatant:** 0 2 15 60 C

---

**Diagram Notes:*

- **T1** and **T2** indicate temperature measurements.
- **C** denotes control conditions.
- **200 μM** is indicated for certain conditions.
- **1200 K** represents a temperature range.

---

*For a detailed analysis, consult the full report for specific conditions and results.*
Figure 9. Western blots of 3.2-12% SDS-PAGE gradient gels of the pellets and supernatants from the μ-calpain digested myofibrils digested in the presence of 200 μM CaCl₂. Blots were incubated overnight at 4°C with a monoclonal antibody to nebulin (NB2). 0, 2, 15, 60 = minutes of incubation, C = 60 minute buffer control. a) Digest done at pH 7.0, 165 mM NaCl, 200 μM CaCl₂. b) Digest done at pH 7.0, 330 mM NaCl, 200 μM CaCl₂. c) Digest done at pH 5.6, 165 mM NaCl, 200 μM CaCl₂. d) Digest done at pH 5.6, 330 mM NaCl, 200 μM CaCl₂.
a  pH 7.0, 165 mM NaCl
   Pellet  Supernatant
   0 2 15 60 C 0 2 15 60 C

Nebulin - •  •

200 μM

b  pH 7.0, 330 mM NaCl
   Pellet  Supernatant
   0 2 15 60 C 0 2 15 60 C

Nebulin - •  •

c  pH 5.6, 165 mM NaCl
   Pellet  Supernatant
   0 2 15 60 C 0 2 15 60 C

Nebulin - •  •

d  pH 5.6, 330 mM NaCl
   Pellet  Supernatant
   0 2 15 60 C 0 2 15 60 C

Nebulin - •  •
Figure 10. Western blots of 3.2-12% SDS-PAGE gradient gels of the pellets and supernatants from the μ-calpain digested myofibrils digested in the presence of 200 μM CaCl2. Blots were incubated for 2 hours at room temperature with a polyclonal antibody to filamin. 0, 2, 15, 60 = minutes of incubation, C = 60 minute buffer control. a) Digest done at pH 7.0, 165 mM NaCl, 200 μM CaCl2. b) Digest done at pH 7.0, 330 mM NaCl, 200 μM CaCl2. Closed arrowhead designates the position of intact filamin. Small arrow designates the position of the large degradation product of filamin. c) Digest done at pH 5.6, 165 mM NaCl, 200 μM CaCl2. Closed arrowhead designates the position of intact filamin. Small arrow designates the position of the large degradation product of filamin. d) Digest done at pH 5.6, 330 mM NaCl, 200 μM CaCl2. Closed arrowhead designates the position of intact filamin. Small arrow designates the position of the large degradation product of filamin.
Figure 11. Western blots of 18% SDS-PAGE gels of the pellets and supernatants from the μ-calpain digested myofibrils digested in the presence of 200 μM CaCl₂. Blots were incubated for one hour at room temperature with a polyclonal antibody to desmin. 0, 2, 15, 60 = minutes of incubation, C = 60 minute buffer control. a) Digest done at pH 7.0, 165 mM NaCl, 200 μM CaCl₂. b) Digest done at pH 7.0, 330 mM NaCl, 200 μM CaCl₂. c) Digest done at pH 5.6, 165 mM NaCl, 200 μM CaCl₂. d) Digest done at pH 5.6, 330 mM NaCl, 200 μM CaCl₂.
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Desmin

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Desmin

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Desmin
Figure 12. Western blots of 18% SDS-PAGE gels of the pellets and supernatants from the \( \mu \)-calpain digested myofibrils digested in the presence of 200 \( \mu \text{M} \) \( \text{CaCl}_2 \). Blots were incubated for one hour at room temperature with a monoclonal antibody to troponin-T. 0, 2, 15, 60 = minutes of incubation, 30 K = 30,000 dalton degradation product of troponin-T, 28 K = 28,000 dalton degradation product of troponin-T, C = 60 minute buffer control. Arrowheads indicate the positions of two isoforms of intact troponin-T. a) Digest done at pH 7.0, 165 mM NaCl, 200 \( \mu \text{M} \) \( \text{CaCl}_2 \). b) Digest done at pH 7.0, 330 mM NaCl, 200 \( \mu \text{M} \) \( \text{CaCl}_2 \). c) Digest done at pH 5.6, 165 mM NaCl, 200 \( \mu \text{M} \) \( \text{CaCl}_2 \). d) Digest done at pH 5.6, 330 mM NaCl, 200 \( \mu \text{M} \) \( \text{CaCl}_2 \).
a pH 7.0, 165 mM NaCl

Pellet | Supernatant
--- | ---
0  | 2  | 15  | 60  | C  | 0  | 2  | 15  | 60  | C

Troponin-T

28 K-

b pH 7.0, 330 mM NaCl

Pellet | Supernatant
--- | ---
0  | 2  | 15  | 60  | C  | 0  | 2  | 15  | 60  | C

Troponin-T

28 K-

c pH 5.6, 165 mM NaCl

Pellet | Supernatant
--- | ---
0  | 2  | 15  | 60  | C  | 0  | 2  | 15  | 60  | C

Troponin-T

30 K-

28 K-

d pH 5.6, 330 mM NaCl

Pellet | Supernatant
--- | ---
0  | 2  | 15  | 60  | C  | 0  | 2  | 15  | 60  | C

Troponin-T
Figure 13. Coomassie stained 18% SDS-PAGE gels of the pellets and supernatants from the μ-calpain digested myofibrils digested in the presence of 100 μM CaCl₂. 0, 2, 15, 60 = minutes of incubation, MHC = myosin heavy chain, C = 60 minute buffer control. Solid triangle designates the position of α-actinin.  a) Digestion done at pH 7.0, 165 mM NaCl. b) Digestion done at pH 7.0, 330 mM NaCl. c) Digestion done at pH 5.6, 165 mM NaCl. d) Digestion done at pH 5.6, 330 mM NaCl.
a) pH 7.0, 165 mM NaCl

b) pH 7.0, 330 mM NaCl

c) pH 5.6, 165 mM NaCl

d) pH 5.6, 330 mM NaCl

Pellet | Supernatant
-----|-----
0 | 0 | 2 | 15 | 60 | C | 0 | 2 | 15 | 60 | C

MHC

Actin

100 µM
Figure 14. Coomassie stained 18% SDS-PAGE gels of the pellets and supernatants from the $\mu$-calpain digested myofibrils digested in the presence of 200 $\mu$M CaCl$_2$. 0, 2, 15, 60 = minutes of incubation, MHC = myosin heavy chain. Solid triangle designates the position of $\alpha$-actinin.  
a) Digestion done at pH 7.0, 165 mM NaCl, C1 = 60 minute enzyme control, C2 = 60 minute buffer control.  
b) Digestion done at pH 7.0, 330 mM NaCl, C1 = 60 minute enzyme control, C2 = 60 minute buffer control.  
c) Digestion done at pH 5.6, 165 mM NaCl.  
d) Digestion done at pH 5.6, 330 mM NaCl, C = 60 minute buffer control.
200 μM

a  pH 7.0, 165 mM NaCl

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MHC

Actin

b  pH 7.0, 330 mM NaCl

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MHC

Actin

c  pH 5.6, 165 mM NaCl

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MHC

Actin

d  pH 5.6, 330 mM NaCl

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MHC

Actin
GENERAL SUMMARY

From the studies contained in this body of work, several conclusions can be made. First, it can be concluded that the method of preparation (purified myofibrils vs. whole muscle homogenates) had little effect upon the rate of degradation of the intact forms of the proteins titin and nebulin.

Secondly, under conditions of low pH and temperature, μ-calpain was shown to produce very similar, if not the same degradation products of the myofibrillar proteins, titin, nebulin, filamin, desmin, and troponin-T that are produced under conditions of postmortem aging. In the postmortem aged samples, it appears that postmortem tenderization does not depend on the degradation of one single protein, rather tenderization is more closely linked to structural changes occurring in several key regions of the muscle cell. The proteins examined in this study are located in different regions of the muscle cell, and most have been implicated as being important in maintaining the structure and function of the muscle cell in some manner. In addition, these proteins are located at regions that appear to be affected during postmortem aging, including areas near the Z-line and in the I-band. Degradation of proteins such as desmin and filamin, located at the periphery of the Z-line, may disrupt the lateral register of the myofibrils themselves as well as their attachments to the cell membrane. Degradation of the proteins within the myofibril that are associated with the thick and thin filaments may allow lateral movement to occur within the sarcomere of postmortem aged samples. Titin, nebulin and troponin-T, through their ability to directly interact with, or modulate the interaction between, proteins of the thick and thin filaments and/or the Z-line have the opportunity to play key roles. Disruption of these proteins,
especially titin and nebulin, may allow for further physiochemical changes that lead
to myofibril fragmentation and ultimately tenderization.

As seen in this study, \( \mu \)-calpain has the ability under postmortem conditions
of relatively low pH and temperature, to degrade titin, nebulin, filamin, desmin and
troponin-T in much the same manner as was seen in naturally aged myofibrils.
This further implicates \( \mu \)-calpain as being a catalyst for the changes occurring in
postmortem muscle.

Thirdly, the proteins examined in this study appeared to differ in their
susceptibility to proteolysis by \( \mu \)-calpain. Under the majority of the conditions used,
the proteins titin, nebulin and desmin appeared to be the most susceptible to \( \mu \)-
calpain digestion. This difference in susceptibility especially, at the higher pH and
ionic strength conditions could arise from pH/ionic strength induced conformational
changes in the proteins. These changes may alter the susceptibility of the proteins
to calpain digestion by rendering specific cleavage sites more or less accessible.

The pH/ionic strength conditions may also affect the calpain molecule. It is
possible that at 100 \( \mu \)M Ca\(^{2+}\), under the pH/ionic strength conditions of pH 7.0, 330
mM NaCl and 4°C, conformational changes in the calpain molecule itself could
occur that may affect the ability of \( \mu \)-calpain to digest its substrate myofibrillar
proteins.

The fact that at 4°C, \( \mu \)-calpain retains greater activity under the conditions of
pH 5.6, 330 mM NaCl than it had under the conditions of pH 7.0, 330 mM NaCl on
all five of the proteins examined, may indicate a synergistic relationship between
pH and ionic strength and their effect on calpain activity. It is possible that
conformational changes of the enzyme induced by the lowered pH outweigh the
effects of increased ionic strength under the conditions examined.
On the basis of these results, the low pH and temperature, and relatively higher ionic strengths found in postmortem muscle would not appear to inhibit the \( \mu \)-calpain-induced degradation of the five proteins studied. This study further implicates \( \mu \)-calpain in postmortem protein degradation.

Finally, the fact that many factors affect the observed activity of \( \mu \)-calpain on myofibrillar proteins under conditions of physiological temperature (37°C) was demonstrated by this study. In addition to ionic strength, which has been shown to reduce the activity of \( \mu \)-calpain against the myofibrillar proteins, the other factors of pH and Ca\(^{2+}\) concentration exhibit unique effects on protein degradation by \( \mu \)-calpain at this temperature. This study may indicate that \( \mu \)-calpain exhibits a unique conformation or stability at the conditions of pH 7.0, 100 \( \mu \)M Ca\(^{2+}\) and 37°C that suppresses the activity of the enzyme under this set of conditions. While the effect of these conditions on the structure of the substrate proteins is not known, this study indicates the need to further investigate the physiological parameters of pH and temperature in more studies to more carefully elucidate the mechanism of calpain control in muscle cells.

This study shows that many factors can come into play when examining the delicate interactions between the \( \mu \)-calpain enzyme and its substrate myofibrillar proteins. In order to more fully appreciate the intricacies of the calpain system and its influence on tenderness, researchers need to focus not only on the effects of the environment on the enzyme, but also on the substrate proteins. Studies need to be done that take into consideration the temperature, pH and ionic strength conditions found in living tissue and in meat products in order to elucidate the role of \( \mu \)-calpain in myofibrillar protein turnover and postmortem protein degradation. More research is needed to determine precisely, the ideal conditions for myofibrillar
protein degradation by the calpains if the system is to be manipulated to enhance tenderness or muscle growth.
ACKNOWLEDGMENTS

I would like to express sincere appreciation to my major professor, Dr. F.C. Parrish, Jr. for all of his advice and support throughout my graduate career. His council has been invaluable in guiding my professional career.

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