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Myofibrillar protein degradation patterns and structural changes in skeletal muscle from electrically stimulated Bos taurus and Bos indicus crossbred cattle

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Myofibrillar protein degradation patterns and structural changes in skeletal muscle from electrically stimulated Bos taurus and Bos indicus crossbred cattle

by

Chiung-Ying Ho

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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Iowa State University Ames, Iowa

1995
Myofibrillar protein degradation patterns and structural changes in skeletal muscle from electrically stimulated *Bos taurus* and *Bos indicus* crossbred cattle

Chiung-Ying Ho

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Degradation of titin, nebulin, desmin and troponin-T, structural changes in nonstimulated (NS) and electrically stimulated (ES) bovine skeletal muscle and comparison of these changes in Angus x Jersey (AxJ) cattle (*Bos taurus* cross) with the changes in Brahman x Simmental (BxS) cattle (*Bos indicus* cross) were determined. Myofibrils for SDS-PAGE and Western blots and intact muscle samples for transmission electron microscopy were prepared at 0, 1, 3, 7, 14 and 28 days postmortem (PM). In SDS-PAGE, ES slightly accelerated the degradation of intact titin (T1 band), nebulin, desmin and troponin-T in AxJ samples. In Western blots of AxJ samples, ES enhanced T1 degradation, the appearance of a 38 kDa desmin degradation product and the accumulation of the 30 kDa polypeptide but had no detectable affect on nebulin degradation. Both SDS-PAGE and Western blots of BxS samples showed that ES had no effect on degradation of T1, nebulin, desmin and troponin-T but slightly enhanced the accumulation of the 30 kDa polypeptide. These four proteins were degraded faster in AxJ than in BxS samples. ES accelerated the appearance of wide I-band fractures and increased the frequency of narrow I-band fractures in all samples and of intermediate and wide I-band fractures through day 7 in AxJ samples. In BxS samples, ES
accelerated the appearance and frequency of all three types of I-band fractures through 14 days PM. All three types of I-band fractures were seen sooner in NS AxJ than in NS BxS samples. The frequency of all types of I-band fractures was greater in all AxJ than in all BxS samples. Twice as many intermediate and wide I-band fractures were present at 3, 7, 14 and 28 days in AxJ as in BxS samples. Z-line degradation occurred but was unaffected by ES or by breed. In conclusion, ES slightly accelerated the degradation of titin, nebulin, desmin and troponin-T in AxJ samples only and accelerated the appearance of wide I-band fractures in AxJ samples. ES caused all three types of I-band fractures to appear sooner in BxS than in NS BxS samples.
# TABLE OF CONTENTS

## GENERAL INTRODUCTION

Dissertation Organization 3
Literature Review 3

## IDENTIFICATION OF THE 30 KDA POLYPEPTIDE IN POST MORTEM SKELETAL MUSCLE AS A DEGRADATION PRODUCT OF TROPONIN-T

Summary 37
Introduction 39
Materials and Methods 40
Results 43
Discussion 49
Conclusion 52
Acknowledgements 53
References 54

## EFFECT OF ELECTRICAL STIMULATION ON POSTMORTEM TITIN, NEBULIN AND DESMIN DEGRADATION AND ULTRASTRUCTURAL CHANGES IN BOVINE LONGISSIMUS MUSCLE

Abstract 59
Introduction 61
Materials and Methods 62
Results 66
Discussion 86
Implications 94
Literature Cited 95
CHANGES IN TITIN, NEBULIN, DESMIN, AND TROPONIN-T AND IN ULTRASTRUCTURE RESULTING FROM POSTMORTEM ELECTRICAL STIMULATION OF BOS INDICUS CROSSBRED CATTLE

Abstract 103
Introduction 104
Materials and Methods 106
Results 109
Discussion 125
Implications 132
Literature Cited 133
GENERAL SUMMARY 140
REFERENCES CITED 144
ACKNOWLEDGMENTS 165
GENERAL INTRODUCTION

The excellent progress that has been made in understanding the structure of intracellular components of skeletal muscle cells, especially the myofibril (Craig, 1994), has been coupled with the use of sophisticated immunoelectron microscope studies to locate specific proteins in the myofibril. This information has enhanced our understanding of how living skeletal muscle functions and also has formed the basis for molecularly-oriented investigations of myofibrillar changes in muscle postmortem (PM). For example, it has been reported that several muscle proteins, especially titin, nebulin, desmin, and troponin-T (TN-T), are degraded during PM storage. In myofibrils, titin is an elastic filamentous protein that extends from the Z line to the M line; nebulin comprises inextensible filaments that are closely associated with, or part of, thin filaments; α-actinin is an integral Z-line protein; desmin is the major component of intermediate filaments that connect Z lines of adjacent myofibrils; and TN-T is a subunit of troponin which is located on actin filaments. Degradation or loss of these proteins has been implicated in the loss of myofibrillar integrity, thus, increasing meat tenderness. Several studies have investigated protein changes in bovine muscle PM. Olson and Parrish (1977) studied TN-T degradation, Hwan and Bandman (1989) studied desmin degradation and Fritz and Greaser (1991) studied titin and nebulin degradation. Each of these studies implicated these proteins in the loss of myofibrillar integrity. Bandman and Zdanis (1988) found that, between 2 and 3 weeks after slaughter, no undegraded titin was present. Uytterhaegen et al. (1992) showed that aging and/or electrical
stimulation (ES) increased the degradation of titin and TN-T. However, Fritz et al. (1993) reported that titin content does not distinguish tough from tender beef during PM storage.

Another type of PM change is structural change. Stromer et al. (1967) used electron microscopy to show that sarcomere shortening and both Z-line and M-line degradation are characteristics of PM bovine skeletal muscle. Marsh et al. (1974) found that extensive cold shortening of bovine muscle produced I-band fractures adjacent to the Z line. Both Savell et al. (1978) and Takahashi et al. (1987) reported that electrical stimulation caused the formation of contraction nodes in bovine muscle, and that sarcomeres in the internodal zones were stretched or fractured, thus potentially increasing meat tenderness. These earlier studies used either SDS-gel analysis or structural analysis but not both.

The purpose of the study reported in this dissertation was to compare PM changes in nonstimulated and ES longissimus muscle from Bos taurus crossbred cattle and from Bos indicus crossbred cattle. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots were used to detect changes in titin, nebulin, α-actinin, desmin, and TN-T, and transmission electron microscopy was used to determine structural changes.
Dissertation Organization

This dissertation includes three papers. The purpose of the first paper is to identify the origin of the 30 kDa polypeptide in PM bovine skeletal muscle. This paper has been published in Biochimie and is included here with the permission of the Societe francaise de biochimie et biologic moleculaire/Elsevier. The goal of the second paper is to investigate the effect of electrical stimulation on titin, nebulin, α-actinin and desmin degradation and ultrastructural changes in bovine skeletal muscle from *Bos taurus* crossbred cattle. The third paper compares these protein degradation patterns and ultrastructural changes in *Bos taurus* crossbred cattle with the changes in *Bos indicus* crossbred cattle. Both papers were submitted for publication. The three papers are preceded by a General Introduction and will be followed by a General Summary section. References cited in the General Introduction follow the General Summary section.

Literature Review

Skeletal muscle structure

Skeletal muscle also is called striated muscle because it is comprised of an alternating arrangement of light and dark bands that show a striation pattern in the light microscope. The basic components of an entire skeletal muscle are muscle fibers which also are called muscle cells. These are long, cylindrical cells with tapering ends that are 1 to 40 mm in length and 10 to 100 μm in diameter. Muscle fibers are multinucleated cells (100-200 nuclei per cell) with the nuclei just beneath
the outer fiber membrane which is often called the sarcolemma. The endomysium, a very thin sheet of connective tissue, is immediately adjacent to the sarcolemma. The perimysium, a thicker extension of the endomysium, surrounds groups of muscle fibers to form muscle bundles. The size of the bundles is responsible for the visual perception of muscle texture. The epimysium, a thick connective tissue layer that is continuous with the perimysium, surrounds the entire muscle (for reviews, see Goll et al., 1984; Squire, 1986a).

Components of muscle cells

Muscle fibers or cells, as cells in other tissues, contain an outer cell membrane or sarcolemma, mitochondria, sarcoplasmic reticulum, lysosomes, ribosomes and a Golgi apparatus. In addition to these subcellular organelles, muscle fibers also contain contractile organelles known as myofibrils. Myofibrils are elongated protein threads, 1 to 3 \( \mu \)m in diameter, each enveloped by sarcoplasmic reticulum membranes and oriented with their axis parallel with the long axis of the muscle cell. Myofibrils extend from one end of the muscle cell to the other. Myofibrils occupy about 80-87% of the interior of skeletal muscle cells. The light and dark bands of adjacent myofibrils are in register, thus producing a cross-striated cell or fiber. The dark band is anisotropic or birefringent in polarized light and is called the A band. The light band is isotropic or weakly birefringent in polarized light and is called the I band. A dark line called the Z line bisects the 1-\( \mu \)m-long I band. A light area in the center of the A band is known as the H zone, and bisecting this band is the dark M line. A sarcomere is the contractile unit of striated muscle, is the distance from one Z line
often is 2.5 to 2.8 \( \mu \text{m} \) long in resting mammalian muscle (Goll et al., 1984; Craig, 1994).

The sarcomere is widely accepted as being constructed of two sets of parallel and interdigitated protein filaments (thick and thin filaments) that are discontinuous and inextensible. Thick filaments in mammalian skeletal muscle are 1.6 \( \mu \text{m} \) long and are 14 to 16 nm in diameter with tapered ends. Thin filaments are 1 \( \mu \text{m} \) long and 6 to 8 nm in diameter. Thin filaments extend from their anchor point at the Z line through the space between thick filaments to the edge of the H-zone. Each of the thick filaments bears a large number of regularly spaced lateral projections termed cross-bridges which are 5 to 6 nm in diameter and approximately 18 to 19 nm long (Goll et al., 1984). Thick filaments contain myosin and C-protein, and thin filaments contain actin, tropomyosin, troponin and possibly cap Z and gelsolin. Protein components of the Z line include actin, \( \alpha \)-actinin, zeugmatin, cap Z, amorphin, Z-protein, Eu-actinin, Z-nin, 220 kDa and 32 kDa protein (Vigoreaux, 1994). Filamin also is presumed to be associated with the Z line (Goll et al., 1984). Desmin filaments at the periphery of Z lines connect Z lines in adjacent myofibrils and Z lines in peripheral myofibrils to the sarcolemma. The M line, a transverse structure at the center of the A band that connects thick filaments at their center, contains M-protein, myomesin and creatine kinase. Titin comprises a third filament system which extends from the Z line to the M line (Fürst et al., 1989) and is the major component of an elastic cytoskeletal lattice that coexists with thick and thin filaments. Nebulin is associated with or may be a part of thin filaments and may
serve as a template to regulate thin filament length (Kruger et al., 1991).

Muscle cells also contain two sets of membrane-bounded tubules, the transverse system (T system) and the longitudinal system (L system) which surround and lie between the myofibrils. The T system is about 0.3% of the total volume of the muscle fiber and is a tubular system formed by invaginations of the sarcolemma that occur periodically along the length of the muscle fiber. In mammalian skeletal muscles, T tubules are found near the A-I junction and conduct a signal (action potential) from the sarcolemma to the interior of the muscle cell to the sarcoplasmic reticulum. The L system tubules extend from one T tubule to the next and form a tubular network around each myofibril. This tubular network is also known as the sarcoplasmic reticulum, is about 3% of the volume of a muscle cell and is responsible for regulating free calcium levels inside the cell. The sarcoplasmic reticulum membrane contains an ATP-driven calcium pump protein that allows the reticulum to accumulate calcium against a concentration gradient (Lytton and MacLennan, 1992).

**Structural proteins in myofibrils** Myosin, with a molecular weight of ~520,000, is about 43% of the myofibrillar mass. The myosin molecule consists of two heavy chains (~230 kDa) and four light chains (~20 kDa). Myosin has actin-activated ATPase activity, can bind strongly to actin and can form thick filaments at low ionic strength (Squire, 1986c). The configuration of the myosin molecule is a long rod with two pear-shaped heads at one end. The entire molecule is 165 to 170 nm long. The rod portion of the molecule is 155 to 160 nm long and
approximately 2 nm in diameter. This rod portion contains two polypeptide chains that are more than 95% α-helical. The two heads are asymmetric and are approximately 6.5 nm wide, 16.5 nm long and 4 nm thick (Rayment et al., 1993). The myosin molecule can be cleaved by the proteolytic enzymes, chymotrypsin or trypsin, into two fragments named light meromyosin (LMM) and heavy meromyosin (HMM). The tail portion of the original myosin molecule is the source of LMM which has no ATPase activity and can not bind to actin but can form smooth-surfaced filaments that have diameters similar to those of thick filaments. The head and part of the tail of the original myosin molecule give rise to HMM which has ATPase activity and binds to actin, but can not form filaments. Incubating with trypsin for longer time will split HMM into fragments called subfragment 1 (HMM-S1) and subfragment 2 (HMM-S2) (Squire, 1973). Two HMM-S1 asymmetric fragments are produced for each HMM-S2 molecule. The two HMM-S1 fragments are the two heads from one original myosin molecule. HMM-S1 has ATPase activity, binds to actin, contains two of the four small polypeptide chains found in the original myosin molecule but can not form filaments. HMM-S2 is the short myosin tail on HMM, has no ATPase activity, can not bind to actin and can not form filaments. The properties of HMM-S1 indicate that the active sites for myosin ATPase activity and the actin binding sites are both located in the heads of the myosin molecule and that each myosin molecule has two of these sites (Goll et al., 1984; Pearson and Young, 1989a; Lowey, 1994).
C-protein is a thick filament-associated protein with a molecular weight of about 140,000. In myofibrils, C-protein constitutes 2.5 to 3% of myofibrillar protein. In electron micrographs, bovine skeletal muscle C-protein appears as short rods with a relatively uniform length of about 50 nm and 4 nm in diameter (Fürst et al., 1992). Chicken skeletal muscle C-protein preparations contain V- or U-shaped rods (Swan and Fischman, 1986). Analysis of a cDNA clone has shown that the C-protein molecule contains sequence motifs that resemble immunoglobulin C2 domains and fibronectin type III repeats and that C-protein is a member of the immunoglobulin superfamily (Einheber and Fischman, 1990). C-protein is located in seven to nine stripes spaced 43 nm apart in the middle third of each half of the A band and binds to the HMM-S2 and LMM portions of the myosin molecule. C-protein also binds to F-actin and to native thin filaments *in vitro*, and its interaction with regulated thin filaments is Ca\(^{2+}\)-sensitive (Moos, 1981; Yamamoto, 1986). The location of C-protein in the A band and its ability to bind to thin filaments have raised the possibility that C-protein may modulate muscle contraction. When C-protein is added to myosin filaments, their actin-activated ATPase activity is modified. At very low ionic strength, the ATPase is strongly inhibited by C-protein whereas, at physiological ionic strength, C-protein slightly activated the ATPase (Yamamoto and Moos, 1983). More recently, Fürst et al. (1992) reported that C-protein binds strongly to myosin, myosin rods and purified titin (T2) *in vitro* and suggested that C-protein could connect titin filaments to multiple sites in the A band.
M-protein, with a molecular weight of 165,000, is a structural component of the M line in striated muscle and comprises 3 to 5% of the myofibrillar mass. Electron microscopy of rotary-shadowed M-protein showed that it is an elongated molecule with a length of 36 nm and a width of 4.1 nm (Woodhead and Lowey, 1982). The cDNA sequence of M-protein predicts a 1450-amino acid polypeptide with a calculated molecular weight of 163,000. The sequence contains five copies of fibronectin type III repeats that are in the middle part of the predicted molecule. Two of six copies of the immunoglobulin C2 repeats are located toward the N-terminus and the remaining four copies are positioned near the C-terminus. This shows that M-protein, along with other thick filament-associated proteins such as titin and C-protein, is also a member of the immunoglobulin superfamily (Noguchi et al., 1992). M-protein and two other proteins, myomesin (185 kDa) and the homodimeric MM-type isoenzyme of creatine kinase (86 kDa) were reported to be localized at the M line. Grove et al. (1985) found that M-protein and myomesin were consistently localized at the M line in both chicken skeletal and cardiac muscle fibers during differentiation and that myomesin was present before M-protein could be detected. Strehler et al. (1983) suggested that MM-creatine kinase is probably a major protein involved in the formation of M4 and M4' M-bridges. Masaki and Takaiti (1974) reported that M-protein is tightly bound to myosin at near physiological conditions, but Woodhead and Lowey (1983) showed that the direct interaction of M-protein or creatine kinase with myosin or with S-2 is, if present at all, very weak in vitro.
Actin, a 42 kDa protein, constitutes about 20% of the myofibrillar mass. Actin is a highly conserved globular protein that consists of 374 amino acids and that polymerizes into filaments. The actin molecule contains only one polypeptide chain and is referred to as G-actin. The overall dimensions of the actin molecule are 3.5 x 5.5 x 5.5 nm (Mannherz, 1992). Each G-actin normally binds one molecule of ATP and one molecule of a divalent cation which have been hypothesized to contribute to the stability of the globular form (Nagy and Jencks, 1962).

Polymerization of actin, however, can take place without the presence of ATP and divalent cations (Kasai et al., 1965). The assembly of actin into filaments can be divided into four steps: (1) activation - salt-binding and conformational changes in G-actin monomers are involved and also possibly the binding of fresh ATP to the monomer; (2) nucleation - the formation of oligomers having a higher probability of growing into filaments than dissociating back to monomers; (3) elongation - the addition and removal of monomers from each end of the filament; (4) annealing - the end-to-end joining of two shorter filaments to form a longer filament. Any of these steps can be reversed. During or immediately following assembly, the ATP associated with actin is hydrolyzed to ADP and inorganic phosphate; the ADP remains associated with the actin aggregated in the filament, but the inorganic phosphate does not (Pollard and Cooper, 1986). Actin filaments, also known as F-actin, consist of a double-stranded, right handed helix, with significant intersubunit contacts along the two long-pitch (75 nm/turn) helixes. A strand of overlapping tropomyosin molecules extends the length of the F-actin filament and lies in each of
the two grooves created by the F-actin helix. Troponin binds periodically along the tropomyosin filament and is a complex of three subunits (-C, -I and -T).

Tropomyosin is about 4 to 6% of the myofibrillar mass and, together with troponin, plays a central role in contractile regulation. The tropomyosin molecule is an elongated α-helical structure about 40 nm long and 2 nm in diameter that contains two polypeptide subunits associated in register in a coiled-coil. Two types of subunits occur in skeletal muscle, α (32 kDa) and β (34 kDa), and the proportions of each subunit type appear to be tissue specific. The β subunit contains 284 amino acid residues which are highly conserved throughout evolution. The coiled-coil interaction between the tropomyosin subunits is stabilized by both hydrophobic interactions between the nonpolar side chains and electrostatic interactions between the charged side chains. Alteration of carboxy terminal residues of tropomyosin adversely affects the interaction between tropomyosin and actin and suggests that the overlap of tropomyosin molecules is important to tropomyosin polymerization and binding to actin (Zot and Potter, 1987).

The fitting of 41 nm long tropomyosin molecules into the spiraling grooves of the F-actin filament results in an axial tropomyosin repeat of 38.5 nm and in having one tropomyosin molecule span seven actin monomers (Zot and Potter, 1987). Sequence periodicities in polar and non-polar residues in outer helical positions of the coiled-coil of rabbit striated muscle α-tropomyosin are sufficiently regular to suggest that seven or 14 quasi-equivalent actin binding sites could exist (Phillips et al., 1986). Hitchcock-DeGregori and Varnell (1990) reported that, by making
deletions in chicken striated α-tropomyosin cDNA with oligonucleotide-directed mutagenesis, they found a 14-fold periodicity is sufficient for regulation and consistent with the presence of two sets of seven α and seven β quasi-equivalent actin-binding sites. Phillips et al. (1986), however, suggested that interactions between tropomyosin and actin are not so strictly limited, and tropomyosin is continually changing connections with actin, producing a net effect of relaxation or contraction. Trombitas et al. (1990) showed that labeling with a polyclonal antibody to chicken heart tropomyosin produced 23 transverse I-band stripes with a periodicity of 38.4 nm. The stripe adjacent to the Z line was not labeled. Monoclonal antibodies to chicken heart tropomyosin showed an extra 24th stripe immediately adjacent to the Z line, implying that a unique antigenic site on tropomyosin is accessible only near the Z line.

Troponin comprises 4 to 6% of the myofibrillar mass and is required for the Ca^{2+} dependence of actomyosin ATPase activity during muscle contraction. Troponin is a particle consisting of a globular head and a thin filamentous tail with a total length of 25.4 nm. The axial size of the globular head is 8.3 nm and the length of the tail is 17.1 nm (Ohtsuki et al., 1988). A troponin molecule contains one of each of three different subunits: the 37 kDa subunit which strongly binds to tropomyosin is called TN-T; the 24 kDa subunit which can inhibit the actin-myosin interaction is called troponin-I (TN-I); and the 18 kDa subunit which binds calcium ions is called troponin-C (TN-C) (Squire, 1986b).

Troponin-T, the largest of the three troponin subunits, links the troponin
complex to tropomyosin and also interacts with TN-I and TN-C. Troponin T consists of 259 amino acid residues and is a highly polar molecule because of a large number of acidic side chains near its N-terminus and basic residues near its C-terminus (Zot and Potter, 1987). Troponin-T is a 16.9 nm long filamentous particle. Troponin T1, one of two chymotryptic subfragments of TN-T, is an 11.6 nm long filamentous particle. Antibody to troponin T1 forms transverse striations that are spaced 38 nm apart along the thin filament (Ohtsuki et al., 1988).

Tropomyosin binding sites occur at residues 71 to 151 and 197 to 259 of the TN-T sequence, and the TN-C binding site is near the C-terminus of TN-T between residues 159 and 259 (Zot and Potter, 1987).

Troponin-I consists of 179 amino acids and has an actual molecular weight of 20,864 based on its sequence (Wilkinson and Grand, 1975). A segment from residues 102 to 135 contains 12 basic side chains and another segment from residues 5 to 27 contains six basic side chains. Both of these segments are thought to be involved in regulating thin filaments (Zot and Potter, 1987).

Troponin-I can directly bind actin and inhibits the interaction of actin with myosin, and the inhibition is enhanced in the presence of tropomyosin (Craig, 1994). Troponin-I also can bind TN-C with or without Ca^{2+}. Residues 1 to 21 and 96 to 116 of the TN-I sequence could bind to TN-C. The TN-T binding site may occur in the heptad repeat region between residues 57 to 106 of TN-I (Zot and Potter, 1987).
Troponin-C is composed of 159 residues, is a very acidic protein because of its high content of glutamic and aspartic acid residues and has a calculated molecular weight of 17,965 (Collins et al., 1977). It has four Ca\(^{2+}\)-binding sites: two low-affinity sites that selectively bind Ca\(^{2+}\) over Mg\(^{2+}\), and two high-affinity sites that bind Ca\(^{2+}\) and Mg\(^{2+}\) competitively. These four homologous regions are found in the TN-C sequence, and each region represents a helix-loop-helix Ca\(^{2+}\)-binding domain, referred to as an "EF-hand". These Ca\(^{2+}\)-binding domains consist of an \(\alpha\)-helical region on each side of a 12-residue loop that contains the six ligands responsible for coordination of a Ca\(^{2+}\) ion. The crystal structure of TN-C showed that the molecule is a 7.3 nm long helical protein (Herzberg and James, 1988) and that the N-terminal Ca\(^{2+}\)-specific sites and the C-terminal Ca\(^{2+}\)-Mg\(^{2+}\) sites are separated by a long nine-turn \(\alpha\)-helix, thus forming a dumbbell shaped molecule. Muscle contraction is regulated by the Ca\(^{2+}\)-specific sites, and the Ca\(^{2+}\)-Mg\(^{2+}\) sites probably always have either Ca\(^{2+}\) or Mg\(^{2+}\) bound to them. The binding of divalent cations to the Ca\(^{2+}\)-Mg\(^{2+}\) sites could strengthen the interaction between troponin subunits. The homologies between the DNA sequences of TN-C, calmodulin and parvalbumin provide evidence that all three proteins are derived from a common precursor molecule which had multiple Ca\(^{2+}\)-binding sites (Zot and Potter, 1987).

Titin, with a molecular mass of \(-3000\) kDa, is the largest polypeptide so far identified and comprises \(-10\%\) of the myofibrillar mass. The titin molecule is string-like, is approximately 1 \(\mu\)m long, and \textit{in vivo} spans from the M line to the Z line. Much of the I-band region of titin is an elastic connection between the thick
filament and the Z line, and forms a third type of sarcomere filament in addition to thick and thin filaments. These connections may center the A band in the sarcomere and provide structural continuity in relaxed myofibrils. The A band region of titin may be attached to the thick filament or associated proteins, where it could serve as a molecular ruler regulating thick filament length and assembly (Labeit et al., 1990). The A-band titin sequence consists almost entirely of a repetition of two types of motifs termed class I and class II. These motifs each contain about 100 amino acids and are similar to type III fibronectin and C-2 immunoglobulins, respectively. Both motifs tend to fold and form separate globular domains. Such motifs are present in several evolutionarily divergent muscle proteins (e.g. C-protein, skelemin, and chicken smooth muscle myosin light chain kinase), all of which may interact with myosin. The class I and II domains in the A band region of titin are arranged in a regular eleven domain pattern or super-repeat, I-I-I-II-I-I-II-I-I-II. The super repeat spans ~44 nm in situ which is very similar to the 43 nm spacing of crossbridges and thus the packing of myosin molecules in thick filaments (Trinick, 1992). Fürst et al. (1989) showed that monoclonal antibodies to titin identified quasi-repeats in the titin molecule near the M line that were characterized by a 42-43 nm repeat spacing.

Tan et al. (1993) demonstrated that the cDNA sequences from the region of titin located in the A band adjacent to the A-I junction had a similar repeating pattern of fibronectin type III and immunoglobulin C2 motifs to those located in the central region of the A band. Gautel et al. (1993) cloned and sequenced the C-
terminal region of the titin molecule, which is at the M-line end of the molecule and which binds to the 165 and 190 kDa M-line proteins. In contrast to the repeating titin motif patterns in the rest of the A band, the titin sequence from the M line showed a more complex structure of immunoglobulin-C2 repeats, separated by unique interdomain insertions. One insertion consists of four KSP repeats analogous to the multi-phosphorylation repeats of neurofilament subunits H and M. Gautel et al. (1993) suggested that titin C-terminal phosphorylation by SP-specific kinases was regulated during differentiation, and that this may control the assembly of the M line during myogenesis. Recently, Maruyama et al. (1993) cloned and sequenced the I-band region of titin and found it contained immunoglobulin C2 motifs and regions highly homologous with neurofilament H and M subunits. Labeit et al. (1992) found that expressed A-band titin fragments could bind to the LMM portion of the myosin molecule and to C-protein. Later, Soteriou et al. (1993) also reported that ostensibly intact titin purified from rabbit skeletal muscle could bind to whole myosin, myosin rod, LMM, C-protein, X-protein and AMP-deaminase.

In most vertebrate skeletal muscles, there are two forms of titin: T1 (mother molecule) and its proteolytic product, T2. Takahashi et al. (1992) reported that T1 was degraded into T2 and a 1200 kDa fragment when rabbit myofibrils were treated with a solution containing 0.1 mM CaCl₂ and 30 μg leupeptin/ml. The proteolytic product, T2 is localized in the A band (Kawamura et al., 1995), and the 1200 kDa fragment is localized in the I band near the Z line (Kimura et al., 1992). The average lengths of T1, T2 and the 1200 kDa fragment were estimated to be
approximately 1250, 920, and 360 nm, respectively (Suzuki et al., 1934). Nave et al. (1989) also demonstrated that T2, in shadowed preparations, appeared as extremely long (~900 nm) flexible, slender strands (3 to 4 nm wide) with axial periodicity and a globular head at one end. More recently, Kawamura et al. (1995) reported that T2 was degraded into 400 kDa and 1700 kDa fragments after treatment with α-chymotrypsin, and that epitopes recognized by polyclonal antibodies to the 400 kDa fragment were scattered slightly into the I band from their original I-band location near the edge of the A band in stretched sarcomeres.

Nebulin, a family of giant sarcomere matrix proteins of 700 to 900 kDa, is 1.05 to 1.3 μm in length in most vertebrate skeletal muscles, comprises about 3% of myofibrillar mass and has been proposed to constitute a set of inextensive longitudinal filaments which are attached to the Z line and are coextensive with the actin filaments (Wang and Wright, 1988; Chen et al., 1993). The cDNA sequence of nebulin has shown that a motif of 35 conserved amino acids repeats throughout most of the molecule. Groups of seven of the 35-residue motifs form a 245 residue super repeat that is ~38 nm long. The repeating 35-residue motifs are likely to be largely α-helical. Since the ratio of actin : tropomyosin : troponin in thin filaments is 7:1:1, and the thin filament has a periodicity of 38.5 nm, the seven-fold character of the super-repeat suggests that successive discrete α-helical domains in the nebulin molecule bind to successive actin subunits and/or to tropomyosin and troponin which are arranged periodically on the thin filament. The nebulin molecule would then lie in the groove(s) of the actin filament similar to tropomyosin (Trinick,
Nave et al. (1990) showed that α-actinin bound to nebulin and suggested that nebulin could form a fourth filament system in skeletal muscle that might extend to the Z line. Labeit et al. (1991) proposed that nebulin could interact with both actin and tropomyosin in thin filaments and that nebulin acts as a protein-ruler to regulate precise thin filament assembly. Recently, Wright et al. (1993) used immunoelectron microscopy to localize three site-specific monoclonal antibodies to cloned human nebulin fragments in rabbit and human skeletal muscle and found that nebulin has its C-terminal end anchored at the Z line and spanned the full length of the thin filament.

Jin and Wang (1991a, b) showed that a cloned human nebulin fragment from the N-terminal region could bind to myosin, C-protein and actin but did not bind to tropomyosin and troponin. Chen et al. (1993) found that the interaction of an expressed nebulin fragment and actin promoted nucleation of actin polymerization, reduced actin depolymerization and stabilized the actin nuclei. Chen and Wang (1994) studied the conformational state of an expressed nebulin fragment and found that, in contrast to the predicted high α-helical content, the nebulin fragment in aqueous solutions only showed 10% α-helix, but the α-helix content could be increased to 50% by increasing the trifluoroethanol concentration to 67%. Thus Chen and Wang (1994) suggested that the interaction of nebulin with actin is probably different from the tropomyosin-actin interaction. Tatsumi et al. (1992) found that chicken nebulin filaments had a molecular mass of about 700 kDa and were split into 200-, 180-, 40-, 33-, and 23-kDa subfragments when treated with a
solution that contained 0.1 mM CaCl$_2$ and 30 μg leupeptin/ml. The 200-, 40- and 23-kDa subfragments can bind calcium and may be responsible for the calcium-induced fragmentation.

α-Actinin is a major Z-line protein that comprises about 2% of the myofibrillar mass and is visualized as a long rod-shaped molecule in the electron microscope, 3 to 4 nm wide by 30 to 40 nm in length (Podlubnaya et al., 1975; Suzuki et al., 1976). It is an anti-parallel homodimer with a subunit molecular mass of 94 to 103 kDa. The α-actinin molecule from chicken skeletal muscle contains 897 amino acids (Arimura et al., 1988). Three domains, an N-terminal region which contains a highly conserved actin-binding domain, four internal 122 amino acid spectrin-like repeats which may be responsible for the formation of the anti-parallel dimer and a C-terminal region which contains two EF-hand calcium binding motifs exist in the molecule. The four internal repeats of α-actinin also have extensive homology with dystrophin (Davidson and Critchley, 1988). For a review on α-actinin structure and functions see Blanchard et al. (1989). Goll et al. (1991) reported that α-actinin could bind and cross-link actin filaments in vitro but hypothesized that native α-actinin could not, by itself, directly cross-link actin filaments in the Z line. It is possible that α-actinin may act as a stabilizer of thin filaments and/or an organizer to help anchor thin filaments from apposing sarcomeres, determine directionality of thin filaments and enhance contractile-promoting activity of actin. Other possible roles for α-actinin may be to anchor nebulin filaments, titin filaments or other cytoskeletal components. (Robson et al., 1981; Vigoreaux, 1994)
Desmin comprises less than 1% of the myofibrillar mass, is located around the periphery of the Z line and connects Z lines of adjacent myofibrils (Robson et al., 1981). Desmin filaments appear as long, flexible strands in vitro with a diameter of 10 nm (Huiatt et al., 1980; O'Shea et al., 1981). Desmin is a type III sequence intermediate filament protein and has a molecular mass of ~53 kDa. The desmin molecule contains structural principles common to cytoplasmic intermediate filaments of vertebrates and contains an N-terminal region, a central rod domain and a C-terminal region. The central α-helical rod domain is remarkably conserved in its size, secondary structure and its sequence. The rod domain consists of approximately 310 amino acids, with about 280 residues in an α-helical conformation. It has four α-helical segments that contain a quasi-heptad sequence of the form (a-b-c-d-e-f-g)^n where more than 75% of the a and d positions are occupied by apolar residues and positions b, c, e, f and g are usually polar or charged residues. This heptad sequence favors the formation of a coiled-coil. The four α-helical segments (1A, 1B, 2A and 2B) are separated by three non-helical linkers: L1 connects 1A and 1B, L1-2 connects 1B and 2A and L2 connects 2A and 2B (Steinert and Roop, 1988). The rod domain is flanked at both ends by shorter non-helical regions. The N terminal domain contains non-acidic residues, and ~15% of the residues are arginine (Geisler and Weber, 1982). This domain is the site for proteolysis (Kaufmann et al., 1985) and enzymatic phosphorylation (Geisler and Weber, 1988). It is also known that the N-terminus but not the C-terminus is required for assembly of desmin into filaments (Kaufmann et al., 1985).
Muscle contraction  H. E. Huxley and Hanson (1954) and A. F. Huxley and Niedergerke (1954) independently suggested that muscle contraction is a result of the sliding of filaments past each other which caused a greater overlap of thick and thin filaments without changing the length of either type of filament. During muscle contraction, the H zone narrows and eventually disappears as thin filaments slide into this area, and the I band also narrows because the ends of thick filaments move closer to the Z lines. The stimulus for contraction of skeletal muscle is an electrical impulse transmitted via a nerve to the motor end plate to the cell membrane and on to the T tubules. The stimulus from the T tubules causes the release of Ca$^{2+}$ from the lateral cisternae of the sarcoplasmic reticulum. The continuity of each T tubule with the cell membrane is the mechanism to stimulate an entire muscle simultaneously. This elevates the free intracellular Ca$^{2+}$ from $10^{-8}$ M to $10^{-6}$ or $10^{-5}$ M. The increased level of free Ca$^{2+}$ triggers molecular events in thin filaments which results in muscle contraction (Goll et al., 1984; Judge et al., 1989a).

A widely quoted mechanism of calcium regulation of muscle contraction is called the steric blocking model. At low calcium concentrations ($10^{-8}$ M or less), no Ca$^{2+}$ is bound to TN-C, the affinity of TN-C for TN-I and TN-T is low, TN-I is bound tightly to actin but loosely to TN-T, and TN-T is bound tightly to tropomyosin. Tropomyosin is located out of the groove of the double-stranded actin helix, in a position where it might partly block the site on actin that binds myosin. As the Ca$^{2+}$ concentration is increased from approximately $10^{-7}$ to $10^{-5}$ M, Ca$^{2+}$ binds to TN-C
and induces changes in the troponin complex. The affinity of TN-C for TN-I and TN-T is high, TN-I is bound tightly to TN-T but is dissociated from actin, and TN-T still binds to tropomyosin. Tropomyosin moves back into the grooves of the double-stranded actin helix, and the myosin-binding site on actin is exposed. Myosin can then bind to actin, and contraction develops and continues. At the end of the stimulus for contraction, \( \text{Ca}^{2+} \) is pumped back into the sarcoplasmic reticulum. The energy for pumping is produced by the hydrolysis of ATP. With the progressive lowering of the \( \text{Ca}^{2+} \) concentration to \( 10^{-8} \) M, \( \text{Ca}^{2+} \) is removed from TN-C. Troponin-I then binds to actin and forces the tropomyosin back out of the grooves of the actin filament (Goll et al., 1984; Payne and Rudnick, 1989; Craig, 1994).

**Postmortem skeletal muscle**

The most prominent early postmortem (PM) changes in the physical characteristics of skeletal muscle are stiffening and loss of extensibility of the muscle that is also called the onset of rigor mortis. These changes are generally accompanied by the loss of ATP and a pH decline.

**Biochemical changes** After exsanguination, oxygen stores are depleted and energy metabolism is switched from the aerobic pathway to the anaerobic pathway. Glycogen continues to break down to pyruvate, and pyruvate is converted to lactic acid. Since the circulatory system is non-functional, lactic acid continues to accumulate in the muscle until all the muscle glycogen is exhausted.
The accumulation of lactic acid causes a lowering of pH in PM muscle. A normal pH decline in muscle is from pH 7.4 in a living animal to an ultimate pH of about 5.5-5.6 after about 24 hr PM (Judge et al., 1989). Adenosine triphosphate (ATP) is produced as glycogen is degraded in muscle. After the depletion of glycogen, creatine phosphate (CP) and creatine phosphokinase are used for the rephosphorylation of adenosine diphosphate (ADP) to ATP. When all of the CP stores are depleted, the level of ATP becomes insufficient to prevent crossbridging between the thick and thin filaments, and the muscle begins to lose its extensibility; the rapid phase of rigor onset has replaced the delay phase (Marsh, 1981; Judge et al., 1989b; Pearson and Young, 1989b).

Physical changes accompanying the onset of rigor mortis include loss of elasticity, loss of extensibility, shortening and tension development. Measurements of extensibility are frequently used to define the development of rigor mortis. Bendall (1973) stated that the time course of rigor changes consists of three phases: (1) a delay phase during which the extensibility of the muscle remains constant and high, lasts about 11 hr in rabbit muscle held at 17°C and obtained from well fed animals. (2) the rapid or onset phase occurs when CP is depleted, actomyosin begins to form, and extensibility of the muscle decreases rapidly. This phase lasts until the completion of rigor mortis. (3) in the postrigor phase, the muscle is relatively inextensible until endogenous and/or exogenous proteolytic enzymes attack the muscle. The loss of water-holding capacity accompanied by a shrinkage of muscle also occurs during the rigor process. The amount of the loss
of water holding capacity often depends on the rate and extent of the pH drop. When muscle pH is high, the water binding capacity of PM muscle is similar to that of living muscle; however, when muscle pH declines rapidly during PM storage, water binding capacity of PM muscle decreases and is lower than that of living animals (Judge et al., 1989b).

In PM muscle, several structural proteins such as titin, nebulin, desmin and TN-T are degraded. The degradation of these proteins has been implicated in the loss of myofibrillar integrity, thus increasing meat tenderization. Early studies of titin degradation suggested that, as PM time increased, the amount of titin was greatly reduced in PM muscle (Maruyama et al., 1977; Takahashi and Saito, 1979). Bandman and Zdanis (1988) also found that 2 to 3 weeks after slaughter, no undegraded titin could be detected in bovine semitendinosus muscle. Taylor et al. (1995) showed that degradation of both titin and nebulin had occurred during the first 24 hr PM in bovine rectus abdominis muscle. Locker and Wild (1984), however, reported that little titin was degraded in bovine sternomandibularis muscle during prolonged storage at 15°C. Fritz and Greaser (1991) also showed that some intact titin was still present in bovine psoas major muscle stored at 4°C for 14 days. Lusby et al. (1983) indicated that titin was degraded during the first seven days PM in bovine longissimus muscle, but the breakdown rate of titin was greater at 37°C than at 2° or 25°C. It has been reported that the degradation of titin may relate to meat tenderness. Both Paterson and Parrish (1987) and Huff-Lonergan et al. (1995) observed that titin degraded faster in tender than in tough
bovine muscle. Fritz et al. (1993), however, reported that titin content did not distinguish tough from tender beef and that titin primarily was a single protein band in SDS gels prepared from whole muscle samples 48 hr PM and was a doublet at 16 days PM. Ringkob et al. (1988) and Fritz and Greaser (1991) used immunofluorescence microscopy to show that 9D10 mAb to titin stained 2 bands per sarcomere in bovine psoas myofibrils prepared from 3 hr PM muscle and 4 bands per sarcomere in 48 hr PM myofibrils. Because the conversion from T1 to T2 was unnecessary for the change from two to four bands, Fritz and Greaser (1991) suggested that titin structure may be altered within 48 hr PM or proteolysis or alteration of a protein to which titin is bound may have occurred. During PM storage, nebulin was degraded faster than titin in bovine skeletal muscle (Lusby et al., 1983; Fritz and Greaser, 1991). Paterson and Parrish (1987) found that, in bovine muscle, very little nebulin was detected at 7 days PM.

Another protein change accompanying the PM tenderization of meat is the degradation of TN-T (Penny and Dransfield, 1979) and the simultaneous appearance on SDS gels of a 30 kDa component (Olson and Parrish, 1977). Dąbrowska et al. (1973) first reported that the 30 kDa polypeptide was present in isolated troponin preparations, and the amount could be increased if the preparation was treated with trypsin. Olson et al. (1977) also indicated that incubation of purified troponin with calpain caused degradation of TN-T to a 30 kDa polypeptide and implied that the 30 kDa polypeptide originates from TN-T. Cheng and Parrish (1977) reported that calcium treatment of bovine muscle showed more
rapid degradation of TN-T and the earlier appearance of a 30 kDa polypeptide during PM storage. MacBride and Parrish (1977) observed that a 30 kDa polypeptide was present in tender beef but was not present in tough beef at 1 day PM, and suggested that the appearance of a 30 kDa polypeptide can be used as an index of meat tenderness. Buts et al. (1989) and Uytterhaegen et al. (1992) found that by using SDS-PAGE and a semi-quantitative analysis, the disappearance of TN-T and the appearance of a 30 kDa band were related by negative correlation coefficients of -0.814 and -0.71, respectively. Wheeler and Koohmaraie (1994) observed that TN-T degradation could be detected at 24 hr PM and was extensive at 72 hr PM in ovine longissimus muscle. Whipple et al. (1990) reported that in skeletal muscle from *Bos taurus* crossbred cattle, a 30 kDa polypeptide was clearly evident by 14 days PM; however, in muscle from *Bos indicus* crossbred cattle, a 30 kDa polypeptide still could not be seen at 14 days PM. The 30 kDa polypeptide was unambiguously identified with an immunoblot approach as a degradation product of TN-T by Ho et al. (1994).

In addition to the appearance of the 30 kDa polypeptide during TN-T degradation, several other TN-T degradation products have also been reported. Pommier et al. (1987) demonstrated that electrical stimulation altered the rate of degradation of TN-T and that aging of veal longissimus muscles enhanced the appearance of two new bands in the 25 to 35 kDa region. Mikami et al. (1990) found that ES enhanced the degradation of TN-T and the appearance of 30 kDa and 33 kDa polypeptides during aging.
Tropomyosin is also degraded during PM storage. Johnson and Bowers (1976) reported that tropomyosin was absent in 3 hr PM turkey breast muscle. Arakawa et al. (1970a, b), however, found that yields of both α-actinin and a tropomyosin-troponin fraction from rabbit muscle were not changed by PM storage and that these two protein fractions prepared from muscle stored 8 days at 25°C or at various temperature-pH combinations were functionally active and exhibited normal sedimentation patterns.

Z-line degradation is one of the molecular and morphological changes that have been observed in PM muscle. Desmin and α-actinin are the major proteins that have been associated with or are localized in the Z line. Stromer et al. (1974) and Olson et al. (1977) found the amount of α-actinin present in muscle PM did not change during aging. Yates et al. (1983), however, observed that a loss of α-actinin occurred in 1 day PM bovine muscle at 4°C. Bechtel and Parrish (1983) reported that, in bovine longissimus muscle, the amount of α-actinin diminished but was still present after 14 days PM storage at 37°C. Hwan and Bandman (1989) found that α-actinin was degraded slowly at 4°C, and that degradation products of α-actinin could not be detected in blots until 2 week PM. Degradation of α-actinin was more rapid if muscle samples were stored at 25°C or 37°C. In contrast, desmin was easily degraded at 4°C, and little undegraded desmin remained in 3 week PM bovine muscle. Koohmaraie et al. (1991) found that at 1 day PM, desmin was degraded most extensively in pork muscle and less extensively in lamb and beef muscle. Wheeler and Koomaraie (1994) reported that, in ovine longissimus
muscle, the desmin degradation rate was similar to the rate for TN-T, was
detectable at 24 hr PM and was extensive at 72 hr PM. Desmin was degraded
much slower in muscle from *Bos indicus* crossbred cattle than in *Bos taurus*
crossbred cattle and could still be seen at 14 days PM in muscle from *Bos indicus*
crossbred cattle (Whipple et al., 1990).

Several studies have reported that myosin (Arakawa et al., 1976; Bechtel and
Parrish, 1983; Bandman and Zdanis, 1988) and actin (Bechtel and Parrish, 1983)
were not degraded during aging at 4°C but were degraded in PM muscle stored at
37°C (Arakawa et al., 1976; Bandman and Zdanis, 1988). Yates et al. (1983) also
found a significant decrease in amount of myosin and a concomitant appearance of
material in the 50-95 and 28-32 kDa regions in PM bovine muscle stored for 1 day
at 37°C. Penny and Ferguson-Pryce (1979) showed that myosin was degraded by
approximately 50% at pH 4.4-4.8 at 25°C but was not degraded above pH 6.0 in
bovine longissimus muscle. Yates et al. (1983) confirmed that incubation of bovine
longissimus samples at pH 5.4 resulted in greater loss of myosin heavy chain than
incubation at pH 7.0 and concluded that pH was more important than temperature
in myosin heavy chain degradation.

**Ultrastructural changes** The major structural changes in muscle cells
during PM storage include sarcomere shortening, Z-line degradation, I-band
fractures and intracellular membrane-bounded organelle degradation. Sarcomere
shortening and increased myofibrillar spaces can be observed during early PM
storage time. Stromer et al. (1967, 1974) reported that sarcomeres shortened
during the first 6 to 8 hr after death and indicated that PM shortening in bovine muscle occurs via a sliding of filaments and is structurally identical to muscle contraction. Henderson et al. (1970) studied PM changes in bovine, porcine and rabbit muscle and also found that a variable amount of sarcomere shortening occurred during the first 24 hr PM. Davey and Gilbert (1969) and Will et al. (1980) observed a loss of adhesion between adjacent myofibrils and increased space between myofibrils in bovine muscle fibers 24-30 hr PM.

Z-line degradation also can be seen during early PM storage. Davey and Gilbert (1967, 1969) found that aged bovine myofibrils had lost Z-line structure and were likely to fracture at, or near, the Z line. Gann and Merkel (1978) showed that a slight loss of Z-line material already occurred at 1 hr PM and that additional Z-line degradation was evident at 48 hr PM in white myofibers of bovine longissimus muscle. Greaser et al. (1969) showed that the major structural change in 24 hr PM porcine myofibrils was breakage in the Z line and loss of material from the Z line and the adjacent thin filaments. In 2 days PM turkey breast muscle, Z lines already appeared diffuse and somewhat irregular (Johnson and Bowers, 1976). Henderson et al. (1970) and Stromer et al. (1974) reported that Z lines were gradually degraded in bovine, porcine and rabbit muscle, and the rate and extent of Z-line degradation were accelerated by higher storage temperature. As PM time increased, a loss of Z-line density (Davey and Dickson, 1970) and longitudinal splitting in the Z line (Young et al., 1980-81) could be observed in bovine skeletal muscle. In more recent studies, Wheeler et al. (1990) and Taylor et al. (1995),
however, claimed that no Z-line breakage or significant loss of Z-line material was observed in 14 or 16 day PM bovine muscle samples, respectively.

Another major structural change in PM muscle is I-band fractures adjacent to the Z line. Davey and Dickson (1970) and Young et al. (1980-81) described that a weakening of the myofibrillar structure at the junction of the I band with the Z line was evident in bovine skeletal muscle as PM time increased. Breaks in the I band adjacent to the Z line began to appear after 3 days PM (Taylor et al., 1955) and became increasing prevalent with long periods of PM storage (Ouali, 1990). Wheeler et al. (1990) found that the frequency of I-band fractures was lower in tough animals than in tender animals.

Degradation of membrane-containing structures such as the sarcolemma, mitochondria, nucleus and sarcoplasmic reticulum during PM storage is also well-documented. Reed et al. (1966) found that, in early postrigor porcine muscle, the sarcolemma had lost its uniform appearance and showed a system of regularly spaced bars or a sheet containing holes of various shapes and sizes and suggested that the sarcolemma was a very labile system that may change rapidly after death. Osner (1966) reported that the chicken muscle sarcolemma did not become permeable to proteins by passing through rigor unless the muscle was under tension on the carcass. Abbott et al. (1977) showed that the porcine muscle sarcolemma was quite diffuse at 1 day, later became separated from the muscle fibers and, by 8 days PM, the sarcolemma had lost all structural integrity. The sarcolemma was ruptured, and the basement membrane was lifted away from the
sarcolemma and/or broken in both 24 hr PM control and electrically stimulated bovine muscle (Will et al., 1980). In a recent study, Taylor et al. (1995) indicated that, in PM bovine skeletal muscle, the sarcolemma was broadening and was detached from myofibrils in about half the fibers at 24 hr PM. By 3 days PM, the sarcolemma was separated from the myofibrils by a space in all fibers.

In bovine muscle after 4 hr PM at 37°C, mitochondria and other membranes were also disrupted and appeared as large vacuoles (Henderson et al., 1970). Will et al. (1980) showed that swollen mitochondria which contained ruptured cristae, swelling of the sarcoplasmic reticulum, general swelling of the entire muscle cell and shrunken nuclei were already present in both control and electrically stimulated 24 hr PM bovine muscle. Greaser et al. (1969) found that mitochondria isolated from normal porcine muscle at 24 hr PM were very swollen with a very low matrix density; however, the appearance of the heavy sarcoplasmic reticulum fraction did not change from 0 to 24 hr PM. Dutson et al. (1974) also reported that, in porcine white fibers at 24 hr PM, mitochondria had less distinct cristae, had lost most of the dense background material between the cristae and some appeared as open vesicles. Triads or transverse tubules were absent, and the sarcoplasmic reticulum was no longer recognizable. Abbott et al. (1977) also noted that porcine muscle mitochondria underwent PM cristae aggregation and ultimately lost cristae entirely. Nuclei became smaller and chromatin was more concentrated by 8 days PM. The sarcolemma was diffuse and separated from the muscle fibers at 1 day and, by 8 days PM, had lost all structural integrity.
Postmortem electrical stimulation of skeletal muscle

A number of researchers have clearly demonstrated that electrical stimulation (ES) not only improved meat tenderness but also increased the desirability of lean meat color (Savell et al., 1977, 1978; McKeith et al., 1982; Eikelenboom and Smulders, 1986) and prevented cold shortening of ovine muscle (Carse, 1973) and of bovine muscle (Takahashi et al., 1984). A general review on the effects of various muscle treatments, including ES, on properties of muscle PM has been written by Smulders and van Laack (1992).

Effect of ES on biochemistry of muscle

An indicator of the rate of PM glycolysis in muscle is the rate of pH decrease in that muscle. Muscle pH begins to decline soon after slaughtering the animal and continues over a period of about 24 hr or more, depending on the species, the muscle and both the antemortem and postmortem treatments(s), until the ultimate pH is reached. ES accelerates the rate of PM glycolysis in muscle (Forrest and Briskey, 1967; McLoughlin, 1970). Takahashi et al. (1984) showed that the intramuscular pH decreased about 0.57 ± 0.12 unit during a 5-min ES period, compared with 0.05 ± 0.07 unit in the controls. Takahashi et al. (1984) also demonstrated that very rapid pH decrease is associated with less tender muscle and ascribed this phenomenon to reduced proteolytic activity at the lower intramuscular pH levels. This research group subsequently recommended that a 3 hr PM intramuscular pH of 5.9-6.3 should be targeted to achieve optimum tenderness (Smulders et al., 1990). Wu et al. (1985) reported that the 2 hr PM intramuscular pH in ES steer carcasses was 5.79
compared with 6.53 in controls and 5.93 in ES bull carcasses vs. 6.36 in controls. Pommier et al. (1987) reported that low-voltage (45V) ES of veal carcasses effectively reduced the 45 min and 4 hr PM pH by about 0.6 unit. Marsh et al. (1987) found that both cooling rate and ES, but not ES alone, determined the rate of pH decline and indicated that the effect of ES on tenderness is highly dependent on the subsequent cooling rate.

Several studies have shown that ES also promotes the activity of lysosomal and calpain enzymes and enhances the proteolytic breakdown of the myofibrillar proteins. Dutson et al. (1980) reported that ES accelerated rigor onset which causes rapid acidification to release and activate lysosomal enzymes β-glucuronidase and cathepsin C. Wu et al. (1985) showed that ES could increase the free activities of lysosomal enzymes, β-glucuronidase, cathepsin B and cathepsin H in both bull and steer carcasses. Pommier et al. (1987) also found that ES increased the amount of cathepsin D in a soluble fraction prepared from veal longissimus muscle homogenates but decreased the rate of degradation of TN-T in PM bovine muscle. Etherington et al. (1990) examined extractable chicken muscle proteases (cathepsins B, D and L, μ- and m-calpains) and glycosidase (β-D-glucuronidase and N-acetyl-β-D-glucosaminidase) in ES, glycogen-depleted and control muscle at 0 and 48 hr PM. The activities of cathepsins B, D and L did not decline between 0 and 48 hr PM in ES samples but μ-calpain activity did drop 83%. The activity of μ-calpain also decreased 58% in control muscle and 63% in glycogen-depleted muscle during the first 48 hr PM. Ducastaing et al. (1985) also
found that ES drastically reduced the activity of \( \mu \)-calpain by 80% at 4 hr PM in beef muscle. Injecting beef muscle cylinders with inhibitors of cathepsins B, D, H, and L had no affect on tenderization, but Uytterhaegen et al. (1994) found that injecting Triton X-100 and Ca\(^{2+}\) did increase tenderization and degradation of myofibrillar proteins. This led Uytterhaegen et al. (1994) to conclude that the calpains rather than cathepsins were important in beef tenderization PM. A closely related opinion that lysosomal enzymes are not uniquely involved in ES-induced tenderization has long been held by Marsh (1981, 1983) and was more recently emphasized by Marsh et al. (1987). For a recent review on the possible roles of muscle proteinases in meat tenderness, see Valin and Ouali (1992).

Dransfield et al. (1992a) confirmed that levels of cathepsins B and L and \( \beta \)-glucuronidases were unchanged in PM bovine muscle and found that 73% of \( \mu \)-calpain activity was lost in the first 24 hr if intramuscular pH was 6.2 and the ES muscle was held at 15°C. The most tender ES bovine muscle at 24 hr PM was produced when muscle was stored at 15°C until the pH was 6.0 and then increasing the temperature to 30°C (Dransfield et al., 1992b). A theoretical model for the control of \( \mu \)-calpain by PM pH and temperature has been proposed by Dransfield (1992) and claims to predict 68% of the variation in toughness in ES and NS bovine pectoralis profundus muscles.

Babiker (1985) reported that, in ES muscle, degradation of myosin increased during the first 24 hr as storage temperature increased to 40°C and that a 30 kDa band increased in amount until 30°C and decreased at 40°C. Kang et al. (1983)
found that, in ES rabbit muscle, more intense bands at 95, 30 and 27 kDa were present sooner PM than in control muscle. Uytterhaegen et al. (1992) reported that ES accelerated TN-T degradation in PM bovine muscle but that degradation of titin, nebulin and filamin was not affected by ES. Mikami et al. (1990) also showed that the degradation of TN-T and the appearance of the 30 kDa polypeptide were seen sooner in ES samples than in control samples. Geesink et al. (1992), however, found that ES had no affect on the intensity of the 30 kDa polypeptide in either control or clenbuterol-treated bovine muscle during PM storage.

Effect of ES on ultrastructure of muscle

Cassens et al. (1963a) found that contraction nodes (CN) in porcine muscle were associated with extremely rapid PM glycolysis and may also be caused by physical disturbance such as pricking or cutting muscle fibers at the time of death (Cassens et al., 1963b). Rapid PM glycolysis as indicated by an accelerated pH drop may occur during or in association with ES and thus cause the formation of CN. Savell et al. (1978) reported that CN, stretched areas on either side of the CN and physical disruption of myofibrils were present in 20-24 hr PM ES bovine muscle samples and speculated that the disruption could improve meat tenderness. ES samples had less well defined I bands and Z lines in the CN, but stretched or broken sarcomeres existed on either side of the CN. Takahashi et al. (1984) reported that normal-frequency high-voltage (60 Hz, 500V) ES produced extensive fracturing that was visible with the light microscope with breaks appearing approximately every 6 mm of fiber length. An electron microscope study of structural changes in bovine
longissimus dorsi muscle showed that 60 Hz, 500V ES caused widespread formation of CN and many stretched or fractured sarcomeres in the internodal zones, but stimulation with 2 Hz, 500V caused no structural changes in the myofibril (Takahashi et al., 1987). George et al. (1980) observed CN with both the light and electron microscopes and that CN began to form 1.5 to 24 hr after stimulation (depending on the muscle) of bovine muscle. Unfortunately, George et al. (1980) incorrectly identified the CN as due to a deposition of denatured sarcoplasmic proteins. Sorinmade et al. (1982) also found that in stimulated bovine longissimus muscle, CN, superstretched myofibrils and torn or fragmented filaments around the Z line were also detected at 2 days PM but not in control muscle. In contrast to these studies, Fabiansson and Libelius (1985) found that CN occurred in both control and ES muscle samples and stated that CN were a common artifact in improperly restrained tissue samples that were fixed in cold fixatives; therefore they were not a specific effect of ES. Several studies have reported that there were no significant differences in sarcomere lengths between control and ES bovine muscle samples (Savell et al., 1978; Takahashi et al., 1984; Uytterhaegen et al., 1992). George et al. (1980), however, found that stimulated muscles had longer sarcomeres than the controls. In each of these studies, the selection of sarcomeres to be measured and/or the measurement method was incompletely described thus making it difficult to compare results.
IDENTIFICATION OF THE 30 KDA POLYPEPTIDE IN POST MORTEM SKELETAL MUSCLE AS A DEGRADATION PRODUCT OF TROPOinin-T

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Summary - Although a 30 kDa polypeptide frequently is seen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of post mortem (pm) skeletal muscle and in turn is used as an indicator of proteolysis, its origin has not been conclusively identified. We used antibodies to selected myofibrillar proteins, including some known to be degraded pm, to identify this polypeptide. The left side of eight beef carcasses was electrically stimulated (ES) within an hour after slaughter, and the right side served as the nonstimulated (NS) control.

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Abbreviations: 30 kDa, peptide with molecular mass of 30 000 Da; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ES, electrically stimulated; NS, nonstimulated; LL, longissimus lumborum; pm, post mortem; TN-T, troponin-T; mAb, monoclonal antibody; mA, milliampere; PVDF, polyvinylidene fluoride; BSA-PBS, bovine serum albumin-phosphate buffer solution; TN-I, troponin-I.
The longissimus lumbarum (LL) muscle was removed from the carcass at 24 h pm and was stored at 2°C. Myofibrils were prepared from the LL muscle immediately after stimulation (0 day) and from the stored muscle sample at 1, 3, 7, 14 and 28 days pm for analysis of SDS-PAGE and Western blots. By SDS-PAGE, troponin-T (TN-T) decreased in amount more rapidly pm in ES samples than in NS samples. By SDS-PAGE, a 30 kDa band increased and became a prominent band by 7 days pm in both NS and ES samples. A monoclonal antibody (mAb) to TN-T labeled purified TN-T, as well as the TN-T in myofibrils, a prominent 30 kDa polypeptide and a family of lower molecular weight polypeptides in pm muscle. This mAb also labeled a 30 kDa band that had been electrophoretically purified from pm muscle. The 30 kDa band in blots of myofibrils was prominent from day 3 through 28 in both ES and NS samples, but was exaggerated in the ES samples. Antibodies to other myofibrillar proteins (titin, nebulin, α-actinin and desmin) did not label the 30 kDa band. We conclude, on the basis of labeling with a mAb to TN-T, that the prominent 30 kDa polypeptide often observed in pm bovine skeletal muscle is a degradation product of TN-T and, furthermore, that an entire family of lower molecular weight polypeptides (one of which is the 30 kDa polypeptide) originates from TN-T.

**Key Words:** troponin-T/ 30 kDa polypeptide/ bovine muscle/ proteolysis
Introduction

Immediately upon death, a number of distinct changes in the metabolism of muscle cells are set in motion [1, 2]. These changes include evidence of proteolysis in the myofibrillar and cytoskeletal apparatus [1-4]. The post mortem (pm) proteolysis is thought to result primarily from continued action of the calpain system [2, 5]. Many papers have described the appearance of a 30 kDa polypeptide in pm muscle. Some of these reports have related the presence and amount of this 30 kDa polypeptide to an indication of the rate and extent of pm proteolysis. A 30 kDa polypeptide was detected in several bovine muscles pm at about the same time that troponin-T (TN-T) was decreasing in the same muscles [6]. Soaking intact cubes of bovine muscle in a calcium solution accelerated both the TN-T degradation and the appearance of a 30 kDa polypeptide when compared with non-calcium-treated controls or with oxalate-treated samples [7]. This suggested that calpain may be involved in this reaction. Greater amounts of a 30 kDa polypeptide were found in pm samples from young bovine animals than in samples from older animals [8]. The way in which muscle is treated pm can alter significantly the extent of protein degradation. For instance, electrical stimulation of bovine carcasses accelerates both the degradation of TN-T and the appearance of a 30 kDa polypeptide [3, 9].

Although several papers suggest a temporal relationship between degradation of TN-T and appearance of a 30 kDa polypeptide, none of them actually clearly identified the origin of the 30 kDa polypeptide. A recent review [10] emphasized
that the origin of the 30 kDa polypeptide has not been unambiguously determined.

In pm muscle, actin and myosin are not degraded [2]. Several key myofibrillar proteins, however, are degraded simultaneously but at differing rates [11]. To better understand the pm changes in myofibrillar proteins and how these changes relate to properties of the original muscle, it is important to know precisely which protein is the source of this prominent polypeptide. The objective of this study was to positively identify the source of the 30 kDa polypeptide.

Materials and Methods

Sample preparation

The left sides of 8 market weight crossbred steers (Angus x Jersey) were each electrically stimulated (ES) with 200 V, 20 Hz for 15 sec for 3 stimulation periods with a 30-sec interval between stimuli. The objective of this stimulation was to achieve a 3-h pm muscle pH of 5.9 [12]. The right side of each carcass was a nonstimulated (NS) control. A 50-gm sample (0 day) was removed from the lateral portion of the longissimus lumborum (LL) muscle opposite the 13th rib immediately after stimulation. For the 3-h pm muscle pH determination, a 1-gm sample was removed from the same portion of the LL muscle and was homogenized in 10 ml of 5 mM sodium iodoacetate in 150 mM KCl (pH 7.0). A 5-cm thick cross section of the LL muscle posterior from the 13th rib was removed from the carcass at 1 day pm, was stored at 2°C and was used for subsequent samples.
Myofibrils were prepared from the LL muscle immediately after stimulation (0 day) and at 1, 3, 7, 14 and 28 days _pm_ by the method of Fritz et al [13]. The LL muscle samples were homogenized and washed in a wash buffer [50 mM KCl, 2 mM EDTA, 4 mM MgCl₂, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 20 mM Tris-HCl, pH 7.0] 8 times and were collected by centrifugation at 3000 xg for 5 min after each wash. A rigor buffer [75 mM KCl, 2 mM EGTA, 2 mM MgCl₂, 10 mM KH₂PO₄, pH 7.0] was then used for an additional 3 washes. Myofibrils were resuspended in equal part of the rigor buffer and glycerol and stored at -20°C.

*Electrophoretic purification of the 30 kDa polypeptide*

An 18% Tris-glycine slab gel (acrylamide : N, N'-methylenebisacrylamide= 200:1) with a 5% stacking gel (acrylamide : N, N'-methylenebisacrylamide= 37:1) [13, 14] was run at 15 mA for 11 h to electrophoretically separate the 30 kDa band present in a 14-day myofibril sample. After electrophoresis, the gel was washed with 4 changes of deionized water and stained with 0.05% Coomassie blue prepared in deionized water. After staining for 40 min at room temperature, the gel was washed with several changes of water for an hour total. When the bands were visible, the 30 kDa band was excised with a scalpel. The excised band was rinsed in deionized water for a few minutes with several changes of water; it then was diced with a scalpel and further fragmented by passing the diced band through a 5-ml syringe without a needle. The gel fragments were then resuspended in glycerol and were re-electrophoresed in an 18% Tris-glycine slab gel.
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Myofibrillar proteins from NS and ES muscle samples were routinely analyzed in a 15% Tris-glycine slab gel with a 5% stacking gel (acrylamide : N, N'-methylenebisacrylamide= 37:1) [13, 14]. The gel was run at 10 mA for 17 h.

Western blot analysis

Proteins were transferred from the slab gel to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA) by a semidry transfer method [15]. After transfer, the membrane was incubated in a 5% bovine serum albumin-phosphate buffer solution (BSA-PBS) for 30 min at 37°C and then washed 3 times in a 0.1% BSA-PBS solution for 5 min at room temperature. Monoclonal antibodies (mAbs) to TN-T (JLT-12) (Sigma Chemical Company, St. Louis, MO), nebulin (NB2) (Sigma Chemical Company, St. Louis, MO) and titin (9D10) and polyclonal antibodies to desmin and α-actinin were used as primary antibodies. The membrane was incubated with one of the primary antibodies for 2 h at room temperature, washed 3 times in 0.1% BSA-PBS for 5 min, incubated with immunogold-labeled secondary antibody for 2 h at room temperature, washed twice in 0.1% BSA-PBS for 5 min each and twice in deionized water for 1 min each. The gold label was enhanced by silver staining [16].

Preparation of selected myofibrillar proteins used as markers/standards

A sample of myosin was prepared from bovine LL muscle by the procedure of Hammond and Goll [17]. Troponin was prepared from bovine LL muscle, purified by the methods of Arakawa et al [18-20] and Eisenberg and Kielley [21], and then
separated into its three subunits T (37 kDa), I (24 kDa) and C (20 kDa) by the procedure of Margossian and Cohen [22]. Tropomyosin was prepared by the procedure of Arakawa et al [18-20] and Eisenberg and Kielley [21]. A partially purified sample of desmin was prepared by the method of O'Shea et al [23].

Results

The average 3-hr pH for NS muscle was 6.25 and for ES muscle was 5.91, which is virtually identical to the optimum ES muscle pH recommended for maximizing pm proteolysis by Smulders et al [12]. Characterization of the mAb to TN-T (fig 1a, b) showed that this antibody clearly and strongly recognizes the TN-T band in the purified TN-T sample (lane T) and the TN-T band in 0-day myofibrils (lane Mf). The TN-T mAb also reacts with a small amount of 30 kDa band and some other lower molecular weight polypeptides in both the purified TN-T sample (b, lane T) and the 0-day myofibril sample (b, lane Mf). SDS-PAGE and Western blot identification of the 30 kDa polypeptide (fig 1c, d) revealed that mAb to TN-T reacts strongly with TN-T in the 0-day sample (d, lane 1), with the 30 kDa band in the 14-day sample (d, lane 2) and with the 30 kDa band (d, lane 3) electrophoretically purified from a 14-day pm myofibril sample.

SDS-PAGE analysis of bovine myofibrils from NS muscle (fig 2a) showed four major changes during pm storage in proteins migrating below myosin heavy chains. First, the TN-T band decreases in amount, noticeably so by day 7, and is barely detectable by day 14. Second, a 30 kDa polypeptide increases in amount and is
quite prominent by day 7. Third, the troponin-I (TN-I) band decreases noticeably in amount by day 7 and evidently migrates as a slightly lower molecular weight band in both the 14- and 28-day samples. Fourth, the very small amount of desmin, (identified as desmin by using a polyclonal antibody to desmin; results not shown) present in skeletal muscle myofibrils [23, 24] decreases in amount as pm time increases. By day 7, the desmin band can hardly be seen.

SDS-PAGE analysis of myofibrils from ES muscle (fig 2c) presented the same four changes in proteins migrating faster than myosin heavy chains, but the timing differed for some of these changes. TN-T degrades more rapidly in ES samples (noticeably so by day 3), is a minor band by day 7 and can not be detected by day 14. The appearance of the 30 kDa band and the degradation of TN-I and desmin during pm storage had a pattern similar to that seen in NS samples. In Western blots (fig 2b, d), labeling with the TN-T antibody showed that density of the TN-T band decreases at similar rates in both NS and ES samples, but that the TN-T band decreases slightly faster in ES than in NS samples. In NS samples, the 30 kDa polypeptide increases in amount through day 3 and then remains about constant in amount in the following samples. In ES samples, the 30 kDa polypeptide shows a progressive increase in amount through 28 days. A 28 kDa band migrating just faster than the 30 kDa protein was quite prominent in the blots of both NS and ES samples (more so in ES), and this band may be that described as the 27 kDa band in pm bovine skeletal muscle by Ouali [1]. A 15 kDa
Figure 1. Characterization of the mAb to TN-T and identification of the 30 kDa polypeptide with mAb to TN-T.  

a. SDS-PAGE (15% gel) of purified TN-T (lane T) and bovine LL myofibrils prepared on 0 day (lane Mf) and stained with Coomassie blue.  
b. Western blot from a duplicate gel reacted with mAb to TN-T; the mAb reacts strongly with the TN-T band in both samples.  
c. SDS-PAGE (18% gel) of bovine LL myofibrils prepared on 0 day (lane 1) and 14 days pm (lane 2) and the 30 kDa band electrophoretically purified from a 14-day myofibril sample (lane 3) and stained with Coomassie blue.  
d. Western blot from a duplicate gel reacted with the mAb to TN-T; labeling with this antibody demonstrates clearly that the prominent 30 kDa polypeptide is a degradation product of TN-T.
Figure 2. Bovine myofibrils prepared at different times $pm$ from NS and ES muscle in SDS-PAGE (15% gel) and Western blots. Lanes 0-28= myofibrils prepared on 0, 1, 3, 7, 14 and 28 days $pm$. Standards were: partially purified myosin (lane M); purified TN-T (lane T); desmin-enriched fraction prepared from bovine muscle (lane D); purified bovine tropomyosin (lane TM); purified bovine troponin-I (lane I); and purified bovine troponin-C (lane C).  

a. By SDS-PAGE analysis of myofibrils from NS muscle, TN-T decreases by day 7 and is present as a very light band at day 14. The 30 kDa polypeptide is first seen in the 3-day sample and is a prominent band thereafter.  
b. Western blot analysis of bovine myofibrils from NS muscle showed strong reaction with mAb to TN-T. Labeling of the intact TN-T band (37 kDa) decreases and labeling of a family of lower molecular weight polypeptides (including a prominent 30 kDa band) increases as time $pm$ increases.  
c. By SDS-PAGE analysis of myofibrils from ES muscle, TN-T decreases noticeably by day 3 and is absent by day 14; the pattern of appearance of the 30 kDa polypeptide is similar to that seen in NS samples.  
d. Western blot analysis of myofibrils from ES muscle showed strong reaction with the mAb to TN-T; ES enhances the accumulation of the 30 kDa polypeptide through 28 days $pm$. The family of TN-T degradation products appeared qualitatively similar to that shown for the NS samples.  

MHC, myosin heavy chain; TN-T, troponin-T; TM, tropomyosin; TN-I, troponin-I; TN-C, troponin-C; MLC-1, 2 and 3, myosin light chains 1, 2 and 3; single arrowhead, 31 kDa band; double arrowhead, 28 kDa band.
polypeptide appears in the 7-day ES sample and becomes prominent in both the
NS and ES 14- and 28-day samples. A striking result of this study was that the TN-
T antibody labeled a relatively large and growing family of lower molecular weight
TN-T degradation products (~35 to 10 kDa) in the pm aged samples. Antibodies to
other myofibrillar proteins (titin, nebulin, α-actinin and desmin) did not label the 30
kDa band (results not shown) or any of the other bands migrating below TN-T that
were labeled by the TN-T antibody. A band (~31 kDa) (fig 2a, c) migrating just
slightly slower than the 30 kDa protein was present in virtually all myofibril samples
analyzed (0 to 28 days), but its identity remains unknown. It was not labeled by the
TN-T mAb. The Western blots were, not unexpectedly, much more sensitive
indicators of changes in TN-T and appearance of the TN-T products than was
analysis by SDS-PAGE. Some of the multiplicity of bands, both in the TN-T region
and in the region of TN-T breakdown products, labeled by the TN-T mAb may
reflect the presence of TN-T isoforms. Many TN-T isoforms are known to be
possible [25], and at least two have been observed in bovine skeletal muscle [26].

Discussion

Troponin-T is generally considered the most proteolytically sensitive subunit
of the troponin molecule [27, 28], which in turn probably results from its rather open
and accessible protein structure [29]. The appearance and accumulation of the 30
kDa polypeptide have been associated by some investigators with the decreased
levels of shear force required to cause muscle disintegration in pm muscle [8].
Incubation of bovine myofibrils with calpain [6] or adding calcium to bovine muscle [7] both resulted in degradation of TN-T and appearance of a 30 kDa polypeptide. The relatively small amounts of a 30 kDa polypeptide present in isolated troponin preparations also could be increased if the isolated preparation was digested with trypsin [30]. The disappearance of TN-T and the appearance of the 30 kDa band have been related by a significant negative correlation [3, 31]. As pointed out in these papers [3, 31], however, a highly significant negative correlation by itself does not establish a direct cause and effect relationship between TN-T and the 30 kDa polypeptide. Similarly, the ostensible temporal relationship between TN-T and the 30 kDa polypeptide [6, 8] does not identify the parent protein of the 30 kDa polypeptide.

We have used antibodies to titin, nebulin, α-actinin, desmin and most importantly TN-T to identify the 30 kDa band. We chose antibodies to titin, nebulin, desmin and TN-T because all of these proteins are degraded and diminish in amount in muscle pm [11, 32]. We also included antibodies to α-actinin, which supposedly is not degraded by calpain [33], because α-actinin is a major component of skeletal muscle Z lines, the myofibrillar structures that are the first locus of pm structural alterations [34]. The mAb to TN-T is the only antibody from this group that recognized the 30 kDa band. In Western blots, the mAb to TN-T showed a strong recognition of the TN-T band in both purified TN-T and 0-day myofibril samples (fig 1b) of the 30 kDa polypeptide and of other lower molecular weight TN-T degradation products in pm bovine myofibrils (fig 2b, d). To further
identify the origin of the 30 kDa band, we re-electrophoresed the 30 kDa polypeptide that was electrophoretically purified from the 14-day NS sample (fig 1c, d). The excised band migrated as a single band and was labeled strongly with the mAb to TN-T. This provides additional direct evidence that the 30 kDa polypeptide that accumulates in pm bovine skeletal muscle comes from TN-T.

We used electrical stimulation in our study because it has been reported that it accelerates changes in TN-T, and it also served as a marker of sensitivity of our procedure. In SDS-PAGE (fig 2a, c), TN-T decreased in amount faster in ES than in NS samples, in agreement with the results of Mikami et al [9] and Uytterhaegen et al [3]. In contrast to the results of Mikami et al [9] and Uytterhaegen et al [3], the 30 kDa polypeptide did not appear earlier in our ES samples, a result that is in agreement with data reported by Geesink et al [35]. Different electrical stimulation or myofibril preparation protocols or different gel systems may have caused variation in the appearance and/or detection of the 30 kDa band in ES samples.

The much greater sensitivity of the Western blots (fig 2b, d) confirms that the density of the TN-T band decreased slightly faster in ES than in NS samples and that it still was present as a very light band at day 28 in both NS and ES samples. Concurrently, a relatively large family of lower molecular weight TN-T degradation products was labeled by the mAb to TN-T [1]. Some of these degradation products of TN-T may co-migrate with other proteins and thus may not be as easily detected by SDS-PAGE. In the ES samples shown in fig 2d, the amount of the 30 kDa band increased progressively through 28 days pm. The increase in amount of the 30
kDa polypeptide in ES samples observed from 1 h through 12 days pm [3] supports the pattern of increase seen in fig 2d.

We have shown that degradation of TN-T and appearance of the 30 kDa protein and related TN-T degradation products are early events in pm skeletal muscle. Most investigators have simply used appearance of the 30 kDa protein as a measure or indicator of proteolysis, rather than as one of the underlying causes of the well-known structural alteration that occurs in or near the myofibrillar Z-line region pm. We believe their assumption should not be accepted without question. Indeed, TN-T is both an important regulatory and structural component of skeletal muscle thin filaments [27, 36], and thin filaments in turn are anchored to the Z line, a site of early pm changes [11, 34]. Degradation of those TN-T subunits located immediately adjacent to the Z line may be involved in fragmentation of pm muscle. It has already been shown that alteration in TN-T causes disruptions of Z lines [37], and it is also well known [25] that changes in expression of isoforms of TN-T and α-actinin, the protein comprising the Z-lattice filament [38], are coordinated.

Conclusion

On the basis of these results, we conclude that TN-T in pm muscle is degraded into a family of smaller polypeptides and that one of these is a prominent polypeptide with a $M_r=30\,000$. Labeling the 30 kDa band electrophoretically purified from pm bovine skeletal muscle myofibrils with a mAb to TN-T positively identifies the 30 kDa polypeptide as a degradation product of TN-T. SDS-PAGE
analysis shows that ES slightly accelerates the degradation of TN-T. Western blots show that the amount of the 30 kDa polypeptide remains relatively constant in NS samples from day 3 through day 28, but increases progressively from 1 to 28 days in ES samples. Degradation of TN-T and appearance of the prominent 30 kDa, a TN-T degradation product, are excellent indicators of pm proteolysis. Because TN-T is an important structural and regulatory component of skeletal muscle thin filaments [36] and because thin filaments, in turn, are anchored to the Z line, a site of early pm structural change [34], degradation of those subunits located closest to the Z line may be involved in pm fragmentation of skeletal muscle.

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EFFECT OF ELECTRICAL STIMULATION ON POSTMORTEM TITIN, NEBULIN AND DESMIN DEGRADATION AND ULTRASTRUCTURAL CHANGES IN BOVINE LONGISSIMUS MUSCLE¹,²

A paper submitted to the Journal of Animal Science

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ABSTRACT: Electrical stimulation (ES) of bovine carcasses has been hypothesized to increase the activity of some proteolytic enzymes that may degrade key structural proteins in muscle cells, cause fractures and breaks in muscle fibers, and enhance meat tenderness. The objective of this study was to compare postmortem (PM) changes in the muscle proteins, titin, nebulin, α-actinin, and desmin and in myofibrillar structure in nonstimulated (NS) and ES bovine skeletal muscle. The left side of eight beef carcasses was stimulated within 1

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h of death, and the right side was the NS control. Myofibrils for SDS-PAGE and samples for transmission electron microscopy were prepared from the longissimus muscle at 0, 1, 3, 7, 14 and 28 d PM. In SDS-PAGE, titin migrated as three closely spaced bands in both NS and ES 0-d samples. The slowest migrating band, T1 (intact titin) decreased slightly faster in ES samples and was absent from both NS and ES samples at 7 d. The fastest migrating band, T2 (degraded titin) increased in amount through 3 d and was still present at 28 d. In Western blots of ES samples, a titin monoclonal antibody (mAb) identified a more heavily labeled large family of degradation products, which migrated faster than myosin heavy chains, than in NS samples. In SDS-PAGE of NS samples, intact nebulin decreased by 3 d and disappeared by 7 d, but in ES samples, the nebulin band decreased by 1 d and was nearly absent by 3 d. Blots labeled with a mAb to nebulin showed that the intact nebulin band was nearly absent by 3 d in both NS and ES samples. SDS-PAGE results showed that, by 3 d after death, the amount of intact desmin decreased slightly faster in ES samples. Blots labeled with a polyclonal antibody to desmin showed that a more heavily labeled desmin degradation product, a 38 kDa band, was present in ES than in NS samples. Postmortem degradation of α-actinin was not detected in this study. Contraction node (CN) formation, stretching of conjoined sarcomeres adjacent to the nodes, increased frequency of I-band fractures, and accelerated appearance of wide I-band fractures adjacent to the Z-line were characteristics of ES muscle. We conclude that titin, nebulin, and desmin, but not α-actinin, are degraded PM. Electrical stimulation slightly
accelerated the degradation of titin, nebulin and desmin, produced both CN and a higher frequency of I-band fractures, and enhanced the earlier appearance of wide I-band fractures adjacent to the Z-line.

**Key Words:** Titin, Nebulin, Desmin, α-Actinin, Electrical Stimulation, Electron Microscopy.

**Introduction**

Early postmortem (PM) electrical stimulation (ES) increases meat tenderness and promotes the activity of some endogenous proteolytic enzymes, including μ-calpain (Dransfield et al., 1992; Smulders and van Laack, 1992; Uytterhaegen et al., 1992). In myofibrils, titin is a very long filamentous protein that extends from the Z-line to the M-line and links thick filaments to the Z-line; nebulin comprises inextensible filaments that are closely associated with, or part of, thin filaments; α-actinin is an integral Z-line protein; and desmin is the major component of intermediate filaments that associate with Z-lines (for reviews, see Robson et al., 1981, 1991; Robson and Huiatt, 1983). Degradation of some of these structural proteins has been implicated in the loss of myofibrillar integrity, thus, increasing meat tenderness (Huff-Lonergan et al., 1995; Taylor et al., 1995). Robson et al. (1980) and Koohmaraie et al. (1991) for example, suggested that PM meat tenderness may be attributed, in part, to desmin degradation. Uytterhaegen et al. (1992) reported that aging and/or ES increased the degradation of titin and troponin-T, but that ES had no effect on sarcomere length. We also found that
troponin-T decreased more rapidly PM in ES bovine muscle, and that the amount of
the 30 kDa degradation product of troponin-T was greatly increased in ES samples
(Ho et al., 1994). Electron microscopy studies have shown that ES caused the
formation of contraction nodes (CN), and that sarcomeres in the internodal zones
were stretched or fractured in bovine muscle (Takahashi et al., 1987). Sarcomere
shortening and both Z-line and M-line degradation were characteristics of PM
bovine skeletal muscle (Stromer et al., 1967, 1974). Changes in proteins that
comprise cytoskeletal filaments in muscle, or anchoring structures for these
filaments, therefore may be very important in determining the properties of PM
muscle.

The objectives of this study were: (1) to investigate the extent of degradation
of titin, nebulin, α-actinin, and desmin during PM storage of nonstimulated (NS)
and ES bovine skeletal muscle by using SDS-PAGE and Western blots, and (2) to
compare the degradation of these proteins with structural changes in NS and ES
samples by using transmission electron microscopy (TEM).

**Materials and Methods**

*Sample Preparation*

We used one line of crossbred steers (Angus x Jersey), which is different from
most previous studies that have utilized typically heterogeneous feedlot cattle.
Previous studies at Iowa State University (Reiling et al., 1991) have shown that
cattle from this line are genetically predisposed to produce tender muscle. The left
side of eight market weight Angus x Jersey steers was each electrically stimulated with 200 V, 20 Hz for 15 s for three stimulation periods with a 30 s interval between stimuli. The right side of each carcass was a NS control. The electrical stimulation and sampling procedures were previously described for these eight animals in Ho et al. (1994). Myofibrils were prepared from the longissimus muscle (LM) immediately after stimulation (0 d) and at 1, 3, 7, 14, and 28 d PM by using the method of Fritz et al. (1989). Muscle samples for TEM also were taken from the LM immediately after stimulation (0 d) and at 1, 3, 7, 14, and 28 d PM. Thin strips of muscle were cut parallel to the longitudinal fiber axis and were isometrically clamped before the ends of the strips were severed. Tissue samples were doubly fixed in Karnovsky's fixative (Karnovsky, 1965) at 2°C for 3 h and then in 1% osmium tetroxide for 1.5 h. Dehydration in graded acetones was followed by embedding in Epon-Araldite resin. Two blocks were selected at random from each sample for sectioning. Thin sections were mounted on 300-mesh grids and were positively stained with methanolic uranyl acetate and Reynold's lead citrate (Stromer and Bendayan, 1988). All sections were examined at 80 kV in a JEOL JEM-100CXII electron microscope. Images were recorded on Kodak SO-163 electron image film. The frequency of I-band fractures was determined by first scanning alternate rows of grid openings and then counting both the total number of myofibrils and the total number of each fracture type in sections that covered 15 to 20 randomly selected grid openings per grid. Two grids were counted per sample treatment and time for each animal. Frequencies reported are averages
over all animals for each treatment and sampling time. Sarcomere lengths were measured on at least eight randomly selected negatives from each sample. Length of sarcomeres in CN and stretched sarcomeres adjacent to CN were not included in the average sarcomere length for each sample. Sarcomere lengths in these two regions were measured and reported separately.

**Preparation of Selected Myofibrillar Proteins Used as Markers/Standards**

Titin and nebulin were prepared from bovine LM and were purified by the methods of Wang (1982) and Wang and Wright (1988). Myosin, desmin, tropomyosin and troponin-T, -I and -C standards were prepared as we previously described (Ho et al., 1994).

**SDS-PAGE Analysis**

Three gel systems were used to analyze both NS and ES muscle samples in order to resolve proteins that varied widely in molecular mass: 1) an 8% Tris-glycine slab gel (8 cm high x 10 cm wide x 1.5 mm thick) (acrylamide : N, N'-methylenebisacrylamide = 200 : 1, wt/wt) with a 2.57% stacking gel (acrylamide : N, N'-methylenebisacrylamide = 5.7 : 1, wt/wt) was run at 8 mA for 6 h and was used to detect PM changes in titin and nebulin; 2) an 18% Tris-glycine slab gel (16 cm high x 18 cm wide x 1.5 mm thick) (acrylamide : N, N'-methylenebisacrylamide = 100 : 1, wt/wt) with a 5% stacking gel (acrylamide : N, N'-methylenebisacrylamide = 37 : 1, wt/wt) was run at 10 mA for 17 h and was used to monitor the titin and nebulin degradation products which migrate faster than myosin heavy chain (MHC); 3) a 15% Tris-glycine slab gel (16 cm high x 18 cm wide x 1.5 mm thick) with a 5%
stacking gel (both with an acrylamide : N, N'-methylenebisacrylamide = 37 : 1, wt/wt) was run at 10 mA for 17 h and was used to detect desmin and α-actinin degradation (Laemmli, 1970; Fritz, et al., 1989).

Western Blot Analysis

Proteins were transferred from the slab gel to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA) by a wet transfer method (Towbin et al., 1979; Ho et al., 1994). After transfer, the membrane was incubated in a 5% (wt/vol) bovine serum albumin-phosphate buffer solution (BSA-PBS) for 30 min at 37°C and then washed three times in a 0.1% (wt/vol) BSA-PBS solution for 5 min at 25°C. Two titin monoclonal antibodies (mAb), 9D10 (Greaser antibody, Developmental Studies Hybridoma Bank) and T12 (Boehringer Mannheim, Indianapolis, IN), NB2 (Sigma Chemical Company, St. Louis, MO) to nebulin, universal intermediate filament antibody (IFA) (Pruss antibody, American Type Culture Collection; Pruss et al., 1981) and monospecific polyclonal antibodies (pAb) to bovine α-actinin and bovine skeletal muscle desmin were used as primary antibodies. Each membrane was incubated with a primary antibody for 2 h at 25°C except for mAbs T12 and NB2, which were incubated for 18 h at 4°C and then washed three times in 0.1% (wt/vol) BSA-PBS for 5 min. Incubation with the immunogold-labeled second antibody was done for 2 h at 25°C except for blots labeled with mAbs T12 and NB2, which were incubated for 18 h at 4°C. After immunogold labeling, membranes were washed twice in 0.1% (wt/vol) BSA-PBS for 5 min each, twice in deionized water for 1 min each, and were silver enhanced by
the method of Moeremans et al. (1989).

Results

Titin

SDS-PAGE analysis of both NS and ES samples with an 8% gel (Fig. 1a, b) showed that three closely spaced titin bands are present at 0 d. The slowest migrating band is T1 (intact titin) and the fastest band is T2 (a major, ~2,400 kDa degradation product of titin; Robson et al., 1991). The band migrating between T1 and T2 has been referred to as T1-2, and also may be a transient degradation product of titin (Huff-Lonergan et al., 1995). The T1 band decreases slightly faster in ES samples and disappears by 7 d in both NS and ES samples. The T2 band increases in amount through 3 d and is still present at 28 d. In NS samples, an ~1,200 kDa band is present at 0 d and is barely detectable by 3 d. In ES samples, the ~1,200 kDa band decreases slightly faster than in NS samples and is nearly absent by 1 d. Examination of 18% gels of both NS and ES samples (Fig. 2a, b) showed that ~130 and ~39 kDa bands increase from 3 d to 7 d and remain as prominent bands at 28 d.

Western blots from 8% gels (Fig. 3a, b) with mAb 9D10 showed that the T1 band, a very light band near the top of the blot, disappears by 7 d in NS samples (Fig. 3a) and is nearly absent by 3 d in ES samples (Fig. 3b). The T2 band decreases by 7 d and can not be detected by 28 d in both NS and ES samples, although a T2 band was still identified by SDS-PAGE (Fig. 1a, b) (see Discussion).
A large family of degradation products (~1,900 to 22 kDa) seen in blots from 8% and 18% gels (Fig. 3a, b and 4a, b, respectively) increases as PM time increases. A more heavily labeled family of degradation products that migrated between MHC and ~67 kDa is evident in ES samples (Fig. 4b). In Fig. 4a and 4b, an ~39 kDa band that is recognized by mAb 9D10 increases in the 1- and 3-d samples and still persists at 28 d in both NS and ES samples.

Two titin bands are very lightly labeled by the T12 mAb in blots from 8% gels (Fig. 5). As PM time increases, the T1 band decreases and cannot be detected by 3 d in ES samples (Fig. 5b) and by 7 d in NS samples (Fig. 5a). At 28 d, the T2 band is absent in both NS and ES samples (Fig. 5a, b) (see Discussion). A small group of lower-molecular-weight titin degradation products (~1,550 to 750 kDa) is also recognized by T12 in both NS (Fig. 5a) and ES (Fig. 5b) samples. Initially the molecular weights of the products range from ~1,550 to ~1,200 kDa and decrease to a single ~750 kDa band at 28 d (Fig. 5a, b). Blots from 18% gels showed that T12 labeled no titin degradation products below MHC except for a very light band/zone in the range of 190 to 110 kDa in the 28-d ES sample (results not shown).

**Nebulin**

In SDS-PAGE of NS samples (Fig. 1a), the intact nebulin band decreases by 3 d and is barely detectable by 7 d but is nearly absent by 3 d in ES samples (Fig. 1b). Blots from 8% gels with mAb NB2 confirm that the intact nebulin band is
Figure 1. Eight percent gels of myofibrils prepared at different times PM from NS (a) and from ES (b) bovine muscle. Lane Nb = nebulin standard. Lane T = titin standard. T1 = intact titin. T2 = an -2,400 kDa titin degradation product. MHC = myosin heavy chain. T1 and intact nebulin both disappear slightly faster in ES myofibrils. An ~1,200 kDa band (arrow) present at 0 d also decreases faster in ES samples.

Figure 2. Eighteen percent gels of myofibrils prepared at different times PM from NS (a) and from ES (b) bovine muscle. Lane TM = tropomyosin standard. Lane TN-T = troponin-T standard. Lane D = desmin-enriched standard. Lane M = partially purified myosin standard. Lane TN-I = troponin-I standard. Lane TN-C = troponin-C standard. MHC = myosin heavy chain. MLC-1, 2, 3 = myosin light chains 1, 2 and 3. These higher percent gels show that ~130 kDa (asterisk) and ~39 kDa (arrow head) components increase in amount as PM time increases. When compared with TN-C, which is immediately below TN-I, TN-I decreases somewhat with increasing PM time.
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**1a**

**1b**

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**2a**

**2b**
Figure 3. Western blots prepared from 8% gels of NS (a) and ES (b) samples and labeled with 9D10 titin mAb. Lane T = titin standard. T1 = intact titin. T2 = an ~2,400 kDa titin degradation product. 1900 kDa - 1200 kDa = region of gel where proteins from approximately 1,900 to 1,200 kDa migrate. Antibody labeling shows that T1 (arrow heads) disappears faster in ES myofibrils. Several lower molecular weight bands are also labeled.

Figure 4. Western blots prepared from a single 18% gel of NS (a) and ES (b) samples and labeled with 9D10 titin mAb. MHC = myosin heavy chain. A large family of lower molecular weight bands is labeled more heavily in ES samples than in NS samples.
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3a

4a

4b
Figure 5. Western blots prepared from 8% gels of NS (a) and ES (b) samples were labeled with T12 titin mAb. Lane T = titin standard. T1 = intact titin. T2 = an ~2,400 kDa titin degradation product. 1550 -750 kDa = region of gel where proteins from approximately 1,550 to 750 kDa migrate. This antibody also indicates that T1 (arrow heads) disappears faster in ES myofibrils. An approximately 1,200 kDa band (arrow) is also recognized by mAb T12.

Figure 6. Western blots prepared from 8% gels in NS (a) and ES (b) samples and labeled with NB2 nebulin mAb. Lane Nb = nebulin standard. Although results of SDS-PAGE (Fig. 1a, b) suggested that intact nebulin disappears faster in ES myofibrils, labeling with mAb NB2 showed little difference in nebulin degradation between NS and ES samples. Several lower molecular weight bands (~600 to ~380 kDa) are also labeled from 3 to 28 d PM.
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Nebulin-380 kDa

-740-1660 kDa

5a 5b

6a 6b
virtually absent by 3 d in NS samples (Fig. 6a) and in ES (Fig. 6b) samples. A family of lower molecular weight nebulin degradation products (-600 to -380 kDa) appeared from 3 to 28 d in both NS and ES samples. Western blots from 18% gels showed that no nebulin degradation product (below MHC) is recognized by mAb NB2 in both NS and ES samples except for a light band/zone (190 to 100 kDa) that appears at 28 d in ES samples (results not shown).

Desmin and α-Actinin

The desmin band in 15% SDS-PAGE of NS samples is a light band by 3 d and disappears by 7 d (Fig. 7a). In ES samples, the desmin band is barely detectable by 3 d (Fig. 7b). Similar results on desmin degradation were observed in 18% SDS-PAGE (Fig. 2a, b), i.e., desmin degrades slightly faster PM in ES samples. In 15% gels (Fig. 7) an unidentified prominent band migrates faster than desmin, but in 18% gels (Fig. 2) a similar band migrates slower than desmin. This unidentified band in each gel system is unaffected by PM storage and stains with Stains-all which indicates that it is a glycoprotein or a phosphoprotein. Glycoproteins and/or phosphoproteins frequently migrate anomalously in SDS-PAGE conditions, e.g., neurofilaments M and H (Kaufmann et al., 1984) and triadin (Knudson et al., 1993a, b). A pAb to desmin recognized both the desmin band and an ~43 kDa desmin degradation product in the desmin-enriched fraction used as the standard in the Western blot from the 15% gel (Fig. 8). After 1 d PM, there is a steady decrease in the desmin band until 28 d when the desmin band can hardly be seen in both NS and ES samples. An ~38 kDa band is very lightly labeled in NS samples (Fig. 8a).
but is present as a more heavily labeled desmin degradation product from 7 to 28 d in ES samples (Fig. 8b). Although labeling with intermediate filament mAb (IFA), as expected, produced less intense labeling of desmin than with the more sensitive desmin pAb, IFA also showed that the desmin band decreased (i.e., it disappears by 3 d in both NS [Fig. 9a] and ES samples [Fig. 9b]). SDS-PAGE (Fig. 2a, b and 7a, b) and Western blot (Fig. 10a, b) analysis of both NS and ES samples indicated that there is little α-actinin change PM.

**Ultrastructural Changes in NS and ES Muscle**

*Sarcomere Length and Appearance of Contraction Nodes.* Immediately after death, sarcomeres in NS muscle samples are well ordered (Fig. 11a) and have an average length of 2.5 ± 0.1 μm (n = 66). Immediately after ES, contraction nodes (CN) (Fig. 11b, c) and stretched sarcomeres (Fig. 11d) around the CN are formed in seven animals, and all sarcomeres were moderately shortened in the remaining animal (results not shown). The CN, and stretched sarcomeres around the CN, were seen in all samples through 28 d PM (Fig. 11e) from four animals but could not be detected at 1 d PM or in subsequent samples in the other three animals that had CN at 0 d. The number of CN ranges from 0 to 4 per grid opening in 0-d ES samples and did not change through 28 d PM in the four animals still exhibiting CN at 1 d PM. The number of sarcomeres per myofibril in a CN ranges from 2 to 10 and averages 4. These extremely shortened sarcomeres are 1.1 ± 0.1 μm long (n = 82) and contain curved Z-lines, no I-bands, and thick filaments that are in contact with Z-lines (Fig. 11c). Stretched sarcomeres in the region adjacent to a CN within
Figure 7. Fifteen percent gels of myofibrils prepared at different times postmortem from NS (a) and from ES (b) bovine muscle. Lane M = partially purified myosin. Lane TN-T = troponin-T standard. Lane D = desmin-enriched standard. Lane TM = tropomyosin standard. Lane TN-I = troponin-I standard. Lane TN-C = troponin-C standard. MHC = myosin heavy chain. MLC-1, 2, 3 = myosin light chains 1, 2 and 3. Desmin decreases as PM time increases, but α-actinin is not affected by stimulation or PM time.

Figure 8. Western blots prepared from 15% gels of NS (a) and ES (b) samples were labeled with polyclonal anti-desmin. Lane D = desmin-enriched standard. The desmin band decreases similarly in NS and ES samples. Increased labeling of lower molecular weight bands especially a 38 kDa band in ES samples, is seen as PM time increases.
Figure 9. Western blots prepared from 15% gels of NS (a) and ES (b) samples and labeled with IFA mAb. Lane D = desmin-enriched standard. Although this antibody produces lower levels of labeling than the polyclonal anti-desmin antibody (Figure. 8a, b), the decrease in desmin content as PM time increases is evident.

Figure 10. Western blots prepared from a single 15% gel of NS (a) and ES (b) samples were labeled with polyclonal anti-α-actinin. Electrical stimulation and PM time do not alter the α-actinin content of myofibrils.
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Desmin-

9a

9b

α-Actinin-

10a

10b
Figure 11. Structure of NS and ES bovine skeletal muscle. (a). NS 0-d muscle shows the regular banding pattern characteristic of relaxed skeletal muscle. Bar = 2 \mu m.  
(b). An ES 0-d muscle fiber contains a contraction node (CN) and stretched sarcomeres (S), but an adjacent fiber contains relaxed sarcomeres. Bar = 4 \mu m.  
(c). Higher magnification of part of a contraction node in a 0-d stimulated fiber. Thick filaments of the A-band are in contact with Z-lines (Z). Bar = 0.5 \mu m.  
(d). Myofibrils that contain contraction nodes also contain stretched sarcomeres such as this in adjacent regions. Note the wide I-band centered on the Z-line (Z) and the irregular edges of the A-band (A). Bar = 1 \mu m.  
(e). Contraction nodes (CN) often are still present at 28 d in ES samples. Bar = 4 \mu m.
the same myofibril are $3.2 \pm 0.2 \mu m$ long ($n = 73$) and contain wide I-bands, crooked Z-lines and M-lines and A-bands with uneven edges (Fig. 11d). Sarcomeres at greater distances from both a CN and the associated stretched sarcomeres within the same myofibril, and in myofibrils without a CN (4 to 6 myofibrils away), in ES samples at 0 d are similar to the sarcomeres observed in the 0-d NS samples and have a sarcomere length of $2.6 \pm 0.1 \mu m$ ($n = 72$). At 1 d PM, NS samples have a sarcomere length of $1.8 \pm 0.1 \mu m$ ($n = 48$), compared with a sarcomere length of $2.0 \pm 0.2 \mu m$ ($n = 47$) in ES samples. No further change in sarcomere lengths occurred in both NS and ES samples from 1 to 28 d.

Changes in the I-Band/Z-Line Region. Degradation of Z-lines per se progresses similarly in both NS and ES samples as time PM increases. At 1 d PM, one to three discontinuities or gaps in the Z-line, narrower I-bands, double overlap of thin filaments at the center of sarcomeres and increased intermyofibrillar spaces are evident in both NS (Fig. 12a-1) and ES samples. The extent of Z-line structural degradation that typifies 3 d PM samples (Fig. 12a-2) is evident when compared with a typical Z-line in 0-d samples (Fig. 12a-3). Overlapping, interconnected filaments characterize Z-lines seen in longitudinal sections of 0-d samples (Fig. 12a-3). By 3 d PM, the fibrillar structure has been replaced by an amorphous band that is not uniform in density and that is adjacent to I-band fractures (Fig. 12a-2). By 28 d PM, Z-lines are wider, are amorphous with more irregular edges, are less dense and often have 2 to 5 gaps in a typical Z-line (results not shown).

In addition to these characteristic PM changes in Z-line structure, three types
of I-band fractures were observed adjacent to Z-lines. The frequency of these I-band fractures was influenced by both ES and storage time. Narrow I-band fractures (Fig. 12b), ranging from 0.1 \( \mu m \) to 0.8 \( \mu m \) wide and involving 1 to 6 contiguous myofibrils, are present in 2 to 3\% of 1-d NS myofibrils and in 5 to 10\% of ES myofibrils. These fractures in 1-d samples are spanned by a few filaments that have diameters less than or equal to the diameter of thin filaments. At 3 d PM, narrow I-band fractures can be detected in 2 to 10\% of myofibrils in NS samples and in 5 to 15\% of myofibrils in ES samples. At 7 d PM, narrow I-band fractures, with or without filaments spanning the fracture zone, were observed in 3 to 10\% of the myofibrils in NS samples and in 5 to 20\% of myofibrils in ES samples. At 14 d PM, narrow I-band fractures with or without spanning filaments increased in frequency and can be seen in 5 to 20\% of the myofibrils in NS samples and in 10 to 30\% of the myofibrils in ES samples. At 28 d PM, all narrow I-band fractures have no filaments spanning the fracture zone and are present in 9 to 20\% of the myofibrils in NS samples and in 10 to 30\% of the myofibrils in ES samples.

Neither intermediate width I-band fractures (Fig. 12c), ranging from 0.8 to 3.5 \( \mu m \) wide and involving 3 to 12 contiguous myofibrils, nor wide I-band fractures (Fig. 12d), which range from 4 to more than 20 \( \mu m \) wide, involve 6 to 35 contiguous myofibrils and often contain released sarcomeres, can be detected in 3 d NS samples but are present in 10 to 30\% of the myofibrils in 3 d ES samples. At 7 d PM, both intermediate and wide I-band fractures were found in 15 to 30\% of myofibrils in NS samples and in 20 to 50\% of myofibrils in ES samples. The
Figure 12. The Z-line and I-band changes in PM bovine skeletal muscle. (a-1).
NS muscle at 1 d PM contains shortened sarcomeres and Z-lines that have discontinuities (arrows). Bar = 1 μm. (a-2). Z-lines lose their fibrillar structure and become amorphous by 3 d in ES samples. I-band fractures adjacent to Z-lines (arrow) are readily seen. Bar = 0.2 μm. (a-3). Z-lines in 0-d NS samples contain overlapping and interconnected filaments that give Z-lines a distinct fibrillar structure. Bar = 0.2 μm. (b). Narrow I-band fractures (arrows) adjacent to the Z-line with filaments spanning the fracture zone are present in ES samples at 1 d. Bar = 1 μm. (c). Intermediate width I-band fracture (arrow) in NS 7-d muscle samples. Bar = 2 μm. (d). Wide I-band fractures (arrow) in NS 28-d muscle samples frequently contain sarcomeres that are randomly oriented (double arrow head). This orientation implies that sarcomeres released from adjacent myofibrils are present in the fracture zone. Bar = 2 μm.
combined frequency of intermediate and wide I-band fractures increased to 30 to 70% of myofibrils in both 14 d NS and ES samples. Wide I-band fractures were seen in 40 to 85% of myofibrils at 28 d in both NS and ES samples.

Discussion

Effect of ES on Degradation of Titin, Nebulin, α-Actinin and Desmin During PM Storage

Several investigators (e.g., Dransfield, 1992; Dransfield et al., 1992; Koohmaraie, 1992; Uytterhaegen et al., 1994) have suggested that μ-calpain activity in PM muscle causes many of the proteolytic alterations in key structural proteins and that this, in turn, is related to tenderization. It also has been suggested that one of the effects of ES is to increase the amount of proteolytic enzyme activity in bovine muscle (Dransfield, 1992; Dransfield et al., 1992; Smulders and van Laack, 1992; Uytterhaegen et al., 1992). One objective of our experiments was to determine the effect of ES on proteins believed to be involved in muscle integrity. Reports from several laboratories have indicated that titin was degraded during PM storage (Lusby et al., 1983; Bandman and Zdanis, 1988; Huff-Lonergan et al., 1995; Taylor et al., 1995) and also that the degree of titin degradation may be associated with increasing meat tenderness (Paterson and Parrish, 1987; Huff-Lonergan et al., 1995). Our SDS-PAGE results also showed that the T1 band of titin degraded during PM storage, decreased slightly faster in ES samples, and that the T2 band increased in amount and was still present at 28
d (Fig. 1a, b). However, Fritz et al. (1993) found that there is no relationship between titin content on SDS gels and tenderness. The \( -1,200 \) kDa band (Fig. 1b), supposedly a fragment of T1 as described by Takahashi et al. (1992), is also degraded slightly faster in ES samples. However, ES does not accelerate the appearance of either a 130 or a 39 kDa band (cf. Fig. 2a and b). The source of the 130 kDa band is not known, but it is possible that the 130 kDa band is a fragment of higher molecular proteins such as titin, nebulin or M-line components.

Western blots labeled with mAb 9D10 also showed that the intact titin band decreased slightly faster in ES than in NS samples (Fig. 3a, b). However, a band with a mobility similar to T2, which is present in 8% gels of both NS and ES samples and labeled by mAb 9D10 at early times PM did not label this band in NS and ES 28 d samples (Fig. 1a, b). Either the epitope recognized by 9D10 evidently is not available in this band in the 28-d samples, or the amount of T2 present at 28 d has decreased in amount sufficiently that it has dropped below the sensitivity of the blot labeling. Western blots labeled with mAb 9D10 show that a large family of degradation products (below MHC to 22 kDa) is labeled (Fig. 4a, b), one of which is a 39 kDa band that increases during the first 7 d PM. The 39 kDa band, which migrates slightly faster than actin, has been hypothesized to be creatine phosphokinase (Pommier et al., 1987) and seemingly was identified as a 43 kDa peptide by Uytterhaegen et al. (1992).

Labeling with mAb T12 also demonstrated that T1 decreases slightly faster in ES samples (cf. Fig. 5a and b). Again, a band with the relative mobility of T2 that
was labeled at early times PM was present at 28 d in the 8% gel of NS (Fig. 1a) and ES samples (Fig. 1b), but not recognized by mAb T12. It is possible that the epitope recognized by mAb T12, which is near the Z-line, is so close to the cleavage site that the putative T2 in the 28-d samples may have had the epitope lost (e.g., further cleavage of polypeptides in the T2 band may not be resolved in the SDS-PAGE system) or altered and thus was not recognized by the antibody. It also is possible that the amount of the T2 at 28 d PM may have fallen below the detection limit in the Western blot analysis with mAb T12. The mAb T12 does label several bands with a relative mobility down to approximately 750 kDa (Fig. 5a, b) but does not label many of the lower molecular weight components in blots from 8% gels that were recognized by mAb 9D10 (Fig. 3a, b). Others (e.g., Fritz and Greaser, 1991; Taylor et al., 1995) also have obtained Western blot results that indicate variable degrees of labeling and some inconsistencies. The blot analyses suggest that a qualitative rather than quantitative interpretation is more appropriate.

The intact nebulin band in SDS-PAGE (Fig. 1) decreases slightly faster in ES than in NS samples and disappears more rapidly than titin in both NS and ES samples (Fig. 1a, b). This is consistent with the more rapid degradation of nebulin than titin in PM bovine muscle reported by Lusby et al., 1983; Locker and Wild, 1984; and Fritz and Greaser, 1991. In both NS and ES samples, a family of lower molecular weight nebulin degradation products (~600 to ~380 kDa) (Fig. 6a, b) was recognized by mAb NB2, which is in agreement with the results of Fritz and
The SDS-PAGE results showed that desmin, the major component of intermediate filaments that connect adjacent myofibrils at their Z-line levels and the Z-lines of the peripheral layer of myofibrils to the cell membrane skeleton (Robson, 1989; Stromer, 1990), decreased slightly faster in ES samples (cf. Fig. 7a and b) and was barely detectable at 3 d. Robson et al. (1984) reported that at 1 d PM, approximately 10 to 25% of intact desmin had been lost, and nearly all desmin was gone by 7 d in bovine muscle stored at 15°C. Koohmaraie et al. (1991) found that desmin was already degraded by 1 d in pork, beef and lamb muscle and was more extensively degraded in pork than in beef and lamb muscle. These findings are in agreement with our SDS-PAGE results that desmin degradation occurred early during PM storage. A pAb to desmin, however, indicated that intact desmin disappears at similar times in NS and ES samples but that ES enhances the amount of a desmin degradation product, a 38 kDa band (Fig. 8b), between 7 and 28 d PM. The ostensible discrepancy between the SDS-PAGE and blot data may be due to a masking effect by the antibody labeling and the silver enhancement steps that may have obscured small decreases in the desmin band in blots. This explanation is supported by increased amounts of a 38 kDa polypeptide that are labeled by the desmin antibody as PM time increases. Our blot results with the polyclonal Ab are consistent with those of Hwan and Bandman (1989) who reported that desmin fragments existed by 4 d in muscle stored at 4°C, and that little intact desmin remained at 3 wk PM. Labeling with IFA also showed that intact desmin
was degraded PM. Although labeling with IFA mAb suggested that desmin disappeared sooner (by 3 d) in both NS and ES samples in comparison with blot results obtained with desmin pAb (Fig. 8), no degradation products were detected during PM storage (Fig. 9a, b). This is likely a result of the much lower labeling sensitivity of this mAb (IFA) in comparison with that of the much higher sensitivity of the desmin pAb in blots. In both SDS-PAGE and Western blots, the amount of intact $\alpha$-actinin does not change in NS or ES samples during PM storage, which is consistent with the results of Stromer et al. (1974) and Uytterhaegen et al. (1992). This also is consistent with the results of Goll et al. (1991) who showed that calpain digestion of myofibrils releases intact $\alpha$-actinin. We earlier reported (Ho et al., 1994) that in comparison to NS, ES caused earlier PM degradation of troponin-T and a significant increase in the amount of its 30 kDa degradation product.

**Effect of ES on ultrastructural changes during PM storage**

Electrical stimulation improves meat tenderness (Savell et al., 1977) and has been hypothesized to do so by preventing cold-shorting (Takahashi et al., 1984), by producing fractures and breaks in muscle fibers (Savell et al., 1978; Takahashi et al., 1987), by producing an intermediate rate of glycolysis (Smulders et al., 1990) or by a combination of these factors (for review, see Smulders and van Laack, 1992). Seven animals had prominent CN immediately after stimulation (Fig. 11b). These CN consisted of 2 to 10 highly contracted sarcomeres per myofibril similar to those described by Savell et al. (1978) and Sorinmade et al. (1982). George et al. (1980), however, suggested, on the basis of histological examination, that the
formation of CN was due to denaturation of sarcoplasmic proteins and not to muscle fiber contraction or disruption. The observation by George et al. (1980) that CN were birefringent in polarized light indicates that CN are sites of accumulation of thick filaments, which are responsible for A-band birefringence in muscle. Our thin, longitudinal sections through a CN clearly show densely packed thick filaments in the CN (Fig. 11c). Muscle from four animals still contained CN at 28 d PM, but three animals had no CN at 1 d PM. Cassens et al. (1963a, b) also found the absence of CN in porcine muscle at onset of rigor mortis or 24 h PM and suggested that when ES is not too intense, the process of CN formation is reversible, but when ES is intense, CN are formed before rigor onset, and the formation of CN becomes irreversible. The existence of CN in muscle at 1 d PM has been reported by Savell et al. (1978) and Takahashi et al. (1987), at 2 d PM by Sorinmade et al. (1982), at 4 d PM by McKeith et al. (1980) and at 5 d PM by George et al. (1980). None of these groups sampled at longer PM time than 5 d, so it is not known if CN persisted in their samples. Sarcomere lengths in 0 d ES muscle CN were 44% of rest length sarcomeres in control muscle, but stretched sarcomeres adjacent to a CN within the same myofibril were 128% of rest length sarcomeres. At 1 d PM, there was little difference in sarcomere length between NS and ES muscle samples. Although Savell et al. (1978) and Uytterhaegen et al. (1992) both reported that ES had no effect on sarcomere length in 20 to 24 h PM muscle samples, sarcomeres were measured by Savell et al. (1978) in isolated myofibrils and by Uytterhaegen et al. (1992) in ground muscle samples. Because
of the very different sample preparation techniques, comparisons with our data are, at best, problematic.

Alterations near, or changes in, Z-lines and increased intermyofibrillar spaces with the loss of lateral attachments between Z-lines of adjacent myofibrils during PM storage reported here are consistent with previous observations on bovine muscle (Stromer et al., 1967; Davey and Gilbert, 1967, 1969; Davey and Dickson, 1970; Henderson et al., 1970; Sorinmade et al., 1982). Our results also showed that Z-lines were no longer continuous structures by 1 d (Fig. 12a-1), and many became amorphous by 3 d (Fig. 12a-2). Taylor et al. (1995), however, reported that Z-lines remained almost unchanged ultrastructurally after 16 d PM. It is possible that the section thickness, the staining method, and the relatively low magnification chosen by Taylor et al. (1995) may have obscured important ultrastructural changes in Z-line structure in their samples. The extent or rate of progression of Z-line changes per se and the development of intermyofibrillar spaces were very similar in NS and ES samples.

Fractures in the l-band adjacent to the Z-line result in Z-lines still attached to thin filaments on one side of the fracture. I-band fractures adjacent to the Z-line have also been noted by Taylor et al (1995) in non-homogenized bovine biceps femoris muscle that was stored 4 d or longer at 4°C and in homogenized bovine biceps muscle used for determining the myofibrillar fragmentation index. Davey and Dickson (1970) also reported that breaking at the l-band/Z-line junction and occasionally at the edge of the A-band occurred in bovine muscle during aging.
Ouali (1990) described l-band fractures in bovine muscle after 15 d PM as being mainly at the \( N_2 \) line, but occasionally near the A-I junction. These observations suggest that the l-band adjacent to the Z-line is a structurally labile site. We characterized these fractures as narrow, intermediate or wide, depending on the axial width of the fracture. The frequency of these fractures is affected by ES and by time PM. Narrow fractures occur at 1 d PM in both NS and ES samples but with greater frequency in ES samples. The frequency of narrow fractures in ES muscle gradually increases from 5 to 10% at 1 d PM to 10 to 30% at 28 d PM. Intermediate and wide l-band fractures are seen sooner PM and with greater frequency in ES samples. Taylor et al. (1995) suggested that degradation of titin and nebulin may contribute to the increased l-band fractures during PM storage. Our results also indicated that the disappearance of T1 and nebulin are closely related temporally to the appearance of both intermediate and wide l-band fractures. In NS samples, T1 and intact nebulin (Fig. 1a) disappeared at 7 d, and concomitantly both intermediate and wide l-band fractures were first seen at 7 d. In ES samples, T1 and nebulin (Fig. 1b) were nearly gone at 3 d, and both types of l-band fractures also appeared earlier at 3 d. Despite the uncertainty of the causative agent(s), the progressive increase in both l-band fracture frequency and width in ES samples compared with NS samples and during PM storage suggests that these fractures may contribute to improved tenderness. Because we have observed significant ultrastructural changes both inside the Z-line and beside it, we believe it may be premature to conclude that only those changes near the Z-line
are important (Taylor et al., 1995). It seems reasonable to us that alterations occurring in the Z-line itself (e.g., degradation or loss of function of Z-line components), which are many (Robson et al., 1991), may release titin and/or nebulin from their Z-line attachment sites and cause the fractures.

From our results, we conclude that titin, nebulin and desmin, but not α-actinin, are degraded PM. Taken together with our earlier report (Ho et al., 1994), we have shown by SDS-PAGE analysis that ES slightly accelerated the degradation of titin, nebulin, desmin, and troponin-T during PM storage. Electrical stimulation produced CN and variable sarcomere lengths in 0-d samples. Electrical stimulation also increased the frequency of I-band fractures in ES muscle samples. Wide I-band fractures adjacent to the Z-line were seen sooner PM in ES than in NS bovine muscle. Z-line degradation occurred at similar rates in NS and ES samples. Formation of both I-band fractures and CN are the two principal structural alterations caused by ES.

Implications

One result of electrical stimulation is an increase in meat tenderness. We have shown that electrical stimulation slightly accelerates the degradation of the cytoskeletal proteins, titin, nebulin, and desmin in postmortem muscle. We also found that electrical stimulation increased the frequency of three types of myofibrillar I-band fractures (narrow, intermediate and wide) and caused wide I-band fractures to appear sooner postmortem. Electrical stimulation may improve
meat tenderness by a combination of mechanical disruption and enhanced proteolysis.

**Literature Cited**


Longissimus dorsi from Holstein veal calves fed a corn or barley diet. Meat Sci. 21:203.


CHANGES IN TITIN, NEBULIN, DESMIN, AND TROPONIN-T AND IN ULTRASTRUCTURE RESULTING FROM POSTMORTEM ELECTRICAL STIMULATION OF BOS INDICUS CROSSBRED CATTLE^{1,2}

A paper submitted to the Journal of Animal Science

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ABSTRACT: The effect of electrical stimulation (ES) on degradation of titin, nebulin, desmin, and troponin-T (TN-T), and on structural changes in the longissimus muscle (LM) from Brahman x Simmental (B x S) cattle (Bos indicus cross) were determined. The left side of seven B x S beef carcasses was stimulated within 1 h postmortem (PM), and the right side was the nonstimulated (NS) control. Myofibrils for SDS-PAGE and samples for transmission electron microscopy were prepared from the LM at 0, 1, 3, 7, 14, and 28 d PM. SDS-PAGE

^1We thank Mary Sue Mayes for assistance in preparing TEM samples, ISU Meat Lab personnel for slaughtering the animals, and Darrel E. Goll for generously providing polyclonal antibodies to bovine α-actinin. This research was supported in part by the Beef Industry Council of the National Live Stock and Meat Board and by USDA NRICGP Award 92-37206-8051.


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and Western blots showed that the T1 band of titin was absent at 14 d in both NS and ES samples. In SDS-PAGE, intact nebulin was nearly gone by 7 d, but in blots, nebulin decreased by 7 d and was absent by 14 d in both NS and ES samples. The desmin band could still be seen as a light band at 28 d in SDS-PAGE and in Western blots of both NS and ES samples. A decrease in TN-T and a concomitant increase in the 30 kDa polypeptide were observed in both NS and ES samples. Western blots with a mAb to TN-T confirmed that TN-T decreased at similar rates in NS and ES samples, but showed that the 30 kDa polypeptide was more heavily labeled in ES samples from 7 to 28 d. Contraction nodes were present in 0 d ES samples and were still observed in 28 d ES samples. Narrow, intermediate and wide I-band fractures were seen earlier and at a greater frequency in ES than in NS samples. Therefore, ES had no detectable affect on titin, nebulin, desmin or TN-T degradation, but accelerated the appearance and enhanced the frequency of three types of I-band fractures in these *Bos indicus* crossbred cattle.

**Key Words:** Titin, Nebulin, Desmin, Troponin-T, Electrical Stimulation, Electron Microscopy.

**Introduction**

Muscle from *Bos indicus* crossbred cattle has been characterized as more variable and less tender than muscle from *Bos taurus* crossbred cattle (Winer et al., 1981; Koch et al., 1982; Wheeler et al., 1990). As the percentage of *Bos*
*indicus* inheritance increased, the level of tenderness decreased (Crouse et al., 1989; Williams et al., 1994). It also has been reported that calpastatin activity was higher in a *Bos indicus* cross than in a *Bos taurus* cross (Whipple et al., 1990; Shackelford et al., 1991). Whipple et al. (1990) speculated that the higher calpastatin activity might slow desmin degradation and appearance of the 30 kDa polypeptide in less tender muscle from a *Bos indicus* cross. MacBride and Parrish (1977) also observed that a 30 kDa polypeptide appeared earlier in tender than in tough bovine muscle. Huff-Lonergan et al. (1995) showed that, during postmortem (PM) storage, titin and nebulin were degraded more slowly in tough than in tender bovine muscle. Fritz et al. (1993), however, reported that titin content did not distinguish tough from tender beef.

Electrical stimulation (ES) has been used to increase meat tenderness in beef, lamb, and goat carcasses (Savell et al., 1977) and has produced fractures and breaks in muscle fibers (Savell et al., 1978; Takahashi et al., 1987). Crouse et al. (1987) reported that ES markedly improved tenderness of muscle from *Bos indicus* crossbred cattle. Wheeler et al. (1990) also found that at 14 day PM in nonstimulated (NS) carcasses, fewer l-band fractures were present in muscle samples from *Bos indicus* than from *Bos taurus* cattle. The objectives of this study were: (1) to observe the degradation patterns of titin, nebulin, desmin, and troponin-T (TN-T), and structural changes during PM storage of both NS and ES bovine muscle from Brahman x Simmental (B x S) cattle (*Bos indicus* cross) by using SDS-PAGE, Western blots, and transmission electron microscopy (TEM); (2)
to compare the protein degradation and structural changes in B x S cattle with the changes in Angus x Jersey (A x J) cattle (Bos taurus cross) that were reported previously by Ho et al. (1994, 1995).

Materials and Methods

Sample Preparation

The left side of seven market weight B x S crossbred steers was stimulated with 200 V, 20 Hz for 15 or 20 s for three stimulation periods with a 30 s interval between stimuli. The right side of each carcass was a NS control. Sampling procedure and the 3-h pH measurement were previously described in Ho et al. (1994). Myofibrils were prepared immediately after stimulation (0 d) and at 1, 3, 7, 14, and 28 d PM from the longissimus muscle (LM) stored at 2°C by using the method of Fritz et al. (1989). Muscle samples for TEM were taken from the LM at the same PM times. Procedures for TEM sample preparation, measurement of sarcomere length and the frequency of I-band fractures were done as described in Ho et al. (1995).

Preparation of Selected Myofibrillar Proteins Used As Markers/Standards

Preparation of titin, nebulin, myosin, desmin, tropomyosin, and troponin-T and -C standards was done as described in Ho et al. (1994, 1995).

SDS-PAGE Analysis

Two gel systems were used to analyze both NS and ES myofibrils in order to resolve proteins that varied widely in molecular mass: 1) an 8% acrylamide Tris-
glycine slab gel (15 cm high x 10 cm wide x 1.0 mm thick) (acrylamide : N, N'-methylenebisacrylamide = 200 : 1, wt/wt) with a 2.57% acrylamide stacking gel (acrylamide : N, N'-methylenebisacrylamide = 5.7 : 1, wt/wt) was run at 8 mA for 15 h and was used to obtain separation of intact and degraded titin bands and to monitor PM changes in titin and nebulin; 2) an 18% acrylamide Tris-glycine slab gel (16 cm high x 18 cm wide x 1.5 mm thick) (acrylamide : N, N'-methylenebisacrylamide = 100 : 1, wt/wt) with a 5% acrylamide stacking gel (acrylamide : N, N'-methylenebisacrylamide = 37 : 1, wt/wt) was run at 10 mA for 17 h and was used to detect α-actinin, desmin and TN-T degradation and titin and nebulin degradation products, which migrate faster than myosin heavy chain (MHC) (Laemmli, 1970; Fritz et al., 1989).

Western Blot Analysis

Monoclonal antibodies (mAbs) 9D10 (Greaser antibody, Developmental Studies Hybridoma Bank) and T12 (Boehringer Mannheim, Indianapolis, IN) both to titin, JLT-12 to TN-T and NB2 to nebulin (both from Sigma Chemical Company, St. Louis, MO), and polyclonal antibodies (pAb) to α-actinin and desmin were used as primary antibodies. The procedure for blotting and immunogold-silver staining was previously described in Ho et al. (1994), and was used with the pAb to α-actinin and desmin and the mAbs to 9D10 and TN-T. Because the labeling signals from mAbs T12 to titin and NB2 to nebulin were very low in blots from 8% gels with the immunogold-silver staining method, the chemiluminescence method, which has greater sensitivity, was used for mAbs T12 and NB2. The enhanced
The chemiluminescence (ECL) procedure used was provided by the manufacturer (Amersham, Arlington Heights, IL). In the latter procedure, after transfer, the membrane was incubated in a 5% blotto-phosphate buffered saline, .1% Tween (PBS-T) for 1 h at 25°C and then washed 3 times in PBS-T for 10 min at 25°C. Then each membrane was incubated with primary antibody (mAbs T12 to titin or NB2 ) for 18 h at 4°C and then washed 3 times in PBS-T for 10 min. Incubation with horseradish peroxidase-labeled goat anti-mouse antibody was done for 1 h at 25°C. The membrane was then washed once for 15 min and four times for 5 min each with PBS-T, and was incubated in the ECL reagent for 1 min at 25°C. The protein bands on the membrane were detected with an autoradiography film (Hyperfilm ECL, Amersham, Arlington Heights, IL).

Tenderness Measurements

Tenderness measurements of steaks were done by using a star probe attachment (a 5-pointed star with a maximum diameter of 9 mm) for an Instron model 4502 (Instron, Canton, MA). Steaks 2.5 cm thick were removed from the carcass at 24 h PM. Steaks were stored at 2°C for 72 to 96 h and six star probe determinations (raw) were done on each raw steak at 2°C. After the star probe determinations (raw) were completed, the steaks were vacuum packaged and aged at 2°C for 12 d before being frozen at -30°C. The steaks were stored at -30°C for 2 to 3 mo, and then thawed for 24 h at 2°C. Steaks were broiled in a General Electric Model CB-60 electric broiler to an internal temperature of 60°C. Steaks were turned when the internal temperature reached 40°C. Steaks were held at
20°C for 2 to 3 h before six star probe determinations (cooked) at previously unprobed sites were done. All star probe determinations were done at a crosshead speed of 250 mm/min. Peak force was recorded when the probe had penetrated 80% of the thickness of the steak.

Results

The 3-h pH and Tenderness Measurements

The average 3-h pH for NS muscle was 6.37; it was 6.01 for ES muscle. The 3-h pH in ES muscle was in the range (5.9 to 6.3) recommended by Smulders et al. (1990) for maximizing PM proteolysis. The star probe was used in this study to detect tenderness differences because in three previous studies on pork loins from 3,500 animals, the correlation between the star probe and sensory panel tenderness values averaged .8 (K. J. Prusa, Iowa State University, personal communication). Star probe measurements of raw NS steaks gave an average resistance value of 2.80 kg, and for ES steaks the average was 2.39 kg. Star probe measurements of cooked NS steaks averaged 4.82 kg, and for ES steaks the average was 4.22 kg.

Effect of ES on Protein Degradation Patterns in Muscle from B x S Cattle

SDS-PAGE of muscle samples in an 8% gel (Fig. 1) showed that the T1 band (intact titin) decreases by 7 d and disappears by 14 d, and the T2 band (a major, ~2,400 kDa degradation product of titin; Robson et al., 1991) increases in amount through 14 d and is still present at 28 d in both NS and ES samples. The titin
bands migrating between T1 and T2, which can be seen on these long gels, also disappear by 14 d PM. A light -1,200 kDa band is present at 0 d and is essentially absent by 7 d in both NS and ES samples. The intact nebulin band is present at 0 d and can hardly be seen at 7 d in both NS and ES samples. The 18% gels of both NS and ES samples (Figs. 2a and 2b) showed that an -130 kDa band (asterisk) is not obvious until 28 d PM, and an -39 kDa band (arrowhead) is a prominent band at 3 d and increases in amount through 28 d. Desmin decreases in both NS and ES samples during PM storage and is a very light band by 28 d (note that, in these gels, desmin migrates close to, and just below, a heavier band that remains through the aging period). Troponin-T (two isoforms are present and decrease similarly; Ho et al., 1994) decreases in amount from 3 to 14 d and is nearly absent by 28 d in both NS and ES samples. A 30 kDa band (double arrowhead) shows a marked increase by 14 d and is a very prominent band in 28 d NS and ES samples. In Western blots from 8% gels (Fig.3), labeling with titin mAb 9D10 showed that the T1 band disappears from both NS and ES samples by 14 d, and the T2 band can hardly be seen at 28 d. A large family of degradation products (-1,900 to 22 kDa) in both NS and ES samples, including an -39 kDa band, becomes more prominent with PM time as seen in 9D10 blots from 8% and 18% gels (Fig. 3 and Figs. 4a and 4b, respectively).

Because an -39 kDa band has previously been hypothesized to be creatine phosphokinase (Pommier et al., 1987), which may be released from M lines during PM degradation (Ouali, 1990), we attempted to identify this band. A similar
Figure 1. Eight percent gel of myofibrils prepared from both NS and ES bovine muscle. Lane T = titin standard. Lane N = nebulin standard. T1 = intact titin. T2 = an ~2,400 kDa titin degradation product. MHC = myosin heavy chain. In both NS and ES samples, the T1 band disappears by 14 d, and the T2 band becomes very prominent by 14 d. The minor titin bands migrating between T1 and T2 also disappear by 14 d PM. Intact nebulin is nearly gone by 7 d. An ~1,200 kDa band present from 0 d to 3 d is absent by 7 d in both NS and ES samples.

Figure 2. Eighteen percent gels of myofibrils prepared from NS (a) and from ES (b) bovine muscle. Lane TM = tropomyosin standard. Lane TN-T = troponin-T enriched standard. Lane D = desmin-enriched standard. Lane M = partially purified myosin standard. MHC = myosin heavy chain. TN-I = troponin-I. TN-C = troponin-C. MLC-1, 2, 3 = myosin light chains 1, 2 and 3. In both NS and ES samples, desmin and TN-T decrease as PM time increases and can still be seen as light bands at 28 d. The minor band above and the prominent band below TN-T are TN-T isoforms that also decreases similarly to TN-T. The 30 kDa polypeptide (double arrowhead) becomes prominent by 14 d in both NS and ES samples. An ~30 kDa band (asterisk) does not become obvious until 28 d PM in NS and ES samples. An ~39 kDa band (arrowhead) can be seen at 3 d and increases in amount as PM time increases in NS and ES samples.
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Figure 3. Western blot prepared from an 8% gel of NS and ES samples labeled with 9D10 titin mAb and a goat anti-mouse IgM-gold probe. Lane T = titin standard. T1 = intact titin. T2 = an -2,400 kDa titin degradation product. Antibody labeling shows that T1 is absent at 14 d in both NS and ES samples. A family of lower molecular weight titin degradation products that increases with time PM is also labeled.

Figure 4. Western blots prepared from 18% gels of NS (a) and ES (b) samples were labeled with 9D10 titin mAb and a goat anti-mouse IgM-gold probe. A large family of lower molecular weight titin degradation products that increases with time PM is labeled in both NS and ES samples. A 39 kDa band increases as PM time increases and becomes prominent by 7 d in both NS and ES samples.
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- MHC: 32 kDa
- 32 kDa
- 22 kDa
- 22 kDa

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4b
quantity of purified rabbit creatine phosphokinase (Boehringer Mannheim, Indianapolis, IN) and an ~39 kDa band present in 28-d bovine myofibrils were analyzed by SDS-PAGE and Western blots. In SDS-PAGE (Fig. 5a), rabbit creatine phosphokinase migrates slightly faster than an ~39 kDa band in the PM myofibril sample, and in blots (Fig. 5b) both the creatine phosphokinase band and an ~39 kDa band are labeled by 9D10. These results are difficult to interpret unambiguously, but it is possible that the purified creatine phosphokinase sample contains a titin degradation product or that mAb 9D10 can also recognize an epitope on creatine phosphokinase.

A Western blot from an 8% gel labeled with T12 mAb showed that, in both NS and ES samples, T1 and T2 can not be detected by 14 d, and several lower molecular weight titin degradation products (below ~1,550 kDa) are recognized by T12 through 28 d (Fig. 6). Blots from 18% gels showed that T12 mAb labeled no titin degradation products below myosin heavy chain (MHC) (results not shown).

A blot from an 8% gel labeled with mAb NB2 (Fig. 7) demonstrated that the intact nebulin band decreases by 7 d and is absent by 14 d in both NS and ES samples. Several lower molecular weight nebulin degradation bands (~600 to ~380 kDa) are present from 7 to 28 d in both NS and ES samples. Western blots from 18% gels showed that no nebulin degradation products below MHC are recognized by mAb NB2 in both NS and ES samples (results not shown).

In Western blots, a mAb to TN-T labels the TN-T bands (for explanation of multiplicity of TN-T bands, see Ho et al., 1994) and a family of TN-T degradation
products (-35 to 28 kDa) in both NS and ES samples (Figs. 8a and 8b). The intact TN-T band that comigrates with the purified TN-T standard and its two isoforms, one light band migrating just above the TN-T band and the other prominent band migrating just below the TN-T band, decrease in amount through 28 d in both NS and ES samples. A 30 kDa polypeptide increases in amount through 28 d in both NS and ES samples and is somewhat more heavily labeled in ES samples from 7 to 28 d (Fig. 8b). A 28 kDa band, which may be homologous with the 27 kDa band described by Ouali (1992), is also present through 28 d in both NS and ES samples (Figs. 8a and 8b).

Western blots labeled with a pAb to desmin (Figs. 9a and 9b) demonstrated that some intact desmin can still be seen as a light band by 28 d, and no lower molecular weight desmin degradation product was detected in either NS or ES samples. Results from SDS-PAGE (Figs. 2a and 2b) and Western blots (results not shown) showed that PM storage did not noticeably affect α-actinin in either NS or ES samples.

**Effect of ES on Ultrastructural Changes in Bovine Muscle from B x S Cattle**

Sarcomeres in 0-d NS muscle samples contain distinct A-bands, I-bands, and Z-lines (Fig. 10a) and have an average length of 2.5 ± 0.2 μm (n = 62). In 0-d ES muscle, contraction nodes (CN) formed in five animals that received three 20-sec periods of ES (Fig. 10b). The first two animals used in this study received three 15-sec stimuli as we had done previously with *Bos taurus* cattle (Ho et al., 1994), but an inadequate pH decrease required a subsequent increase in stimulation.
Figure 5. Labeling of a 39 kDa band with mAb 9D10 to titin and a goat anti-mouse IgM-gold probe. CK = purified rabbit creatine phosphokinase. 28d = 28-d PM bovine myofibrils. MHC = myosin heavy chain. (a) An 18% gel of purified CK and the 28-d PM myofibrils. (b) Western blot from a duplicate gel reacted with mAb 9D10 to titin. Both CK and a 39 kDa band in the 28-d sample are recognized by 9D10 titin mAb.

Figure 6. Western blot prepared from an 8% gel of NS and ES samples labeled with T12 titin mAb. Lane T = titin standard. T1 = intact titin. T2 = an ~2,400 kDa titin degradation product. No bands in the T1 and T2 region can be detected at 14 d in both NS and ES myofibrils. This antibody also labels lower molecular weight titin degradation products (below ~1550 kDa) from 0 to 28 d PM. A major ~750 kDa band is marked.

Figure 7. Western blot prepared from an 8% gel of NS and ES samples labeled with NB2 nebuiin mAb. Intact nebuiin decreases by 7 d and is absent at 14 d in both NS and ES samples. Several lower molecular weight bands (~600 to ~380 kDa) with comparable mobility in NS and ES samples are also labeled from 7 to 28 d PM.
Figure 8. Western blots prepared from 18% gels of NS (a) and ES (b) samples were labeled with TN-T mAb. The TN-T band that comigrates with the purified TN-T standard (not shown) and the other two TN-T isoforms (one above, one below) decrease as PM time increases and are light bands at 28 d. A 30 kDa polypeptide is somewhat more heavily labeled from 7 d to 28 d in ES samples.

Figure 9. Western blots prepared from a single 18% gel of NS (a) and ES (b) samples labeled with polyclonal anti-desmin. The desmin band changes little and is still present at 28 days in both NS and ES samples.
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*Image 8a and 8b show samples of Troponin-T. Image 9a and 9b show samples of Desmin.*
duration. Contraction nodes were not seen in the 0-d samples from the first two animals that received the 15-sec stimulation. At 1 d PM, however, CN were present in muscle from six animals and from 3 through 28 d, CN were seen in muscle from all seven animals (Fig. 10C). We believe that the slower ATP decline in the first two animals may be responsible for the delayed appearance of CN. The number of CN ranges from 0 to 7 per grid opening in ES samples from 0 d to 28 d PM. In 0-d ES samples, the extremely shortened sarcomeres in CN have an average length of 1.2 ± 0.2 μm (n = 70), and stretched sarcomeres in the region adjacent to a CN within the same myofibril are 3.0 ± 0.2 μm (n = 52) long. Sarcomeres at greater distances from a CN and in myofibrils without a CN (2 to 6 myofibrils away) are similar to the sarcomeres seen in the 0-d NS samples and have an average sarcomere length of 2.6 ± 0.2 μm (n = 64). The sarcomere length in 1-d NS samples is 1.8 ± 0.1 μm (n = 78), and in 1-d ES samples, the sarcomere length away from nodes is 1.9 ± 0.2 μm (n= 82). During PM storage, sarcomere lengths were similar in NS and ES samples away from nodes, and were 1.7 ± 0.1 μm (n = 86) at 28 d in both NS and ES samples.

As PM time increases, intact Z-lines with a fibrillar structure as seen in 0-d samples (Fig. 10d) are progressively degraded, and by 3 d PM, Z-lines become amorphous, and 1 to 3 discontinuities or gaps occur in a typical Z-line (Fig. 10e). Evidence that Z-line degradation begins soon after slaughter is shown in Fig. 10f, where one to two discontinuities or gaps are seen in most Z-lines in NS muscle sampled 1 d PM. Z-line degradation is similar in both NS and ES samples, and by
28 d PM, Z-lines are amorphous with uneven edges and have two to five gaps in a
typical Z-line in both NS and ES samples (results not shown).

During PM storage, three types of I-band fractures adjacent to Z-lines
sequentially appeared in both NS and ES samples but appeared sooner and at
higher frequency in ES than in NS samples. In ES samples, narrow I-band
fractures with filaments in the fracture zone were already present in 1 to 2% of 1-d
myofibrils and had increased to 2 to 14% in 3-d myofibrils. From 7 to 28 d PM, 3 to
20% of ES myofibrils had narrow I-band fractures, some with but others without
filaments in the fracture zone. In NS samples, narrow I-band fractures, with or
without filaments in the fracture zone (Fig. 10g) were not seen until 7 d PM. The
frequency of narrow I-band fractures in NS muscle remained at 2 to 15% of
myofibrils from 7 through 28 d PM. Intermediate and wide I-band fractures were
present in 5 to 20% of 3-d ES myofibrils and in 5 to 30% of 7-d ES myofibrils. In
contrast to this, intermediate and wide I-band fractures were not detected in NS
myofibrils until 14 d PM (Fig. 10h), when the fractures were present in 5 to 20% of
myofibrils. Electrically stimulated muscle sampled at 14 d had intermediate and
wide I-band fractures in 10 to 40% of myofibrils. That same frequency of
intermediate and wide I-band fractures (10 to 40%) was observed in both ES and
NS samples at 28 d PM.
Figure 10. Structural changes in PM bovine skeletal muscle. (a) A NS 0-d sample shows uniform A-bands (A) and I-bands (I) and intact Z-lines (Z) in relaxed sarcomeres. Bar = 2 \( \mu m \). (b) ES 0-d muscle contains contraction nodes (CN), and the sarcomeres in the nodes are extremely shortened. Bar = 2 \( \mu m \). (c) Contraction nodes (CN) can still be seen in 28-d ES samples. Bar = 2 \( \mu m \). (d) Z-lines in 0-d NS samples contain a distinct fibrillar structure. Bar = 0.2 \( \mu m \). (e) Z-lines in 3-d ES samples become amorphous and have discontinuities (arrows). Bar = 0.5 \( \mu m \). (f) Z-lines in 1-d NS muscle already show a discontinuous structure (arrows). Bar = 1 \( \mu m \). (g) During PM storage, narrow I-band fractures (arrows) can not be seen until 7 d in NS samples. Bar = 2 \( \mu m \). (h) Intermediate width (arrowhead) and wide I-band (long arrow) fractures appear later, and can be detected by 14 d in NS samples. Bar = 3 \( \mu m \).
Discussion

Despite the relatively widespread use of ES on beef carcasses, there is limited agreement on how ES may be improving muscle tenderness. Although several investigators (Winer et al., 1981; Koch et al., 1982; Wheeler et al., 1990) agree that muscle from cattle with *Bos indicus* breeding is less tender than muscle from cattle with *Bos taurus* breeding, limited information is available on the effects of ES on muscle structure or on myofibrillar proteins in *Bos indicus* cattle. The objectives of this study were to determine the effect of ES on specific muscle proteins and on muscle structure in *Bos indicus* cattle and to compare these changes with those caused by ES of *Bos taurus* cattle in previous studies (Ho et al., 1994, 1995)

Protein changes in NS and ES samples

Our SDS-PAGE results showed that ES did not accelerate degradation of titin, nebulin, α-actinin, desmin, and TN-T and also had no detectable affect on the rate of disappearance of an ~1,200 kDa band and the appearance of an ~130 and a 39 kDa band in B x S samples. However, ES somewhat enhanced the accumulation of the 30 kDa polypeptide in B x S samples during PM storage as shown by Western blotting (Fig. 8). The T1 band (intact titin) was gone by 14 d PM in both NS and ES samples, which is in agreement with Bandman and Zdanis (1988) who observed that, by two to three wk PM, no undegraded titin could be detected in bovine semitendinosus muscle stored at 4°C. Huff-Lonergan et al. (1995) found that in bovine muscle, T1 was absent or nearly absent by 7 to 14 d, and Taylor et al.
and Taylor et al. (1995) also observed that T1 in bovine muscle was entirely degraded to the T2 form between 24 and 72 h PM. The PM degradation of titin reported by Bandman and Zdanis (1988), by Huff-Lonergan et al. (1995) and by Taylor et al. (1995) is consistent with our results from NS B x S samples reported here and from NS *Bos taurus* A x J cattle observed previously (Ho et al., 1995). Fritz and Greaser (1991), however, reported that after two wk PM, some undegraded titin could still be seen in bovine psoas major muscle stored at 4°C. The muscles used in those studies were from cattle of unknown breeding, which prevents definitive comparison with our results from *Bos indicus* crossbred cattle.

Several studies have suggested that the extent of titin degradation may be associated with increasing meat tenderness during aging (Locker et al., 1977; Takahashi and Saito, 1979; Paterson and Parrish, 1987). Fritz et al. (1993) reported that the titin content of bovine muscle as measured by SDS-PAGE had no relationship to meat tenderness, but later reports (Huff-Lonergan et al., 1995; Taylor et al., 1995) have raised concerns about the identification of intact titin by Fritz et al. (1993).

In both NS and ES B x S samples, the intact nebulin band disappeared more rapidly than titin during PM storage, which is in agreement with the observations of Lusby et al. (1983), Locker and Wild (1984), and Fritz and Greaser (1991) on PM bovine muscle of unknown heritage. The degradation patterns of nebulin in Western blots from both NS and ES B x S samples (Fig. 7) are similar to the results of Fritz and Greaser (1991). Huff-Lonergan et al. (1995) found that intact nebulin
was almost gone at 3 d in tender bovine muscle but was not absent until 7 d in tough bovine muscle. Their findings are consistent with our results in NS samples from A x J (Ho et al., 1995) and from B x S cattle, respectively.

Valin and Ouali (1992) reported that the amount of a 30 kDa band is significantly correlated with a mechanical (r = .90) or biochemical (r = .94) meat tenderness index, and suggested that the accumulation of the 30 kDa polypeptide during aging is a useful index for meat tenderness. MacBride and Parrish (1977) also found that at 1 d PM, a 30 kDa band was present in tender bovine muscle but was absent in tough bovine muscle and suggested that the difference between tough and tender meat was closely associated with the appearance of a 30 kDa polypeptide. Although our SDS-PAGE results (Fig. 2) showed no obvious difference between NS and ES B x S samples in either TN-T degradation or 30 kDa polypeptide accumulation, our sensitive Western blot results (Fig. 8) showed that ES somewhat enhanced the accumulation of the 30 kDa polypeptide in B x S samples during PM storage. We previously demonstrated that the 30 kDa polypeptide comes from TN-T (Ho et al., 1994). The somewhat enhanced accumulation of the 30 kDa band in B x S samples (Fig. 8b) suggests that ES may slightly accelerate TN-T degradation. Mikami et al. (1990) and Uytterhaegen et al. (1992) both observed that ES enhanced the disappearance of TN-T and the appearance of the 30 kDa polypeptide in muscle from *Bos taurus* cattle by SDS-PAGE analysis. Geesink et al. (1993), however, reported that ES did not enhance the accumulation of the 30 kDa polypeptide in either control or clenbuterol-treated
bovine muscle from *Bos taurus* crossbred veal calves. Our previous SDS-PAGE results from A x J (*Bos taurus* cross) samples showed that ES slightly accelerated TN-T degradation but did not detect any enhancement of the appearance of the 30 kDa polypeptide (Ho et al., 1994). Taken together with the current study, the effect of ES on TN-T and the 30 kDa polypeptide seems to be somewhat variable.

Although desmin is extremely susceptible to a variety of proteolytic enzymes including calpain (O'Shea et al., 1979; Robson and Huiatt, 1983), and ES reportedly can increase the activity of proteolytic enzymes in bovine muscle (Dransfield, 1992; Dransfield et al., 1992; Smulders and van Laack, 1992; Uytterhaegen et al., 1992), our SDS-PAGE (Fig. 2) and Western blot results (Fig. 9) showed that ES did not accelerate desmin degradation in B x S samples during PM storage. We could still detect a desmin band at 28 d PM by SDS-PAGE analysis of B x S samples (Fig. 2). Western blots from B x S samples showed little or no changes in desmin during 28 d storage of NS and ES B x S samples (Figs. 9a and 9b). This is in contrast with our previous Western blot results from A x J samples, which showed that desmin decreased during storage and was absent by 28 d PM (Ho et al., 1995). In agreement with our results, Koohmaraie et al. (1991) previously showed that desmin was almost gone at 14 d PM in muscle samples from *Bos taurus* crossbred cattle. Whipple et al. (1990) also found that, by 14 d PM, desmin was absent in samples from *Bos taurus* crossbred cattle but was still present in muscle samples from crossbred cattle that contained a higher percentage of *Bos indicus* breeding. Robson et al. (1984) reported that desmin
was nearly absent by 7 d in bovine semitendinosus muscle, but the muscle was stored at 15°C. Hwan and Bandman (1989) found little undegraded desmin at three wk PM in bovine semitendinosus muscle stored at 4°C, but the study did not specify the breed of cattle used; thus, the relationship to our results is unclear.

To summarize, ES did not accelerate the PM degradation of titin, nebulin, desmin or TN-T in muscle from B x S cattle. In contrast, ES slightly accelerated the PM degradation of these proteins in muscle from A x J cattle (Ho et al., 1994, 1995). In NS muscle samples, titin, nebulin, desmin, and TN-T were degraded more slowly in B x S muscle than we observed previously in A x J muscle (Ho et al., 1994, 1995). Degradation products with molecular masses of 130 kDa, 39 kDa and 30 kDa appeared later during storage of B x S samples than in A x J samples. A 38 kDa desmin degradation product and a 15 kDa TN-T degradation product were present in A x J samples but were not detected in any B x S samples.

Structural Changes in NS and ES samples

Valin and Ouali (1992) indicated that, among many biochemical and ultrastructural changes involved in meat tenderization, I-band fractures at or near the N₂ line are the key factor for improving meat tenderness. Savell et al. (1978) and Takahashi et al. (1987) found that ES caused the formation of CN, that sarcomeres in the internodal zones were stretched or fractured, and that, under suitable treatment conditions, tenderness was increased. Our results showed that, in B x S samples, ES not only caused the formation of CN and I-band fractures but also accelerated the appearance and increased the frequency of three types
(narrow, intermediate, and wide) of I-band fractures. I-band fractures in B x S samples appeared later PM and/or at a lower frequency than in the A x J samples studied previously (Ho et al., 1995). This observation is consistent with Wheeler et al. (1990), who reported that, at 14 d PM, more I-band fractures were present in muscle samples from Bos taurus cattle than from Bos indicus cattle.

Electrical stimulation did not, however, enhance Z-line degradation and had no effect on sarcomere length in B x S samples when compared with NS samples. We found that Z-lines were degraded progressively and at similar rates during PM storage in both the B x S and the A x J samples (Ho et al., 1995). The progressive loss of Z-line structure during PM muscle storage is a well-established phenomenon (Stromer et al., 1967; Davey and Gilbert, 1967, 1969; Davey and Dickson, 1970; reviewed by Goll et al., 1977; Koohmaraie, 1992). It is possible that differences in sample preparation or sampling for structural evaluation may have caused Wheeler et al. (1990) to conclude that no significant loss of Z-line material occurred by 14 d PM in muscle from either Bos taurus or Bos indicus cattle, and Taylor et al. (1995) to find no changes in Z-lines at 16 d PM.

The positive relationship between I-band fractures and meat tenderness is an attractive hypothesis (Savell et al., 1978; Takahashi et al., 1987) that is supported by our data. Star probe measurements on both raw and cooked B x S steaks gave lower average force values for ES than NS steaks. The incidence of narrow I-band fractures was greater in ES samples at each sampling time. Intermediate and wide I-band fractures in ES B x S samples were seen sooner and at greater frequencies
than in NS samples during the first 14 d of PM storage. Similarly, in the ES A x J samples studied previously (Ho et al., 1995), narrow I-band fractures were consistently more numerous at each sampling time, and intermediate and wide I-band fractures were also seen sooner and at greater frequencies during the first 7 d PM. The observation by Whipple et al (1990) that NS Bos indicus LM is less tender than NS Bos taurus LM correlates well with the consistently lower incidence of I-band fractures in our B x S samples. This is especially notable in 28 d PM samples when the incidence of intermediate and wide I-band fractures in B x S samples was less than half that observed in A x J samples (Ho et al., 1995).

Our data suggest that there may be a relationship between the degradation of certain proteins and the appearance of I-band fractures in B x S samples. The T1 band of titin in NS samples was absent at 14 d PM (Fig. 1) and both intermediate and wide I-band fractures were first seen at 14 d PM (Fig. 10h). Nebulin was nearly gone by 7 d PM in NS samples, and narrow I-band fractures were not seen until 7 d PM. At variance with this pattern is the observation that T1 and nebulin degraded at the same rate in ES as in NS B x S samples, but all types of I-band fractures appeared sooner in ES samples. We previously found that the disappearance of T1 and nebulin were closely related temporally to the appearance of intermediate and wide I-band fractures in both NS and ES A x J samples (Ho et al., 1995).

In conclusion, titin, nebulin, desmin, and TN-T degraded at similar rates PM in NS and ES B x S muscle samples. Titin, nebulin, desmin, and TN-T degraded
slower in both NS and ES muscle samples from B x S cattle than from A x J cattle. Z-line degradation occurred during PM storage but was unaffected by ES. I-band fractures occurred sooner in ES B x S muscle samples. The frequency of all I-band fracture types was higher in ES samples at each sampling time except at 28 d PM where the frequency of intermediate and wide fractures was identical.

Implications

The results of this paper indicate that the rate of titin, nebulin, desmin, and troponin-T degradation and the appearance and the frequency of narrow, intermediate and wide of I-band fractures may contribute to the differences in meat tenderness between Bos taurus and Bos indicus crossbred cattle. In Brahman x Simmental samples, electrical stimulation enhanced the appearance and the frequency of all three types of I-band fractures but had no detectable influence on degradation of these key structural proteins. This suggests that improved meat tenderness by electrical stimulation may be principally due to the mechanical disruption in muscle from Brahman x Simmental cattle.
133

Literature Cited


GENERAL SUMMARY

The major objectives of this study were: (1) to use antibodies to selected myofibrillar proteins to positively identify the source of the 30 kDa polypeptide; (2) to investigate the effect of electrical stimulation on postmortem (PM) changes in titin, nebulin, desmin and troponin-T and in myofibrillar structure in bovine skeletal muscle from Angus x Jersey (AxJ) (*Bos taurus* cross) - a tender line of cattle; (3) to determine the degradation patterns of titin, nebulin, desmin and troponin-T and structural changes during PM storage of both nonstimulated (NS) and electrically stimulated (ES) bovine muscle samples from Brahman x Simmental (BxS) (*Bos indicus* cross) - a less tender line of cattle and to compare these changes with the changes in muscle samples from AxJ cattle. SDS-PAGE to follow the changes of these proteins during PM storage, Western blots with specific antibodies to positively identify these proteins and their degradation products, and transmission electron microscopy of muscle samples to determine structural changes all were used on the samples included in this study.

The results reported in paper one (muscle samples from AxJ cattle) showed that intact troponin-T was degraded to a family of smaller polypeptides and that a principal component of this family is the 30 kDa polypeptide. Labeling the 30 kDa band electrophoretically purified from PM bovine muscle with a troponin-T mAb positively identified the 30 kDa polypeptide as a degradation product of troponin-T. This is the first conclusive identification of the origin of the 30 kDa polypeptide. In SDS-PAGE, ES slightly accelerated the degradation of troponin-T, and in Western
blots, ES enhanced the accumulation of the 30 kDa polypeptide during PM storage. Degradation of troponin-T and the concomitant appearance of the 30 kDa polypeptide, a troponin-T degradation product, are excellent indicators of PM proteolysis. Because troponin-T is an important component of thin filaments which are anchored to the Z line, which, in turn, is a site of early PM structural alteration, degradation of troponin-T near the Z line may be involved in PM tenderization.

The data in paper two (muscle samples from AxJ cattle) demonstrated that titin, nebulin and desmin, but not α-actinin, were degraded PM. Taken together with the results of paper one, SDS-PAGE analysis showed that ES slightly accelerated the degradation of titin, nebulin, desmin and troponin-T during PM storage. ES caused the formation of contraction nodes and produced variable sarcomere lengths in 0 day muscle samples. The frequency of narrow, intermediate and wide l-band fractures was higher in ES than in NS muscle samples at each sampling time except at 14 and 28 days PM where the frequency of intermediate and wide l-band fractures was identical in NS and ES samples. Wide l-band fractures were seen sooner PM in ES than in NS muscle samples. Z-line degradation occurred at similar rates in NS and ES samples. Formation of both contraction nodes and l-band fractures are the two principal structural alterations caused by ES.

The results included in the third paper (muscle samples from BxS cattle) showed that titin, nebulin, desmin and troponin-T were degraded at similar rates in NS and ES samples, but Western blot analysis demonstrated that accumulation of
the 30 kDa polypeptide was higher in ES than in NS samples. In agreement with many previous studies, Z-line degradation which included loss of fibrillar structure and the formation of transverse gaps or spaces in the Z line occurred during PM storage but was unaffected by ES. Narrow, intermediate and wide l-band fractures occurred sooner PM in ES than in NS muscle samples. The frequency of all three types of l-band fractures was higher in ES samples at each sampling time except at 28 days PM where the frequency of intermediate and wide l-band fractures was identical in NS and ES samples.

When comparing these two lines of cattle, titin, nebulin, desmin and troponin-T degraded slower in NS and ES BxS samples than in NS and ES AxJ samples. SDS-PAGE analysis showed that ES slightly accelerated the degradation of titin, nebulin, desmin and troponin-T in AxJ samples but had no detectable affect on these proteins in BxS samples. In Western blots, ES enhanced the accumulation of the 30 kDa polypeptide in muscle samples from both lines of cattle during PM aging. Because we demonstrated that the 30 kDa band comes from troponin-T, this result suggests that ES may slightly accelerate troponin-T degradation in both lines of cattle. Z lines were degraded at similar rates PM in NS and ES samples from both lines of cattle. Narrow, intermediate and wide l-band fractures were seen sooner in NS AxJ samples than in NS BxS samples. ES accelerated the appearance of only wide l-band fractures in AxJ samples but accelerated the appearance of all three types of l-band fractures in BxS samples. The frequency of all l-band fracture types was higher in ES samples from both lines of cattle except
at 14 and 28 days in AxJ samples and at 28 days in BxS samples where frequency of intermediate and wide fractures were identical in NS and ES samples. The frequency of all three types of I-band fractures in both NS and ES AxJ samples was higher than in NS and ES BxS samples. Intermediate and wide I-band fractures were present at twice the frequency in 3, 7, 14 and 28 day NS and ES AxJ samples than in BxS samples.

Overall, the results of this study suggest that differences in meat tenderness between *Bos taurus* and *Bos indicus* crossbred cattle may be due mainly to the appearance and the frequency of three types of I-band fractures but also to the extent of titin, nebulin, desmin and troponin-T degradation during PM storage. ES may improve meat tenderness by mechanical disruption, increased proteolysis of these structural proteins or a combination of both in muscle samples from *Bos taurus* crossbred cattle but principally by mechanical disruption in muscle samples from *Bos indicus* crossbred cattle.
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