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Effect of postmortem storage on degradation of the recently discovered myofibrillar protein titin in bovine longissimus muscle

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Lusby, Mary Lee Gragg

**EFFECT OF POSTMORTEM STORAGE ON DEGRADATION OF THE
RECENTLY DISCOVERED MYOFIBRILLAR PROTEIN TITIN IN BOVINE
LONGISSIMUS MUSCLE**

Iowa State University

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Effect of postmortem storage on degradation of the recently
discovered myofibrillar protein titin in bovine longissimus muscle

by

Mary Lee Gragg Lusby

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
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LIST OF ABBREVIATIONS

A	angstroms
CAF	calcium activated factor
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPNP	1,2-epoxy-3(p-nitrophenoxy)propane
g	acceleration due to gravity = $980 \text{ cm} \cdot \text{sec}^{-2}$
M_r	molecular weight
org	microbial organisms
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
SDS-PAGE	polyacrylamide gel electrophoresis in presence of SDS
Tris	tris-(hydroxymethyl)-aminomethane
TSA	trypticase soy agar
w/v	weight/volume
v/v	volume/volume

INTRODUCTION

An extremely large myofibrillar protein was recently discovered in chicken and rabbit skeletal muscle by Wang et al. (1979) and named titin. Other researchers (Maruyama et al., 1976a, b, 1977a, b, 1981b, Toyoda and Maruyama, 1978) have also noted the existence of very large elastic-like proteins, which they named connectin, that may be responsible for at least some of the structural continuity and integrity of muscle. As defined by electrophoretic analysis, titin is a high molecular weight doublet ($M_r \sim 1.0 \times 10^6$) found in both vertebrate and invertebrate muscles (Wang et al., 1979). Although antibody localization patterns with specific titin antibodies are seemingly complex to interpret, they suggest that titin is a major structural protein in the myofibril (Wang et al., 1979). It has been postulated by Wang and Ramirez-Mitchell (1979, 1981, 1982a, 1983) that titin may be the major cytoskeletal protein comprising a set of very thin, longitudinal running elastic filaments in the sarcomere. Thus, titin may be important in maintaining the structural integrity of the muscle cell. Titin has also been shown to be highly susceptible to proteolytic degradation by endogenous proteases (Wang, 1982a, b; Wang and Ramirez-Mitchell, 1983; Zeece et al., 1983). There have been several reports over the last thirty years of a set of filaments that are distinct from the well-known thick and thin filaments. Locker and his associates (Locker and Leet, 1975, 1976a, b; Locker et al., 1977; Locker, 1982) refer to these additional filaments as "gap filaments" and have suggested that connectin (or titin) is the protein constituting these filaments.

Furthermore, these workers contend that the structural condition of gap filaments controls meat tenderization.

Because titin may have a cytoskeletal role in muscle cells and is susceptible to proteolysis, its degradation postmortem may be important in tenderization and in the improvement of other quality characteristics of muscle that occur during the aging process. Tenderness is one of the most important quality characteristics in meat palatability. It is well-known that a number of variables affect meat tenderness; however, the increase that occurs in tenderness during postmortem storage is still not fully understood. This increase in tenderness during storage is usually considered a consequence of changes in myofibrillar components of muscle. In fact, in muscles having very little connective tissue, the myofibrillar proteins may be responsible for 90-95% of the variation in tenderness (Stromer et al., 1974). Furthermore, the term "actomyosin toughness" has been used in an effort to emphasize the major role myofibrillar proteins assert in meat tenderization (Marsh and Leet, 1966). It is widely accepted that the mechanism of degradation of myofibrillar proteins during postmortem storage is the action of endogenous proteases such as cathepsins and calcium activated factor (CAF). Olson et al. (1977) were the first to provide evidence that CAF was the protease causing fragmentation of myofibrils at or near the Z-line and the degradation of troponin-T to a 30,000 dalton component. Moreover, Olson and Parrish (1977) devised a method to objectively determine myofibril fragmentation that accounted for about 50% of the variation in beefsteak tenderness. MacBride and Parrish (1977) showed that a 30,000 dalton component was found only in

tender beef longissimus muscle. Consequently, MacBride and Parrish (1977) introduced the term "myofibril fragmentation tenderness" to explain the fragmentation of the myofibril at or near the Z-line during postmortem storage of conventionally aged bovine muscle. More information about the extent and role of proteolysis and the proteins undergoing degradation in postmortem muscle, however, is needed to provide clarity to the mechanism of tenderization and the changes in processing characteristics of meat.

Because titin is a major myofibrillar protein and the proteolytic degradation of this extremely large polypeptide during postmortem storage may be related to meat tenderization and quality, a study was conducted with the following four primary objectives:

1. Discover if titin in bovine muscle was present and similar to titin identified by Wang et al. (1979) in chicken and rabbit skeletal muscle.
2. Determine the localization of titin in skeletal muscle.
3. Investigate the characteristics of titin from different muscles, muscle fiber types and species.
4. Examine the effects of postmortem aging time and temperature on the integrity of titin polypeptides in bovine skeletal muscle.

LITERATURE REVIEW

Muscle Structure and Biochemistry of Myofibrillar Proteins

A review of the fundamental structural features of skeletal muscle and the biochemistry of myofibrillar proteins is necessary in order to provide a more comprehensive understanding to the contribution of the muscle protein titin in postmortem tenderization of meat. The following brief discussion of skeletal muscle structure is based on reviews of the microstructure of muscle and muscle components by Briskey and Fukazawa (1971), Huxley (1972) and Gould (1973).

Skeletal muscle is surrounded by a connective tissue sheath, termed the epimysium. At various locations, the epimysium invaginates into the body of the muscle, separating it into muscle bundles or fasciculi. A layer of connective tissue, the perimysium, then surrounds each fasciculus. From the perimysium, a fine connective tissue, designated as the endomysium, anastomoses and surrounds each muscle fiber (cell). The endomysium lies just outside the cell membrane that is often referred to as the sarcolemma.

The smallest unit of structure in a muscle that can give normal physiological response is the single muscle fiber (Huxley, 1972). Muscle fibers are long, cylindrical, nonbranching cells with tapering conical ends. The lengths of the muscle fibers can measure up to several centimeters, depending on the lengths of the muscles from which they are derived. Muscle cell diameters characteristically range from 10 to 100 μm . Each fiber is multinucleated, formed by fusion of mononucleated myoblasts during embryonic development. Skeletal muscle fibers contain the normal constituents of many other cell types such as mitochondria (sarcosomes), Golgi apparatus,

ribosomes and glycogen granules. They also contain an elaborate internal membrane system, the sarcotubular system, which is involved in regulation of the contractile mechanism. The contractile components of the muscle cell are highly organized structures known as myofibrils. Myofibrils are elongated protein threads, 1 to 3 μm in diameter, lying parallel to one another along the long axis of the muscle cell. Each myofibril travels the entire length of the cell. Myofibrils have no membranes surrounding them; however, they exist as structural entities because they are insoluble at the ionic strength of the muscle cell.

Myofibrils are cross-striated and consist of alternating light and dark bands when viewed in the phase microscope. The overall striated appearance of the skeletal muscle cell is due to the precise alignment of the light and dark bands of adjacent myofibrils. In the polarizing microscope, the light bands are weakly birefringent (isotropic) and are termed the I-bands. The dark bands are strongly birefringent (anisotropic) and are called the A-bands. Bisecting the center of the I-band is a dark line called the Z-line or Z-disk. A lighter zone exists in the center of each A-band, approximately 0.35 μm wide at rest length, and is known as the H-zone. A narrow dense line of protein, the M-line, is observed with electron microscopy in the center of the H-zone. The distance between one Z-line and the next Z-line is defined as a sarcomere which represents the repeating contractile unit of a myofibril. Sarcomeres vary in length from about 2.3 to 2.8 μm in normal rest length muscle and from 1.8 to 2.0 μm when muscle is contracted. Page (1968) identified two dense lines, termed N-lines, on either side and parallel to the Z-lines. The N_1 -line is narrow, 0.05 μm , and is fixed in position during contraction, 0.1 -

0.2 μm from the center of the Z-line. In contrast, the N_2 -line, which is broader (up to 0.15 μm) and located between the N_1 and the edge of the A band, varies in position as the sarcomere length changes (Franzini-Armstrong, 1970). The N_2 -line, however, maintains the same proportional distance from the Z-line and M-line at different sarcomere lengths (Franzini-Armstrong, 1970; Locker and Leet, 1976b; Wang, 1981).

Examination with the electron microscope has shown that myofibrils are composed of long filaments (myofilaments) lying side by side and extending longitudinally parallel with the long axis of the myofibril. Huxley (1953) first described the striations of these myofibrils as a double array of interdigitating thick and thin filaments. The thin filaments (60 - 80 A in diameter, 1.0 μm long) are attached at one end to the Z-line and extend into the A-band between adjacent thick filaments. The thick filaments (140 - 160 A in diameter, 1.5 μm long), however, comprise the A-band and are not attached to the Z-line. The I-band contains only the thin filaments and the Z-line, whereas in the A-band the thin filaments, thick filaments, H-zone and M-line are visible (Huxley, 1957, 1963, 1972).

Muscle cells have a specialized sarcotubular system which consists of two sets of membrane-limited tubules (Franzini-Armstrong, 1973). The transverse system (T-system or T-tubules) consists of tubules formed by periodic invaginations of the sarcolemma and which run into the interior of the cell in a direction perpendicular to the long axis of the muscle fiber. T-tubules occur at the level of the Z-lines in some muscles such as amphibian skeletal muscles, and at the level of every A-I junction in others such as in mammalian skeletal muscles. They function to spread nerve impulses quickly from the sarcolemma to myofibrils. The longitudinal

system (sarcoplasmic reticulum), the smooth endoplasmic reticulum of skeletal muscle cells, is an extensive network of membrane-limited tubules that surrounds each myofibril and that extends from one T-tubule to the next. As the sarcoplasmic reticulum membranes approach the T-tubule, they coalesce to form a large sac termed the lateral cisternae adjacent to each side of the T-tubule. A fenestrated collar around the center of the A band is also formed by the anastomoses of the longitudinal system from both ends of the A-band. The T-tubule with lateral cisternae on either side of it is called a triad. The membranes of the sarcoplasmic reticulum are able to accumulate Ca^{2+} against a concentration gradient. After receiving a nerve impulse, depolarization of the membrane occurs and Ca^{2+} is released into the muscle cell. The free Ca^{2+} is reaccumulated in the lateral cisternae when membranes are repolarized. The release and reaccumulation of Ca^{2+} serves to initiate or terminate contraction.

Muscle contraction occurs when the interdigitating thick and thin filaments slide past one another to cause shortening. The sarcomere length decreases during contraction, but the length of the thick and thin filaments remains constant (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954). As the sarcomere shortens, the H-zone narrows as the thin filaments penetrate further into the A-band. The H-zone eventually disappears if the thin filaments from each side of the sarcomere reach each other in the center of the A-band. Also during contraction, the I-band narrows as the ends of the thick filaments approach the Z-lines.

Muscle proteins have been classified into three groups based on their solubility in salt solutions of various ionic strengths: sarcoplasmic,

myofibrillar and stromal proteins (Szent-Gyorgi, 1960; Briskey, 1967; Goll et al., 1969).

Sarcoplasmic proteins make up 30-35% of the total muscle proteins (Stromer et al., 1974) and are soluble in a neutral salt solution having an ionic strength below 0.2. The sarcoplasm contains over 100 different proteins including myoglobin and metabolic enzymes.

The myofibrillar proteins, which are soluble in salt solutions having an ionic strength of 0.4 to 1.5, constitute 50-58% of the total muscle proteins and are therefore the largest class of muscle proteins (Briskey, 1967). In this protein fraction, there are at least 12 myofibrillar proteins in skeletal muscle, namely, myosin, actin, tropomyosin, troponin, titin (connectin), nebulin, α -actinin, desmin, filamin, C-protein and two M-line proteins.

Myosin is the primary protein component of the thick filament (Hanson and Huxley, 1953) and represents 45% of the myofibrillar proteins (Greaser et al., 1981). Myosin is characterized by its actin-binding ability, by the possession of an actin-activated ATPase and by its ability to self-assemble into thick filaments at the intracellular ionic strength of the muscle cell. The native myosin molecule consists of a long double-stranded, α -helical rod-like tail connected to two globular heads (Lowey et al., 1969). The tails of adjacent myosin molecules aggregate to form the shaft of the thick filament and the heads of the myosin molecules are bent outward to form the cross-bridges. Muscle myosin has a molecular weight of 470,000 and consists of two large subunits of approximately 200,000 daltons each and four smaller subunits (light chains) ranging in molecular weight from 16,000 to 25,000 (Lowey and Risby, 1971).

Actin represents 20% of the total myofibrillar proteins (Greaser et al., 1981) and is the major component of the thin filaments. Globular or G-actin is a single polypeptide chain with a molecular weight of approximately 42,000. One G-actin molecule contains one molecule of Ca^{2+} and one of ATP, and polymerizes to form a double helical filamentous, or F-actin, polymer by the addition of 0.1 M KCl or 1 mM Mg^{2+} (Laki, 1971). In contrast to striated muscle cells where essentially all of the actin is present as F-actin, both globular and filamentous actins are present in a large number of other cell types. The interaction of myosin and actin in muscle cells activates the Mg^{2+} -dependent myosin ATPase resulting in a contractile response. This contractile response continues until the ATP is depleted. Actin, along with forming the backbone of each thin filament, also interacts with tropomyosin and troponin. The Ca^{2+} -dependent interaction of myosin and actin is controlled by tropomyosin and troponin, the regulatory proteins in striated muscle (Ebashi and Endo, 1968).

Tropomyosin, a rod-shaped protein molecule located in each of the two grooves of the F-actin helix in thin filaments (Spudich et al., 1972), makes up 5% of the total myofibrillar proteins (Greaser et al., 1981). The tropomyosin molecule consists of two polypeptide chains arranged to form a two stranded coiled-coil (Crick, 1953; Cohen and Szent-Gyorgyi, 1957; Mak et al., 1980). Two major polypeptide forms can be detected electrophoretically by SDS-PAGE in skeletal muscle; α -tropomyosin has a molecular weight of approximately 33,000 while β -tropomyosin has a molecular weight of approximately 36,000 (Cummins and Perry, 1973). Different muscle types, such as red and white, have been shown to have very different ratios of the two forms of tropomyosin (Cummins and Perry, 1974).

Troponin is a globular protein having a molecular weight of 80,000 and it constitutes 5% of the total myofibrillar protein (Greaser et al., 1981). The troponin molecule is composed of three polypeptide chains present in equimolar concentrations, each having a specific function (Greaser and Gergely, 1971; Potter and Gergely, 1974). As determined by SDS-PAGE, troponin-T (TN-T), troponin-I (TN-I) and troponin-C (TN-C) have molecular weights of approximately 37,000, 24,000, and 18,000, respectively. TN-T attaches the troponin complex to tropomyosin. TN-I, with the assistance of tropomyosin and the other troponin subunits, inhibits actomyosin APTase by inhibiting actin-myosin interaction. TN-C is capable of reversibly binding calcium ions. A nervous stimulus results in an influx of Ca^{2+} ions from the sarcoplasmic reticulum whereupon the TN-C subunit binds the released Ca^{2+} ion, causing a conformational change to occur in the polypeptide. TN-I is then released from actin, there is movement of the tropomyosin strands into the actin grooves of the thin filament which exposes myosin binding sites on the actin filament, and actin and myosin interact repetitively to cause shortening of the sarcomere (Parry and Squire, 1973; Weber and Murray, 1973).

Immunofluorescence studies have shown that α -actinin is associated with actin and is the major protein limited in myofibrillar location to the Z-line (Schollmeyer et al., 1973; Suzuki et al., 1976). The molecule makes up 2% of the myofibrillar protein (Greaser et al., 1981) and is about 3.5 nm wide and 40 nm long (Suzuki et al., 1976). α -Actinin, first purified by Robson et al. (1970), contains approximately 75% α -helical secondary structure and is composed of two subunits with equal molecular weights (100,000). It has been postulated that in vivo, α -actinin has a structural

role of anchoring thin filaments from opposing sarcomeres at the Z-line (Craig-Schmidt et al., 1981; Yamaguchi et al., 1983). Desmin (O'Shea et al., 1979, 1981; Robson et al., 1981, 1983) and filamin (Wang et al., 1975; Bechtel, 1979; Zeece et al., 1979) with subunit molecular weights of approximately 55,000 and 250,000, respectively, each represents less than 1% of the total myofibrillar protein. Desmin comprises a set of intermediate filaments, 10 nm, and evidence suggests that these intermediate filaments connect adjacent myofibrils at the Z-line level (Huiatt et al., 1980; O'Shea et al., 1981; Richardson et al., 1981; Robson et al., 1980, 1981, 1983). Filamin, initially isolated from avian smooth muscle by Wang et al. (1975), is a high molecular weight actin-binding protein with two subunits of 250,000 daltons. Although filamin was recently localized by indirect immunofluorescence in the Z-line region of chicken skeletal muscle (Bechtel, 1979), its function at the Z-line is not known.

C-protein represents 2% of the total myofibrillar proteins (Greaser et al., 1981) and has a subunit molecular weight of about 140,000. The C-protein molecule is localized in the thick filament in seven bands, about 43 nm apart, in each half of each bipolar thick filament (Craig and Offer, 1976). Various roles have been suggested for C-protein such as influencing myosin cross-bridge movement, effecting the actual actin-myosin interaction, and maintaining the thick filament shape during muscle contraction (Moos et al., 1978; Starr and Offer, 1978; Moos and Feng, 1980; Moos, 1981).

The M-line proteins make up less than 3% of the myofibrillar proteins (Greaser et al., 1981) and are also associated with the thick filament. When longitudinal sections of muscle are examined by electron microscopy, the M-line appears as three to five lines perpendicular to the long axis

of thick filament. The M-line structure contains two major protein components, creatine kinase, with a molecular weight of approximately 43,000 (Walliman et al., 1978) and myomesin with a subunit molecular weight of approximately 170,000 (Trinick and Lowey, 1977; Eppenberger et al., 1981).

High molecular weight myofibrillar proteins, titin and nebulin, have recently been isolated from skeletal muscle and may account for approximately 10-15% of the myofibrillar proteins (Wang et al., 1979). Studies of Maruyama et al. (1981b) indicated that some of the high molecular weight components of the highly insoluble elastic protein isolated from muscle, connectin, are identical to titin. Titin and nebulin with molecular weights of approximately 1.0×10^6 and 5×10^5 , respectively, as determined by SDS-PAGE (Wang and Williamson, 1980; Maruyama et al., 1981b; Wang, 1981) along with connectin will be reviewed in subsequent sections.

The stromal proteins make up 10-20% of the total muscle protein and are insoluble in neutral aqueous solvents (Stromer et al., 1974). These proteins comprise the connective tissue located in the epimysium, perimysium and endomysium (Goll, 1962). Collagen is the major fibrous element of connective tissue and is perhaps the most abundant single protein in mammals, representing up to 20-25% of the total body protein (Seifter and Gallup, 1966).

The basic molecular unit of collagen is tropocollagen. The tropocollagen molecule is a fairly rigid rod with a molecular weight of 300,000, length of 280-300 nm and a diameter of 1.4 - 1.5 nm. Within this molecule are a group of three polypeptide chains, each of a molecular weight of

100,000, coiled around one another to form a triple-helical structure (Rice et al., 1964). Proline and hydroxyproline make up approximately one-fourth of the amino acids in the collagen molecule and prevent easy rotation in the regions where they are located. The higher the content of proline and hydroxyproline, the higher the resistance of the molecule to heat or chemical denaturation. The collagen fiber is, however, easily converted to a soluble form, gelatin, by heating in aqueous solution. Formation of intermolecular covalent bonds between tropocollagen molecules is a characteristic of collagen maturation. Mature collagen fibrils may be represented as assemblies of tropocollagen molecules joined together by a variety of intermolecular covalent cross-linkages to form asymmetrical polymer networks. No significant increase in the amount of collagen occurs as animal age increases (Goll, 1965; Hill, 1966). Intermolecular cross-linking, however, increases with animal maturity, significantly decreasing collagen solubility until eventually it can only be dissolved using strong denaturants (Goll et al., 1964a; Hill, 1966; Herring et al., 1967; McClain, 1976).

Third Set of Filaments in the Myofibril

For many years, researchers have speculated on the existence of an elastic protein component within the myofibril that would account for the elastic properties of the muscle during passive stretch. One of the earliest reports on this subject was by Huxley and Hanson in 1954. Upon observing that myofibrils were reversibly extended after myosin removal, they proposed that a set of very fine elastic filaments provided continuity between two sets of actin filaments in the sarcomere. These elastic

filaments were termed "S" filaments because they stretched (Huxley and Hanson, 1954), and it was suggested that the "S" filaments were cross-linked to myosin filaments at some point in the H-zone (Hanson and Huxley, 1955). The S-substance was estimated by interference microscopy to be 3% of the myofibrillar protein (Huxley and Hanson, 1957). Huxley and Peachey (1961) first obtained evidence of the proposed "S" filaments while investigating the ability of stretched muscle fibers to contract. They observed fine filaments connecting the ends of the two major sets of filaments. Some reservations were later expressed by Huxley (1962) based on his observations of double overlap of the thin filaments of the I-band which could not easily accommodate the "S" filaments postulated to connect the ends of opposite I filaments across the H-zone.

In the 1960s, several groups described filaments visible in electron micrographs of highly stretched or extracted muscle that were apparently distinct from the thick and thin sets of filaments. Sjöstrand (1962) stretched frog semitendinosus muscle in situ to the point where a gap developed between the A-band and I-band filaments. Fine filaments were observed bridging this gap that were thinner than the filaments of the I-band. The thickness of these thin filaments varied; the thinnest measured 30 A or less in diameter compared with 70 A for actin filaments. These thin filaments appeared to be continuous with A-band filaments at one end and with I-band filaments at the other. The sliding filament model for muscle contraction (Huxley and Hanson, 1954; Hanson and Huxley, 1955), however, assumes no such continuity of the A- and I-band filaments. Huxley (1964) later suggested that the filaments observed by Sjöstrand (1962) were either simply due to a superimposition of filaments lying in two dif-

ferent planes or were the "S" filaments proposed earlier (Huxley and Hanson, 1954, 1957; Hanson and Huxley, 1955; Huxley and Peachey, 1961) and that they were withdrawn from the A-band or were stuck to the sides of the A-band filaments.

Carlsen et al. (1965) examined stretched glycerol-extracted rabbit psoas muscle fibers in which the A- and I-band filaments were detached and separated by a gap. "Gap" filaments were observed forming connections between the A- and I-filaments. These researchers did not observe thin gap filaments continuing into the spaces between the A-filaments. Carlsen et al. (1965) also demonstrated that sarcomeres stretched to the point where there was no overlap between thick and thin filaments were still able to contract. And if shortening was initiated from sarcomere lengths at which there was a gap, a narrowing of the I-band was brought about by a curling of the I-filaments at the boundary between the A- and I-bands. It was concluded that the contraction of fibers, stretched beyond the point of overlap of the thick and thin filaments, was not passive process imposed by neighboring, less stretched fibers (Carlsen et al. (1965).

Auber and Couteaux (1963) were the first to provide a definitive description of a third type of filament in striated muscle. They found a set of "C" filaments in insect fibrillar flight muscle which connected the ends of the myosin-containing thick filaments to the Z-lines and suggested that these filaments could be important in the contractile mechanism. This observation was also made by Garamvolgyi (1965) who demonstrated that no gap appeared in insect flight muscle at sarcomere lengths at which the overlap of thick and thin filaments ceased. Instead of a gap, a band more

dense than the I-band appeared indicating that its substance originated only from the A-band and not from the I-band which had a much lower optical density at all sarcomere lengths. In 1977, Ullrick and coworkers conducted experiments designed to determine the presence or absence of extensions of the thick filaments of Z disks in vertebrate and invertebrate striated muscle. They performed serial cross sections of stretched frog sartorius and chameleon tongue muscles and of waterbug flight muscles, and made myofibrillar filament counts at the level of the A-band and I-Z junction. Ullrick et al. (1977) concluded that thick filament extensions were not present in vertebrate muscle; however, they did exist in invertebrate flight muscle.

It is generally accepted that some type of connecting filaments exists in invertebrate flight muscle. There is much more controversy, however, concerning their presence in vertebrate muscle. Huxley (1960) used rabbit psoas muscle to make total filament counts in the A- and I-bands of serial sectioned myofibrils. His results indicated that the number of filaments in the I-band agreed closely with the number of thin actin filaments in the A-band. There was no evidence for the existence of additional filaments in the I-band extending from the tapered ends of the myosin filaments. In 1968, however, Guba et al. (1968) counted filaments per unit area in the A-band, the A-I junctional area and in the I-band of rabbit psoas muscle serial cross sections. From their tabulations, these researchers concluded that there were too many filaments in the I-band to be accounted for solely by the thin filaments. They suggested that a continuity of the thick filaments existed through the I-band to the Z-line.

Recently, structures called "end filaments" were observed when low

ionic strength buffers were used to dissociate separated thick filaments of vertebrate muscle into three subfilaments (Trinick, 1981). After the dissociation was performed, end filaments were observed at the termination of the subfilaments. One end filament was evident for every three subfilaments. The end filaments were approximately 850 A in length, 50 A in width and showed transverse striations with a periodicity of 42 A. Trinick (1981) noted that the end filaments were not observed on the thick filaments before dissociation which implied that they were in some way folded back inside the thick filament tips.

Saide (1981) isolated a structural protein of connecting filaments, which linked thick filaments to the Z-line in insect fibrillar muscle. Glycinerated flight muscle treated with antiserum to this protein demonstrated enhanced density from the ends of the thick filaments to the I-Z junction regardless of sarcomere length. Saide (1981) also determined that the protein of the connecting filaments migrated on SDS gels with an apparent molecular weight of 360,000 and named it projectin.

Locker and Leet (1975) noticed that when bovine sternomandibularis muscle was stretched to five times rest length, a gap occurred between the A- and I-bands. This gap was usually spanned by thin "gap filaments" which appeared to be continuous with the thick filaments. These researchers determined that although gap filaments were not uniform along their lengths, they had about the same diameter as the thin filaments, but were less numerous (Locker and Leet, 1976a). Also during stretching, a marked lengthening of the A-band was observed, arising from both dislocation and stretch of the thick filaments with half sliding in one direction and half in the other. The I-band, however, appeared to remain unchanged (Locker and

Leet, 1975). The proposed explanation for this phenomenon was that in the unstretched sarcomere half the thick filaments were anchored, each by a gap filament, to one end of the sarcomere, and half to the other in a regular pattern. Upon excessive stretch, these gap filaments would act as transmitters of the applied force to the A-band, resulting in the dislocation and stretch of the thick filaments (Locker and Leet, 1975). Gap filaments were observed to be highly elastic, surviving extension of the sarcomere to up to five times the length in excised muscle, at which point they appeared to be the only component maintaining structural continuity in the sarcomere (Locker and Leet, 1975; Locker and Leet, 1976a).

Huxley and Hanson (1954) noted that stretched myofibrils sprang back to near rest length even after myosin had been extracted. Locker and Leet (1976a) demonstrated that this still happens when both myosin and actin have been extracted, although great disorganization of filaments occurred. After extraction of stretched muscle fibers, the surviving gap filaments ran from an overlap in the center of the sarcomere through the Z-line (Locker and Leet, 1976a). dos Remedios and Gilmour (1971, 1978) also had reported "residual filaments" left after extraction of rabbit psoas muscle. These filaments were somewhat variable in diameter (40 - 70 A) and ran between Z-lines.

Locker and Leet (1976a) postulated that each gap filament forms a core to a thick filament, emerging at only one end of the thick filament and running through the Z-line to terminate as a core to a second thick filament in the adjacent sarcomere. In this way, gap filaments would form the elastic component in muscle, providing continuity of structure through the Z-line, but not through the sarcomere.

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When muscle goes into rigor, the bridges between actin and myosin impose a low extensibility. At about 18% extension and loads of 0.9 - 1.6 kg/cm², muscle reaches a point where it continues to extend readily without further loading. This is designated as the "yield point" of the muscle (Locker and Daines, 1975; Locker and Carse, 1976; Locker and Wild, 1982a). Locker and Wild (1982a) found the histological appearance of yielded muscle to vary with rigor temperature and between fibers, but in general the response was due to fissures opening up at random across I-bands of adjacent myofibrils. It was postulated that at the yield point, the I-filaments either broke near the Z-line or were pulled out of the Z-line as the gap filaments stretched. The mechanism of yielding was considered to be a failure of the actin filaments, or their attachments, followed by elastic extension of gap filaments with the I-bank (Locker and Wild, 1982a). If muscle was postmortem aged for 1 day at 15°C before loading, the yield point fell drastically with loads of 0.1 - 0.2 kg/cm², and the I-bands broke along the A-I junction. It appeared that both I-filaments and gap filaments were degraded by proteolysis and failed together at low loads (Locker and Wild, 1982a).

The aspect of meat tenderness is usually concerned with the properties of heat-denatured muscle proteins. Heating has a profound effect on the chemical, structural and palatability characteristics of meat. Increasing internal temperature of doneness has been shown to reduce palatability, especially tenderness (Machlik and Draudt, 1963; Schmidt et al., 1970; Bouton and Harris, 1972; Parrish et al., 1973b; Bouton et al., 1974). The most prominent change noted during heating of muscle tissue is the degradation of the two major structural components: the collagenous and myofibril-

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Heating to an internal temperature of 60°C has been determined to result in maximal beef tenderness (Machlik and Draudt, 1963; Schmidt et al., 1970). Goll et al. (1964b) proposed that optimal tenderness at this temperature was related to the shrinkage and partial solubilization of bovine muscle collagen initiated at 60°C. Cheng and Parrish (1976) and Schmidt and Parrish (1971) found that in bovine longissimus muscle brought rapidly to 60, 70 and 80°C, endomysial connective tissue shrinkage was initiated at 60°C and it was completed at approximately 70°C. Perimysial connective tissue shrinkage, however, required internal temperatures of 70°C and higher before any significant fiber changes were observed (Schmidt and Parrish, 1971).

Myosin and actin undergo denaturation and shrinkage early in cooking. (Hamm, 1966). Schmidt and Parrish (1971) demonstrated that heating bovine longissimus muscle to 60°C caused loss of M-line structure, initiation of disintegration and coagulation of thick and thin filaments and further myofibrillar protein shrinkage. By heating the muscle to 70 and 80°C, more disintegration of thin filaments and coagulation of thick filaments occurred. At 90°C, an amorphous structure resulted. Some filamentous structure was observed to survive in longitudinal sections of the I-band, while in cross sections, some thin filaments were apparent although somewhat obscured by a coagulum (Schmidt and Parrish, 1971). Locker et al. (1977) have proposed that gap filaments, unevenly coated with coagulated actin, were the primary source for the observed coagulum. Davey and Graafhuis (1976) have demonstrated with muscle which had gone into rigor at twice excised length that gap filaments survived cooking for 40 min. at

80°C. In raw muscle, a small gap appeared (0.1 μm) and was spanned by thin gap filaments. According to Locker and Daines (1980), these thin filaments could not be I-filaments since they had been completely withdrawn from the A-band at this degree of stretch. They noted, however, the possibility that these thin filaments were thin aggregates of myosin molecules. After cooking, the thick and thin filaments retracted and coagulated, increasing the gap to 0.6 μm (Davey and Graafhuis, 1976). In this enlarged gap, the gap filaments appeared to be well-preserved and extended from the thick filament. Contrary to muscle cooked at excised length, the actin had not disintegrated but aggregated into thicker filaments. Locker et al. (1977) suggested that the gap filaments acted as a focus for the aggregation of actin filaments.

According to Locker (1982), gap filaments were denatured steadily during an hour at 60°C. He proposed that gap filaments then lost the extreme extensibility of their native state, but retained a more moderate extensibility, which along with that of denatured collagen, conferred the pseudo-elastic character of cooked meat. Therefore, although gap filaments became cooked at 60°C, they remained strong while A- and I-filaments were rapidly losing any significance to strength.

Davey and Graafhuis (1976) determined that if muscle stretched to twice excised length was aged for 3 days postmortem at 25°C, the gap filaments disappeared leaving complete gaps on cooking. Since the filaments were visible before cooking, Davey and Graafhuis (1976) postulated that aging must modify their resistance to heat rather than cause their dissolution.

There is some evidence that gap filaments are vulnerable to

proteolytic enzymes. dos Remedios (1969; cited in Locker and Leet, 1976a) recorded that his "residual filaments" were attacked by trypsin although they were resistant to many protein solvents. Huxley and Hanson (1954) noted that the "ghosts" of myofibrils left after myosin extraction disintegrated if the fibers had been treated with trypsin during preparation of myofibrils. It was also shown by Goll et al. (1971) that the in vitro action of trypsin on myofibrils simulated the effects of post-mortem aging, by weakening the actin-myosin interaction. When muscle fibers were stretched to 3-5 times excised length on microscope slides and were treated with a dilute trypsin solution, all fibers snapped within 1-3 min. (Locker et al., 1977). Because muscle fibers depend on gap filaments for their continuity of structure according to the model of Locker and Leet (1976a), Locker et al. (1977) concluded that gap filaments were vulnerable to trypsin. Locker and coworkers (1977) repeated the trypsin experiments on overstretched fibers using a crude bovine calcium activated factor (CAF) preparation. As with trypsin, a general loss of structure was observed with irregularities appearing in the spacings of the striations and separations occurring between myofibrils. A further experiment was carried out using muscle glycerinated at twice excised length, incubated in a crude CAF preparation and cooked (Locker et al., 1977). In the CAF-CA²⁺ sample before cooking, only a diffuse remnant of Z-line was observed and some sarcomeres revealed gap filaments between the A- and I-bands. During cooking, however, the sample disintegrated completely (Locker et al., 1977). The destruction of gap filaments on cooking, however, was almost as severe in the presence of EDTA as in the presence of CA²⁺ at pH 7. This observation

suggested that both neutral proteases and cathepsins were active (Locker, 1982). Locker et al. (1977) postulated that muscle proteases attacked the gap filaments to a degree not evident in the raw state, but caused their destruction on cooking.

It has been generally held that the I-filament (Marsh and Carse, 1974) or Z-line (Davey and Gilbert, 1967, 1969; Takahashi et al., 1967; Parrish et al., 1973a; Stromer et al., 1974; Olson et al., 1976) is the weak link in the sarcomere. According to the gap filament model proponents (Locker and Leet, 1976a; Locker et al., 1976, 1977; Locker and Daines, 1980; Locker, 1982; Locker and Wild, 1982a, b), however, the gap filaments become the important link. Locker et al. (1976) proposed that gap filaments determine tensile strength of the myofibril in the raw or cooked state and are therefore important to tenderness. Since myosin filaments fuse on heating and become too strong to break while actin filaments are destroyed, it appears that only the gap filaments and collagen define tensile strength in cooked muscle at excised length or 100% stretch (Locker et al., 1977; Locker, 1982).

Connectin

Considerable effort has been made to isolate an elastic protein which can be identified with a third type of filament in striated muscle. It is well-known that there is an elastic component in muscle fibers in the absence of cell membranes (Maruyama et al., 1980). In fact, Maruyama et al. (1976b) determined that passive elasticity remained in skinned fibers of frog skeletal muscle after extraction of contractile and other soluble proteins. Maruyama (1976) isolated a highly insoluble intracellular elastic

protein and named it connectin. Furthermore, they proposed that this newly discovered elastic protein is responsible for structural continuity and tension transmission in skeletal muscles (Maruyama et al., 1976b, 1977b).

To isolate connectin, Maruyama et al. (1977b) utilized an exhaustive extraction procedure. The residues of skeletal and heart muscles remaining after myosin, actin and regulatory proteins had been extracted were further extracted with either 0.1 N NaOH or 1% sodium dodecyl sulfate (SDS) with subsequent phenol treatment to remove nucleic acids and some glycoproteins. They reported that connectin tended to form large compact aggregates in vitro, especially in the presence of salt. Only treatments with Triton X-100 were found to result in partial dispersion of the aggregates and entanglements of very thin filaments of about 20 A in diameter were observed after this treatment. The connectin solubilized in 0.1% SDS showed amorphous aggregates; however, filamentous structures were not clearly observed.

Connectin was found to comprise approximately 5% of the total myofibrillar proteins in rabbit psaos muscle and contained about 5% lipids and 1% sugars (Maruyama et al., 1977b). Depending on whether the SDS or alkali treatment was utilized for the extraction procedure, the protein had a slightly different amino acid composition (Maruyama et al., 1977b). Connectin preparations isolated from both skeletal and cardiac muscles of various species of vertebrates, avian smooth muscle and also from invertebrate skeletal muscle, had very similar amino acid compositions (Maruyama et al., 1977c; Maruyama et al., 1978). It was further determined that antisera against connectin had no species specificity (Maruyama et al., 1977b).

It is of particular interest that the connectin content in cardiac myofibrils is much greater than in skeletal myofibrils, being approximately 18% of the total myofibrillar protein (Maruyama et al., 1977a). The abundance of connectin in cardiac myofibrils may be responsible for keeping the sarcomere lengths of myofibrils short at rest (Maruyama et al., 1977a). The shortened sarcomeres may account for the exceptionally effective tension generation of cardiac muscle on passive stretching due to blood inflow (Maruyama et al., 1977a). Locker and Daines (1980), however, found no significant difference between the connectin content of heart and skeletal muscle tissue.

A connectin-like elastic protein was isolated from the cortical layers of the sea-urchin egg (Maruyama et al., 1976a) suggesting that the elastic protein may play a general role in maintaining cell shape. This protein has been obtained also from cell membranes of erythrocytes. Fluorescent antibody staining suggested that it was located on the cytoplasmic surface of the membrane (Maruyama et al., 1977c) and the elastic protein from nonmuscle cells is very similar to muscle connectin (Maruyama et al., 1977b).

Maruyama (1976) compared connectin with noncollagenous reticulin, an extracellular glycoprotein of the basement membranes present in the endomysium of muscle. These two proteins had similar amino acid compositions, however, they differed in solubility and antigenicity. The amino acid composition of connectin was also found to be entirely different from that of collagen or elastin (Kimura et al., 1978; Maruyama et al., 1977c).

Another unique characteristic about connectin is that it seems to be an extremely large myofibrillar protein. Maruyama et al. (1977b) determined that although a small portion of connectin soluble in 1% SDS moved

as bands having molecular weights of about 90,000 and 45,000 daltons, the bulk of the protein did not move onto 10% polyacrylamide gel during electrophoresis. Even when solubilized, the major portion of connectin was too large to enter a 10% gel. Researchers have concluded that connectin must be highly heterogeneous in composition (Maruyama et al., 1977b, 1980; King and Kurth, 1980). The smallest protein component of connectin was later found to have a molecular weight of 43,000 daltons, and although it had the same mobility as actin or 10S-actinin, this protein differed from the other proteins in its extractability from myofibrils (Maruyama et al., 1980). The large portion of the high molecular weight polypeptides in connectin preparations electrophoresed slowly on 2.5% acrylamide gels with a mobility similar to that of a tetramer of myosin heavy chains, suggesting an apparent molecular weight of approximately 800,000 (Maruyama et al., 1980).

The high molecular weight, low solubility and elastic properties of connectin suggest that it may be a post-translational covalently cross-linked product of one or several different smaller polypeptides (Maruyama et al., 1977b; Fujii et al., 1978). Fujii et al. (1978) reported that connectin from chicken breast muscle contained reducible compounds, aldimine forms of lysinonorleucine and histidino-hydroxymerodesmosine, derived from lysine and hydroxylysine aldehydes. It was concluded by Fujii et al. (1978) that connectin and connective tissue proteins, collagen and elastin, share the same copper-dependent lysyloxidase mediated cross-linking system. Borohydride reduction of connectin prepared from carp muscle also yielded products which may have originated from connectin cross-links (Kimura et al., 1979). It was postulated that the connective tissue cross-linking enzyme, lysyl oxidase, was also present in skeletal muscle

tissue (Fujii et al., 1978). In fact, Maruyama and Shimada (1978) claimed that the content of connectin in lathrytic rat muscle was lower than in control muscle. Lathyrism, a connective tissue disease, is caused by an inhibition of lysyl oxidase necessary in the formation of cross-links of lysine derivatives in collagen and elastin (Siegel and Martin, 1970). Maruyama and Shimada (1978) suggested that the decrease in connectin content in lathrytic muscle, determined as an insoluble residue after various extraction processes, was due to increased solubility of connectin by inhibited cross-link formation. Furthermore, Fujii and Kurosu (1979) found a progressive decrease in the content of reducible cross-links of connectin with age and concluded that this was associated with conversion to more stable cross-links by a process similar to that occurring in collagen (Bailey and Robins, 1976). In contrast to these observations, Robins and Rucklidge (1980) have recently reported that no lysine-derived reducible cross-links were detected in connectin obtained from either skeletal or cardiac muscles, although some hexitol-lysine derivatives were present. These researchers also suggested that although the copper-dependent enzyme, lysyl oxidase, undoubtedly initiates cross-linking of extracellular collagen in muscle, its activity on cross-linking connectin within an intracellular compartment containing high concentrations of carnosine and anserine, which chelate copper, is unlikely.

Recently, however, Fujii and Maruyama (1982) have reaffirmed their finding of the lysine-derived cross-link, aldimine form of lysinonorleucine. They claimed that the failure of Robins and Rucklidge (1980) to detect this compound may have been due to the treatment of the samples with a crude collagenase preparation, which resulted in complete digestion of

connectin. Gruen et al. (1982) investigated a second mechanism in which cross-links may be formed from lysine side chains involving transglutaminase catalyzed formation of ϵ (γ -glutamyl) lysine cross-links (Folk and Finlayson, 1977). Low levels of this isopeptide have been reported in myofibrils, but the particular proteins cross-linked have not been identified (Folk and Finlayson, 1977). Gruen et al. (1982) found no evidence for the presence of ϵ (γ -glutamyl) lysine or other isopeptide cross-links in connectin. In contrast to the findings of Maruyama and Shimada (1978), SDS gel electrophoresis of Gruen et al. (1982) did not reveal any difference in connectin between normal and lathrytic muscle. From these results, Gruen and coworkers (1982) concluded that lysyl oxidase does not initiate cross-link formation in connectin. Although connectin may be covalently cross-linked by some unknown mechanism, Gruen et al. (1982) suggested that the protein is synthesized as a single polypeptide chain.

Maruyama et al. (1977b) first demonstrated the net-like structure of connectin in frog skeletal muscle using thick and thin filament extracted myofibrils. As a result, the extracted myofibrils were stained uniformly by fluorescent antibodies against connectin. In addition, electron microscopic observations revealed that the connectin structure was a net consisting of very thin filaments of less than 2 nm in diameter (Maruyama et al., 1977b; Toyoda and Maruyama, 1978). When myosin and actin were completely removed from frog cardiac myofibrils (Toyoda and Maruyama, 1978) and from rabbit psaos myofibrils (dos Remedios and Gilmour, 1978), a filamentous material running between Z-lines remained which appeared to prevent dissolution of the "ghost" myofibrils. The amino acid composition of the KI-extracted frog cardiac myofibrils was similar to that of connectin,

although not identical (Toyoda and Maruyama, 1978). Toyoda and Maruyama (1978) claimed that the difference in amino acid compositions was probably due to the presence of Z-line material in the extracted myofibrils. It was proposed that in situ thin connectin filaments are cross-linked to form elastic nets linking neighboring Z-lines (Toyoda and Maruyama, 1978; dos Remedios and Gilmour, 1978). Maruyama et al. (1980) cut oblique sections of contracted cardiac myofibrils and observed connectin nets in all sections with the electron microscope. They concluded that connectin nets were three dimensional and covered the entire longitudinal region between Z-lines in a myofibril. Thus, the elasticity of living skeletal muscle may be largely dependent on the continuous net structure of connectin (Takahashi and Saito, 1979).

Locker and Daines (1980) suggested that connectin may be the protein component of gap filaments. However, electron micrographs indicated that gap filaments existed as discrete filaments (Locker and Leet, 1976a), whereas connectin appeared as an entangled network (dos Remedios and Gilmour, 1978; Toyoda and Maruyama, 1978). This difference in structural geometry may arise because of sample preparation, as gap filaments were observed in sections of whole muscle, whereas connectin was obtained as a residue after extraction of other proteins (King and Kurth, 1980).

Locker and Daines (1980) reported that Maruyama's model, with connectin existing as a three-dimensional network within myofibrils, but external to the thick and thin filaments (Maruyama et al., 1980), would impede the smooth functioning of the myosin head-actin interactions during contraction. Gruen et al. (1982) found that in developing fetal muscle, myosin heavy chains were observed some weeks earlier than connectin. These researchers

claimed that this evidence, along with the known susceptibility of connectin to hydrolysis (Kimura et al., 1979; King et al., 1981; Maruyama et al., 1981a; Takahashi and Saito, 1979) suggested that connectin existed in an exposed environment rather than as a core to the thick filament reported by Locker and coworkers (Locker and Leet, 1976a; Locker and Daines, 1980).

Maruyama et al. (1980), utilizing indirect immunofluorescence, demonstrated that the A-band regions and the Z-lines of chicken breast myofibrils were strongly fluorescent while I-band regions were weakly stained with fluorescent antiserum against connectin. From this observation they suggested that connectin was present through the entire sarcomere. When Maruyama et al. (1981b) later treated intact myofibrils from chicken skeletal muscle with antiserum against connectin, the A-I junctions were strongly fluorescent. The middle region of the A-bands and the whole I-bands were faintly stained, but the Z-lines were not fluorescent. These results were in contrast to their previous work (Maruyama et al., 1980) where Z-lines were stained in addition to the A-I junctions, middle region of the A-band and the whole I-band, however, Z-line fluorescence was not reproducible. Maruyama et al. (1981b) proposed that the connectin antiserum produced in their earlier experiments (Maruyama et al., 1980) must have contained some proteins located in the Z-lines which resulted in Z-line staining. Upon extraction of myosin from chicken breast myofibrils (Maruyama et al., 1981b), some protein materials were accumulated at both edges of the A-band. When these myofibrils were treated with fluorescent antiserum against connectin, the A-I junction area was still fluorescent and the center of the A-band was less fluorescent than the edges. Maruyama et al. (1981b) demonstrated that on the removal of the

bulk of myosin filaments, the connectin antiserum staining at the area of the A-I junction was wider than in nonextracted myofibrils. When myofibrils were extracted with KI to remove both the myosin and actin filaments, dense lines appeared at both sides of the Z-lines. These results were similar to the observations of Granger and Lazarides (1978). These dense lines were strongly stained and there were fluorescent filaments between the neighboring Z-lines, but Z-lines were not stained (Maruyama et al., 1981b).

After alkali and phenol treatments, isolated connectin was found to be very insensitive to proteolytic enzymes (Maruyama et al., 1977b). However, in intact myofibrils, connectin was digested by trypsin, chymotrypsin, papain, nagarse and serine protease. Pepsin and calcium activated factor (CAF) did not degrade connectin (Maruyama et al., 1981a). Maruyama and coworkers (1980) reported it was not surprising that connectin in situ was easily attacked by most proteases because it forms three-dimensional nets in myofibrils which would be very accessible to proteolytic attack. However, after the extraction of actin and myosin filaments, the connectin nets become closely associated with each other (Maruyama et al., 1980). In this way, connectin becomes very insensitive to proteases, presumably due to steric hindrance to proteolytic enzyme binding (Maruyama et al., 1981a).

Endogenous proteases have been implicated in the tenderization of meat during postmortem aging as a result of their attack on Z-lines (Davey and Gilbert, 1967, 1969; Takahashi et al., 1967; Parrish et al., 1973a; Penny, 1974; Stromer et al., 1974; Olson et al., 1976; Parrish, 1978) gap filaments (Locker et al., 1977), or connectin (Takahashi and

Saito, 1979). Numerous proteases may contribute to the tenderization of meat, but the relative contribution of each remains to be determined. CAF, Cathepsin B and Cathepsin D occur in muscle and have been shown to attack myofibrillar proteins in vitro (King and Harris, 1982).

Young et al. (1980-81) demonstrated the progressive changes in connectin during postmortem muscle storage at 15°C. Bovine sternomandibularis muscle was sampled at death and up to 6 days postmortem and was separated into three salt soluble fractions: phosphate soluble, concentrated KI soluble and guanidine-HCl soluble. Connectin was partly lost from the guanidine fraction during postmortem storage, possibly through increased solubility in the KI fraction. Young et al. (1980-81) concluded that disintegration of the cytoskeletal network can account for the postmortem changes in the physical properties of muscle and for increased tenderness after cooking of aged meat.

An interesting aspect related to meat quality is that vertebrate skeletal muscle loses its elasticity with postmortem time and this loss appears to be closely associated with the tenderization of meat that occurs during aging (Takahashi and Saito, 1979). They determined that the connectin content of rabbit psoas and chicken breast skeletal muscles decreased with postmortem storage time and this decrease coincided well with the loss of muscle elasticity. The rate of decrease, however, varied with the source of muscles. As the amount of connectin fell to zero during postmortem storage, the connectin network structure observed between Z-lines vanished (Takahashi and Saito, 1979). These researchers concluded that the continuous net structure of connectin was responsible for about 30% of the

total elasticity of living skeletal muscle and its degradation was responsible for the postmortem tenderization of meat.

Heating meat for 20 min. at 60°C resulted in the degradation of connectin to products with molecular weights in the region of 45,000 daltons (King and Kurth, 1980). The extent of connectin breakdown was greater at a low pH and these researchers concluded that pH change, rather than other factors, most likely resulted in the increasing breakdown of connectin with postmortem time. It was found that the initial rate of connectin degradation was higher at 60°C than at 50, 70, or 80°C (King and Kurth, 1980). Lutalo-Bosa and MacRae (1969) determined that proteolytic activity is retained at temperatures as high as 60°C. Davey and Gilbert (1974) later presented evidence that the rate of proteolysis in meat reached a maximum at 60-65°C, with rapid inactivation of proteases at higher temperatures.

Cheng and Parrish (1979), however, suggested that CAF degradation of Z-lines and other myofibrillar proteins was stimulated during heating. The effect of CAF activity on the modification of myofibrillar proteins during heating could be additive to the tenderizing effects of CAF on muscle during postmortem aging. In view of the known high activity of acid cathepsins at 60°C (Penfield and Meyer, 1975), King and Kurth (1980) concluded that these enzymes may also contribute to the breakdown of connectin during cooking at 60°C.

Because there are specific inhibitors of some proteases, such inhibitors may be used to discriminate between different types of proteases postulated to be involved in meat tenderization. King and Harris (1982) injected two inhibitors of carboxyl proteases into muscle, pepstatin and

EPNP, in order to investigate their effects on heat-induced changes in the structural proteins and the mechanical properties of meat. Pepstatin is a pentapeptide which reversibly inhibits carboxyl proteases by forming a transition-state analogue, while 1,2-epoxy-3 (p-nitrophenoxy) propane (EPNP) esterifies an essential carboxyl group at the active site (Tang, 1976). That carboxyl proteases were active in tenderizing meat at 60°C was demonstrated by the effect of EPNP and pepstatin injections into pre-rigor muscle which resulted in reduced connectin breakdown and increased Warner-Bratzler shear measurements (King and Harris, 1982). Gel electrophoresis showed that connectin was hydrolyzed to a greater extent than other muscle proteins at this temperature and that breakdown of connectin was inhibited by pepstatin and EPNP (King and Harris, 1982; King et al., 1981). King et al. (1981) heated muscle samples at 50-70°C and determined degradation of connectin was inversely related to the ultimate pH of the muscle source, again indicating the role of carboxyl proteases. The greater activity of carboxyl proteases in tissues from older animals was apparently responsible for more extensive connectin degradation (King et al., 1981). King and Harris (1982) concluded that connectin is unlikely to be responsible for toughness in cooked meat because it is extremely susceptible to proteolysis at moderate cooking temperatures.

If connectin is indeed the protein component of the gap filaments, it is difficult to reconcile the extensive breakdown during cooking at 60°C (King and Kurth, 1980) with the apparent stability of gap filaments during heating muscle at excised length or at 100% stretch at 80-100°C (Locker et al., 1977). According to Locker and Daines (1980), in heated muscle,

the gap filaments are the only surviving continuity links in the I-band and their tensile strength determines the myofibrillar component of meat toughness.

Titin and Nebulin

A group of three extremely large ($M_r > 400,000$) myofibrillar proteins (initially referred to as bands 1, 2 and 3, in increasing order of migration as detected by SDS-PAGE) recently discovered by Wang et al. (1979), was found to be present in a wide range of vertebrate and invertebrate striated muscles. As defined by electrophoretic analysis, titin was a pair of very large myofibrillar proteins of similar size determined to be chemically and immunologically distinct from filamin, myosin and actin (Wang and McClure, 1978; Wang et al., 1979). Band 1 and band 2 were found to have apparent molecular weights of 1.4×10^6 and 1.2×10^6 because they comigrated on SDS gels with the heptamer and hexamer of cross-linked myosin heavy chain, respectively (Wang, 1982a). Bands 1 and 2 of the high molecular weight doublet will be referred to as titin herein. Recent studies of Maruyama et al. (1981b) by means of gel electrophoresis, amino acid composition and antibody localization, suggested that the high molecular weight components of typical connectin preparations are identical to titin.

Initial antibody localization and electrophoretic results indicated that titin was present in both skeletal and cardiac muscles, but was absent in smooth muscle cells (Wang et al., 1979). Titin was isolated from chicken skeletal muscle myofibrils in a relatively pure state using agarose gel permeation chromatography in the presence of SDS by taking advantage of titin's unusually large size (Wang et al., 1979; Wang, 1982a).

Titin along with band 3, recently named nebulin by Wang (1981), accounted for approximately 10-15% of total myofibrillar proteins (Wang et al., 1979) on the basis of Folin-Lowry protein determinations (Lowry et al., 1951). Wang (1982b) reported that the amino acid composition of titin was distinct from other myofibrillar proteins while band 1 and band 2 of the titin doublet had similar if not identical compositions. Titin was determined to be enriched in proline, an α -helix breaking residue, as high as 8-9% in some preparations (Wang, 1982b). This would suggest that native titin may not have as extensive α -helical region as myosin heavy chain which contains approximately 2% proline (Wang, 1982b). Wang (1982b) considered the possibility that polypeptides of such large sizes may have been constructed out of covalently linked subunits; however, no chemical evidence has been found to support this view.

After SDS removal, purified chicken breast titin was found to aggregate into an elastic gel composed of very thin filaments (50 - 80 A) of various lengths and was very difficult to redissolve in SDS (Wang and Ramirez-Mitchell, 1979). These researchers concluded that titin was at least partially renatured upon SDS removal and then assembled into filaments which were distinct from myosin and actin filaments. Using rotary and unidirectional shadowing of titin preparations, Wang and Ramirez-Mitchell (1982b) have observed electron microscope images that suggested titin was an extremely long (400-700 nm) and flexible filament. Their micrographs also revealed the presence of somewhat regular, periodic structural areas along the long titin strands.

Intact titin and nebulin were not extracted by a wide range of solutions commonly used to dissolve thick and thin filaments, such as

0.6 M KCl or KI, and therefore represented the major residual proteins of extracted myofibrils (Wang, 1982b). In order to purify and analyze these proteins, it was necessary to use denaturants such as SDS, guanidinium chloride and urea (Wang et al., 1979; Wang, 1982a, b). In KI extracted myofibrils, with the majority of actin, myosin, and their associated regulatory proteins removed, a large number of very thin, longitudinal filaments connecting residual Z-lines were detected by negative staining (Wang and Ramirez-Mitchell, 1979, 1981, 1982a, 1983). Gel electrophoresis showed that the titin doublet was the major component of the extracted myofibril preparation, although small amounts of myosin and actin remained (Wang and Ramirez-Mitchell, 1979, 1981). The residual filaments of the extracted myofibrils resembled those of the titin gels and Wang and Ramirez-Mitchell (1979, 1981, 1982a,) proposed that titin may be the component of putative longitudinal filaments in striated muscle. Similar observations have been made for connectin by others (dos Remedios and Gilmour, 1978; Toyoda and Maruyama, 1978; Locker and Daines, 1980). dos Remedios and Gilmour (1978) interpreted the presence of residual filaments connecting successive Z lines after KI extraction as evidence for the third filaments consisting of cross-linked connectin. Wang and Ramirez-Mitchell (1983) pointed out that failure to recognize translocation of titin during KI-extraction in preparation of samples for electron microscopy may have led to misidentification by these other investigators of residual filaments, possibly 10 nm filaments, between Z structures of myofibrils depleted of thick and thin filaments as connectin (titin). By high-resolution transmission and electron microscopy, Wang and Ramirez-Mitchell (1983) determined that these longitudinal

residual filaments observed by others appeared to connect the peripheries of successive Z-lines and therefore could not be titin filaments which are 2-6 nm in diameter and exist within the sarcomere.

LaSalle et al. (1983) examined titin's localization with polyclonal antibodies to bovine skeletal muscle titin in isolated skeletal myofibrils. Immunofluorescence labeling patterns were somewhat variable; however, labeling in the region of the A-I junction with some labeling extending into the A-band was always demonstrated. Immunofluorescence labeling of thick and thin filament extracted myofibrils indicated translocation of much of titin had occurred as evidenced by intense titin staining on both sides of the residual Z structures, these observations being in agreement with those of Wang and Ramirez-Mitchell (1983).

Wang et al. (1979) observed that when titin antibody was applied to glycerinated chicken breast myofibrils, highly reproducible staining patterns were obtained with standard fluorescent techniques. Antibody localization, however, varied significantly in any given preparation in staining pattern and relative intensity, and appeared to be somehow related not only to sarcomere length (Wang et al., 1979), but also to the way in which the myofibrils were prepared (Wang, 1982b). Generally, immunofluorescent staining indicated that titin was present in the A-I junction, M-line region, Z-line and perhaps throughout the entire A-band (Wang et al., 1979). Wang et al. (1979) concluded from these labeling patterns that: 1) since both M- and Z-lines were labeled, transverse structures contained titin, 2) the A-I junction bands behaved as though the titin-containing structure was coupled to the ends of thick filaments since the distance between the pair of labeled bands bordering each A-band did not change

with sarcomere length and 3) intensity and labeling pattern variation may have resulted from structural or accessibility changes of titin in sarcomeres of different lengths. Titin appeared to be preferentially distributed in the A-band, however, it was noted that staining of the A-I junction extended considerably into the I-band. This labeling pattern was in sharp contrast to that of myosin, which was always confined within the boundaries of the phase-dense A-band (Wang, 1982b). Wang (1982b) concluded that this observation, as well as the resistance of intact titin to extraction by A-band solvents, proved that titin was not a thick filament protein.

Immunofluorescence studies of over-stretched bovine muscle by LaSalle et al. (1983) showed intense titin staining in the gap regions between A- and I-bands. In the electron microscope examination of over-stretched bovine muscle, approximately 3-5 nm filaments were observed spanning the region between A-bands and I-bands. It was observed during electron microscope examination that immunoperoxidase labeled this set of thin filaments and electron-dense reaction products were often present in the outer half of the I-bands and outer edge of A-bands (LaSalle et al., 1983).

Several studies have suggested that titin is highly susceptible to proteolytic degradation (Wang, 1982a, b; Wang and Ramirez-Mitchell, 1983; Zeece et al., 1983). Wang (1982b) reported that within the myofibril, titin was rapidly cleaved by endogenous proteases (especially calcium-activated neutral protease) and by very low concentrations of exogenously added proteases (such as trypsin and chymotrypsin) into a similar set of large degradation fragments. Zeece et al. (1983) also observed titin degradation in bovine longissimus myofibrils in the presence of highly

purified calcium-activated neutral protease. In contrast to these results, Maruyama et al. (1981a) reported that the high molecular weight proteins of connectin (titin) present in chicken skeletal myofibrils were resistant to the action of added calcium-activated neutral protease. Wang, however, demonstrated that the addition of 5 mM EDTA to glycerol-extracting solutions minimized the rapid proteolysis of titin and nebulin that occurred during conventional glycerination procedures (Wang, 1982b; Wang and Ramirez-Mitchell, 1983).

Nebulin, initially referred to as band 3, was determined to have an apparent molecular weight of $5-6 \times 10^5$, corresponding in mobility to the trimer of myosin heavy chain (Wang and Williamson, 1980; Wang, 1981, 1982a, b). Wang et al. (1979) first identified nebulin in vertebrate skeletal myofibrils and it has also been purified from rabbit psoas muscle myofibrils (Wang and Williamson, 1980) and from chicken breast myofibrils (Maruyama et al., 1981a; Ridpath et al., 1982, 1983). Nebulin was purified using gel filtration column chromatography from SDS-solubilized myofibrils (Wang and Williamson, 1980; Wang, 1982a). The amino acid composition of nebulin was similar to that of titin (Maruyama et al., 1981b); however, peptide mapping (Wang, 1981) and immunological studies (Wang and Williamson, 1980; Ridpath et al., 1982) indicated that nebulin was distinct from titin and from other known myofibrillar proteins. Nebulin has been found to be extremely susceptible to proteolytic degradation (Wang and Williamson, 1980; Wang, 1981; Maruyama et al., 1981a; Wang and Ramirez-Mitchell, 1983; Zeece et al. 1983); especially by calcium-activated proteases (Wang, 1981; Maruyama et al., 1981a; Zeece et al. 1983).

Nebulin has been identified by indirect immunofluorescent localization studies as a major protein component of the myofibrillar N_2 -line of skeletal muscle (Wang and Williamson, 1980; Ridpath et al., 1982, 1983). In electron micrographs, the N_2 -line appeared as a dark line crossing the I-band on either side and parallel to the Z-line (Page, 1968; Franzini-Armstrong, 1970). The location of the N_2 -line as a transverse structure in the I-band, where the thin filament array changed from a square lattice at the Z-line to a hexagonal pattern near the A-I junction, suggested that the N_2 -line may regulate thin filament geometry (Franzini-Armstrong, 1970; Locker and Leet, 1976b; Wang and Williamson, 1980; Wang, 1981). When sarcomeres were lengthened, the N_2 -line varied its position, but maintained the same proportional distance from both the Z-line and M-line (Franzini-Armstrong, 1970; Locker and Leet, 1976b; Wang, 1981). This would suggest that the N_2 -lines were not rigidly attached to either the thick or thin filaments, although they may be able to interact with either thick or thin filaments, depending on sarcomere length (Wang and Williamson, 1980). Locker and Leet (1976a) have suggested that the N_2 -line may be attached to the proposed longitudinal elastic filaments that run between Z-lines. Wang and Williamson (1980) put forth the possibility that nebulin and the N_2 -lines were attached directly or indirectly to elastic titin filaments which extended upon lengthening of sarcomeres.

Recently, Wang (1982b) introduced a model where titin and nebulin are the major, if not exclusive, components of a set of longitudinal continuous filaments that connect Z-lines from within the sarcomere. These filaments are elastic and extensible along their length, except where they interact with structures such as thick or thin filaments or M- and

Z-lines. These filaments are also prone to mechanical or proteolytic degradation in discontinuous segments. On the basis of this model, Wang (1982b) interpreted his fluorescent data as meaning that titin was localized in the region between two N_2 -lines across the A-band and that nebulin constituted the small area between the N_2 -line and N_1 -line, a striation approximately $0.15 \mu\text{m}$ away from the center of the Z-line (Franzini-Armstrong, 1970). The appearance of titin and nebulin immunofluorescence staining at areas other than those specified in the model could be explained if breakdown of the elastic longitudinal filament had occurred during myofibril preparation. The elastic filaments would then retract and accumulate around the nearest point where the filament was anchored to an inextensible sarcomeric structure such as thick or thin filaments. Depending on the extent and location of filament damage, the relative intensity and dimension of staining bands could vary accordingly (Wang, 1982b).

To determine the involvement of nebulin in the events of myofibrillogenesis, the localization and rate of accumulation of the protein in primary chicken skeletal muscle cell cultures were investigated (Ridpath et al., 1982, 1983). Nebulin was found to appear during the same period after fusion when muscle-specific myofibrillar proteins are being synthesized (Ridpath et al., 1982, 1983). These results support the theory of Wang and Williamson (1980) that nebulin may function in the organization of the I-band.

MATERIALS AND METHODS

Source of Muscle Tissue

Portions of bovine longissimus, biceps femoris, psoas major, sternomandibularis and porcine longissimus muscles were removed within one hour after exsanguination of market weight animals at the Iowa State University Meat Laboratory. The muscles were packed in ice, taken to the Food Technology Laboratory and used immediately for the preparation of 0-day purified myofibrils and whole muscle samples. Chicken pectoralis major and mixed thigh muscle samples were removed immediately after slaughter of birds obtained from the Iowa State University Poultry Farm. Purified chicken myofibrils and whole muscle samples were immediately prepared.

Titin Isolation and Purification

Highly purified myofibrils (included washes with Triton X-100 to remove membranes) were prepared from at-death muscle by the method of Goll et al. (1974). Titin was isolated from the myofibrils and purified in the presence of sodium dodecylsulfate (SDS) by agarose gel filtration column chromatography according to the procedure of Wang et al. (1979). The major steps of the procedure included: 1) solubilization of the myofibrils (200 mg) by hand homogenization in an equal volume of hot (~100°C) sample buffer [10% (w/v) SDS, 10 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 M Tris-HCl, pH 8.0], 2) a heating of the solubilized myofibrils with stirring in a water bath at 100°C for 3 min.

followed by centrifugation at 88,000 x g for 1 hr. at 25°C to remove traces of insoluble material, and 3) application of the clear supernatant (12 ml) to a Bio-Gel A-50m (Bio Rad Laboratories, Richmond, California) permeation column (2.5 x 90 cm) equilibrated previously with elution buffer [0.1% (w/v) SDS, 5 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.1 M Tris-HCl, pH 8.8]. Fractions of 4.5 ml were collected at a rate of 15 ml/hr., and analyzed for protein concentration by absorbance at 280 nm. Designated fractions were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The chromatographically purified titin was used for amino acid analysis, autoradiography and as an antigen for antibody production.

Amino Acid Analysis

Purified titin samples collected from gel filtration columns were dialyzed against distilled deionized water for three days at room temperature to remove most of the SDS. After concentration with an Amicon Model 12 mini/ultrafiltration cell (Amicon Corporation, Danvers, Massachusetts), duplicate samples were evaporated to dryness and hydrolyzed in constant boiling HCl for 24, 48 and 72 hrs. at 110°C. Amino acid compositions were determined with a Durrum D-400 amino acid analyzer. Serine and threonine values were extrapolated to zero time by linear regression. The 72 hr. hydrolysis values were used for isoleucine and valine. Cysteine and $\frac{1}{2}$ cystine were determined as cysteic acid and methionine was determined as methionine sulfone after performic acid oxidation (Moore, 1963). Tryptophan was determined according to the procedure of Hugli and Moore (1972).

Antibody Preparation and Autoradiography

Antisera against titin were produced in two white female New Zealand rabbits using the immunological procedure of Richardson et al. (1981). Control sera were collected before immunization and tested by indirect immunofluorescence to ensure the absence of autoimmunity to any myofibrillar proteins. Each rabbit was injected twice, 14 days apart, subcutaneously in multiple back sites with approximately 0.3 mg (on each day of injection) of column purified titin that had been concentrated against 70% (w/v) sucrose, 0.1% (w/v) SDS, 5 mM EDTA, 0.5 mM DTT, 0.1 M Tris-HCl, pH 8.8, with an Amicon Model 12 mini/ultrafiltration cell. For both injections, purified titin was suspended as an emulsion in Freund's complete adjuvant (Difco, Detroit, Michigan). Animals were bled at 10 to 14 day intervals starting two weeks after the last of the two injections.

Antisera to titin were tested for specificity by immunautoradiography. Samples of purified bovine longissimus myofibrils, bovine skeletal muscle myosin, porcine stomach filamin, chromatographically purified rabbit skeletal muscle titin provided by Dr. Kuan Wang, University of Texas at Austin, and chromatographically purified bovine longissimus muscle titin were electrophoretically separated on a low percent SDS-polyacrylamide (3.2% acrylamide with no stacking gel) slab gel. Because of their very high molecular weights, rabbit and bovine titins do not enter high percent SDS-polyacrylamide gels. Therefore, it was necessary to use low percent SDS-polyacrylamide gels in order to separate them electrophoretically. Samples run in lanes on one half of the slab gel

were duplicated in lanes on the other half. The gel was cut in half after electrophoresis and one half was stained in Coomassie brilliant blue [0.2% (w/v) Coomassie Brilliant blue, 7% (v/v) acetic acid, 40% (v/v) methanol] for identification of the protein bands. The other half of the slab gel was fixed as described by Olden and Yamada (1977), rinsed with incubation buffer [0.1% (w/v) NaN_3 , 0.15 M NaCl 50 mM Tris-HCl, pH 7.4] and then incubated for 12 hrs. at room temperature with one ml bovine titin antiserum diluted 1:20 with incubation buffer. After washing for at least three hrs. with several changes of incubation buffer, the gel was incubated for 12 hrs. with one ml [^{125}I] protein A diluted 1:20 with incubation buffer. Unbound components were removed by extensive changes of incubation buffer, followed by three washes of the gel with incubation buffer containing 0.1% (v/v) Triton X-100. The autoradiogram was prepared by exposure of the vacuum-dried gel to Kodak X-Omat AR film.

The chloramine-T method described by Greenwood et al. (1963) was utilized for labeling Protein A (Pharmacia Inc., Piscataway, New Jersey) with ^{125}I (Amersham Corporation, Arlington Heights, Illinois). One mg of Protein A suspended in one ml of iodination buffer (0.1 M sodium phosphate, pH 7.2) was mixed with 1 mCi ^{125}I . The labeling reaction was initiated by the addition of 0.25 mg chloramine-T (Eastman Kodak Company, Rochester, New York) in 250 μl iodination buffer. A TCA precipitation was done after 15 sec. to determine the percentage of protein-bound counts. When 50-70% of the radioactivity was precipitable, the addition of chloramine-T aliquots was stopped. At this point, the labeling reaction was stopped by addition of sodium metabisulfite (Fisher Scientific Company, Fair Lawn, New Jersey). Free ^{125}I was removed by the procedure of

Tuszynski et al. (1980). Greater than 85% of the radioactivity in the final product was covalently bound to Protein A as determined by TCA precipitation.

Indirect Immunofluorescence

Bovine and porcine longissimus muscle strips were stretched to 2.3 times rest length before rigor and stored in 50% (v/v) glycerol, 0.02 M potassium phosphate, 0.10 M KCl, pH 7.1, at -25°C until used. Myofibrils were prepared from both unstretched at-death muscle and stretched glycerinated muscle by the procedure of Goll et al. (1974). Indirect immunofluorescence microscopy with bovine titin antiserum used as the primary antibody fraction was performed by F. LaSalle (Iowa State University) according to the procedure of Richardson et al. (1981).

Sample Preparation for Postmortem Studies on Titin

Samples of bovine longissimus muscle were excised within one hr. after exsanguination of ten market weight animals and cut into 2.5 cm thick portions. The muscle portions were stored at 2°, 25° and 37°C. To prevent possible degradation of the protein because of microbial contamination, the muscle samples were wrapped in toweling that had been saturated with an antimicrobial solution (10 mM NaN₃, 100 ppm chloramphenicol, 10 ppm rifamycin), overlaid with Saran film and placed in a covered petri dish. Samples were taken at 0, 1, 3 and 7 days postmortem. At each sampling time, the exterior of the muscle was trimmed to a depth of 5 mm and only the interior portion was used. Both whole muscle homogenates and highly purified myofibrils were prepared from the trimmed sample and

electrophoretically analyzed for the presence of titin on high porosity (3.2% acrylamide with no stacking gel) SDS-polyacrylamide slab gels. Purified myofibrils were prepared using the procedure of Goll et al. (1974). Aliquots of the myofibrils were diluted with SDS sample buffer [2% (w/v) SDS, 10 mM sodium phosphate, pH 7.0; Bechtel and Parrish, 1983] to the appropriate protein concentration and then homogenized with a Kontes glass homogenizer. Whole muscle samples (0.5 g) were scissor-minced and then homogenized (Kontes glass homogenizer) in 14.5 ml of the SDS buffer as described by Bechtel and Parrish, 1983. The diluted whole muscle homogenates were heated with stirring in a boiling water bath for 3 min. and then centrifuged at 1,500 x g for 10 min. to remove any traces of undissolved material. The supernatants of the centrifuged whole muscle samples were utilized for electrophoretic analysis. Protein concentrations of both purified myofibrils and whole muscle samples were determined according to the biuret method (Robson et al., 1968).

Gel Electrophoresis

SDS-PAGE was performed according to the method of Studier (1973) using low percent SDS-polyacrylamide (3.2% acrylamide with no stacking gel) slab gels for titin isolation from gel filtration column chromatography of bovine, porcine and chicken skeletal muscle myofibrils. Titin was detected in purified myofibrils and whole muscle samples from bovine muscle, and from chicken pectoralis major and mixed thigh muscles, using 3.2% SDS-polyacrylamide slab gels. High percent SDS-polyacrylamide (10% acrylamide with 5% stacking gel) slab gels were also utilized for titin isolation from chicken pectoralis major and mixed thigh purified

myofibrils and whole muscle samples. The weight ratio of bisacrylamide to acrylamide was always 1:30 and the dimension of the slab gels were 14 cm x 10 cm x 12 mm. For SDS-PAGE of column purified proteins from bovine sternomandibularis muscle, a long (17 cm x 16 cm x 12 mm) low percent SDS-polyacrylamide (5% acrylamide with no stacking gel) slab gel was utilized. Protein samples for SDS-PAGE were heated at 100°C for 3 min. in 7.5% (v/v) 2-mercaptoethanol, 1.5% (w/v) SDS, 15% (w/v) sucrose, 0.03% (w/v) bromphenol blue, and 15 mM 2-N-morpholinoethanesulfonic acid (pH 6.5) using the procedure of Bechtel (1979). Fifty µg of protein were loaded for each gel sample to detect minor components. Gels were stained with Coomassie brilliant blue [0.2% (w/v) Coomassie brilliant blue, 7% (v/v) acetic acid, 40% (v/v) methanol]. Column purified rabbit titin and nebulin standards were kindly supplied by Dr. Kuan Wang, University of Texas at Austin.

Microbiological Determinations

Microbiological counts were determined for both surface and internal portions of bovine longissimus muscle after storage at 2°, 25° and 37°C for seven days postmortem. Two 25 gm samples, prepared as described previously for postmortem studies on titin, one from the surface and one from the internal area of the muscle, were blended for one min. with 225 ml of peptone water for all storage temperatures to give a 1:10 dilution sample. One ml of the 1:10 sample was diluted with 99 ml of peptone water resulting in a 1:1000 dilution. One ml of the 1:1000 dilution was then plated. Plating 1 ml and 0.1 ml aliquots of the 1:10 dilution gave 1:10 and 1:100 plates, respectively.

The muscle slurries were plated in duplicate on TSA (Trypticase Soy Agar, BBL) and incubated for 48 hrs. at 35°C. After the prescribed incubation period, plates were examined for growth with a Quebec colony counter (Fisher Scientific Company, Fair Lawn, New Jersey). The counting procedure followed the Standard Plate Count Method of Gilliland et al. (1976). The gelatin hydrolysis test was performed for all samples according to the procedures of Smibert and Krieg (1981) and microbial colonies were gram stained.

Comparison of Relative Amounts of Titin and Nebulin Present to Other Myofibrillar Proteins

In order to determine if the amount of titin and nebulin present changes with postmortem storage, purified myofibrils were prepared from at-death bovine longissimus muscle, and from muscle stored for 3 and 7 days postmortem at 25°C. SDS-extracts of the myofibrils were each chromatographed successively on the same Bio-Gel A-50m permeation column according to the method of Wang et al. (1979) described previously, to reduce variations in column packing. The study was done in duplicate using muscle samples from two animals. The relative amounts of titin and nebulin (measured together) in comparison to all of the other myofibrillar proteins were determined from elution profiles by measuring the area under the titin and nebulin peak and the total column profile peak area. This same procedure was utilized for comparing the relative amounts of titin and nebulin in chicken pectoralis major with combined titin and nebulin in mixed thigh muscle samples. Two animals were used for both the pectoralis major and mixed thigh muscle samples.

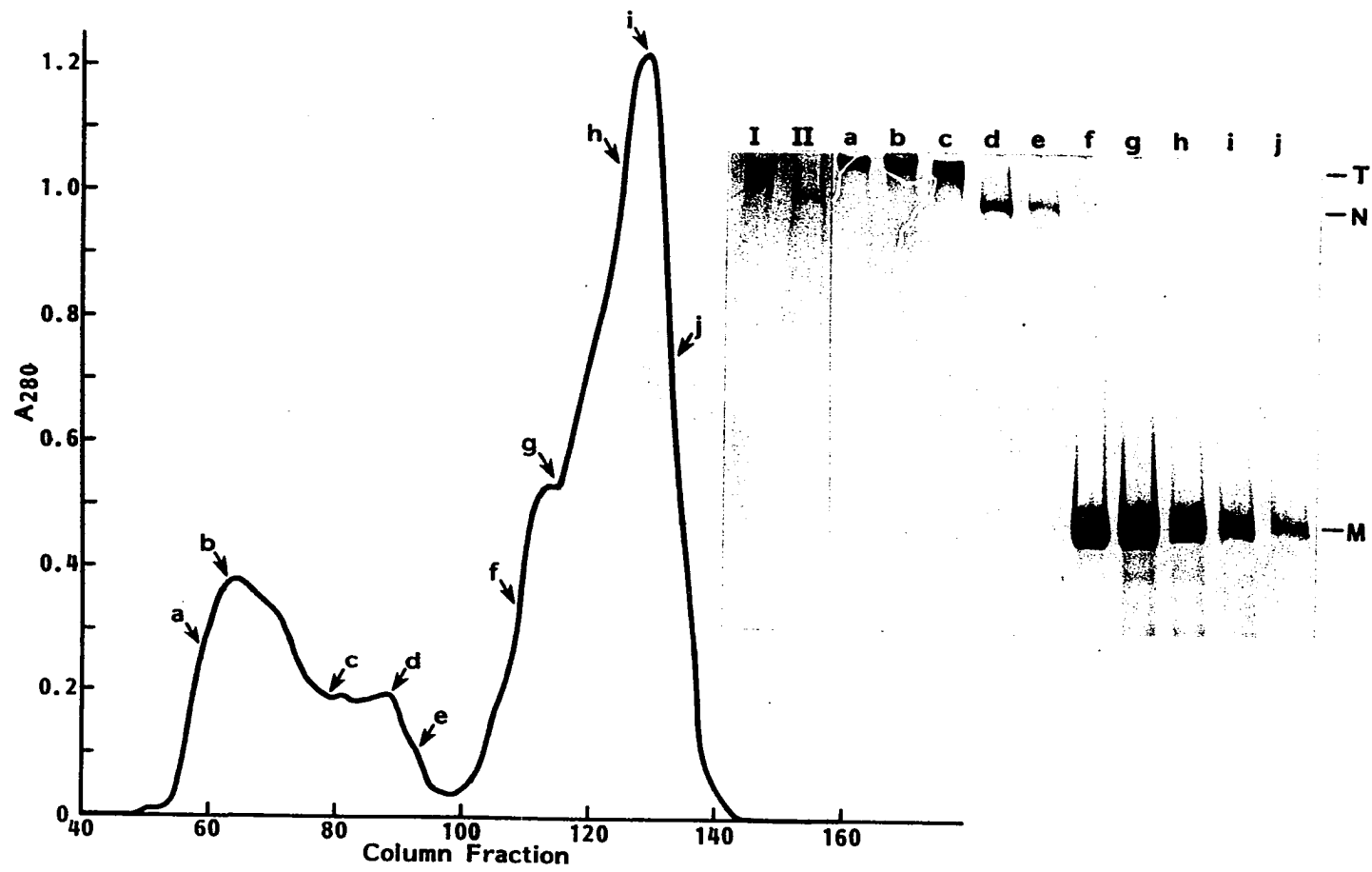
RESULTS AND DISCUSSION

Isolation and Localization of Skeletal Muscle Titin

Initial experiments demonstrated that myofibrils isolated from bovine longissimus muscle contained titin that was similar to the purified titin of chicken and rabbit skeletal muscle discovered by Wang and coworkers (Wang et al., 1979; Wang and Williamson, 1980; Wang, 1982a). A typical elution profile obtained by agarose gel filtration column chromatography of the SDS-extract of purified myofibrils isolated from bovine longissimus muscle is shown in Figure 1. The profile pattern for bovine myofibrils was quite similar to that obtained by Wang et al. (1979) for purified chicken breast muscle titin and nebulin (nebulin was originally referred to as band 3) and also to the column profile for rabbit skeletal muscle titin and nebulin (Wang, 1982a). Using agarose gel filtration, Wang et al. (1979) demonstrated that the leading edge of the first peak in the column elution profile contained titin (band 1 and 2) and the shoulder on the descending edge of this peak was nebulin. Myosin heavy chains and actin (plus the other myofibrillar proteins with molecular weights smaller than actin) were eluted in the second and third peaks, respectively. In the column elution profile of myofibrils isolated from bovine longissimus muscle and the accompanying gel inset (Figure 1), highly purified titin was observed in the leading edge of the first peak, as evidenced by the gel patterns in lane a and b. The descending end of this peak contained a smear of degraded titin as shown in lane c of the gel inset. Nebulin eluted either as a shoulder on the descending edge of the titin peak or as a smaller peak immediately after the titin peak as shown

Figure 1. Agarose gel filtration column profile of the SDS-extract of bovine longissimus muscle myofibrils and accompanying inset of SDS-polyacrylamide slab gel electrophoretic patterns of eluted proteins

Freshly prepared bovine longissimus muscle myofibrils were solubilized in 10% (w/v) SDS, 10 mM EDTA, 0.1 mM PMSF, 0.2 M Tris-HCl, pH 8.0, as described by Wang et al. (1979). The SDS-extract was centrifuged at 88,000 x g for one hr. and the supernatant containing 200 mg protein was loaded onto a Bio-Gel A-50m permeation column (2.5 x 90 cm) previously equilibrated with elution buffer [0.1% (w/v) SDS, 5 mM EDTA, 0.5 mM DTT, 0.1 M Tris-HCl, pH 8.8]. Four and one-half ml fractions were collected at a rate of 15 ml/hr. and each fraction was analyzed for protein by absorbance at 280 nm. The inset shows a 3.2% SDS-polyacrylamide gel (lanes a-j) corresponding to the eluted fractions (lettered arrows a-j) obtained from the SDS-extract of bovine longissimus muscle myofibrils. Twenty μ l of the appropriate fraction were loaded on each lane of the slab gel. The positions of titin, nebulin and of the myosin heavy chains on the gel are identified as T, N and M, respectively. Rabbit titin (I) and nebulin (II) shown in the inset were provided by Dr. Kuan Wang, University of Texas at Austin.



in Figure 1. In this column elution profile (Figure 1), the protein nebulin began to appear in the trailing edge of the first peak as confirmed by the banding pattern observed in lane c. Fairly pure nebulin was found in the smaller peak following the titin peak which corresponded to lanes d and e of the gel inset. Myosin heavy chains were eluted in the large peak following the titin and nebulin peaks as evidenced by the bands seen in lanes f through j. The other myofibrillar proteins of molecular weights lower than myosin heavy chains were included in the last column peak, but because of the high-porosity gels used, these proteins migrated off the bottom of the gel. Furthermore, because of the unusually large size of titin ($M_r \sim 1 \times 10^6$) and nebulin ($M_r \sim 5 \times 10^5$), these proteins do not enter into high percent SDS-polyacrylamide gels (Wang et al., 1979). Therefore, to separate them electrophoretically, it was necessary to use low percent SDS-polyacrylamide gels. With these high-porosity gels (3.2% SDS-polyacrylamide), it was difficult to process these fragile gels throughout staining, destaining, and photography. Wang (1982a) also noted similar difficulties in handling these high-porosity gels.

Wang (1982a) observed that bands 1 and 2 of the titin doublet were partially resolved in the first peak of the gel filtration column profile. Pooling of early fractions of the titin peak resulted in a final preparation enriched in band 1 (Wang, 1982a). Therefore, although titin was observed as a closely spaced doublet in purified myofibrils (Wang et al., 1979), chromatographically purified rabbit titin (lane I of inset in Figure 1) and chromatographically purified bovine longissimus muscle titin (lane a and b of the inset in Figure 1) migrated as a single broad band in our SDS-PAGE system. In our studies, the electrophoretic analysis of

whole-muscle samples and purified myofibrils on 3.2% SDS-polyacrylamide slab gels, titin did not consistently appear as a doublet, as it also often migrated as a single major band. The titin band usually appeared as a closely spaced doublet, however, when only a very light load of protein was electrophoresed. Another possibility for the inconsistent appearance of the closely spaced doublet is that native titin exists as a singular molecular weight species, and the doublet seen on these gels is the result of degradative processes. Wang (1982a,b) suggested that the two bands of the titin doublet are related structurally since limited proteolytic degradation of titin in situ converted band 1 into a major fragment that comigrated with band 2.

On the 3.2% SDS-polyacrylamide slab gel shown in Figure 2, it can be observed that titin purified from myofibrils prepared from at-death bovine longissimus muscle (lane c) comigrated as a single band with a sample of rabbit skeletal muscle titin supplied by Dr. Kuan Wang (lane b). Purified bovine and rabbit muscle titins also comigrated with the titin band in the bovine myofibril sample (lane d). Purified nebulin from rabbit skeletal muscle provided by Dr. Kuan Wang (lane a) comigrated with a corresponding putative nebulin band in the sample of purified bovine myofibrils (lane d). Consequently, the titin and nebulin bands found in myofibrils isolated from bovine longissimus muscle have similar migration patterns on SDS-polyacrylamide slab gels as do titin and nebulin found in rabbit skeletal muscle.

To determine if the amount or the electrophoretic mobility of titin and nebulin varied with different muscles, samples from bovine longissimus

Figure 2. Comparative SDS-polyacrylamide slab gel electrophoretic patterns of purified bovine and rabbit titin, purified rabbit nebulin and the high molecular weight polypeptides present in bovine longissimus muscle myofibrils

Column purified rabbit nebulin (a), column purified rabbit titin (b), column purified bovine titin (c) and purified bovine myofibrils (d), were electrophoresed on a 3.2% SDS-polyacrylamide slab gel by the procedure of Studier (1973). The positions of titin, nebulin, and of the myosin heavy chains on the gel are identified as T, N and M, respectively. Rabbit titin (b) and nebulin (a) were provided by Dr. Kuan Wang, University of Texas at Austin.



dorsi, biceps femoris and psoas major muscles were electrophoretically analyzed (Figure 3). Both whole muscle samples (a) and purified myofibrils (b) were prepared from each muscle and were electrophoresed on a 3.2% SDS-polyacrylamide slab gel. There were only small differences in the amount of titin and nebulin from the different muscles sampled and these differences were most likely due to slight variations in the amount of protein loaded. Also, the migration of titin and nebulin in whole-muscle samples and purified myofibrils from bovine longissimus, biceps femoris and psoas major muscles was similar.

The amino acid composition of chromatographically purified bovine skeletal muscle titin (Table 1) was compared with that of Wang's (personal communication, University of Texas at Austin, Dept. of Chemistry) chromatographically purified rabbit titin and the high molecular weight components of connectin (titin) isolated chromatographically (using the procedure of Wang et al., 1979) from chicken breast myofibrils (Maruyama et al., 1981b). Bovine titin differed from rabbit titin by approximately 20% in its content of lysine, glutamic acid, valine, methionine, and cysteine and by approximately 10% in its content of aspartic acid, threonine, serine, proline, isoleucine and arginine. Despite these differences in amino acid composition, which may be due to species variation or to small technical differences in laboratories, it is believed that the protein isolated from bovine longissimus muscle is titin. Neither bovine or rabbit titin differed greatly from the amino acid composition of chicken breast high molecular weight connectin reported by Maruyama et al. (1981b).

To identify the location of titin in the myofibril structure, antibodies to titin were produced by injecting rabbits with chromatographically puri-

Figure 3. Comparative SDS-polyacrylamide slab gel electrophoretic patterns of whole muscle samples and purified myofibrils from bovine longissimus dorsi, biceps femoris and psoas major muscles

Whole muscle samples (a) and purified myofibrils (b) from bovine longissimus dorsi (LD), biceps femoris (BF) and psoas major (PM) muscles were electrophoresed on a 3.2% SDS-polyacrylamide slab gel using the procedure of Studier (1973). Fifty μg of protein were loaded on each lane of the gel. The positions of titin, nebulin and of the myosin heavy chains on the gel are identified as T, N and M, respectively.

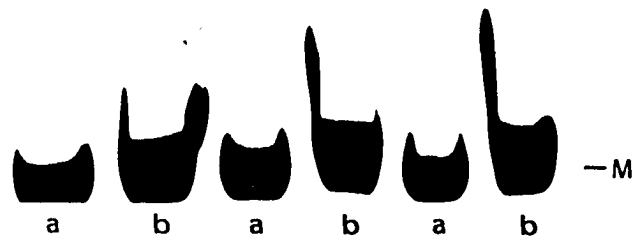
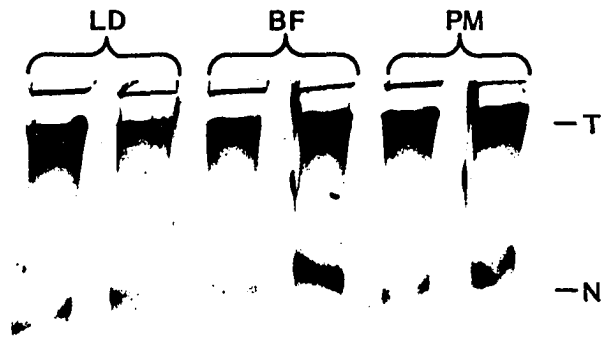


Table 1. Amino acid analysis of bovine titin, rabbit titin and chicken high molecular weight connectin^a

Amino Acid	Bovine Titin ^b	Rabbit Titin ^c	High Molecular Weight Chicken Connectin ^d
Asx	8.5 ± 0.1	9.6	9.3
Thr	6.8 ± 0.1	7.9	6.6
Ser	6.7 ± 0.3	7.4	6.7
Glx	11.6 ± 0.1	13.7	11.8
Pro	8.3 ± 0.1	7.4 ^e	6.7
Gly	7.0 ± 0.4	6.5	7.6
Ala	6.5 ± 0.0	6.6	7.5
Val	9.3 ± 0.2	7.4	7.8
Met	1.2 ± 0.0	0.8	1.6
Ile	5.5 ± 0.1	4.4	5.6
Leu	6.1 ± 0.1	6.5	7.6
Tyr	2.9 ± 0.1	2.8	3.0
Phe	2.6 ± 0.2	2.4	2.9
Lys	8.7 ± 0.1	10.2	7.9
His	1.5 ± 0.1	1.7	1.8
Arg	4.9 ± 0.1	4.4	5.0
Cys	1.3 ± 0.1	0.8	0.6
Trp	1.3 ± 0.2	nd ^f	nd ^f

^aAll values are expressed as mole percent.

^bResults are expressed as means plus or minus the standard error of the means of analyses from three separate column purified titin preparations.

^cData are from Wang, K., University of Texas at Austin, personal communication.

^dData taken from Maruyama et al., 1981b.

^eProline values of 8-9% were reported in Wang, 1982b.

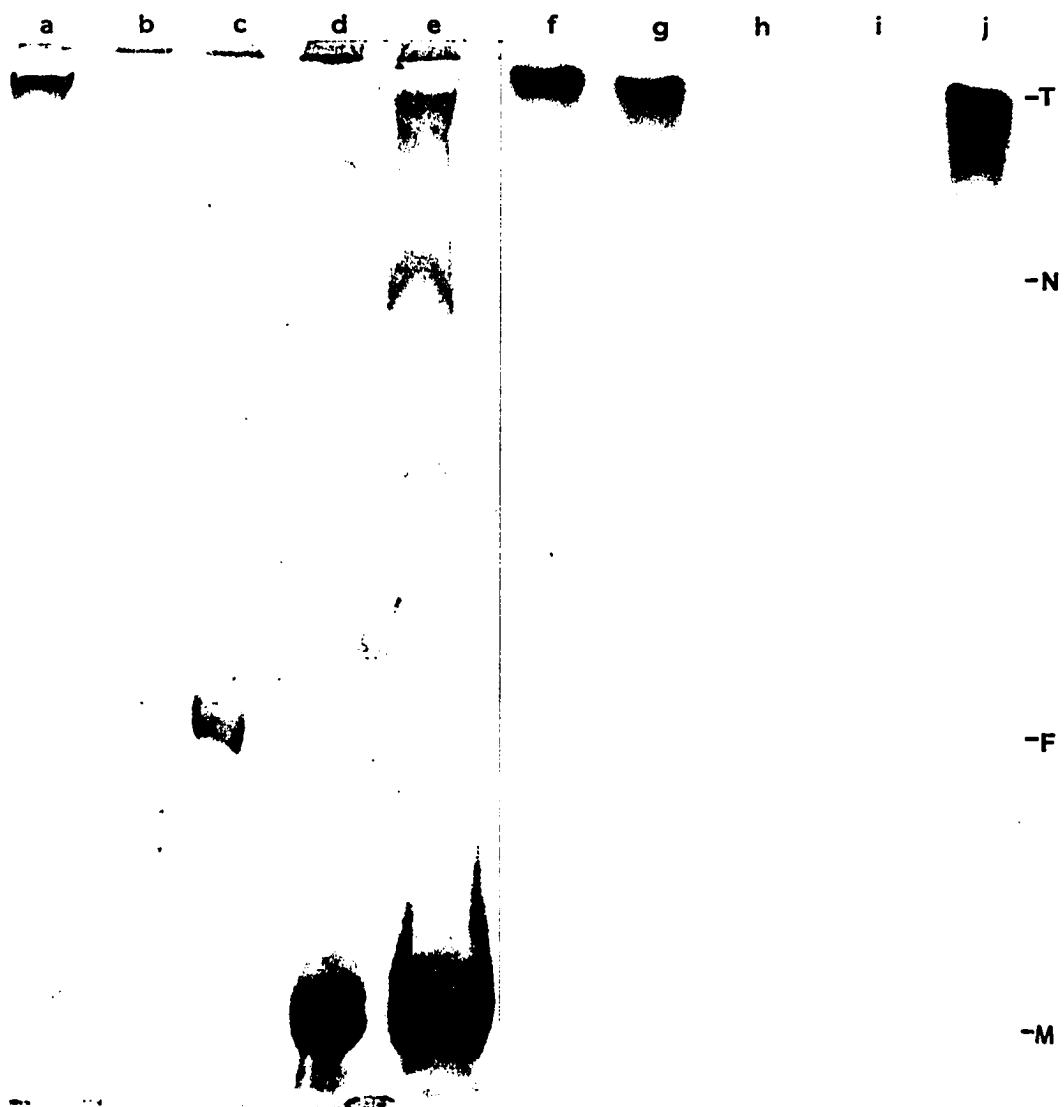
^fnd = not determined.

fied titin from myofibrils isolated from bovine longissimus muscle. Characterization of antiserum against column purified bovine longissimus muscle titin by autoradiography of a 3.2% SDS-polyacrylamide slab gel using [I^{125}] protein A is shown in Figure 4. Protein A can be isolated from the cell wall of Staphylococcus aureus and it interacts with the immunoglobulins of almost all mammals (Surolia et al., 1982). Immunoautoradiographs of chromatographically purified bovine skeletal muscle titin (lanes a and f), chromatographically purified rabbit skeletal muscle titin (lanes b and g), porcine stomach filamin (lanes c and h), bovine skeletal muscle myosin (lanes d and i) and purified myofibrils from bovine longissimus muscle (lanes e and j), prepared using titin antiserum, demonstrated that titin antiserum recognized chromatographically purified bovine skeletal muscle titin, chromatographically purified rabbit skeletal muscle titin (kindly provided by Dr. Kuan Wang, University of Texas at Austin), and titin contained in purified myofibrils from bovine longissimus muscle. The titin antiserum, however, did not cross-react with porcine stomach filamin, bovine skeletal muscle myosin or other high molecular weight proteins of the myofibril. The fact that titin antiserum prepared against chromatographically purified bovine skeletal titin cross-reacted with the sample of chromatographically purified rabbit skeletal titin supplied by Dr. Kuan Wang, reaffirmed that the protein isolated from bovine longissimus muscle was, in fact, titin.

In order to demonstrate that proteins with subunit molecular weights of less than 100,000, which migrated with the dye front or off the low percent SDS-polyacrylamide gels, do not react with titin antiserum, it was also necessary to perform autoradiography with higher percent

Figure 4. Characterization of antiserum prepared against bovine skeletal muscle titin by autoradiography on a low percent SDS-polyacrylamide slab gel using [I^{125}] protein A

For autoradiography, chromatographically purified bovine longissimus muscle titin (a), chromatographically purified rabbit skeletal muscle titin (b), porcine stomach filamin (c) bovine skeletal muscle myosin (d) and purified bovine longissimus muscle myofibrils (e) were electrophoresed on 3.2% SDS-polyacrylamide slab gels using the procedure of Studier (1973). Lanes a, b, c, d and e are the control samples of the stained gel corresponding to lanes f, g, h, i and j, respectively. Lanes f through j represent the autoradiograph of the vacuum-dried gel that had been incubated with titin antiserum followed by [I^{125}] protein A. The positions of titin, nebulin, filamin and of the myosin heavy chains on the gel are identified as T, N and M, respectively. Rabbit titin was provided by Dr. Kuan Wang, University of Texas at Austin.



SDS-polyacrylamide slab gels. Antiserum prepared against column purified bovine longissimus muscle titin was also characterized by autoradiography of a 10% SDS-polyacrylamide slab gel with a 5% SDS-polyacrylamide stacking gel using [I^{125}] protein A (results not shown). Samples of purified myofibrils from bovine longissimus muscle along with purified rabbit skeletal titin were electrophoresed according to the procedure of Studier (1973). In low porosity gels, titin does not enter the 10% SDS-polyacrylamide gel but remains at the top of the 5% stacking gel. Immunoautoradiography using bovine titin antiserum demonstrated that the titin antiserum was bound to titin in the stacking gel, but it was not bound to any lower subunit molecular weight proteins.

With bovine skeletal muscle titin antiserum, titin was localized by indirect immunofluorescence in myofibrils of bovine and porcine longissimus muscle (Figure 5). It was observed that titin antibody localization varied significantly in staining patterns and relative intensity in any given preparation and appeared to be a function of sarcomere lengths. Representative pairs of phase and fluorescence micrographs of the most frequently observed patterns are shown in Figure 5. Indirect immunofluorescence labeling using titin antiserum on myofibrils from unstretched bovine longissimus muscle (Figure 5, a and b) with sarcomere lengths of approximately 2.65 μm exhibited bright staining in the area of the A- and I-band junction of each sarcomere. Some diffuse staining in the central region of the A-band was also observed. Myofibrils from bovine longissimus muscle were prepared from muscle samples that had been stretched pre-rigor to 2.3 times rest length to increase the I-band and sarcomere lengths (Figure 5, c and d). In the myofibrils from stretched bovine longissimus

Figure 5. Indirect immunofluorescence localization of titin in bovine and porcine longissimus muscle myofibrils

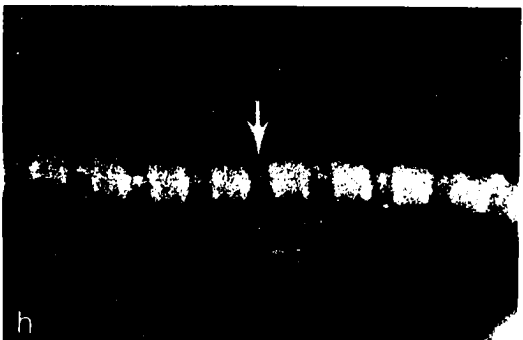
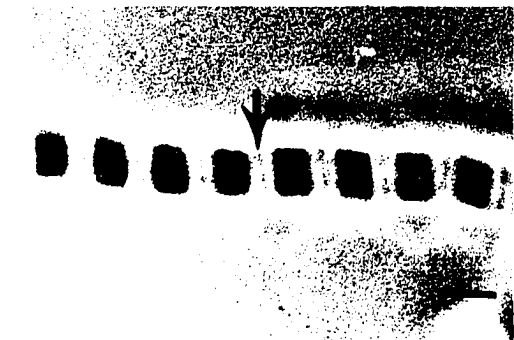
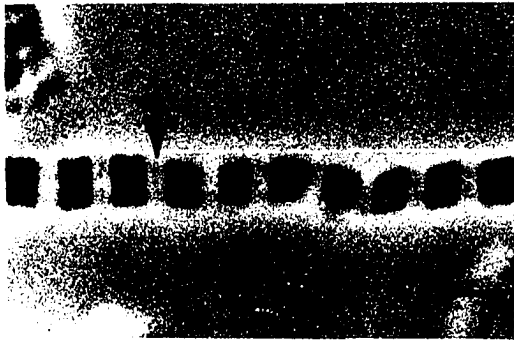
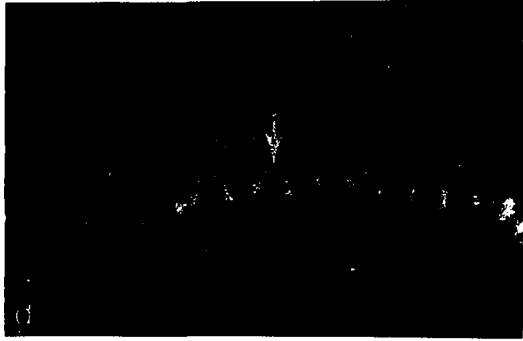
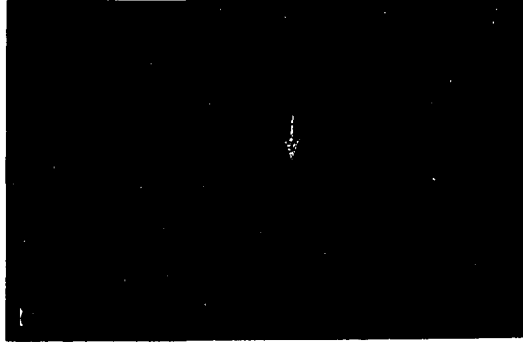
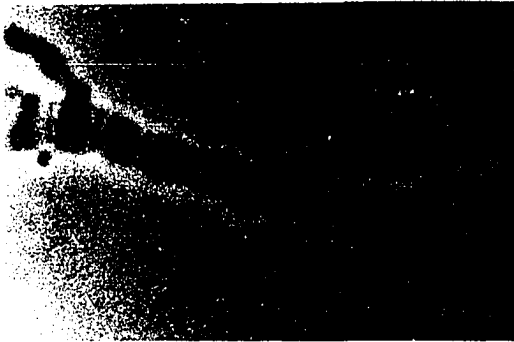
a and b. Phase contrast and fluorescence micrographs, respectively, of bovine longissimus muscle myofibrils treated with titin antiserum. Arrow points to Z line. Sarcomere length ~ 2.65 μm . Note bright fluorescence in the area of the A-I junction and diffuse staining in the central region of the A band.

c and d. Phase contrast and fluorescence micrographs, respectively, of bovine longissimus muscle myofibrils treated with titin antiserum. The myofibrils were prepared from muscle samples that had been stretched pre-rigor to 130% of rest length. Arrow points to Z line. Sarcomere length ~ 3.25 μm . Note bright fluorescence in the area of A-I junction, diffuse staining throughout the A band, with the central region intensely labeled.

e and f. Phase contrast and fluorescence micrographs, respectively, of porcine longissimus muscle myofibrils treated with titin antiserum. Arrow points to Z line. Sarcomere length ~ 2.70 μm . Note the narrow bands of labeling in the area of the A-I junction with diffuse staining throughout the A band.

g and h. Phase contrast and fluorescence micrographs, respectively, of porcine longissimus muscle myofibrils treated with titin antiserum. The myofibrils were prepared from muscle samples that had been stretched pre-rigor to 130% of rest length. Arrow points to Z line. Sarcomere length ~ 3.20 μm . Note bright fluorescence in the area of the A-I junction, intense labeling throughout the entire A band with some punctate fluorescence in the region of the Z line.

Indirect immunofluorescence performed by F. LaSalle (Iowa State University). Bars in each figure represent 2.5 μm .



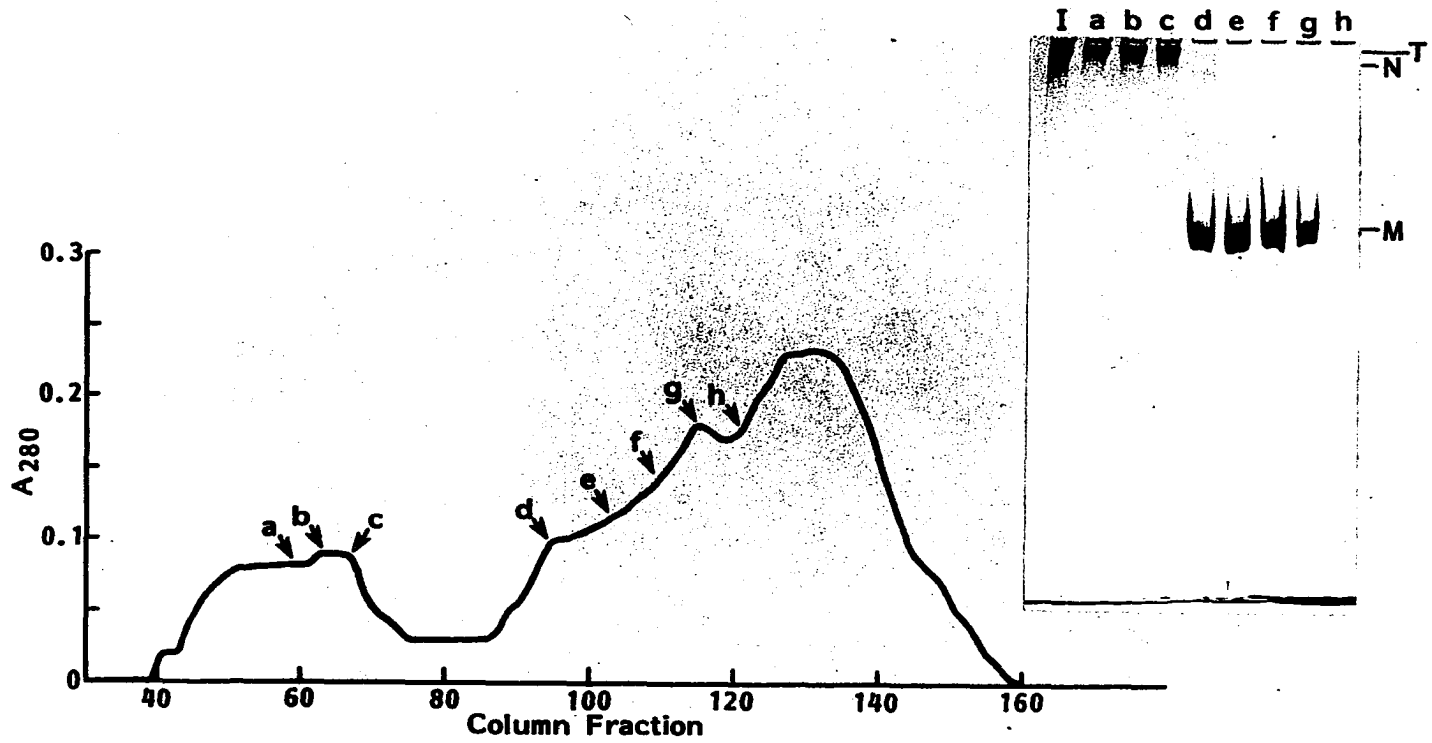
muscle with sarcomere lengths of approximately 3.25 μm , the regions of the A-I junction were again brightly labeled. There was some weak staining throughout the A-band with the central region, presumably due to the H-zone or M-line being intensely labeled. Myofibrils from porcine longissimus muscle with sarcomere lengths of approximately 2.70 μm (Figure 5, e and f) showed fluorescent labeling of the titin antiserum primarily as narrow bands at the area of the junctions of the A- and I-bands. Some diffuse staining was again observed throughout the A-band. However, in myofibrils prepared from porcine muscle samples stretched pre-rigor to 2.3 times rest length (sarcomere length of approximately 3.20 μm), the A-I junctions were intensely labeled with bright staining throughout the entire A-band (Figure 5, g and h). Some punctate fluorescence was also observed in the region of the Z-line. The staining patterns shown in Figure 5 appeared somewhat similar to those reported by Maruyama et al. (1981b) and Wang et al. (1979). Maruyama et al. (1981b) treated intact chicken muscle myofibrils with antiserum against high molecular weight connectin (titin) and found the A-I junctions to be strongly fluorescent. The middle region of the A-bands and the whole I-bands were faintly stained, but the Z-lines were not fluorescent. Wang et al. (1979) also localized titin in chicken breast myofibrils by indirect immunofluorescence and found titin antiserum to react with the myofibril in two specific regions. The junctions of A- and I-bands of each sarcomere were brightly stained and the central region of the A-band, presumably either the H-zone or M-line, was less intensely labeled. There was also weak and diffuse staining throughout the entire A-band. In myofibrils with sarcomere lengths of 2.5 and 2.6 μm , the Z-lines were also stained; however, in

myofibrils with shorter sarcomeres no stain in the Z-line area was detectable.

Because most of the research concerning gap filaments has exclusively utilized highly stretched bovine sternomandibularis muscle (Locker and Leet, 1975, 1976a, 1976b; Locker et al., 1976, 1977; Locker and Daines, 1980), gel filtration chromatography was done using myofibrils from bovine sternomandibularis muscle to determine if there were any differences in the column profiles of myofibrils from bovine longissimus and sternomandibularis muscle. The SDS-extract of myofibrils from bovine sternomandibularis muscle was loaded onto a Bio-Gel A-50m agarose gel filtration column (2.5 x 90 cm) as described by Wang et al. (1979), and resulted in a column profile shown in Figure 6. During the solubilization of the myofibrils with an equal volume of hot (~100°C) sample buffer [10% (w/v) SDS, 10 mM EDTA, 0.1 mM PMSF, 0.2 M Tris-HCl, pH 8.0], it was noted that the viscosity of the resulting solution was extremely high. The high viscosity of the SDS-extract of myofibrils from bovine sternomandibularis muscle in contrast to the SDS-extract of myofibrils from bovine longissimus muscle was most likely due to the high connective tissue content of the sternomandibularis muscle. Nevertheless, a gel filtration chromatography column profile of myofibrils from bovine sternomandibularis muscle (Figure 6) was very similar to that of myofibrils from bovine longissimus muscle (Figure 1). The total protein recovered, however, from the SDS-extract of myofibrils from bovine sternomandibularis muscle was much lower than the SDS-extract of myofibrils from bovine longissimus muscle even though 200 mg of protein was loaded onto the gel filtration column in each case. A long (17 cm x 16 cm x 0.12 cm) 5% SDS-polyacrylamide gel

Figure 6. Agarose gel filtration column profile of the SDS-extract of bovine sternomandibularis muscle myofibrils and accompanying inset of SDS-polyacrylamide slab gel electrophoretic patterns of eluted proteins

Freshly prepared bovine sternomandibularis muscle myofibrils were solubilized in 10% (w/v) SDS, 10 mM EDTA, 0.1 mM PMSF, 0.2 M Tris-HCl, pH 8.0, as described by Wang et al. (1979). The SDS-extract was centrifuged at 88,000 x g for one hr. and the supernatant containing 200 mg protein was loaded onto a Bio-Gel A-50m permeation column (2.5 x 90 cm) previously equilibrated with elution buffer [0.1% (w/v) SDS, 5 mM EDTA, 0.5 mM DTT, 0.1 M Tris-HCl pH 8.8]. Four and one-half ml fractions were collected at a rate of 15 ml/hr. and each fraction was analyzed for protein by absorbance at 280 nm. The inset shows a 5% SDS-polyacrylamide gel (lanes a-h) corresponding to the eluted fractions (lettered arrows a-h) obtained from the SDS-extract of bovine sternomandibularis muscle myofibrils. Fifty μ l of the appropriate fraction were loaded on each lane of the slab gel. The positions of titin, nebulin and of the myosin heavy chains on the gel are identified as T, N and M respectively. Rabbit titin (I) shown in the inset, was provided by Dr. Kuan Wang, University of Texas at Austin.



was used to allow titin and nebulin to enter the gel and also to isolate proteins with subunit molecular weights of less than 100,000 that migrated off the 3.2% SDS-polyacrylamide gels. The lower molecular weight proteins, however, traveled with the dye front, resulting in a band at the bottom of the gel (lanes f, g and h in Figure 6). As in the column profile of myofibrils from bovine longissimus muscle (Figure 1), titin was present and recovered from the first peak after the void volume with nebulin being eluted in the descending edge of the titin peak of myofibrils from bovine sternomandibularis muscle (Figure 6). Gel electrophoretic patterns added further evidence that titin and nebulin were present in myofibrils from bovine sternomandibularis muscle (observed in lanes a, b and c, and b and c respectively). Myosin heavy chains and other myofibrillar proteins of lower molecular weights were eluted in a broad asymmetrical second peak. It was also noted that the samples of bovine sternomandibularis muscle titin (lanes a, b and c in Figure 6) comigrated with rabbit skeletal muscle titin (lane I in Figure 6) which was supplied by Dr. Kuan Wang.

Another study was conducted to determine if there were any species variations in column elution profiles of the SDS-extracts of purified myofibrils from bovine longissimus muscle (Figure 1), porcine longissimus muscle (Figure 7), and chicken mixed thigh (Figure 8) and chicken pectoralis major (Figure 9) muscles. A typical elution profile obtained by agarose gel filtration column chromatography of the SDS-extract of purified myofibrils from porcine longissimus muscle (Figure 7) was similar to that of bovine longissimus muscle (Figure 1). Titin appeared in the first peak of the column profile and those fractions eluted as designated correspond to electrophoretic patterns in lanes a through g on the accompanying 3.2%

Figure 7. Agarose gel filtration column profile of the SDS-extract of porcine longissimus muscle myofibrils and accompanying inset of SDS-polyacrylamide slab gel electrophoretic patterns of eluted proteins

Freshly prepared porcine longissimus muscle myofibrils were solubilized in 10% (w/v) SDS, 10 mM EDTA, 0.1 mM PMSF, 0.2 M Tris-HCl, pH 8.0, as described by Wang et al. (1979). The SDS-extract was centrifuged at 88,000 x g for one hr. and the supernatant containing 200 mg protein was loaded onto a Bio-Gel A-50m permeation column (2.5 x 90 cm) previously equilibrated with elution buffer [0.1% (w/v) SDS, 5 mM EDTA, 0.5 mM DTT, 0.1 M Tris-HCl, pH 8.8]. Four and one-half ml fractions were collected at a rate of 15 ml/hr. and each fraction was analyzed for protein by absorbance at 280 nm. The inset shows a 3.2% SDS-polyacrylamide gel (lanes a-k) corresponding to the eluted fractions (lettered arrows a-k) obtained from the SDS-extract of porcine longissimus muscle myofibrils. Twenty-five μ l of the appropriate fraction were loaded on each lane of the slab gel. The positions of titin, nebulin, and of the myosin heavy chains on the gel are identified as T, N and M, respectively. Rabbit titin (I) and nebulin (II) shown in the inset, were provided by Dr. Kuan Wang, University of Texas at Austin.

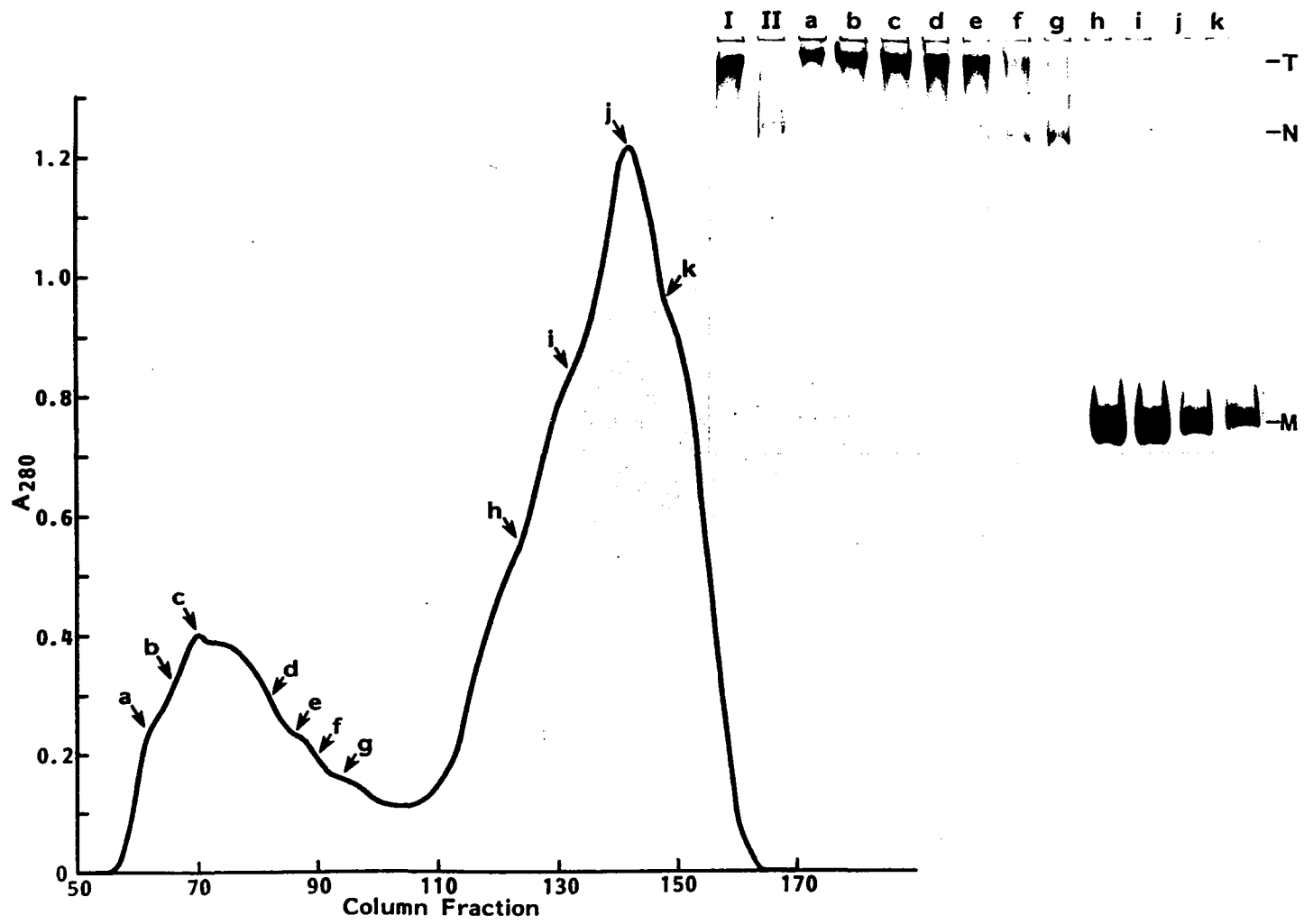


Figure 8. Agarose gel filtration column profile of the SDS-extract of chicken mixed thigh muscle myofibrils and accompanying inset of SDS-polyacrylamide slab gel electrophoretic patterns of eluted proteins

Freshly prepared chicken mixed thigh muscle myofibrils were solubilized in 10% (w/v) SDS, 10 mM EDTA, 0.1 mM PMSF, 0.2 M Tris-HCl, pH 8.0, as described by Wang et al. (1979). The SDS-extract was centrifuged at 88,000 x g for one hr. and the supernatant containing 200 mg protein was loaded onto a Bio-Gel A-50m permeation column (2.5 x 90 cm) previously equilibrated with elution buffer [0.1% (w/v) SDS, 5 mM EDTA, 0.5 mM DTT, 0.1 M Tris-HCl, pH 8.8]. Four and one-half ml fractions were collected at a rate of 15 ml/hr. and each fraction was analyzed for protein by absorbance at 280 nm. The inset shows a 3.2% SDS-polyacrylamide gel (lanes a-k) corresponding to the eluted fractions (lettered arrows a-k) obtained from the SDS-extract of chicken mixed thigh muscle myofibrils. Twenty-five μ l of fractions c, d, h, i, j and k, thirty μ l of fractions b and g, thirty-five μ l of fractions a and e and forty μ l of fraction f were loaded on the slab gel. The positions of titin, nebulin and of the myosin heavy chains on the gel are identified as T, N and M, respectively. Rabbit titin (I) shown in the inset was provided by Dr. Kuan Wang, University of Texas at Austin.

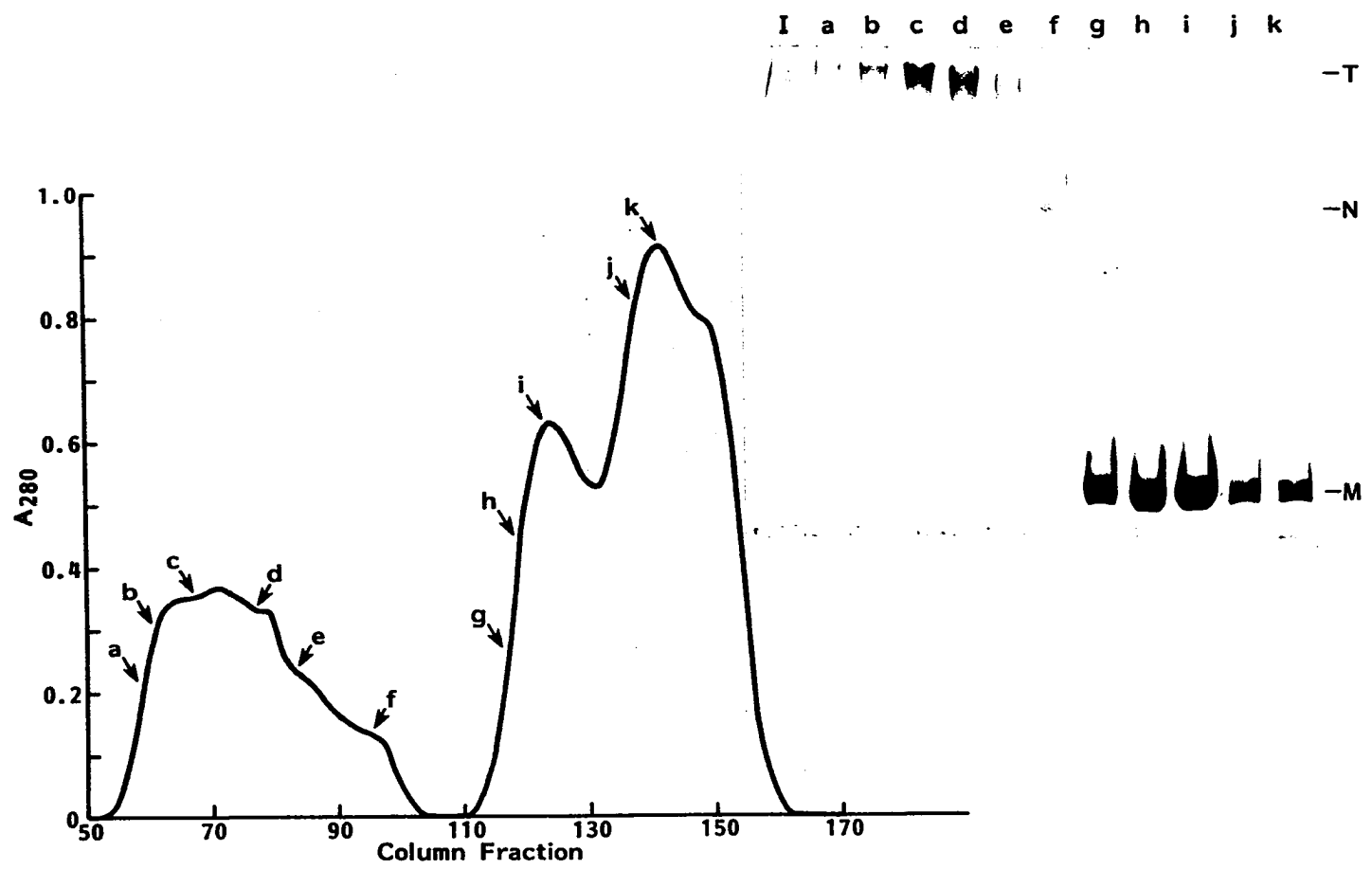
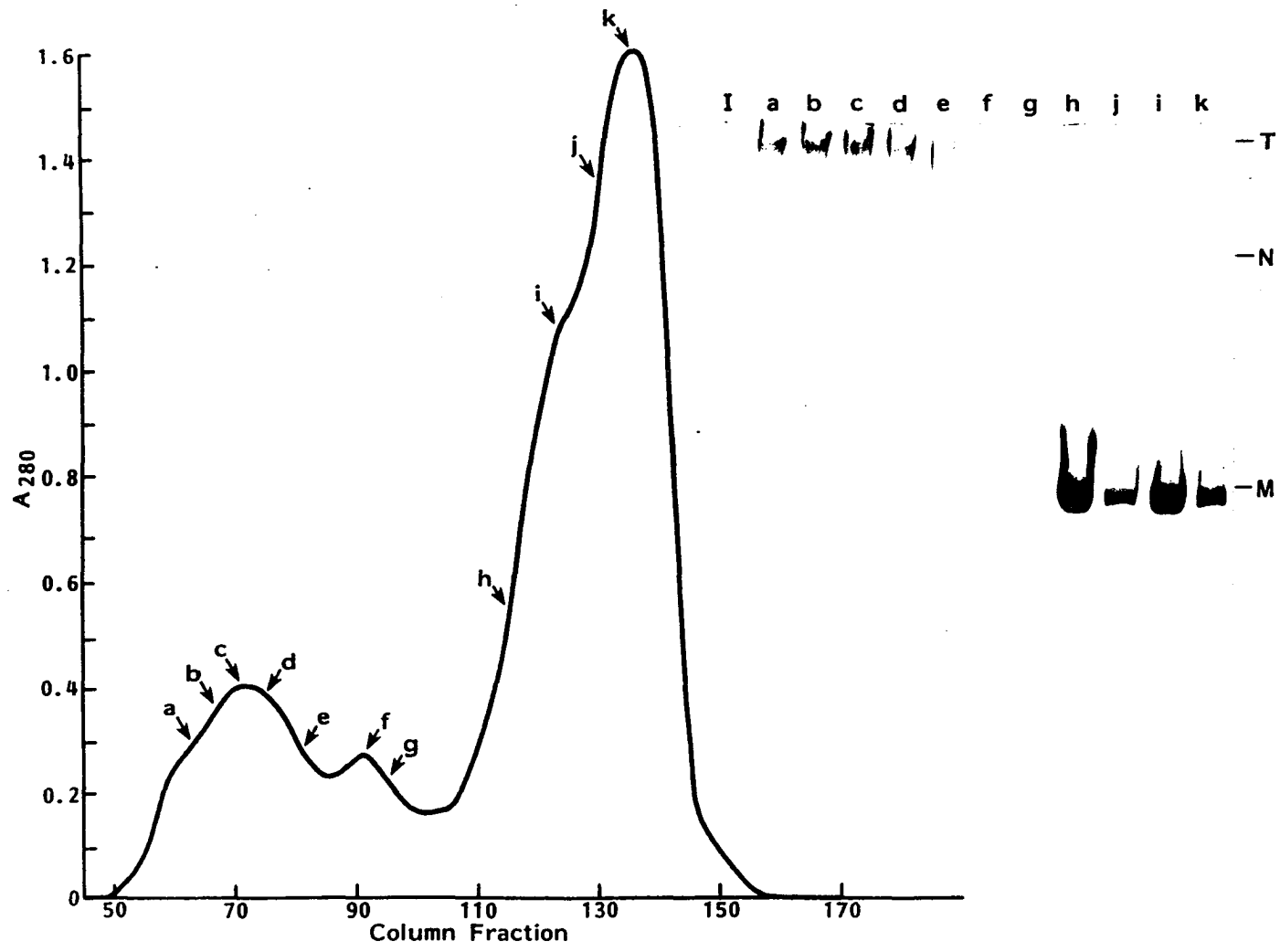


Figure 9. Agarose gel filtration column profile of the SDS-extract of chicken pectoralis major muscle myofibrils and accompanying inset of SDS-polyacrylamide slab gel electrophoretic patterns of eluted proteins

Freshly prepared chicken pectoralis major muscle myofibrils were solubilized in 10% (w/v) SDS, 10 mM EDTA, 0.1 mM PMSF, 0.2 M Tris-HCl, pH 8.0, as described by Wang et al. (1979). The SDS-extract was centrifuged at 88,000 x g for one hr. and the supernatant containing 200 mg protein was loaded onto a Bio-Gel A-50m permeation column (2.5 x 90 cm) previously equilibrated with elution buffer [0.1% (w/v) SDS, 5 mM EDTA, 0.5 mM DTT, 0.1 M Tris-HCl, pH 8.8]. Four and one-half ml fractions were collected at a rate of 15 ml/hr. and each fraction was analyzed for protein by absorbance at 280 nm. The inset shows a 3.2% SDS-polyacrylamide gel (lanes a-k) corresponding to the eluted fractions (lettered arrows a-k) obtained from the SDS-extract of chicken pectoralis major muscle myofibrils. Twenty μ l of fraction k, twenty-five μ l of fractions i and j, thirty μ l of fractions c and d, thirty-five μ l of fractions b, e, f and h, and forty μ l of fractions a and g were loaded on the slab gel. The positions of titin, nebulin and of the myosin heavy chains on the gel are identified as T, N and M, respectively. Rabbit titin (I) shown in the inset was provided by Dr. Kuan Wang, University of Texas at Austin.



SDS-polyacrylamide gel (Figure 7). Highly purified titin appeared in the leading edge of the first peak as shown in lanes a, b and c, of the gel inset, while partially degraded titin appeared on the descending portion of the peak as evidenced by a downward smear shown in lanes d through g on the inset. Nebulin also eluted on the descending edge of the titin peak as confirmed by the banding pattern observed in lanes d through g. Myosin heavy chains eluted in the large second peak as evidenced by the bands seen in lanes h through k. The other myofibrillar proteins with molecular weights lower than myosin heavy chains were included in the last column peak but have traveled off the bottom of the gel. It is evident that chromatographically purified porcine titin (lanes a through g) and nebulin (lanes d through g) comigrated with the samples of chromatographically purified rabbit titin (Lane I) and nebulin (Lane II) supplied by Dr. Kuan Wang.

Along with species variation, possible differences in column profiles due to muscle fiber type were explored. The column elution profiles of SDS-extracts of red muscle (column profile of chicken mixed thigh muscle myofibrils in Figure 8), and white muscle (column profile of chicken pectoralis major muscle myofibrils in Figure 9), were separately loaded onto a Bio-Gel A-50m agarose gel filtration column (2.5 x 90 cm) as described in the Materials and Methods section. Both of the profile patterns from chicken mixed thigh muscle myofibrils (Figure 8) and from chicken pectoralis major muscle myofibrils (Figure 9) were similar to that obtained for bovine longissimus muscle myofibrils (Figure 1). In the column profile of myofibrils from chicken mixed thigh muscle (red) shown in Figure 8, titin was eluted in the first peak after the void volume which corresponds

to lanes a through e on the accompanying slab gel. Highly purified titin was observed only on the ascending edge of this peak as shown in lanes a and b of the gel inset, whereas partially degraded titin was evident near the top of the peak as shown in lanes c and d in Figure 8. More extensive degradation of titin was noted on the descending portion of the titin peak as evidenced by the broad smear shown in lane e of the gel inset. The protein nebulin appeared in a shoulder on the descending edge of the first peak as confirmed by the banding pattern observed in lane f. Myosin heavy chains plus other myofibrillar proteins were eluted in the second and third peaks, respectively; however, proteins with molecular weights of less than 200,000 migrated off the accompanying 3.2% SDS-polyacrylamide gel. Purified skeletal muscle titin (Lane I in Figure 8) supplied by Dr. Kuan Wang, comigrated with chromatographically purified titin from myofibrils of chicken mixed thigh muscle (lanes a through e in Figure 8). Chicken skeletal muscle nebulin prepared from both mixed thigh muscle myofibrils and from pectoralis major muscle myofibrils, consistently migrated in electrophoretic systems with a slightly faster mobility than the rabbit skeletal muscle nebulin supplied by Dr. Kuan Wang, indicating it was approximately 20,000 daltons smaller (results not shown).

As with the column profile for myofibrils from chicken mixed thigh muscle (Figure 8), titin isolated from purified myofibrils from chicken pectoralis major muscle appeared in the first peak of the elution profile. This was confirmed by the banding pattern observed in lanes a through e in Figure 9. Some titin degradation was apparent in both the ascending and descending portions of the titin peak. Extensive degradation of titin, however, was observed only on the lower portion of the trailing edge of

the titin peak which corresponded to lane e of the gel inset. Nebulin eluted as a smaller peak immediately after the titin peak as shown in lanes f and g of the corresponding gel. Myosin heavy chains and the myofibrillar proteins with molecular weights of less than 200,000 that have been eluted off the high porosity gel were found in the second peak of the column profile. The sample of purified rabbit titin in Lane I (supplied by Dr. Kuan Wang) in Figure 9, comigrated with column purified chicken pectoralis major muscle titin.

A preliminary study was conducted to compare the ratio of titin plus nebulin to all of the myofibrillar proteins in red muscle (chicken mixed thigh muscle) with the ratio in white muscle (chicken pectoralis major muscle). Gel filtration column chromatography of myofibrils from chicken mixed thigh and pectoralis major muscles was replicated using muscle samples from two chickens. The total column peak area and the area under the first peak were determined by plotting the elution profile on a grid and counting grid squares under each peak area. The trailing edge of the titin/nebulin peak was extrapolated to zero absorbance. The average (from the two experiments) ratio of the titin/nebulin peak to the total column peak area which contained all of the myofibrillar proteins, was approximately one to four (mean = .26) for myofibrils from chicken mixed thigh muscle and also one to four (mean = .25) for myofibrils from chicken pectoralis major muscle. No attempt was made to measure the amount of titin to nebulin in the initial peak from each profile. No obvious difference in the composition of the titin/nebulin peaks from chicken mixed thigh (Figure 8) and pectoralis major (Figure 9) muscle, however, was indicated by examination of fractions by SDS-PAGE. These results indicated

that the ratio of titin and nebulin to total muscle myofibrillar proteins was the same in red and white muscle.

Wang (1982a,b) noted that both titin and nebulin were extremely susceptible to proteolytic degradation and the rate of degradation varied greatly among species and muscle types. Our study demonstrated that some titin degradation was apparent on gels accompanying column elution profiles of the SDS-extracts of myofibrils from bovine, porcine and chicken skeletal muscle. In the profile patterns of the SDS-extract of myofibrils from bovine longissimus muscle (Figure 1), porcine longissimus muscle (Figure 7) and chicken mixed thigh muscle (Figure 8), titin in the leading edge of the first peak appeared as a sharp, fairly narrow band on 3.2% SDS-polyarylamide gels. On the descending portion of that first peak in column profiles of the SDS-extracts of myofibrils from bovine longissimus muscle (Figure 1) and porcine longissimus muscle (Figure 7), titin electrophoresed on gels as a band with a sharp upper edge, smearing downward to lower molecular weights. For chicken mixed thigh (Figure 8), titin from both the ascending and descending edges near the apex of the titin peak migrated with a sharp upper edge and downward smear on high porosity gels. From the lower portion of the descending edge of this peak, however, the electrophoretic banding pattern of titin was a much broader smear without a distinct upper edge. From the column elution profile for the SDS-extract of myofibrils of chicken pectoralis major muscle (Figure 9), titin did not electrophorese on gels as a narrow band. Some downward smearing of titin on SDS-PAGE was evident in both the ascending portion of the titin peak and the descending edge near the apex of this peak. Extensive degradation was apparent in the migration pattern of titin from the lower portion of

the descending edge of the peak, where the titin band appeared as a broad smear on the accompanying gel. Because of the observed extensive smearing on high porosity gels of titin isolated from chicken pectoralis major muscle, titin from this muscle appeared to be extremely susceptible to breakdown. This extreme susceptibility of titin to degradation was also noted by Wang and Williamson (1980). Chicken breast muscle is protease-rich and appreciable degradation of titin occurs at 4°C within a few hours of homogenization during the preparation of myofibrils (Wang, 1982a).

When chicken mixed thigh and pectoralis major whole muscle samples and myofibrils were analyzed by high-porosity SDS-slab gels (Figure 10), the electrophoretic patterns were quite similar to those obtained by Wang et al. (1979) for chicken breast muscle homogenates and purified myofibrils. Wang et al. (1979) utilized 3.2% SDS-polyacrylamide disc gels to demonstrate that there were three major bands which migrated above myosin heavy chains near the top of the gels: a closely spaced doublet, titin, and a single band with faster mobility, nebulin (nebulin was originally referred to as band 3). In Figure 10, column purified bovine titin (lane a) comigrated on a 3.2% SDS-polyacrylamide slab gel with the titin doublet of chicken mixed thigh whole muscle sample (lane c) and purified myofibrils (lane d) and with the titin doublet of the sample of chicken pectoralis major whole muscle (lane e) and purified myofibrils (lane f). The titin doublet was sometimes observed in preparations of whole muscle samples and purified myofibrils from bovine, porcine, and chicken muscle, however, it was also noted that titin often migrated as a single broad band in these samples. The nebulin bands (Figure 10) in chicken mixed thigh whole muscle samples (lane c) and purified myofibrils (lane d), and

Figure 10. Comparative low percent SDS-polyacrylamide slab gel electrophoretic patterns of bovine titin and skeletal myosin, and whole muscle samples and purified myofibrils from chicken mixed thigh and pectoralis major muscles

Column purified bovine titin (a), column purified bovine skeletal myosin (b), chicken mixed thigh whole muscle (c), purified chicken mixed thigh myofibrils (d), chicken pectoralis major whole muscle (e) and purified chicken pectoralis major myofibrils (f) were electrophoresed on a 3.2% SDS-polyacrylamide slab gel using the procedure of Studier (1973). Fifty μg of protein were loaded on each lane of the slab gel. The positions of titin, nebulin and of the myosin heavy chains on the gel are identified as T, N and M, respectively.

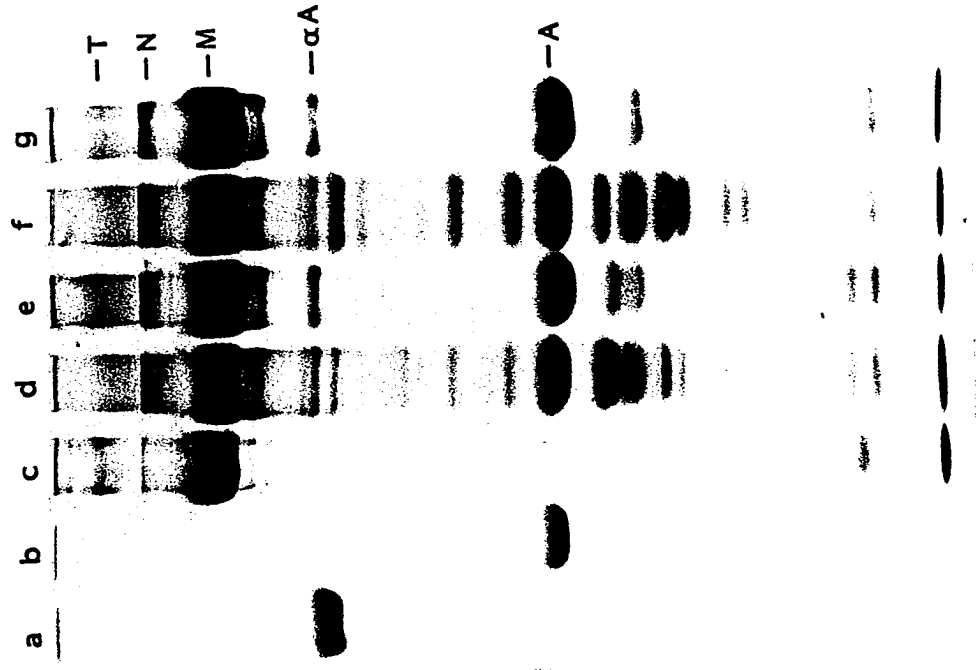


chicken pectoralis major whole muscle samples (lane e) and purified myofibrils (lane f) appeared to have similar electrophoretic mobilities. As would be expected, myosin heavy chains from all samples migrated similarly on the high porosity gel. Proteins with molecular weights lower than approximately 150,000 traveled with the dye front, resulting in a band at the bottom of the gel. There were no obvious differences in the amounts or migration patterns of titin or nebulin from chicken mixed thigh and pectoralis major muscles on 3.2% SDS-polyacrylamide slab gels.

Because proteins with subunit molecular weights of less than approximately 150,000 migrated off the low percent SDS-polyacrylamide gel in Figure 10 and, thus, were not detectable, SDS-PAGE also was performed on a high percent SDS-polyacrylamide gel. Whole muscle samples and purified myofibrils were prepared from chicken mixed thigh and pectoralis major muscles and electrophoresed on a high percent SDS-polyacrylamide (10% acrylamide with 5% acrylamide stacking gel) slab gel (Figure 11). The titin band of chicken mixed thigh whole muscle sample (lane d) and purified myofibrils (lane e) comigrated with titin from chicken pectoralis major whole muscle samples (lane f) and purified myofibrils (lane g). Titin did not enter the 10% SDS-polyacrylamide slab gel in this system, but remained in the stacking gel, as a diffuse band in all samples. The nebulin band migrated in samples from chicken mixed thigh whole muscle (lane d) and purified myofibrils (lane e), and from chicken pectoralis major whole muscle (lane f) and purified myofibrils (lane g), into the top edge of the 10% SDS-polyacrylamide gel. The only apparent difference in the electrophoretic patterns on high percent SDS-polyacrylamide gels between chicken red and white muscle was a protein band that migrated with a slightly faster

Figure 11. Comparative high percent SDS-polyacrylamide slab gel electrophoretic patterns of purified porcine and bovine proteins, and whole muscle samples and purified myofibrils from chicken mixed thigh and pectoralis major muscles

Porcine skeletal alpha actinin (a), porcine skeletal actin (b), bovine skeletal myosin (c), chicken mixed thigh whole muscle (d), purified chicken mixed thigh myofibrils (e), chicken pectoralis major whole muscle (f) and purified chicken pectoralis major myofibrils (g) were electrophoresed on a 10% SDS-polyacrylamide slab gel with a 5% stacking gel using the procedure of Studier (1973). Fifty μ g of protein were loaded on each lane of the slab gel. The positions of titin, nebulin, myosin heavy chains, alpha actinin and actin on the gel are identified as T, N, M, α A, and A, respectively.



mobility than actin ($M_r \sim 42,000$) in chicken mixed thigh purified myofibrils (lane e). This band was absent in purified myofibrils from chicken pectoralis major muscle (lane g). The band is probably troponin T since troponin T isolated from chicken breast and leg muscle has been found to differ in molecular weight as determined by SDS-PAGE. The molecular weight of troponin-T from chicken leg muscle was estimated to be approximately 37,000, whereas that from chicken breast muscle was approximately 44,000 (Wilkinson, 1978). Troponin-T in samples of myofibrils from chicken pectoralis major muscle (lane g) did not appear to migrate as a discrete band. A probable explanation is that troponin-T was not electrophoretically separated from the faster moving actin band, resulting in one band with an estimated molecular weight of 42,000 in chicken pectoralis major muscle samples.

Effect of Postmortem Storage Time and Temperature on Titin Degradation

To determine the effect, if any, of postmortem conditions on the possible proteolytic degradation of titin, both whole muscle samples and purified myofibrils prepared from bovine longissimus muscle, stored for 0, 1, 3 and 7 days postmortem at 2, 25 and 37°C, were analyzed by SDS-PAGE. Prior to storage, muscle samples were wrapped in toweling that had been saturated with an antimicrobial solution (10 mM NaN_3 , 100 ppm chloramphenicol, 10 ppm rifamycin), overlaid with Saran film and placed in a covered petri dish. Microbial counts were made for both surface and internal portions of bovine longissimus muscle after storage at 2, 25 and 37°C for 7 days postmortem to determine if any degradation of muscle proteins that

occurred was due to microbial contamination. Only the internal portions of the muscle samples were utilized for preparation of myofibrils and whole muscle samples. After storage at 2°C for 7 days, the number of microorganisms contaminating the muscle samples was very low. There were less than 10 org/gm of muscle tissue on both the surface and internal areas of these samples. There were, however, marked differences in the number of microorganisms which grew on the surface and interior portions of the muscle after storage for 7 days at both 25° and 37°C. In the 25°C sample, the microbial count on the surface (1.1×10^3 org/gm muscle tissue) was more than 100 times that of the interior of the muscle (95 org/gm muscle tissue). At the higher storage temperature of 37°C, microflora on the muscle surface (2.8×10^4 org/gm muscle tissue) remained more numerous than from the muscle interior (2.4×10^3 org/gm muscle tissue). All the microorganisms observed were colonies of small, white, gram positive cocci. The gelatin hydrolysis test was performed on all samples to determine proteolytic activity; however, all of the microorganisms tested gelatinase negative.

A low storage temperature of 2°C inhibited microbial growth on both the surface and interior portions of the muscle for 7 days of postmortem storage. Storage at 25°C promoted an increase in microbial counts at the muscle surface. Microbial contamination of both the exterior and interior of the muscle occurred in samples that were stored for 7 days at high temperatures (37°C). Since the observed microflora had no gelatinase activity, these organisms did not appear to be proteolytic. Therefore, degradation of titin occurring with increased postmortem storage time and temperature was not believed to be due to microbial contamination.

Because of the insoluble nature of titin, it was sometimes difficult to obtain uniform solubilization of titin in both whole muscle samples and purified myofibrils prepared for SDS-PAGE. Similar difficulties in obtaining uniform solubilization of rabbit and chicken skeletal muscle have been experienced by Wang (1982a,b). He found titin to be extremely difficult to solubilize consistently and quantitatively in SDS. And neither titin nor nebulin was very soluble in aqueous solutions without denaturants. In fact, titin tended to associate irreversibly into a gel-like aggregate even in denaturant solutions (Wang, 1982a,b).

In the at-death (0 day) sample of whole muscle (a) and purified myofibrils (b), titin appeared as a broad band with high molecular weight near the top of the slab gel (Figure 12). This band was confirmed as titin by comigration with a sample of purified titin from bovine muscle and with a purified sample of rabbit titin supplied by Dr. Kuan Wang (not shown). Although not evident in the 0 day sample shown in Figure 12, the titin band usually appeared as a closely spaced doublet if a lighter load of protein was electrophoresed. The top band of the titin doublet always was much more prominent than the lower band in the 0 day samples (not shown). The nebulin band, also evident in the 0 day samples, was faint in the 1 day sample at 2°C and was only slightly detectable in the 25°C samples at 1 day postmortem. After 1 day storage at 37°C, protein bands, possibly from breakdown of titin or nebulin, are observable just beneath the area of the original nebulin band. In the purified myofibrils (b) prepared from the 1 day postmortem sample stored at 2°C, the titin doublet was evident with the lower doublet band appearing as the major titin band and the upper band being the minor band. In the 1 day, 25°C samples, only

Figure 12. SDS-polyacrylamide slab gel of whole muscle and purified myofibrils from bovine longissimus muscle sampled immediately after death and after one day of postmortem storage at 2, 25 or 37°C

Whole muscle samples (a) and purified myofibrils (b) were prepared from at-death (0-day) bovine longissimus muscle and from muscle stored aseptically for one day postmortem at 2, 25 or 37°C. The samples were electrophoresed on a 3.2% SDS-polyacrylamide slab gel using the procedure of Studier (1973). Fifty μ g of protein were loaded on each lane of the slab gel. The positions of titin, nebulin and of the myosin heavy chains on the gel are identified as T, N and M, respectively.



a trace of the upper titin band was present, with a concurrent darkening of the lower doublet band. In the samples prepared from muscle stored for 1 day at 37°C, only the lower band of the titin doublet was still present.

Titin seemed to be sensitive to degradation with an increase in post-mortem storage time as well as temperature. These results are illustrated in Figure 13. From muscle stored for 3 days, only the 2°C samples (whole muscle and purified myofibrils) had traces of the top doublet band, along with the more prominent lower band. In the samples stored at 25°C, only the lower band of the doublet was present. There also was some evidence of additional bands migrating in the region between titin and myosin heavy chains, which were more noticeable in the whole muscle sample. At 37°C, only a trace of the lower titin band was still present, together with a new putative degradation product that migrated to a position immediately beneath the original lower titin band. Wang (1982b) reported that in situ degradation of titin yielded closely spaced fragments of similar mobilities to titin or nebulin and it was often difficult to determine whether a protein band migrating closely to titin or nebulin was intact or degraded. It has been shown previously by other workers (Bechtel and Parrish, 1983; Ikeuchi et al., 1980; Penny and Ferguson-Pryce, 1979) that myosin heavy chains are degraded during postmortem storage of bovine muscle at 37°C. This degradation also was quite evident (i.e., less protein migrating as intact myosin heavy chains) in this study as shown in the whole muscle samples (a) and purified myofibrils (b) after storage for 3 days at 37°C.

In the whole muscle samples and purified myofibrils prepared from muscle stored at 2° or 25°C for 7 days, only some of the lower band of the titin doublet remained (Figure 14). The degradation product immediately

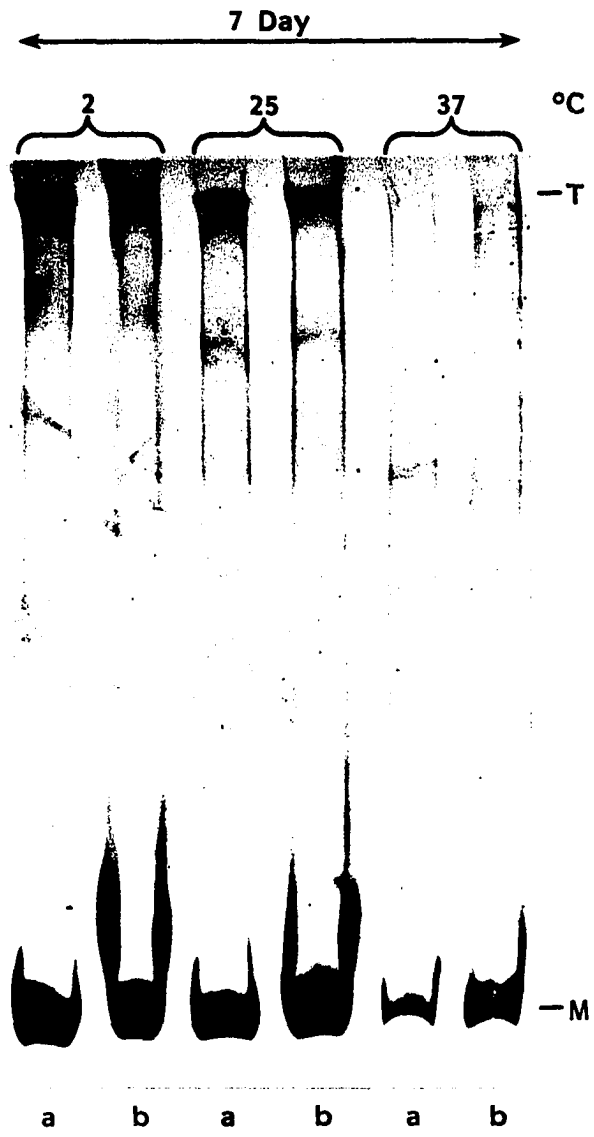
Figure 13. SDS-polyacrylamide slab gel of whole muscle and purified myofibrils from bovine longissimus muscle sampled after three days of postmortem storage at 2, 25 or 37°C

Whole muscle samples (a) and purified myofibrils (b) were prepared from bovine longissimus muscle stored aseptically for three days postmortem at 2, 25 or 37°C. The samples were electrophoresed on a 3.2% SDS-polyacrylamide slab gel using the procedure of Studier (1973). Fifty µg of protein were loaded on each lane of the gel. The positions of titin and of the myosin heavy chains on the gel are identified as T and M, respectively.



Figure 14. SDS-polyacrylamide slab gel of whole muscle and purified myofibrils from bovine longissimus muscle sampled after seven days of postmortem storage at 2, 25 or 37°C

Whole muscle samples (a) and purified myofibrils (b) were prepared from bovine longissimus muscle stored aseptically for seven days postmortem at 2, 25 or 37°C. The samples were electrophoresed on a 3.2% SDS-polyacrylamide slab gel using the procedure of Studier (1973). Fifty µg of protein were loaded on each lane of the slab gel. The positions of titin and of the myosin heavy chains on the gel are identified as T and M, respectively.



below the lower titin band that was first seen after 3 days at 37°C (Figure 13) was evident in the 25°C samples after 7 days of postmortem storage. At 37°C, even this distinct titin putative degradation product as well as other faint bands in the region between titin and myosin heavy chains had disappeared. And again, myosin has been degraded during storage at elevated temperatures as evidenced by comparing the amount of myosin heavy chain bands in the 2° and 25°C samples with the lesser amount of intact myosin band in the 37°C samples (Figure 14).

The results of this study demonstrated that titin was degraded in bovine longissimus bovine muscle postmortem, with the degree of degradation dependent upon storage time and temperature. These results suggest that proteolysis is the cause for the degradation and are consistent with those shown by others that titin (Wang et al., 1979; Wang and Ramirez-Mitchell, 1983) and the high molecular weight components (titin) of connectin (King et al., 1981; Maruyama et al., 1981b) are highly susceptible to proteolytic degradation. According to Wang (1982a,b), both titin and nebulin are extremely prone to proteolytic degradation by endogenous calcium activated proteases.

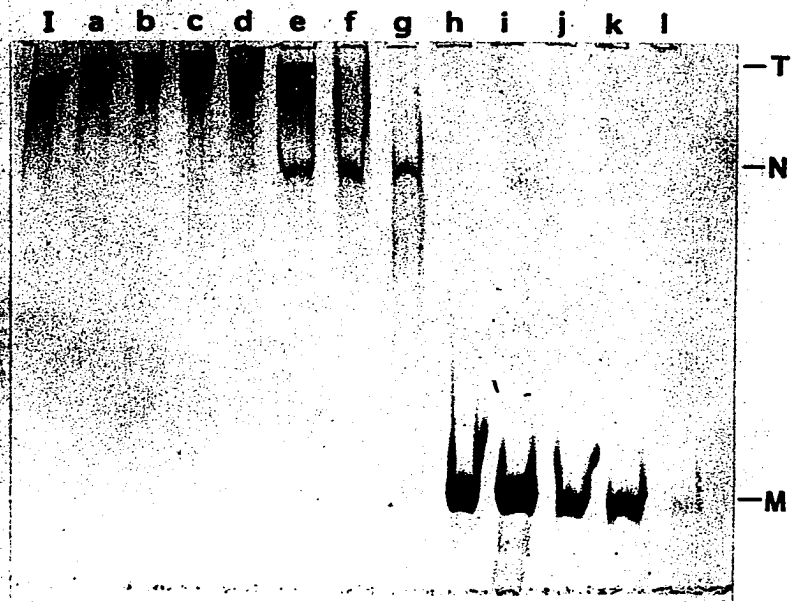
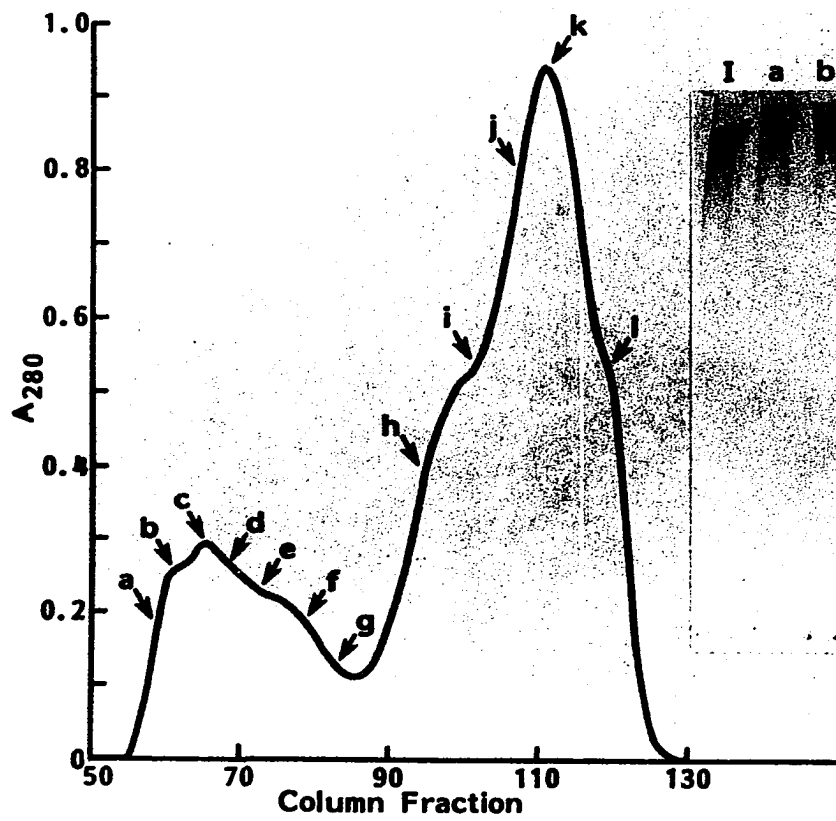
Wang (1982a,b) also noted that proteolytic degradation of titin in situ converts band 1 of the titin doublet into a major fragment that comigrates with band 2. He suggested that the band 2:band 1 weight ratio may be used as a sensitive indicator of the extent of proteolysis and/or protein turnover in muscle (Wang, 1982b). Maruyama et al. (1981b) found, however, that although the high molecular weight components (titin) of connectin were degraded by trypsin, chymotrypsin, papain, nargarse and serine protease, these myofibrillar protein components were not proteolytically degraded by

calcium activated protease. We have recently demonstrated that the titin doublet of bovine longissimus muscle myofibrils is rapidly degraded by the addition of calcium activated neutral protease (Zeece et al., 1983). Some of the degradation products resulting from calcium activated protease digestion of myofibrils were labeled by titin antibodies in western blots of slab gel (Zeece, M. G., R. M. Robson, and F. C. Parrish, 1983, unpublished data, Department of Food Technology, Iowa State University). Thus, degradation of titin by endogenous muscle enzymes may be involved in post-mortem decrease in muscle cell integrity.

Because the previous results indicated that both titin and nebulin are degraded with postmortem storage, a study was conducted to compare the SDS-PAGE of titin and nebulin from the column elution profile of at-death bovine longissimus muscle (0 day) to that of titin and nebulin isolated from muscle stored for 3 and 7 days postmortem at 25°C. Prior to storage, the bovine longissimus muscle samples were wrapped in toweling that had been saturated with an antimicrobial solution (10 mM NaN₃, 100 ppm chloramphenicol, 10 ppm rifamycin), overlaid with Saran film and placed in a covered petri dish. Myofibrils were prepared at each sampling time according to the procedure of Goll et al. (1974). When the SDS-extract of myofibrils prepared from at-death bovine longissimus muscle (prepared by the method of Wang et al., 1979) was loaded onto an agarose gel (Bio-Gel A-50m) filtration column (2.5 x 90 cm) as described by Wang et al. (1979), the resulting column profile (Figure 15) was similar to that observed with the SDS-extract of myofibrils from at-death bovine longissimus muscle from another animal (Figure 1). Highly purified titin was observed in the leading edge of the first peak, as evidenced by the gel patterns in

Figure 15. Agarose gel filtration column profile of the SDS-extract of myofibrils from at-death bovine longissimus muscle and accompanying inset of SDS-polyacrylamide slab gel electrophoretic patterns of eluted proteins

Freshly prepared bovine longissimus muscle myofibrils were solubilized in 10% (w/v) SDS, 10 mM EDTA, 0.1 mM PMSF, 0.2 M Tris-HCl, pH 8.0, as described by Wang et al. (1979). The SDS-extract was centrifuged at 88,000 x g for one hour and the supernatant containing 200 mg protein was loaded onto a Bio-Gel A-50m permeation column (2.5 x 90 cm) previously equilibrated with elution buffer [0.1% (w/v) SDS, 5 mM EDTA, 0.5 mM DTT, 0.1 M Tris-HCl, pH 8.8]. Four and one-half ml fractions were collected at a rate of 15 ml/hr. and each fraction was analyzed for protein by absorbance at 280 nm. The inset shows a 3.2% SDS-polyacrylamide gel (lanes a-l) corresponding to the eluted fractions (lettered arrows a-l) obtained from the SDS-extract of bovine longissimus muscle myofibrils. Twenty-five μ l of fractions h, i, j, k and l, thirty μ l of fractions b, c and d, forty μ l of fractions a and e and 50 μ l of fractions f and g were loaded on the slab gel. The positions of titin, nebulin and of the myosin heavy chains on the gel are identified as T, N and M, respectively. Rabbit titin (I) shown in the inset, was provided by Dr. Kuan Wang, University of Texas at Austin. The identical gel filtration column was used to obtain the column profiles in Figures 16 and 17 in order to reduce variations in column packing. The ratio of the first peak eluted, which contained titin and nebulin, to the total column profile peak area was measured as described in Materials and Methods.



lanes a, b and c. Partially degraded titin was apparent on the descending edge near the top of this peak, appearing as a band with a sharp upper edge with some slight smearing downward to lower molecular weights, as shown in lane d of the gel inset. More degradation of titin was noted on the middle portion of the descending edge of the first peak which corresponded to lane e of the gel inset. In lane e, titin electrophoresed as a band with the upper edge appearing slightly diffuse with extensive downward smearing. In the lower portion of the descending edge of this peak, there was extensive degradation of titin as confirmed by the very light, broad smear observed in lane f. Nebulin appeared as a narrow band in the descending edge of the first peak as confirmed by the banding patterns observed in lanes e, f and g. Some degradation products, possible from the breakdown of titin or nebulin, were observable as a smear just beneath the area of the nebulin band in lane g. Myosin heavy chains along with other myofibrillar proteins of molecular weights lower than myosin, were eluted in the large peak following the titin/nebulin peak.

The column elution profiles of myofibrils isolated from bovine longissimus muscle stored aseptically for 3 and 7 days postmortem at 25°C, are shown in Figures 16 and 17, respectively. The SDS-PAGE from the column elution profile for the 3 day postmortem sample (Figure 16) was very similar to that of the 7 day postmortem sample. In both column profiles, purified titin was observed in the leading edge of the first peak as shown in lanes a and b of the gel insets in Figures 16 and 17. Partially degraded titin appeared as a plateau, on the descending edge of the first peak near the apex as evidenced by the gel pattern in lane c of Figures 16 and 17. In lane c from both column elution profiles, the titin band appeared with

Figure 16. Agarose gel filtration column profile of the SDS-extract of myofibrils from bovine longissimus muscle stored for three days postmortem at 25°C and accompanying inset of SDS-polyacrylamide slab gel electrophoretic patterns of eluted proteins

Purified myofibrils prepared from bovine longissimus muscle stored aseptically for three days postmortem at 25°C, were solubilized in 10% (w/v) SDS, 10 mM EDTA, 0.1 mM PMSF, 0.2 M Tris-HCl, pH 8.0, as described by Wang et al. (1979). The SDS-extract was centrifuged at 88,000 x g for one hr. and the supernatant containing 200 mg protein was loaded onto a Bio-Gel A-50m permeation column (2.5 x 90 cm) previously equilibrated with elution buffer [0.1% (w/v) SDS, 5 mM EDTA, 0.5 mM DTT, 0.1 M Tris-HCl, pH 8.8]. Four and one-half ml fractions were collected at a rate of 15 ml/hr. and each fraction was analyzed for protein by absorbance at 280 nm. The inset shows a 3.2% SDS-polyacrylamide gel (lanes a-j) corresponding to the eluted fractions (lettered arrows a-j) obtained from the SDS-extract of myofibrils prepared from bovine longissimus muscle stored for three days at 25°C. Twenty-five μ l of fractions b, c, g, h, i and j, and forty μ l of fractions a, d, e and f were loaded on the slab gel. The positions of titin, nebulin and of the myosin heavy chains on the gel are identified as T, N and M, respectively. Rabbit titin (I) and nebulin (II) shown in the inset were provided by Dr. Kuan Wang, University of Texas at Austin. The ratio of the first peak eluted, which contained titin and nebulin, to the total column profile peak area was measured as described in Materials and Methods.

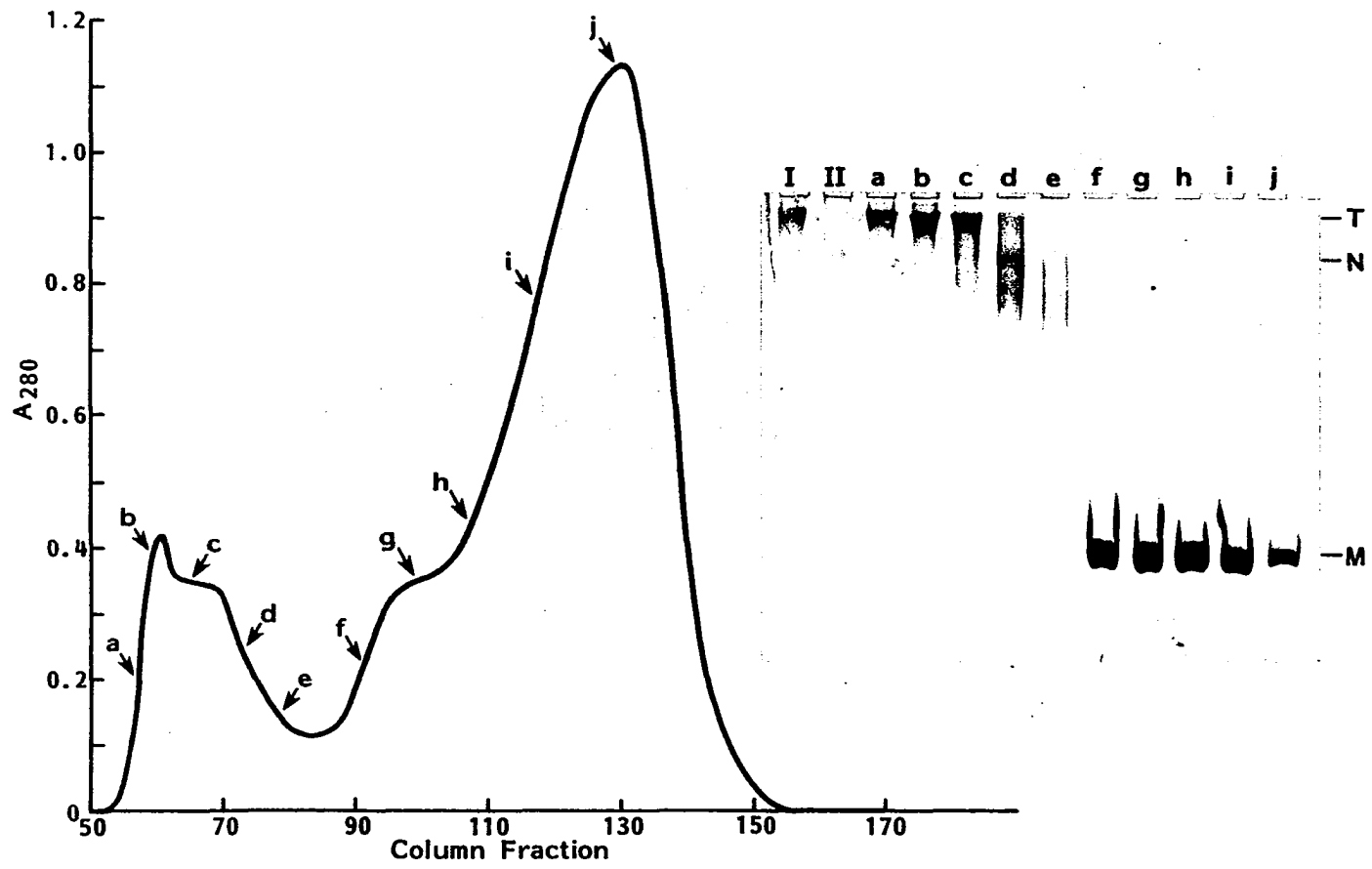
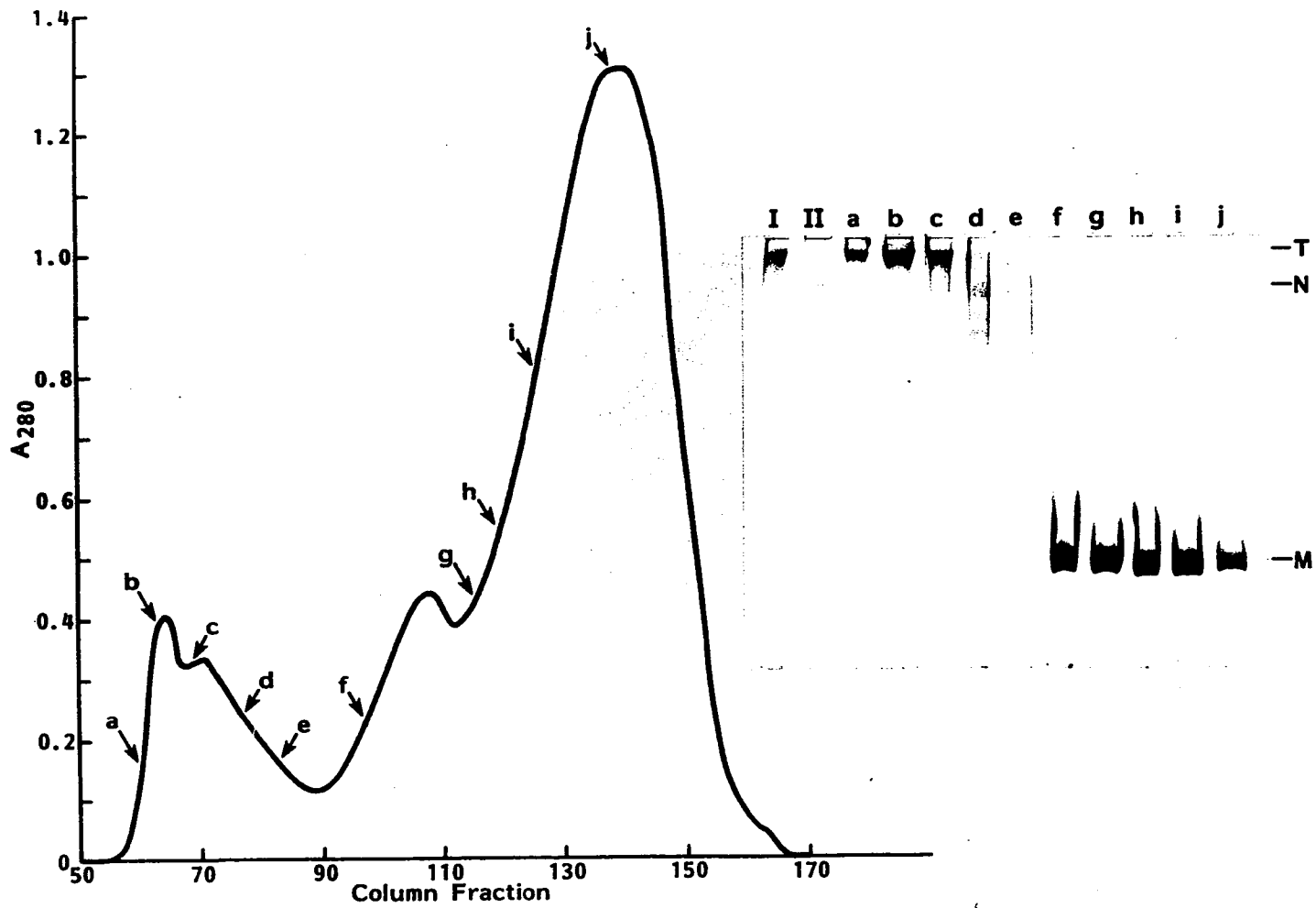


Figure 17. Agarose gel filtration column profile of the SDS-extract of myofibrils from bovine longissimus muscle stored for seven days postmortem at 25°C and accompanying inset of SDS-polyacrylamide slab gel electrophoretic patterns of eluted proteins

Purified myofibrils prepared from bovine longissimus muscle stored aseptically for seven days postmortem at 25°C were solubilized in 10% (w/v) SDS, 10 mM EDTA, 0.1 mM PMSF, 0.2 M Tris-HCl, pH 8.0, as described by Wang et al. (1979). The SDS-extract was centrifuged at 88,000 x g for one hr. and the supernatant containing 200 mg protein was loaded onto a Bio-Gel A-50m permeation column (2.5 x 90 cm) previously equilibrated with elution buffer [0.1% (w/v) SDS, 5 mM EDTA, 0.5 mM DTT, 0.1 M Tris-HCl, pH 8.8]. Four and one-half ml fractions were collected at a rate of 15 ml/hr. and each fraction was analyzed for protein by absorbance at 280 nm. The inset shows a 3.2% SDS-polyacrylamide gel (lanes a-j) corresponding to the eluted fractions (lettered arrows a-j) obtained from the SDS-extract of myofibrils prepared from bovine longissimus muscle stored for seven days at 25°C. Twenty-five µl of fractions b, c, g, h, i and j, and forty µl of fractions a, d, e and f were loaded on the slab gel. The positions of titin, nebulin and of the myosin heavy chains on the gel are identified as T, N and M, respectively. Rabbit titin (I) and nebulin (II) shown in the inset were provided by Dr. Kuan Wang, University of Texas at Austin. The ratio of the first peak eluted, which contained titin and nebulin, to the total column profile peak area was measured as described in Materials and Methods.



a sharp upper border and smeared downward. Titin that was further degraded eluted in the middle of the descending edge of the first peak as determined by the banding pattern observed in lane d of Figures 16 and 17. Titin migrated in this lane as a very broad band with the upper limit appearing somewhat diffuse and with an extensive amount of smearing downward to lower molecular weights. Extensive degradation of titin was noted at the base of the descending edge of the first peak as shown in lane e of Figures 16 and 17 as a very broad smear without well-defined upper and lower edges. Nebulin was not apparent as a discrete band in the descending edge of the first peak that was observed in the 0 day sample of bovine longissimus muscle (Figure 15). With postmortem storage for 3 or 7 days at 25°C, nebulin was probably proteolytically degraded to smaller molecular weight products and these most likely migrated as part of the smear observed in lanes e and f of Figures 16 and 17. Wang (1982a,b) and Maruyama et al. (1981b) noted that nebulin was hydrolyzed in myofibrils treated with calcium activated protease. Myosin heavy chains eluted in the large second peak as evidenced by the bands seen in lanes f through j in Figures 16 and 17. The last peak of both column elution profiles also included other myofibrillar proteins with molecular weights lower than myosin heavy chains but have migrated off the bottom of the accompanying gels. Titin degradation in the bovine longissimus muscle stored for 3 days at 25°C as noted from the column elution profile (Figure 16) does not appear to be different from that observed after storage for 7 days postmortem (Figure 17) at 25°C.

A preliminary study was done to compare the ratio of titin plus nebulin to all of the myofibrillar proteins in at-death bovine longissimus muscle with the ratio in bovine longissimus muscle stored for 3 and 7 days

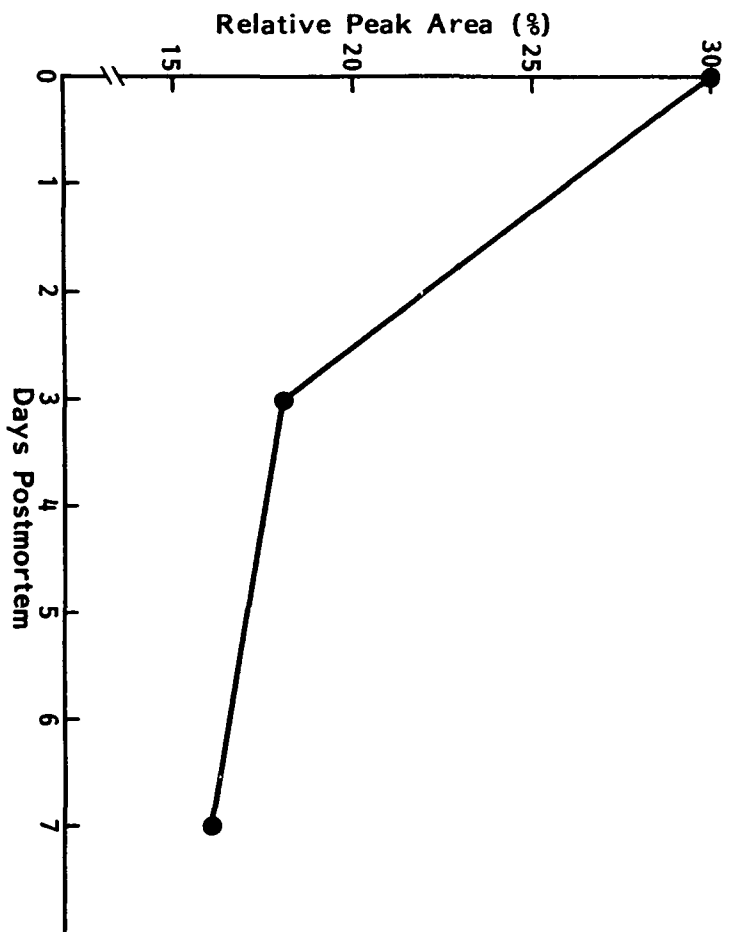
postmortem at 25°C. Gel filtration column profiles of SDS-extracts of myofibrils from at-death bovine longissimus muscle and bovine longissimus muscle stored for 3 and 7 days postmortem were obtained as described by Wang et al. (1979). This experiment was replicated using muscle from two animals. The total column peak area and the area under the first peak were determined by plotting the elution profile on a grid and counting grid squares under each peak area. The trailing edge of the titin/nebulin peak was extrapolated to zero absorbance. The average (from the two experiments) ratio of the titin/nebulin peak to the total column peak area which contained all of the myofibrillar proteins, was two to six (mean = .30) for myofibrils from at-death bovine longissimus muscle, approximately one to six (mean = .18) for muscle stored for 3 days at 25°C and approximately one to six (mean = .16) for muscle stored for 7 days at 25°C. No attempt was made to measure the amount of titin to nebulin in the first peak of each column profile. Examination of fractions by SDS-PAGE shown in the insets indicated that the titin/nebulin peaks from at-death bovine longissimus muscle (Figure 15) differed in composition from those in muscle stored for 3 days (Figure 16) and 7 days (Figure 17) at 25°C.

The content of titin and nebulin in at-death bovine longissimus muscle and in muscle stored 3 and 7 days postmortem at 25°C, expressed as a percentage of relative peak area in bovine longissimus muscle, is shown in Figure 18. A sharp decrease in titin and nebulin content occurred by 3 days postmortem storage at 25°C. There appears, however, to be only a small amount of further degradation of titin and nebulin occurring by 7 days postmortem storage at 25°C. These preliminary observations

suggest that the ratio of titin and nebulin to total muscle myofibrillar protein rapidly decreased with postmortem storage, with the majority of degradation occurring within 3 days of postmortem storage at 25°C.

Figure 18. Relationship between the relative area of the column profile peak containing titin and nebulin and the days of postmortem storage of bovine longissimus muscle at 25°C

Relative peak areas are expressed as $100 (T/A)$ where T is the area under the titin and nebulin peak in the gel filtration column profile and A is the total column profile peak area. Purified myofibrils prepared from at-death (0-day) bovine longissimus muscle and from muscle stored aseptically for three and seven days postmortem at 25°C, were solubilized in 10% (w/v) SDS, 10 mM EDTA, 0.1 mM PMSF, 0.2 M Tris-HCl, pH 8.0, as described by Wang et al. (1979). Each SDS-extract was centrifuged at 88,000 x g for one hr. and the supernatant containing 200 mg protein was loaded onto a Bio-Gel A-50m permeation column (2.5 x 90 cm) previously equilibrated with elution buffer [0.1% (w/v) SDS, 5 mM EDTA, 0.5 mM DTT, 0.1 M Tris-HCl, pH 8.8]. Four and one-half ml fractions were collected at a rate of 15 ml/hr. and each fraction was analyzed for protein by absorbance at 280 nm. The identical gel filtration column was used for all three samples in order to reduce variations in column packing. From the resulting column profiles, the relative peak area (%) was measured as described in Materials and Methods. The study was done in duplicate using muscle samples from two animals and each point representing relative peak area (%) is an average of two values.



SUMMARY

There have been reports of a third set of filaments, in addition to the well-known thick and thin filaments, visible in electron micrographs of highly stretched or extracted muscle. Locker and associates (Locker and Leet, 1975, 1976a, b; Locker et al., 1977; Locker, 1982) have named these additional filaments "gap" filaments and have proposed that they determine tensile strength in myofibrils and are therefore important to meat tenderness. Recently, Wang et al. (1979) isolated a pair of very large myofibrillar proteins ($M_r \sim 1.0 \times 10^6$), referred collectively to as titin, that may be the major protein of the putative thin longitudinal elastic filaments in the sarcomere. Titin, along with the high molecular weight myofibrillar protein nebulin ($M_r \sim 5.0 \times 10^5$), has been identified in skeletal and cardiac muscles of several vertebrates and invertebrates. These two high molecular weight proteins represent approximately 10-15% of the total myofibrillar proteins in chicken skeletal muscle (Wang et al., 1979). Evidence that titin 1) is a major myofibrillar protein, 2) may function as a cytoskeletal protein in the structural integrity of the sarcomere and, 3) is proteolytically degraded, provides impetus for investigations to be carried out using muscle from meat producing animals, especially beef. The possible proteolytic degradation of titin and nebulin during postmortem storage of beef could be a very significant event in the tenderization process of meat.

The purpose of this research was to isolate and characterize skeletal muscle titin and investigate the degradation of this extremely large polypeptide during postmortem storage. The first objective was to discover if

titin in bovine skeletal muscle was present and similar to titin originally identified by Wang et al. (1979) in chicken and rabbit skeletal muscle. Utilizing the technique of agarose gel filtration chromatography in the presence of SDS, titin and nebulin were purified from myofibrils isolated from bovine longissimus muscle. The resulting profile pattern for bovine skeletal muscle myofibrils was similar to that obtained for chicken (Wang et al., 1979) and rabbit skeletal muscle myofibrils (Wang, 1982a). Low percent SDS-polyacrylamide gels were utilized for analysis of column fractions in order to electrophoretically separate and identify titin and nebulin since these unusually large myofibrillar proteins do not enter into high percent SDS-polyacrylamide gels. The amino acid composition of chromatographically purified bovine skeletal muscle titin was very similar to that of Wang's (personal communication, University of Texas at Austin, Dept. of Chemistry) chromatographically purified rabbit titin and also to the high molecular weight components of connectin (Maruyama et al., 1981b) isolated from chicken breast myofibrils (using the procedure of Wang et al., 1979). Bovine and rabbit skeletal muscle titins were also found to comigrate on SDS-polyacrylamide gels and to cross-react in immunautoradiographs with antibodies specific for titin, reaffirming that the protein isolated from bovine longissimus muscle was, in fact, titin.

The second objective was to determine the localization of titin in skeletal muscle and this was accomplished via indirect immunofluorescence using antiserum prepared against chromatographically purified bovine titin. Interpretation of the immunofluorescence staining patterns with titin antiserum in myofibrils from bovine and porcine longissimus muscle was difficult because titin antibody localization varied significantly in staining

patterns and relative intensity in any given preparation. Primarily, immunofluorescence staining patterns appeared to be a function of sarcomere length as was also noted by Wang et al. (1979) and Wang (1982b). Titin was preferentially localized in the A-I junctional area with some diffuse staining throughout the entire A-band in bovine and porcine longissimus muscle myofibrils. Titin was also localized in the Z-line region of myofibrils from porcine longissimus muscle stretched to 2.3 times rest length. These immunofluorescence staining patterns were similar to those reported for chicken skeletal muscle myofibrils by Maruyama et al. (1981b) and Wang et al. (1979).

The third objective was to investigate the characteristics of titin from different muscles, muscle fiber types, and species. To determine if titin differed in bovine longissimus dorsi, biceps femoris and psoas major muscles, whole muscle samples and purified myofibrils were analyzed by SDS-PAGE. The quantity and electrophoretic mobility of titin and nebulin from all three muscles were similar. Titin was also purified by means of gel filtration chromatography from bovine sternomandibularis muscle and the resulting column elution profile was very similar to that from bovine longissimus muscle.

Possible differences in the electrophoretic mobilities of titin and nebulin due to muscle fiber type were explored. As analyzed by SDS-PAGE, the electrophoretic mobilities of titin and nebulin in whole muscle samples and purified myofibrils from red (chicken mixed thigh) muscle and white (chicken pectoralis major) muscle were similar. There were also no differences in the ratio of titin plus nebulin to total muscle myofibrillar proteins in the SDS-extracts of myofibrils from red and white muscle,

indicating that the quantities of titin and nebulin were the same for both muscle fiber types.

The comparative results obtained by gel filtration chromatography of SDS-extracts of myofibrils from bovine, porcine, and chicken skeletal muscle myofibrils indicated that there were no species variations in the overall column elution profile patterns. The amount of titin and nebulin degradation apparent on gels accompanying column elution profiles of the SDS-extracts of myofibrils from bovine longissimus, porcine longissimus, chicken mixed thigh and chicken pectoralis major muscles varied with species and with muscle fiber type. Wang (1982a, b) also reported that both titin and nebulin were extremely susceptible to endogenous proteolytic degradation, and that the rate of degradation varied greatly among species and fiber type. Titin and nebulin from chicken pectoralis major were found to be the most susceptible to proteolytic degradation. Chromatographically purified titin from chicken pectoralis major muscle did not electrophorese on gels as a sharp, narrow band as was observed for bovine longissimus, porcine longissimus and chicken mixed thigh muscle, but rather as a broad smear.

The fourth objective was to examine the effects of postmortem storage time (0, 1, 3, and 7 days postmortem) and temperature (2, 25 and 37°C) on the integrity of titin polypeptides in bovine longissimus muscle. Both whole muscle samples and purified myofibrils for each storage time and temperature were analyzed by SDS-PAGE to determine possible degradation of titin. Titin migrated as a closely spaced doublet with the top band more prominent than the lower band in at-death samples. With increased post-mortem storage time and temperature, the top band of the titin doublet

gradually disappeared with a concurrent darkening of the lower band. Wang (1982a, b) also observed that proteolytic degradation of titin in situ converted the upper band of the titin doublet into a major fragment that comigrated with the lower band. Only the lower doublet band remained after 7 days storage at 2 or 25°C. A trace of the lower band was present by 3 days of storage at 37°C, along with a new putative degradation product that migrated immediately beneath the original titin band. After 7 days storage at 37°C, both the lower band and degradation products immediately beneath the lower band had disappeared.

The comparative results obtained from the SDS-PAGE of purified titin and nebulin from the column elution profiles of at-death bovine longissimus muscle to muscle stored for 3 and 7 days postmortem at 25°C demonstrated that extensive degradation of these two myofibrillar proteins had occurred by 3 days without any obvious additional degradation at 7 days. A comparison of the ratio of titin plus nebulin to all of the myofibrillar proteins, as analyzed from column elution profiles, also indicated that there was a dramatic decrease in titin and nebulin content by 3 days postmortem storage at 25°C with only a slight amount of further degradation by 7 days. Thus, titin was degraded postmortem, with the degree of degradation dependent on storage time and temperature.

These results suggest that proteolysis is the cause for titin degradation. It has recently been demonstrated by our laboratory that the titin doublet of bovine longissimus muscle myofibrils is rapidly degraded by the addition of calcium activated neutral protease (Zeece et al., 1983) with some of the resulting degradation products capable of being labeled by titin antibodies (Zeece, M. G., R. M. Robson, M. L. Lusby and F. C.

Parrish, Jr. 1983. Unpublished data. Department of Food Technology, Iowa State University). Therefore, degradation of titin by endogenous proteases may be involved in the postmortem decrease in muscle cell integrity and improvement in meat tenderness and quality.

CONCLUSIONS

1. The high molecular weight protein titin was present in bovine skeletal muscle and it could be prepared in purified form.
2. Bovine, rabbit and chicken skeletal muscle titins were closely related or homologous proteins.
3. Titin was preferentially localized in the A-I junctional area with some diffuse staining throughout the entire A-band in bovine and porcine longissimus muscle myofibrils. Titin was also localized in the Z-line region of myofibrils from porcine longissimus muscle stretched to 2.3 times rest length.
4. Titin and nebulin from bovine longissimus dorsi, biceps femoris and psoas major whole muscle samples and purified myofibrils were similar.
5. The column elution profiles of the SDS-extracts of myofibrils from bovine sternomandibularis muscle and from bovine longissimus muscle were very similar.
6. There were no differences in the quantities and electrophoretic mobilities of titin and nebulin from chicken red and white muscle.
7. There were no species (bovine, porcine and chicken) variations in the overall column elution profiles.
8. The amount of titin and nebulin degradation occurring in myofibrils from at-death bovine longissimus, porcine longissimus, chicken mixed thigh and chicken pectoralis major muscles varies with species and with muscle fiber type, with titin and nebulin from chicken pectoralis major being the most susceptible to proteolytic degradation.

9. Titin was degraded in bovine longissimus muscle postmortem with the amount of degradation dependent upon storage time and temperature.
10. The lower band of the titin doublet was the putative degradation product of the upper band, and with further degradation, a new degradation product appeared immediately beneath the lower band.
11. Most of the degradation of titin and nebulin in bovine longissimus muscle had occurred by 3 days postmortem storage at 25°C.

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