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Elisabeth J. Huff-Lonergan
*Iowa State University*, elonerga@iastate.edu

Tomiko Mitsuhashi
*Iowa State University*

Frederick C. Parrish Jr.
*Iowa State University*

Richard M. Robson
*Iowa State University*

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**Abstract**
Purified myofibril (MF) and homogenized whole muscle (WM) samples were prepared from A maturity market steers. Samples were removed at 0, 1, 3, 7, 14, and 28 d postmortem. The MF and WM samples from all steers were analyzed by SDS-PAGE (5% gels) and by Western blot analysis using monoclonal antibodies to titin and nebulin. The rates of degradation of the intact forms of titin and nebulin, with regard to differences dependent on sample type (MF vs WM), were examined. The results showed that there was very little difference in the rate of postmortem degradation of the intact form of titin or of intact nebulin with respect to the two types of samples examined. Analysis of MF and WM preparations revealed that titin and nebulin were progressively degraded, each at its own rate, with nebulin degrading faster, as postmortem storage time increased. Examination of MF and WM samples showed that the intact form of titin (T1) was absent at the same time postmortem in both sample types. Intact nebulin was not detected in MF and WM preparations at the same time postmortem with respect to sample type examined. Our results indicate that either purified MF or WM samples can be used satisfactorily to analyze the rate of degradation of the intact forms of both titin and nebulin.

**Keywords**
Biochemistry Biophysics and Molecular Biology, Titin, Nebulin, Postmortem changes, Beef, SDS-PAGE, Immunoblotting

**Disciplines**
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**Comments**
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# Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blotting Comparisons of Purified Myofibrils and Whole Muscle Preparations for Evaluating Titin and Nebulin in Postmortem Bovine Muscle[1,2]

**Elisabeth Huff-Lonergan*,†,3, Tomiko Mitsuhashi*, F. C. Parrish, Jr.*,4, and Richard M. Robson*,†**

Muscle Biology/Meat Science, Departments of *Animal Science and of †Biochemistry and Biophysics, Iowa State University, Ames 50011

**ABSTRACT:** Purified myofibril (MF) and homogenized whole muscle (WM) samples were prepared from A maturity market steers. Samples were removed at 0, 1, 3, 7, 14, and 28 d postmortem. The MF and WM samples from all steers were analyzed by SDS-PAGE (5% gels) and by Western blot analysis using monoclonal antibodies to titin and nebulin. The rates of degradation of the intact forms of titin and nebulin, with regard to differences dependent on sample type (MF vs WM), were examined. The results showed that there was very little difference in the rate of postmortem degradation of the intact form of titin or of intact nebulin with respect to the two types of samples examined. Analysis of MF and WM preparations revealed that titin and nebulin were progressively degraded, each at its own rate, with nebulin degrading faster, as postmortem storage time increased. Examination of MF and WM samples showed that the intact form of titin (T1) was absent at the same time postmortem in both sample types. Intact nebulin was not detected in MF and WM preparations at the same time postmortem with respect to sample type examined. Our results indicate that either purified MF or WM samples can be used satisfactorily to analyze the rate of degradation of the intact forms of both titin and nebulin.

Key Words: Titin, Nebulin, Postmortem Changes, Beef, SDS-PAGE, Immunoblotting

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## Introduction

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

Recent studies have differed considerably in the reported rate of PM degradation of these two proteins, especially titin (c.f., Fritz et al., 1993, with Huff-Lonergan et al., 1995 and Taylor et al., 1995). One

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[3]Present Address: Dept. of Animal and Dairy Sciences, Auburn Univ., Auburn, AL 36849.

[4]To whom correspondence should be addressed: 214 Meat Lab.

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possibility for the differences may be in the type of sample examined, namely purified (MF) or whole muscle (**WM**) preparations (Fritz et al., 1993; Huff-Lonergan et al., 1995). Thus, the objective of this study was to determine whether evaluation of the rate of PM degradation of intact titin (designated as T1) and nebulin was significantly influenced by analysis of purified MF vs WM homogenates.

## Materials and Methods

### *Sources and Storage of Muscle*

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

### *Myofibril Preparation*

Myofibrils from at-death (0-d) and PM aged LT muscle were purified at 2°C according to the myofibril preparation procedure of Goll et al. (1974) as modified by Huff-Lonergan et al. (1995). Protein concentrations were determined by using the biuret procedure as modified by Robson et al. (1968). The MF samples, suspended in 5 m*M* Tris-HCl, pH 8.0, were diluted to 3.2 mg/mL and then 1 vol of each sample was immediately combined with .5 vol of 25°C sample buffer/tracking dye solution (3 m*M* EDTA, 3% [wt/vol] SDS, 30% [vol/vol] glycerol, .003% [wt/vol] pyronin Y, and 30 m*M* Tris-HCl, pH 8.0) (Wang, 1982) and .1 vol of 2-mercaptoethanol for a final protein concentration of 2 mg/mL. The samples were heated at 50°C for 20 min before loading onto polyacrylamide gels.

### *Whole Muscle Preparation*

The WM samples were prepared by using a modification of the method of Bechtel and Parrish (1983). A .4-g sample of LT tissue (from at-death and PM aged samples) was knife-minced, added to 10 mL of a solution (25°C) containing 2% (wt/vol) SDS, 10 m*M* sodium phosphate buffer, pH 7.0, and homogenized with a motor-driven Dounce homogenizer. The sample was then centrifuged at 1,500 × *g* for 15 min at 25°C to remove traces of insoluble components. Protein concentrations of the supernatants were determined by using the bicinchoninic acid (**BCA**) method (Smith et al., 1985). Samples were diluted with water to 6.4 mg/mL and then prepared for SDS-PAGE as described for MF. The final concentration of the WM samples in tracking dye was 4 mg/mL.

### *Gel System*

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

### *Transfer Conditions*

Gels used for transfer were equilibrated for 15 min at 4°C in a transfer buffer containing 25 m*M* Tris, 192 m*M* glycine, 2 m*M* EDTA, 15% (vol/vol) methanol, and .1% (wt/vol) SDS. The SDS was used in the transfer buffer to aid in mobilization and transfer of very-high-molecular-weight proteins from the gel to the membrane. Samples were blotted onto a nitrocellulose membrane by using a constant voltage setting of 90 V for 150 min.

### *Western Blotting*

After transferring, the membranes were incubated for 1 h at 25°C in blocking solution (80 m*M* $Na_2HPO_4$, anhydrous, 20 m*M* $NaH_2PO_4$, 100 m*M* NaCl, .1% [vol/vol] polyoxyethylene sorbitan monolaurate [Tween-20], 5% [wt/vol] non-fat dry milk) prepared as described by the manufacturer of the chemiluminescent Western blotting detection system subsequently used (Amersham, Arlington Heights, IL). Blots were incubated in solutions containing dilutions of primary antibodies in a solution (PBS-Tween) identical to the blocking solution except non-fat dry milk was not added. Primary antibodies used in the Western blotting procedure included monoclonal anti-nebulin (NB2, Sigma Chemical Co., St Louis, MO) diluted 1:5,000 in PBS-Tween (vol/vol), and monoclonal anti-

titin (3D2, prepared against bovine skeletal muscle titin) cell culture supernatant diluted 1:10 in PBS-Tween (vol/vol). Blots were incubated with the primary antibodies overnight at 4°C. Blots were washed three times, 10 min per wash, in PBS-Tween. Bound primary antibodies were labeled with goat-anti-mouse IgG horseradish peroxidase conjugated secondary antibodies (A2554, Sigma Chemical) diluted 1:5,000 in PBS-Tween (vol/vol), for 30 min at 25°C. Blots were rinsed in PBS-Tween three times, 10 min per wash, before detection. A chemiluminescent detection system was used as described by the supplier (Amersham) to detect labeled protein bands.

### *Titin Purification*

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

## Results

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

### *Titin*

Upon examination of the major titin bands on Coomassie stained 5% gels (Figure 1), few differences were noted between the two types of preparation (compare a with b and c with d in Figure 1). In both the MF and WM samples at 0 d PM, T1 was the major form of titin present. At early times PM (e.g., 3 d PM), titin appeared as a distinct doublet, with the lower band, T2, more evident at 3 d than at 1 d. In both the MF and WM samples, the T1 form of titin was present for essentially the same period of time PM (3 d in the sample shown). We did rarely observe a slightly increased tendency for the doublet pattern of titin to be present at earlier times PM in MF samples than in the WM samples from the same steer (e.g., steer #2, Figure 1, c). However, the T2 band in the MF samples was always faint at 0 d and at early times PM (1 d in the samples shown) and at least some of the T1 form was present for the same total length of time (3 d PM) regardless of whether MF or WM samples were examined. The T2 band (large-molecular-weight degradation product of titin) was the most prominent band at 3 d PM in both MF and WM samples and underwent little additional change as detected by SDS-PAGE over the remainder of the 28-d PM aging period. Differences in the rate of appearance, or the relative intensity of the T2 band, were not evident by SDS-PAGE (Figure 1) regardless of whether the MF or WM samples from the same steer were compared. We also found that the degradation product, termed the 1,200 kDa polypeptide (Matsuura et al., 1991), appeared at the same time PM (3 d) in both the MF and WM samples.

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

### *Nebulin*

Essentially no differences attributable to the type of preparation (MF or WM) examined by SDS-PAGE were found in the rate of degradation of intact nebulin (Figure 1). At the time points examined, nebulin was degraded in MF samples at a rate equal to that seen in WM samples, with intact nebulin absent by 3 d PM in both the samples shown. Major qualitative differences in results were not observed between MF and WM samples (Figure 3) when subjected to the sensitive chemiluminescent Western blotting technique, using a monoclonal antibody specific for nebulin. Again, intact nebulin, as detected by the antibody, was present over the same time period in both MF and WM samples. Slight differences in the degradation products from nebulin in the two sample types could be noted at 3 to 7 d PM, but most of the same bands were detected in both MF and WM samples. Thus, the sample type (i.e., MF or WM) made little difference in determining the apparent overall rate of nebulin degradation.

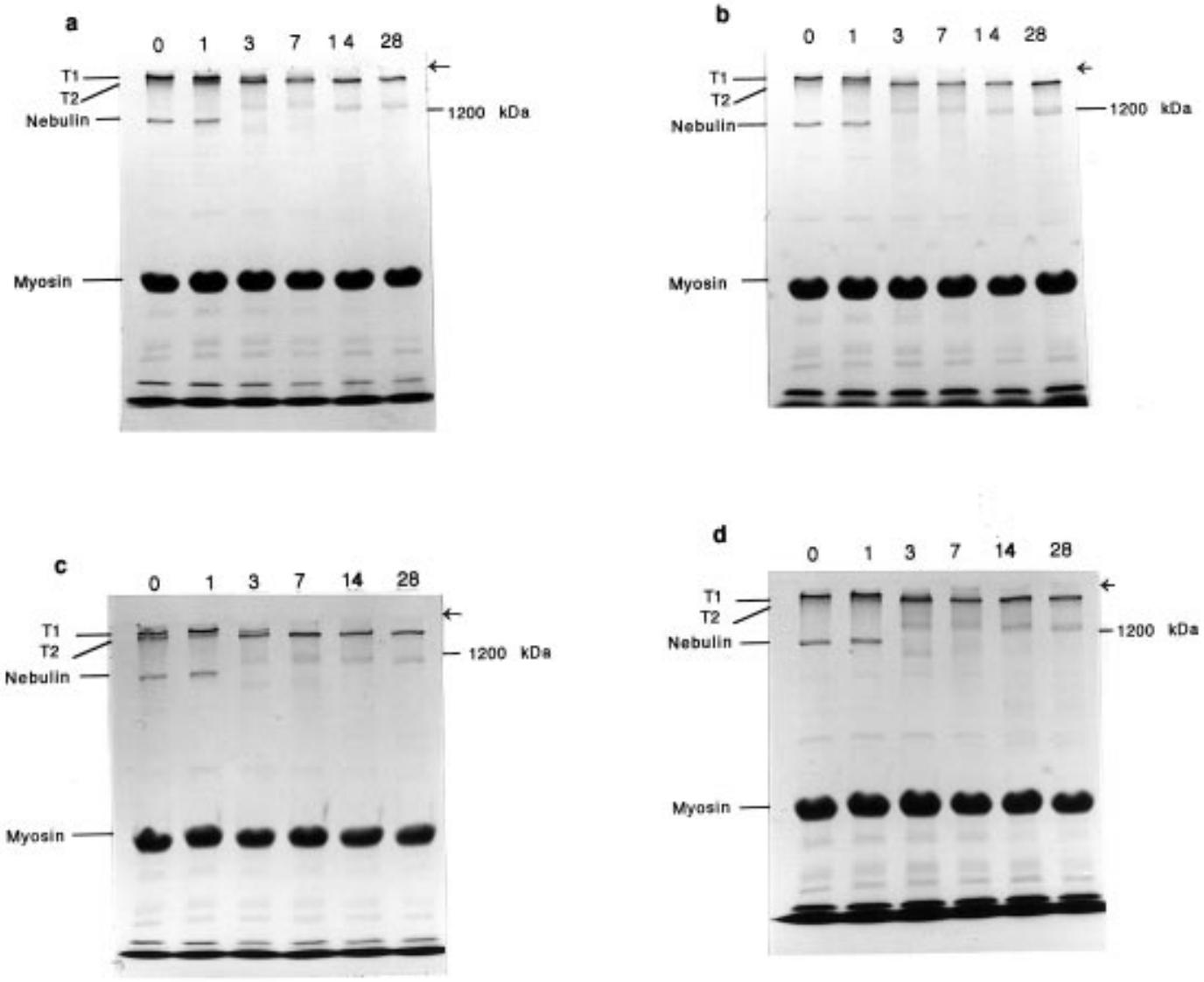


Figure 1. Coomassie-stained 5% gels of purified myofibrils and whole muscle samples from two bovine samples at 0, 1, 3, 7, 14, and 28 d postmortem. (a) Steer #1, purified myofibrils, (b) Steer #1, whole muscle samples, (c) Steer #2, purified myofibrils, (d) Steer #2, whole muscle samples. Abbreviations are as follows: T1 = intact titin, T2 = large (approximately 2,400 kDa) degradation product of intact titin. Myosin = myosin heavy chain. Arrows indicate the tops of the gel wells. 0–28 across the tops of the gels refers to days postmortem.

## Discussion

Meat represents one of the most structurally complex food products produced. One quality attribute of meat that has been explored for a number of years is tenderness. One of the structural proteins suggested by some (Robson et al., 1991; Huff-Lonergan et al., 1995; Taylor et al., 1995) to play at least a partial role in tenderness development is titin. Interest in the role titin may play in meat tenderness was initiated by observations that a third filament system in the skeletal muscle sarcomere, termed gap filaments (Locker and Leet, 1975), was degraded during aging (Davey and Graafhuis, 1976) or by proteolytic treatment in vitro (Locker et al., 1977). Since then, it has been shown that gap filaments are composed of titin (Locker, 1987). Nebulin, through its interaction with both the Z-line and the thin filaments, could also play a role in maintaining the structural integrity of the myofibril (Robson et al., 1991) and, thus, its degradation may influence the PM development of tenderness.

Biochemical techniques have often been employed in an effort to determine changes or differences in structural and regulatory proteins found in muscle and meat that might help explain observed differences in tenderness (e.g., Parrish et al., 1973; Lusby et al., 1983; Fritz and Greaser, 1991). Among the techniques used are SDS-PAGE (e.g., Koohmaraie et al., 1984;

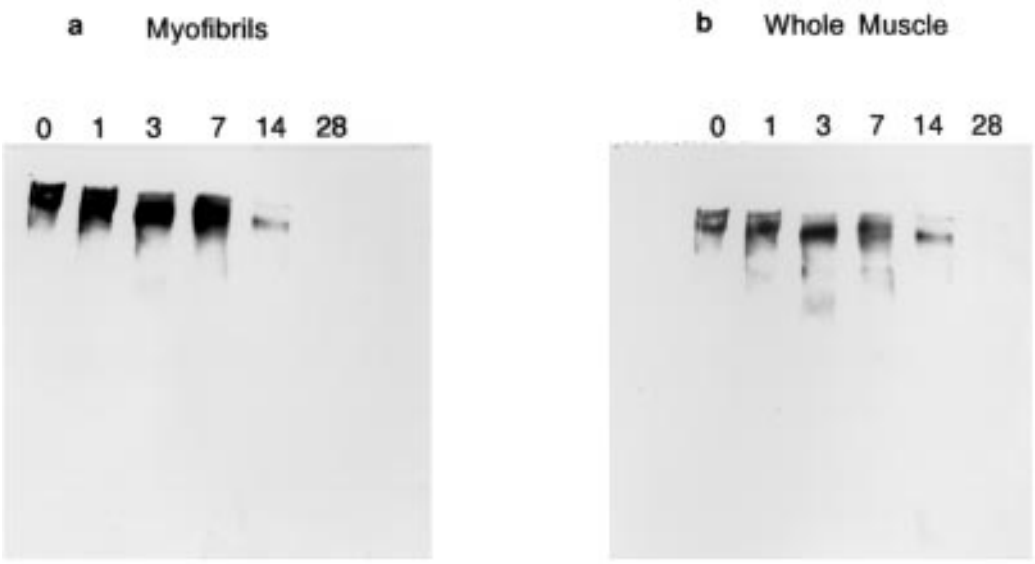


Figure 2. Western blots of purified myofibrils (a) and whole muscle samples (b) from a bovine sample (steer # 1 in Figure 1) at 0, 1, 3, 7, 14, and 28 d postmortem labeled with a monoclonal antibody to titin. 0–28 across the tops of the blots refers to days postmortem.

Huff-Lonergan et al., 1995) and Western blotting (Bandman and Zdanis, 1988; Fritz and Greaser, 1991; Taylor et al., 1995). Although these techniques are sensitive, there is considerable variation in methods used among the studies. In this study, attention was focused specifically on the type of sample examined. Purified MF are prepared by using several washing and differential centrifugation steps (Goll et al., 1974; Huff-Lonergan et al., 1995). The resulting samples are suspensions of MF almost free of sarcoplasmic and connective tissue proteins. Because MF samples are largely devoid of sarcoplasmic proteins, they provide easier interpretation of SDS-PAGE gels when lower-molecular-weight (< 200 kDa) proteins are examined. However, a proposed problem in the use of MF samples is the potential for a variable degree of

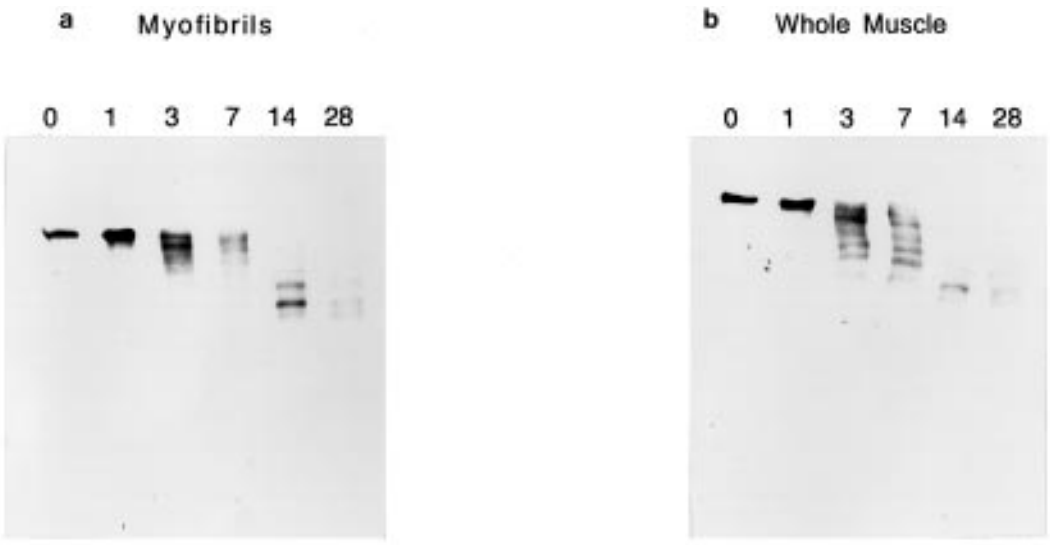


Figure 3. Western blots of purified myofibrils (a) and whole muscle samples (b) from a bovine sample (steer # 1 in Figure 1) at 0, 1, 3, 7, 14, and 28 d postmortem labeled with a monoclonal antibody to nebulin. 0–28 across the tops of the blots refers to days postmortem.

protein loss and/or loss of degradation products of myofibrillar proteins that could be soluble in the buffers used during the repeated washing and centrifugation steps (Fritz et al., 1993). The WM samples, however, contain not only myofibrillar proteins, but also a complex mixture of sarcoplasmic proteins. Thus, WM samples, albeit much faster to prepare, have the distinct disadvantage of containing large amounts of sarcoplasmic proteins that can make SDS-PAGE examination and interpretation of lower-molecular-weight myofibrillar proteins and degradation products very difficult due to the large number of non-myofibrillar protein bands migrating in the lower molecular weight ranges.

Very few qualitative differences between preparation methods were seen in this study in the rate of appearance of two high-molecular-weight, PM-aging-induced products of titin (T2 and 1,200 kDa polypeptide). This indicates that very little, if any, of these two degradation products is lost during the MF preparation procedure. Taylor et al. (1995) have also recently shown, using a commercially available titin antibody, that there is little if any detectable difference between MF and WM with respect to the degradation products of titin detected by their monoclonal antibody on Western blots of WM and MF samples. In addition, in our study, because the presence of the insoluble intact form of titin (T1) was still detectable for the same period of time PM in both MF and WM preparations, and no difference was obtained between MF and WM samples in the time of disappearance of the intact form of nebulin, it is evident that little, if any, of these two proteins is lost during the MF preparation procedure. The current study showed that, in the cases of titin and nebulin, loss of either intact proteins and(or) high-molecular-weight degradation products in MF preparations was not a significant problem. This study demonstrates that either MF or WM preparation procedures can be successfully used to monitor the intact forms of titin and nebulin and(or) their high-molecular-weight degradation products during postmortem aging.

## Implications

Although titin and nebulin are degraded at different times postmortem, few differences in the overall rate of degradation of intact titin or intact nebulin were detected between purified myofibril and whole muscle samples. Whole muscle samples have the advantage of being easier and quicker to prepare, whereas myofibril samples may be desirable when cleaner preparations of myofibrillar proteins are needed. Both types of samples provide valid means of evaluating the rate of degradation (time of disappearance) of the intact forms of these two proteins. Either method can be used satisfactorily in studies for comparing the rate of degradation of intact titin and intact nebulin in different postmortem samples.

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