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Proteolysis of specific muscle structural proteins by mu-calpain at low pH and temperature is similar to degradation in postmortem bovine muscle

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

Keywords
Biochemistry Biophysics and Molecular Biology, Protein degradation, Calpain, Beef, Tenderness, Aging

Disciplines
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Comments
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Proteolysis of Specific Muscle Structural Proteins by μ-Calpain at Low pH and Temperature Is Similar to Degradation in Postmortem Bovine Muscle[1,2]

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ABSTRACT: Postmortem (PM) and μ-calpain-induced degradation of specific skeletal muscle proteins was monitored by SDS-PAGE and Western blotting. Samples were removed from bovine longissimus thoracis (LT) at approximately 45 min PM for the preparation of at-death (0-d) myofibrils (MF). The LT was excised at 1 d PM, vacuum-packaged, and stored at 2°C. Samples were removed for Warner-Bratzler shear force analysis and biochemical analysis at 1, 3, 7, 14, 28, and 56 d PM. The protease μ-calpain was purified from bovine skeletal muscle and used to digest at-death MF at pH 5.6, 4°C, 100 μ*M* $CaCl_2$. Degradation of the proteins titin, nebulin, filamin, desmin, and troponin-T was monitored in the PM and μ-calpain-digested samples by using SDS-PAGE and Western blotting. The PM samples that had significantly lower shear force (LSF) values ($P < .05$) at 1 d PM exhibited faster degradation of these five proteins than the higher shear force (HSF) samples. In LSF samples, the intact titin band (T1) was absent by 7 d PM and nebulin was absent by 3 d PM. In LSF samples, some filamin was degraded by 3 d PM, but in HSF samples degradation was not apparent until 14 d PM. In LSF samples, desmin was degraded more rapidly PM than in HSF samples. Troponin-T was broken down PM to yield two major polypeptides of approximately 28 and 30 kDa; these polypeptides appeared earlier PM in LSF samples. Degradation products, similar to those observed PM, for all five proteins also were detected in Western blots of μ-calpain-digested MF, suggesting the calpain system plays a key role in PM protein degradation.

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

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Introduction

The postmortem (**PM**) degradation of skeletal muscle proteins and its relationship to meat tenderness and quality have been the subject of considerable research. Several myofibrillar and cytoskeletal proteins have been examined, including titin (Lusby et al., 1983; Fritz and Greaser, 1991; Huff-Lonergan et al., 1995), nebulin (Fritz and Greaser, 1991; Huff-Lonergan et al., 1995; Taylor et al., 1995), filamin (Uytterhaegen et al., 1992, 1994), desmin (Robson et al., 1981; Koohmaraie et al., 1991), and troponin-T (Olson et al., 1977; Whipple and Koohmaraie, 1992; Ho et al., 1994).

The protease μ-calpain (for review of calpain, see Goll et al., 1992) has been implicated as the major causative agent for many of the proteolytic changes that occur as meat is aged. Researchers using enzyme caseinolytic activity, SDS-PAGE, and microscopy of digested myofibrils have shown that μ-calpain retains at least partial activity under PM conditions of low pH and temperature (Koohmaraie et al., 1986, 1987). However, many of the specific protein degradation products resulting from proteolysis under these conditions remain to be characterized.

The objective of this study was twofold. The first objective was to identify degradation products occurring under normal PM aging conditions of five

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myofibrillar and myofibril-associated proteins, namely titin, nebulin, filamin, desmin, and troponin-T, in samples differing in Warner-Bratzler shear force values at early times PM. The second objective was to identify the μ-calpain-induced degradation products of the same five proteins from myofibrils (**MF**) in vitro using postmortem-like conditions of pH 5.6 and 4°C and to compare these degradation products to those products found in naturally aged samples from beef differing in shear force values at 1 d PM.

Materials and Methods

Sources and Storage of Muscle

Six A-maturity market steers were slaughtered at the Iowa State University Meat Laboratory following standard slaughter procedures. The carcasses were not electrically stimulated. A 10-g sample was removed from each of the six steers from the region between the 12th and 13th rib of the longissimus thoracis (**LT**) at approximately 45 min PM (0-d sample). A portion of the LT muscle (.64-cm-thick steak and a 2.54-cm-thick steak) was removed from of each of the six steers at 24 h PM after storage of the carcasses at 4°C (1-d sample). The LT muscles were individually vacuum-packaged and stored at 2°C. Adjacent .64- and 2.54-cm-thick steaks were removed from each of the stored LT muscles at 3, 7, 14, 28, and 56 d PM. The .64-cm-thick samples from each animal were used immediately upon removal for preparation of purified MF. The 2.54-cm steaks were vacuum-packaged and frozen at –20°C until they were used for Warner-Bratzler shear force analysis.

Warner-Bratzler Shear Force Determination

Frozen 2.54-cm-thick steaks aged 1, 3, 7, 14, 28, and 56 d from all steers were thawed at 2°C for 16 h before cooking. All steaks were broiled in a General Electric (Chicago Heights, IL) Model CNO2 industrial broiler set at a temperature of 288°C. The surfaces of the steaks were 10.16 cm from the heat source. The steaks were turned when they reached an internal temperature of 24°C and were removed from the heat at an internal temperature of 65°C. Six 1.27-cm-diameter cores were removed parallel to the muscle fiber direction from the central, medial, and lateral portions of each of the steaks (two cores per location). Cores were sheared through the center, perpendicular to the fiber direction, with a Warner-Bratzler shear device attached to an Instron Universal Testing Device (Model 4502) controlled with a Model 4500 computer assist module (Instron, Canton, MA). Peak shear force values were recorded as kilograms/1.27-cm-diameter core. Means were analyzed with a completely random design with steer in the model (Steele and Torrie, 1980).

Myofibril Preparation

Purified MF from at-death and aged muscle from all steers were prepared at 2°C according to the procedure of Huff-Lonergan et al. (1995). Protein concentrations were determined using the biuret procedure as modified by Robson et al. (1968). The MF were diluted to 3.2 mg/mL. One volume of each sample was immediately combined with .5 vol of 25°C tracking dye (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 β-mercaptoethanol (**MCE**) for a final protein concentration of 2 mg/mL. Samples were heated at 50°C for 20 min before elecrophoresis.

Purification of μ*-Calpain*

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

m*M* MES-Tris (MES = 2-[N-Morpholino]ethanesulfonic acid), pH 6.5. The μ-calpain was eluted from this final column with a linear 0 to 150 m*M* KCl gradient in the equilibration buffer. The purified μ-calpain consisted only of the 80- and 28-kDa subunits when analyzed by SDS-PAGE (results not shown).

Calpain Activity Assay

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

Digestion Procedure

At-death MF from bovine longissimus muscle were prepared according to Huff-Lonergan et al. (1995) and stored in 50% (vol/vol) glycerol at –20°C until used. For each assay, 4 mL of glycerinated MF was spun at 3,100 × *g* for 6 min at 4°C. Pellets were washed with 2 mL of 5 m*M* Tris-HCl, pH 8.0, and then centrifuged at 3,100 × *g* for 6 min at 4°C. The supernatants were removed and the myofibril pellets were washed twice in 2 mL of a 4°C buffer containing 165 m*M* NaCl, 50 m*M* MES-Tris pH 5.6 (pH was adjusted at 4°C with cold 2 *M* Tris). After each wash, the samples were centrifuged at 1,100 × *g* for 6 min. After the final centrifugation, the pellets were resuspended in 2 mL of the same buffer and the pH and the conductivity of the samples were monitored and adjusted (if necessary) to pH 5.6 and 165 m*M* NaCl. Protein concentrations were measured by using the biuret procedure as modified by Robson et al. (1968). Concentrations were adjusted with buffer (165 m*M* NaCl, 50 m*M* MES-Tris, pH 5.6) to 4 mg of protein/mL, 100 μ*M* $CaCl_2$, and 15 m*M* MCE. After the samples were allowed to equilibrate for 5 min in a 4°C circulating water bath, μ-calpain was added at a ratio of 1:800 (wt/wt; μ-calpain:myofibrillar protein). Control samples were the same, except 200 m*M* EDTA was added (final concentration 20 m*M* EDTA) to the myofibril/$CaCl_2$/MCE mixture before the addition of μ-calpain (calpain control), or buffer without μ-calpain was added to the myofibril/$CaCl_2$/MCE mixture (buffer control). Final reaction volumes were 2.5 mL. Samples (.4 mL) were removed after 0, 2, 15, 60, and 120 min of digestion with μ-calpain and added to an aliquot of 200 m*M* EDTA to bring the final concentration of EDTA to 20 m*M* to stop the reaction. All digestions were done in triplicate. Samples were centrifuged at 12,000 × *g* for 15 min at 4°C. Each supernatant was removed, its volume measured, and reserved. An amount of 5 m*M* Tris-HCl, pH 8.0, equal to the amount of supernatant removed was added to resuspend the respective pellets. After thorough resuspension of the MF, pryonin Y tracking dye (3 m*M* EDTA, 3% [wt/vol] SDS, 30% [vol/vol] glycerol, .003% [wt/vol] pyronin Y, 120 m*M* DL-dithiothreitol [**DTT**], and 30 m*M* Tris-HCl, pH 8.0) (Wang, 1982) was added. The final protein concentration of the samples in tracking dye was 2 mg/mL. Each reserved supernatant was added to 2×-concentrated pyronin Y tracking dye at a ratio of 1:1 (vol/vol). Samples were immediately heated at 50°C for 20 min and loaded onto gels.

Gel System

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

Transfer Conditions

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

Western Blotting

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

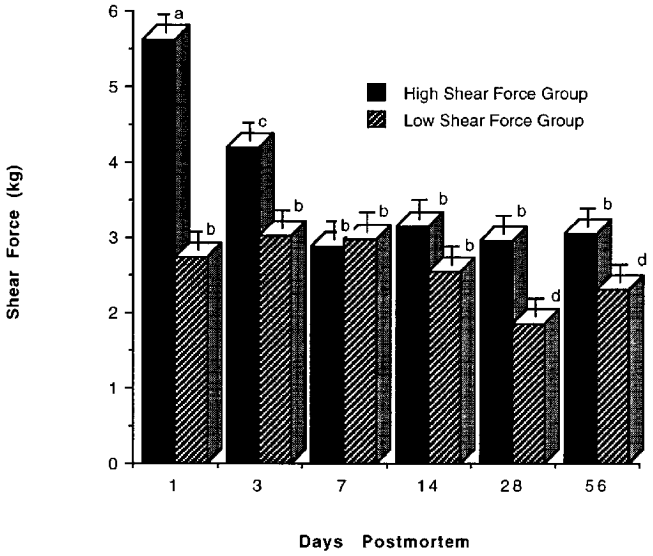

Figure 1. Least squares means and standard errors of the Warner-Bratzler shear force values of the steaks at 1, 3, 7, 14, 28, and 56 d postmortem (PM). The High Shear Force group consisted of samples that were identified as having significantly higher shear force values ($P < .05$) at 1 d PM. The Low Shear Force group consisted of samples that were identified as having significantly lower shear force values ($P < .05$) at 1 d PM. Bars with different superscripts are significantly different ($P < .05$).

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). Titin and nebulin were separated by salt fractionation as described by Wang (1982). Titin was isolated by gel filtration on a 2.6-cm × 90-cm Sephacryl S-500-HR (Pharmacia Biotech, Piscataway, NJ) column.

Results

The steers in this study were classified as having significantly ($P < .05$) lower shear force values (**LSF**) or significantly ($P < .05$) higher shear force values (**HSF**) at 1 d PM and were kept in their respective categories throughout the study. Warner-Bratzler shear force data (Figure 1) showed that while meat from all of the steers decreased significantly ($P < .05$) in shear force after extended PM aging, steaks from

steers in the HSF group underwent a slower decline in shear force than did steaks from steers in the LSF group. The LSF samples, although they already had reduced shear force values, did not undergo further significant ($P < .05$) reduction in shear force values until 28 d PM, indicating the possibility that significant changes affecting shear force values occurred at very early times PM in these samples. The HSF steaks showed a significant ($P < .05$), steady reduction in shear force values 3 d and 7 d PM (Figure 1), suggesting that changes affecting shear force values in these steaks occurred later than in LSF steaks. Examination of the gels and Western blots from the steers in this study revealed that, indeed, proteolytic changes in the myofibrillar/cytoskeletal proteins occurred more slowly in HSF samples than in LSF samples.

All six steers were subjected to analysis by SDS-PAGE and Western blotting. Gels and Western blots shown herein of the MF from aged muscle samples are representative of blots from samples that differed significantly ($P < .05$) in their Warner-Bratzler shear force values at 1 d PM.

Titin and Nebulin

General changes in the large proteins can be seen in the 3.2 to 12% gradient gels in Figure 2. The MF samples from naturally aged steaks with significantly ($P < .05$) LSF values (Figure 2a) exhibited earlier disappearance of the intact forms of titin (**T1**) and nebulin than did the MF samples from steaks with significantly ($P < .05$) HSF values (Figure 2b). In both the respective HSF and LSF samples, nebulin was degraded faster than T1 (Figure 2 a, b). In the MF from the LSF samples (Figure 2a), T1 was absent by 7 d PM, whereas nebulin was absent by 3 d PM. In the MF from the HSF samples (Figure 2b), the same temporal relationship between T1 and nebulin was noted. The MF from the HSF samples possessed some T1 through 7 d of PM aging. No T1 was noted at 14 d PM in any of the HSF samples in this study. Nebulin, on the other hand, was not seen in its intact form beyond 3 d PM in the MF from the HSF samples in this study. Other studies examining the degradation of T1 and nebulin have also noted that nebulin is degraded faster than titin (T1), regardless of the tenderness evaluation or shear force value obtained for the sample (e.g., Huff-Lonergan et al., 1995; Taylor et al., 1995).

When the degradation patterns of the MF prepared from the aged samples (Figure 2a,b) were compared to the gels of the μ-calpain-digested 0-d MF samples (Figure 2c), it was evident that μ-calpain incubation at 4°C, pH 5.6, 100 μM $CaCl_2$ caused many of the same changes. Most notably, nebulin was degraded quickly. The band corresponding to intact nebulin was absent before T1. The T1 band was substantially degraded to predominantly the T2 form after 120 min of incubation. In contrast, the control sample showed no significant degradation at 120 min, indicating the changes were due to μ-calpain and not to the incubation conditions of buffer and temperature.

Western blots probed with a monoclonal antibody to titin showed that the MF from LSF samples (Figure 3a) exhibited earlier PM degradation of T1 than did the MF from the HSF samples (Figure 3b). In addition to T2, the monoclonal antibody detected a titin degradation product that migrated at approximately 1,200 kDa. In the MF from the LSF samples (Figure 3a), the 1,200 kDa polypeptide was detected by 1 d PM, whereas in the MF from the HSF samples (Figure 3b), it was not detected until 3 d PM. As the PM aging time increased further, this 1,200 kDa band decreased in both the MF from the LSF samples (Figure 3a) and the MF from the HSF samples (Figure 3b). The 1,200-kDa polypeptide was not detected by the antibody at or beyond 28 d PM in either the MF from the LSF or the HSF samples, indicating additional degradation of this titin polypeptide degradation product as time PM increased. Some degradation of T2, which could not be easily detected in the gels (Figure 2), also was detected by the Western blotting procedure (Figure 3). By 7 d PM in the MF from the LSF samples (Figure 3a) and by 28 d PM in the MF from the HSF samples (Figure 3b), the titin antibody failed to recognize the T2 band of titin, even though this band could still be seen through 56 d PM on Coomassie-stained gels (see Figure 2 and Discussion).

The blots of the purified MF that had been digested with μ-calpain at pH 5.6, 4°C, 100 μM $CaCl_2$ (Figure 3c) revealed the disappearance of the T1 band by 60 min of incubation. A polypeptide migrating at 1,200 kDa also increased over the incubation period, up to 60 min. By 120 min, however, the latter band was not strongly detected by this antibody. Additionally, as in the MF from the naturally aged samples, the T2 band of titin (Figure 2c) was not recognized by the monoclonal antibody after prolonged times of incubation (120 min) (Figure 3c). The 120 min buffer control (no μ-calpain) did not seem to be significantly different from the 0-time sample, indicating little if any effect of the buffer or temperature on the proteins examined. Failure of the monoclonal antibody to recognize either the 1,200-kDa polypeptide or T2 after extended times PM and after prolonged digestion times (Figure 3) indicates that some additional calpain-induced proteolysis of these two polypeptides occurs, even though this degradation is not easily detectable on the Coomassie-stained gels (Figure 2).

Western blots probed with a monoclonal antibody to nebulin (Figure 4) showed the same trend as that observed in the gels (Figure 2). Nebulin was not recognized by the antibody beyond 1 d PM in the MF from the LSF samples (Figure 4a), but some intact nebulin was detected through 3 d PM in the MF samples from HSF samples (Figure 4b). The MF that

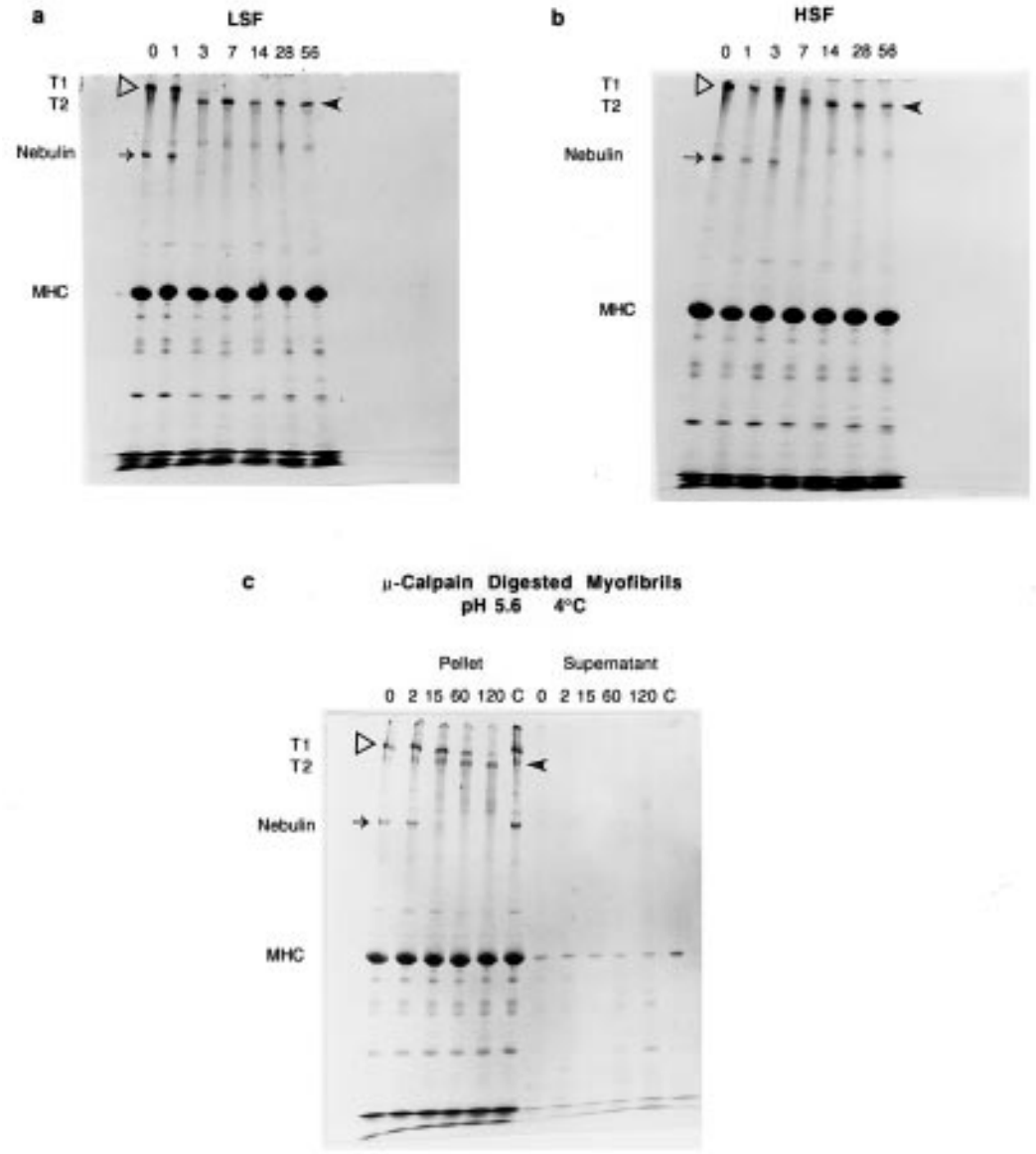

Figure 2. Coomassie-stained 3.2 to 12% gradient SDS-PAGE gels of myofibrils. (a) Myofibrils isolated from bovine muscle samples aged 0, 1, 3, 7, 14, 28, and 56 d postmortem (PM) from a low shear force (LSF) sample. (b) Myofibrils isolated from bovine muscle samples aged 0, 1, 3, 7, 14, 28, and 56 d PM from a high shear force (HSF) sample. 0 to 56 at the tops of the gels refers to days PM. (c) Pellets and supernatants from purified at-death myofibrils incubated at 4°C, pH 5.6, with μ-calpain and sampled after 0, 2, 15, 60, and 120 min of incubation. 0 to 120 at the top of the gel refers to min incubation with μ-calpain. C = buffer control sample after 120 min of incubation at 4°C, pH 5.6. Other abbreviations are as follows: T1 = intact titin, T2 = large (2,400 kDa) degradation product of titin, MHC = myosin heavy chain. Open triangle designates the position of T1. Closed arrowhead designates the position of T2. Small arrow designates the position of nebulin.

were digested with μ-calpain at pH 5.6, 4°C (Figure 4c) showed rapid degradation of nebulin. After 15 min of incubation with calpain, no nebulin was detected by the antibody.

Upon examination of Figures 3 and 4, it also can be seen that in both the MF samples from the aged LSF and HSF samples, as well as in the μ-calpain-digested samples, the degradation of nebulin preceded the degradation of titin. This indicates that nebulin is more susceptible to degradation by endogenous proteases (including μ-calpain) than is titin.

When the MF from aged LSF and HSF samples and the μ-calpain-digested samples were run on 18% gels and transferred to PVDF for Western blotting, neither the monoclonal titin antibody nor the monoclonal nebulin antibody detected any degradation products (results not shown). This suggests that the lower molecular weight degradation products (< approxi-

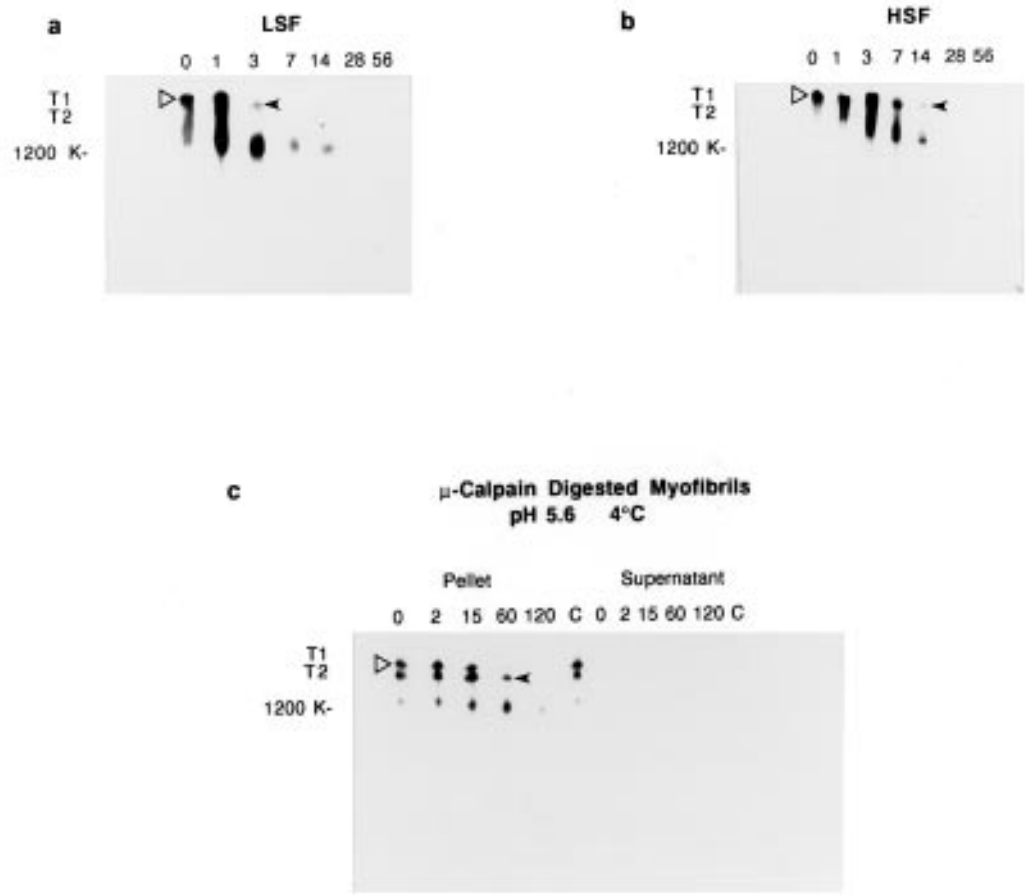

Figure 3. Western blots of myofibril samples run on 3.2 to 12% gradient gels and transferred to PVDF membrane. The blots were incubated with monoclonal titin antibody 4C7 (diluted 1:10 in PBS-Tween) overnight at 4°C. (a) Myofibrils isolated from bovine muscle samples aged 0, 1, 3, 7, 14, 28, and 56 d postmortem (PM) from a low shear force (LSF) sample. (b) Myofibrils isolated from bovine muscle samples aged 0, 1, 3, 7, 14, 28, and 56 d PM from a high shear force (HSF) sample. 0 to 56 at the top of the blot refers to days PM. (c) Pellets and supernatants from purified at-death myofibrils incubated at 4°C, pH 5.6, with μ-calpain and sampled after 0, 2, 15, 60 and 120 min of incubation. 0 to 120 at the top of the blot refers to min of incubation with purified μ-calpain. C = buffer control after 120 min of incubation at 4°C, pH 5.6, and 100 μM $CaCl_2$. Open triangle designates the position of T1. Closed arrowhead designates the position of T2. Abbreviations are as follows: T1 = intact titin, T2 = large (2,400 kDa) degradation product of titin, 1200 K = approximately 1,200 kDa degradation product of titin.

mately 205 kDa) were not recognized by the monoclonal antibodies, or were very small (< approximately 9 kDa) and not resolved on the 18% gels used in this study. No titin or nebulin degradation products that could be recognized by the monoclonal antibodies used in this study were detected in the supernatants of the μ-calpain-digested MF samples run on either the 3.2 to 12% (Figures 3c and 4c) or 18% gels (results not shown). This indicates that it is highly unlikely that any significant degradation products recognized by these two antibodies were lost in the MF preparations from the naturally aged samples.

Filamin

Western blots that were probed with a polyclonal antibody to filamin are shown in Figure 5. The MF samples from the LSF steaks showed the appearance of an approximately 240-kDa degradation product in addition to intact filamin by 3 d PM (Figure 5a). Appearance of this product indicates degradation of some of the intact filamin. In contrast, the approximately 240-kDa degradation product was not apparent in the MF from the HSF samples until 14 d PM (Figure 5b).

When the myofibril samples that were digested with μ-calpain at pH 5.6, 4°C were examined by Western blotting, the polyclonal antibody recognized the presence of an approximately 240-kDa degradation product at 60 min of incubation (Figure 5c). Again, as in MF from the naturally aged samples, the degradation product that was recognized migrated very closely to the intact form, giving the appearance of a doublet. From the results in Figure 5, it is evident

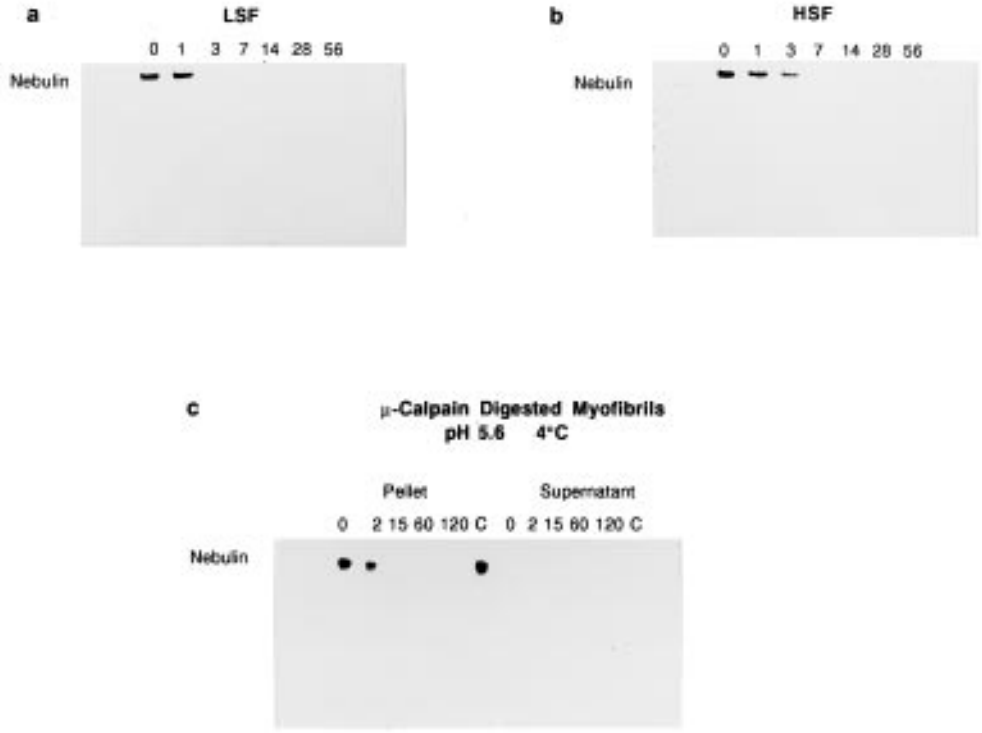

Figure 4. Western blots of myofibril samples run on 3.2 to 12% gradient gels and transferred to PVDF membrane. The blots were incubated with monoclonal nebulin antibody NB2 (diluted 1:5,000) overnight at 4°C. (a) Myofibrils isolated from bovine muscle samples aged 0, 1, 3, 7, 14, 28, and 56 d postmortem (PM) from a low shear force (LSF) sample. (b) Myofibrils isolated from bovine muscle samples aged 0, 1, 3, 7, 14, 28, and 56 d PM from a high shear force (HSF) sample. 0 to 56 at the top of the blot refers to days of PM aging. (c) Pellets and supernatants from purified at-death myofibrils incubated at 4°C, pH 5.6, with μ-calpain and sampled after 0, 2, 15, 60, and 120 min of incubation. 0 to 120 at the top of the blot refers to min of incubation with purified μ-calpain. C = buffer control after 120 min of incubation at 4°C, pH 5.6, and 100 μM $CaCl_2$.

that μ-calpain is capable of producing the same, or at least nearly the same, degradation pattern of filamin as that seen in the MF from the naturally aged samples. No detectable filamin or filamin degradation products were released in the supernatant in the μ-calpain-digested MF (Figure 5c).

Desmin

Western blots of MF from the LSF samples that were probed with a polyclonal antibody to desmin revealed three degradation products (Figure 6a). The first products to appear were detected at 3 d PM and migrated at approximately 45 kDa (band migrating between intact desmin and 38 K band, Figure 6a) and 38 kDa. The 45-kDa polypeptide seemed to be transient and was not detected beyond 14 d PM in the MF from the LSF samples or 28 d PM in the HSF samples, indicating that it, in turn, was further degraded PM. The 38-kDa polypeptide, after it appeared at 3 d PM in MF from LSF samples and at 14 d in the MF from HSF samples, was present throughout the 56-d aging period. Also appearing in the MF from the LSF samples, but not in MF from any of the HSF samples, was a 35-kDa polypeptide. This polypeptide was not seen until 56 d PM in the LSF samples and was very faint. Overall, it was evident that the HSF samples showed slower degradation of desmin and appearance of desmin degradation products (Figure 6b) than did the LSF samples (Figure 6a).

Blots of MF that had been incubated with purified μ-calpain also showed the presence of a 38- and a 35-kDa polypeptide when probed with the polyclonal desmin antibody (Figure 6c). In these blots, the 38-kDa polypeptide appeared first (after 2 min of incubation) and remained detectable throughout the digestion time (120 min). The 35-kDa polypeptide appeared later (after 15 min of incubation) and it remained as a major degradation product through the 120-min incubation. The transient 45-kDa polypeptide, seen in the MF from the naturally aged muscle samples, was not seen in the μ-calpain-digested MF samples. This could be due to degradation of the transient 45-kDa polypeptide before the 2-min sampling time. This was likely because μ-calpain, in the amount added to the purified MF, was able to fully

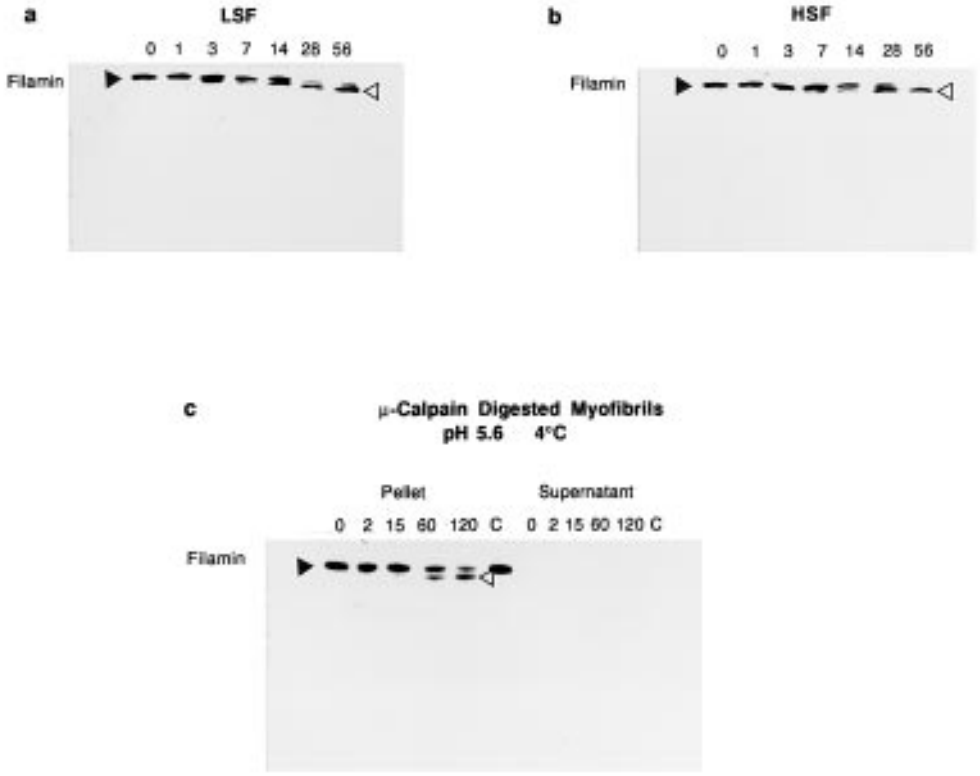

Figure 5. Western blots of myofibril samples run on 3.2 to 12% gradient gels and transferred to PVDF membrane. The blots were incubated with polyclonal filamin antibody (diluted 1:20,000 in blocking solution) for 2 h at 25°C. (a) Myofibrils isolated from bovine muscle samples aged 0, 1, 3, 7, 14, 28, and 56 d postmortem (PM) from a low shear force (LSF) sample. (b) Myofibrils isolated from bovine muscle samples aged 0, 1, 3, 7, 14, 28, and 56 d PM from a high shear force (HSF) sample. 0 to 56 at the top of the blot refers to days of PM aging. (c) Pellets and supernatants from purified at-death myofibrils incubated at 4°C, pH 5.6, with μ-calpain and sampled after 0, 2, 15, 60, and 120 min. 0 to 120 at the top of the blot refers to minutes of incubation with μ-calpain. C = buffer control sample after 120 min of incubation at 4°C, pH 5.6, and 100 μM $CaCl_2$. Closed triangle designates the position of intact filamin. Open triangle designates the position of the large degradation product of filamin.

degrade the intact desmin within 60 min of incubation. The MF from naturally aged samples, on the other hand, continued to show some intact desmin throughout the aging period, indicating a slower rate of degradation of desmin in the MF of PM muscle samples. As was the case for the other proteins examined, no detectable desmin or desmin degradation products were released into the supernatants of the calpain-digested MF (Figure 6c).

Troponin-T

The PM degradation of troponin-T and the appearance of bands migrating at approximately 30 kDa has frequently been reported (MacBride and Parrish, 1977; Olson et al., 1977; Wheeler and Koohmaraie, 1994). Blots of MF in this study that were probed with a monoclonal antibody to troponin-T also showed a difference between the LSF and HSF samples in the degradation of this protein (Figure 7a,b). The troponin-T in the 0-d sample appeared as a closely spaced doublet, which is consistent with troponin isoforms (see Discussion). There were two major PM degradation products (each of these is probably a closely spaced doublet) migrating at approximately 30 and 28 kDa that were labeled by the troponin-T monoclonal antibody. In the MF from the LSF samples (Figure 7a) both the approximately 30 and the 28-kDa bands are prominent by 3 d PM, the 30-kDa band appearing first (1 d). In addition, some intact troponin-T was present on the blots through 14 d PM. The MF from the HSF samples (Figure 7b) showed a marked increase in the amount of time before significant amounts of both the 30- and 28-kDa polypeptides were recognized by the monoclonal antibody. The 30-kDa band (a trace was present at 1 d PM) was not prominently recognized by the troponin-T monoclonal antibody until 7 d PM. The 28-kDa polypeptide was essentially not recognized until 14 d PM. Some intact troponin-T was present on the blots of MF from HSF samples through 28 d PM. The retardation of the PM time of appearance of the 30- and 28-kDa polypeptides

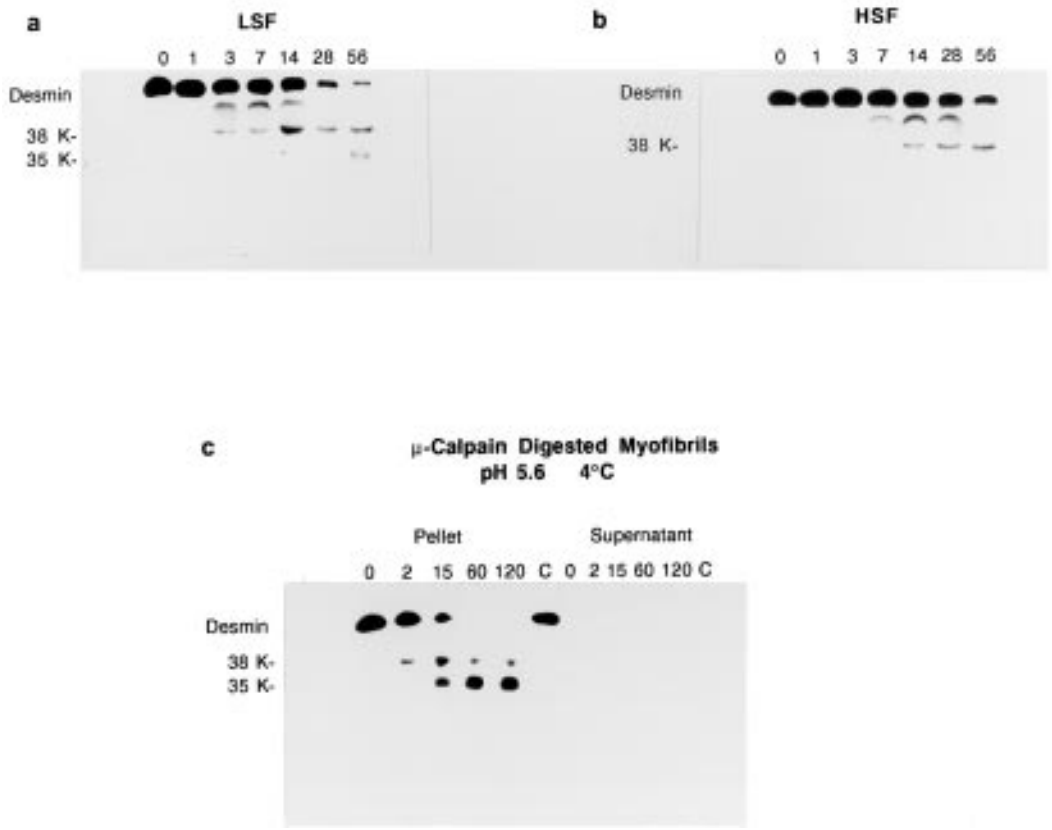

Figure 6. Western blots of myofibril samples run on 18% gels and transferred to PVDF membrane. The blots were incubated with a polyclonal antibody to desmin (diluted 1:15,000 in blocking solution) for 1 h at 25°C. (a) Myofibrils isolated from bovine muscle samples aged 0, 1, 3, 7, 14, 28, and 56 d postmortem (PM) from a low shear force (LSF) sample. (b) Myofibrils isolated from bovine muscle samples aged 0, 1, 3, 7, 14, 28, and 56 d PM from a high shear force (HSF) sample. 0 to 56 at the top of the blot refers to days of PM aging. (c) Pellets and supernatants from purified at-death myofibrils incubated at 4°C, pH 5.6, with μ-calpain and sampled after 0, 2, 15, 60, and 120 min of incubation. 0 to 120 at the top of the blot refers to min of incubation with purified μ-calpain. C = buffer control sample after 120 min of incubation at 4°C, pH 5.6., and 100 μM $CaCl_2$.

and the increased amount of the time that intact troponin-T could be detected on the blots of the MF from the HSF samples are reflective of their higher Warner-Bratzler shear force values.

The purified MF that were digested with purified μ-calpain also showed the development of approximately 30- and 28-kDa bands (Figure 7c). These degradation products appeared within 60 min of incubation with the μ-calpain. The presence of these polypeptides indicate that μ-calpain is capable of producing these degradation products in vitro under PM-like conditions of pH 5.6 and 4°C. No detectable troponin-T or its degradation products were detected in the supernatants of the calpain-digested MF (Figure 7c).

Discussion

We have employed SDS-PAGE and a sensitive Western blotting procedure to demonstrate that μ-calpain degrades five key myofibrillar and cytoskeletal proteins, under PM-like conditions, in a manner very similar to that observed in MF prepared from PM-aged beef samples. Furthermore, we consistently observed, by comparing the rate and degree of PM degradation of the five proteins in MF prepared from LSF and HSF samples with Warner-Bratzler shear force data, that the PM changes in these proteins are related to tenderness. In the LSF samples, changes occurred in all of the proteins at early times PM. In contrast, the HSF generally showed most changes had occurred (e.g. titin, Figure 3b, and nebulin, Figure 4b) or were beginning to occur (e.g. filamin, Figure 5b, desmin, Figure 6b, and troponin-T, Figure 7b) between 3 and 7 d PM, the time point at which the last significant reduction in shear force occurred in the HSF samples. This may indicate that degradation of certain proteins may have a significant effect on the measured shear-force values. Taken *in toto* our results provide evidence in support of the hypothesis that μ-calpain degradation of muscle proteins plays an important role in PM tenderization. The possible

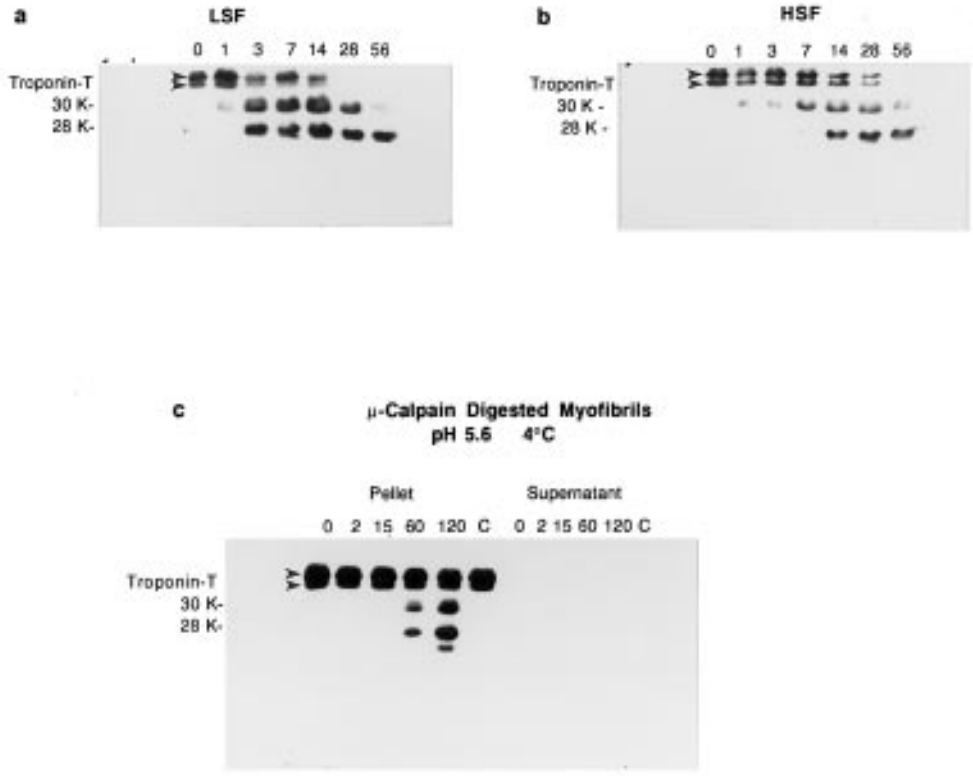

Figure 7. Western blots of myofibril samples run on 18% gels and transferred to PVDF membrane. The blots were incubated with monoclonal troponin-T antibody JLT-12 (diluted 1:20,000 in PBS-Tween) for 1 h at 25°C. (a) Myofibrils isolated from bovine muscle samples aged 0, 1, 3, 7, 14, 28 and 56 d postmortem (PM) from a low shear force (LSF) sample. (b) Myofibrils isolated from bovine muscle samples aged 0, 1, 3, 7, 14, 28, and 56 d PM from a high shear force (HSF) sample. 0 to 56 at the top of the blot refers to days of PM aging. (c) Pellets and supernatants from purified at-death myofibrils incubated at 4°C, pH 5.6, with μ-calpain and sampled after 0, 2, 15, 60, and 120 min of incubation. 0 to 120 at the top of the blot refers to min of digestion with purified μ-calpain. C = buffer control sample after 120 min of incubation at 4°C, pH 5.6, and 100 μM $CaCl_2$. Arrows indicate the position of two isoforms of intact troponin-T.

role(s) of the five proteins in muscle integrity and the importance of the PM changes that occur in each will be discussed in descending order of their molecular mass.

Titin

Titin, an extraordinarily large structural protein (approximately 3,000 kDa), has been shown to span the distance from the Z-line to nearly the M-line (Furst et al., 1988), half of the skeletal muscle sarcomere. One role proposed for titin (for review, see Robson, 1995) is that it may serve to aid in maintaining the overall structural integrity of the myofibril. As part of this proposed function, titin may keep the thick filaments in register and positioned in the middle of the sarcomere. Based on the fact that the I-band portion of titin is not firmly attached to other structures and is elastic in nature, titin also may play roles in maintaining elasticity and resting tension of skeletal muscle cells (Wang et al., 1991, 1993; Robson, 1995). Due to the aforementioned roles of titin in living cells, it is quite conceivable that its degradation PM would lead to weakening of the longitudinal structure of the myofibrillar sarcomere and integrity of muscle. This weakening, in conjunction with other changes in PM muscle, could lead to enhanced tenderness.

The degradation of T1 has been observed in several studies (Lusby et al., 1983; Zeece et al., 1986; Astier et al., 1993). A major degradation product that migrates only slightly faster under SDS-PAGE conditions than T1 is termed T2 (approximately 2,400 kDa) (Kurzban and Wang, 1988). Another titin degradation product that has been observed migrates at approximately 1,200 kDa by SDS-PAGE analysis (Matsuura et al., 1991; Takahashi et al., 1992). This latter polypeptide has been shown to contain the portion of titin that extends from the Z-line to near the putative N_2 line in the I-band (Kimura et al., 1992; Kawamura et al., 1995), although the exact position that the 1,200-kDa polypeptide reaches in the sarco-

mere is still not certain. A polypeptide migrating in the region of 1,200 kDa was observed in the current study. This polypeptide was recognized by the monoclonal antibody (4C7) prepared against intact bovine skeletal muscle titin (Figure 3). The 1,200-kDa polypeptide appeared earlier PM in MF from LSF samples than from HSF samples. This same monoclonal antibody that recognized T1 and the 1,200-kDa polypeptide also initially recognized the other large degradation product, T2. This antibody (4C7), which labels at the A-I junction as determined by immunofluorescence microscopy (results not shown), may recognize a single epitope of titin at the A-I junction, but we cannot rule out other possibilities (e.g., it may recognize closely spaced, repeated epitopes that are common to both the 1,200 kDa and the T2 portion of the molecule). Reports of monoclonal titin antibodies that recognize multiple epitopes along titin are not uncommon (Itoh et al., 1988; Furst et al., 1989). The possibility also exists that the degraded titin polypeptide bands seen in these PM MF also may represent a heterogeneous population of polypeptides, resulting from cleavage on either side of a single epitope, giving rise to fragments migrating at approximately 1,200 kDa and approximately 2,400 kDa (T2), at least some of which contain the epitope recognized by the antibody used in this study. Although we and others identify bands as T1, T2, and 1,200 kDa in our figures, it is possible, if not probable, that each of these bands contains polypeptides of slightly different size that are not resolved (i.e., giant polypeptides with a difference in mass of approximately 50 kDa may migrate closely enough to appear as single bands). The T2 polypeptide was also apparently subsequently degraded or altered PM because the antibody ceased to recognize T2 after prolonged periods of PM storage or μ-calpain digestion. Other researchers have noted a similar decrease, or cessation, of antibody recognition of T2 as time PM increases with certain other titin monoclonal antibodies (C.-Y. Ho, M. H. Stromer, and R. M. Robson, personal communication). Recent studies (Huff-Lonergan et al., 1995; Taylor et al., 1995) suggest a relationship between PM degradation of titin and meat tenderness.

Nebulin

Nebulin, another extremely large (600 to 900 kDa) (Jin and Wang, 1991) structural protein that is found only in skeletal muscle, spans the distance from the Z-line to near the free end of the thin filament. Nebulin, which is thought to be intimately associated with the thin filament and may even constitute part of a composite nebulin/thin filament (Pfuhl et al., 1994; Robson, 1995), may aid in anchoring the thin filament to the Z-line (Wang and Wright, 1988; Komiyama et al., 1992). Degradation of nebulin PM could weaken the thin filament linkages at the Z-line, and(or) of the thin filaments in the nearby I-band regions (Taylor et al., 1995), and thereby weaken the structure of the muscle cell. Nebulin has also recently been shown to be capable of linking actin and myosin (Root and Wang, 1994), and these researchers have proposed that nebulin may also have a regulatory function in skeletal muscle contraction. If the latter role is confirmed, then it is also possible that nebulin's PM degradation may alter actin-myosin interactions in such a way that the alignment and interactions of thick and thin filaments in PM muscle is disrupted. This, too, could lead to an increase in PM tenderization. Nebulin degradation, as seen in this study (Figure 3) and others (Huff-Lonergan et al., 1995; Taylor et al., 1995), does seem to be correlated to PM tenderization, although the exact cause and effect relationship remains to be substantiated.

Filamin

Filamin is a large (M_r = 245,000 in skeletal and cardiac muscle) actin binding protein that has been shown to exist in numerous cell types (Price et al., 1994). Many different isoforms have been shown to exist (Hock et al., 1990). The amount of filamin in skeletal and cardiac muscle is very low (approximately < .1% of the total muscle protein). In skeletal and cardiac muscle, filamin has been shown to be localized at the periphery of the myofibrillar Z-disk, and it may be associated with intermediate filaments in these regions (Price et al., 1994). Thus, postmortem degradation of filamin conceivably could disrupt key linkages that serve to help hold MF in lateral register. Degradation of filamin may also alter linkages connecting the peripheral layer of MF in muscle cells to the sarcolemma by weakening interactions between peripheral myofibrillar Z-disks and the sarcolemma via intermediate filament associations or costameres (Robson, 1995).

This study showed that some filamin was degraded to form an approximately 240-kDa degradation product in both MF from naturally aged muscle and in μ-calpain-digested MF (Figure 4). This same doublet formation (composed of intact and degraded filamin) has been seen in cultured embryonic skeletal muscle cells and was attributed to calpain activity (Kwak et al., 1993). Uytterhaegen et al. (1994) have shown increased degradation of filamin in muscle samples injected with $CaCl_2$, a process that has been shown to stimulate proteolysis and PM tenderization (Koohmaraie et al., 1988b). That the current study shows the degradation of filamin occurs at different rates in the MF from naturally aged muscle with different shear force values suggests that its degradation is either directly involved in tenderization, through disruption of key linkages, or is at least an indicator of PM proteolysis. Further studies that employ a combination of sensitive detection methods (e.g., Western blotting, immunomicroscopy) are needed to determine the role of filamin in skeletal muscle systems and PM tenderization.

Desmin

It has been suggested that desmin, a member of the type III group of intermediate filament proteins (O'Shea et al., 1981; Robson, 1989), which is localized at the periphery of the myofibrillar Z-disk in skeletal muscle (Richardson et al., 1981), plays a role in PM tenderization (Robson et al., 1981; Hwan and Bandman, 1989; Whipple and Koohmaraie, 1991). The desmin intermediate filaments surround the Z-lines of MF, connect adjacent MF at the level of their Z-lines, and the myofibrils to other cellular structures, including the sarcolemma (Yagyu et al., 1990; Robson, 1995). It has been proposed that desmin may be important in maintaining the structural integrity of muscle cells (Robson et al., 1981, 1991). Degradation of structural elements that connect the major components (i.e., the myofibrils) of a muscle cell together, as well as the peripheral layer of myofibrils to the cell membrane, could affect the development of tenderness.

We found, in agreement with others (Robson et al., 1981; Koohmaraie et al., 1984a,b, 1988a; Hwan and Bandman, 1989), that desmin is degraded during PM storage. Furthermore, we found desmin was degraded more rapidly in MF from samples with lower shear force values than from samples with higher shear force values. A major degradation product that was seen in both the LSF and HSF samples was a polypeptide of approximately 38 kDa (Figure 5). This degradation product also appeared in the μ-calpain-digested MF in this study. That the 38-kDa polypeptides produced in both MF prepared from naturally aged samples and in μ-calpain-digested purified MF were detected by the same antibody suggests that these are the same (or a very similar) polypeptide. Thus, μ-calpain may be, at least in part, responsible for production of the 38-kDa polypeptide under normal PM aging conditions. The fact that this polypeptide appeared at earlier times PM in MF from the LSF samples also suggests that its generation may be linked to PM tenderization processes. Whether or not this degradation is directly linked to PM tenderization or is simply an indicator of overall postmortem proteolysis remains to be determined.

Troponin-T

For many years it has been recognized that the degradation of troponin-T and the appearance of polypeptides migrating at approximately 30 kDa are strongly related to, or correlated with, the tenderness of beef (MacBride and Parrish, 1977; Olson and Parrish, 1977; Penny and Dransfield, 1979). It has been shown that purified bovine troponin-T can be degraded by m-calpain in vitro to produce polypeptides in the 30-kDa region (Olson et al., 1977). In addition, polypeptides in the 30-kDa region found in aged bovine muscle specifically have been shown to be products of troponin-T by using Western blotting techniques (Ho et al., 1994). In the current study, increasing PM time was shown to be associated with the appearance of two major bands (each is likely a closely spaced doublet of polypeptides) of approximately 30 and 28 kDa, which were labeled with a monoclonal antibody to troponin-T. In addition, the increasing postmortem aging time was also associated with a loss of troponin-T, as has been reported in numerous studies (e.g., Olson et al., 1977; Koohmaraie et al., 1984b; Ho et al., 1994). The antibody used in this study seems to label two very closely spaced bands corresponding to intact troponin-T. This is likely due to isoforms of troponin-T that are known to exist in skeletal muscle (Briggs et al., 1990; Malhotra, 1994), including specifically bovine skeletal muscle (Clarke et al., 1976; and see discussion in Ho et al., 1994). Both the appearance of the 30- and 28-kDa bands and the disappearance of the intact troponin-T in the MF in this study seem to be very strongly related to the shear force (LSF vs HSF) classification of the original samples. The MF from samples with significantly higher shear force values showed an overall delayed appearance of both the 28- and 30-kDa bands as well as delayed degradation of intact troponin-T (Figure 7), indicating slower degradation of troponin-T in MF from samples with higher shear force values. Under conditions of pH and temperature (pH 5.6 and 4°C) that commonly occur in PM-aged samples, it was observed (Figure 7) that μ-calpain is capable of catalyzing the production of polypeptides migrating at approximately 28 and 30 kDa. This result, coupled with the fact that the degradation products in the MF from naturally aged muscle and the μ-calpain digests are labeled with the same highly specific monoclonal antibody, suggests that these degradation products could be the same (or very similar) polypeptides and supports the hypothesis that μ-calpain is at least partly responsible for the PM degradation of troponin-T and the concomitant production of the 28- and 30-kDa polypeptides.

Degradation of troponin-T may simply be an indicator of overall PM proteolysis (i.e., it occurs as meat becomes more tender). However, because troponin-T is an integral part of skeletal muscle thin filaments (Greaser and Gergely, 1971; Flicker et al., 1982), its role in PM tenderization may warrant more careful examination as has been suggested recently (Ho et al., 1994; Uytterhaegen et al., 1994; Huff-Lonergan et al., 1995; Taylor et al., 1995). Indeed, the troponin-T subunit makes up the elongated portion of the troponin molecule and through its interaction with tropomyosin aids in regulating the thin filament during skeletal muscle contraction. It is conceivable that PM degradation of troponin-T and disruption of its interactions with other thin filament proteins aid in the disruption of the thin filaments in the I-band, possibly leading to fragmentation of the myofibril and overall muscle integrity. Taylor et al. (1995) recently

have shown that MF in PM bovine muscle are broken in their I-bands. Because troponin-T is part of the regulatory complex that mediates actin-myosin interactions, it is also conceivable that its PM degradation may lead to changes involving thick and thin filament interactions. Regardless of whether or not troponin-T aids in disruption of the thin filament in the I-band, alters thick and thin filament interactions, or simply reflects overall protein degradation, its degradation and appearance of polypeptides in the 30-kDa region seem to be an indicator of beef tenderness.

The degradation of all five of the proteins examined in this study occurred more quickly in MF prepared from LSF samples than in MF prepared from HSF samples. In the MF prepared from LSF samples, major changes took place between 1 to 3 d PM, whereas in MF prepared from HSF samples the major changes took place considerably later PM and over a longer period of time, generally between 3 and 14 d PM, coinciding with the significant decrease in Warner-Bratzler shear force values. The results of our study, taken *in toto*, suggest that PM tenderization does not depend on the degradation of one single protein, but rather that tenderization is linked to protein biochemical and structural changes occurring in many key proteins and regions of the muscle cell. Just as the overall integrity and function of muscle cells does not depend on a single protein, but rather on the coordinated interaction of several proteins, the structural weakening of muscle cells PM also must not depend on the degradation of a single myofibrillar or other cytoskeletal protein. The proteins examined in this study are located in different regions of the muscle cell, and most have been implicated in some manner as being important in maintaining the structure and function of the muscle cell (Robson, 1995; Taylor et al., 1995). In addition, these proteins are located at regions that seem to be affected during PM aging, including areas at or near the Z-line and in the I-band (Taylor et al., 1995). Degradation of proteins such as desmin and filamin, located at the periphery of the Z-line, may disrupt the lateral register and integrity of the MF themselves as well as the attachments of the peripheral layer of MF to the sarcolemma. Degradation of the proteins within the myofibril that are associated with the thick and thin filaments may allow lateral movement or breaks to occur within the sarcomeres of PM aged samples. Titin, nebulin, and troponin-T, by their ability to directly interact with, or modulate the interaction between, major proteins of the thick and thin filaments and(or) the Z-line, have the opportunity to play key roles in muscle cell integrity (Huff-Lonergan et al., 1995; Robson, 1995; Taylor et al., 1995). Disruption of these proteins, especially titin and nebulin, may trigger further physicochemical and structural changes that result in myofibril fragmentation and loss of muscle cell integrity, and ultimately in tenderization of the muscle. That the proteins in this study were degraded more quickly in MF prepared from LSF than in MF prepared from HSF samples indicates that degradation of these proteins are related to PM tenderization.

As we observed in this study, μ-calpain has the ability under PM-like in vitro conditions of relatively low pH and temperature to catalyze the degradation of titin, nebulin, filamin, desmin, and troponin-T into many of the same degradation products produced in MF from naturally aged muscle samples. This further implicates μ-calpain as a catalyst for at least some of the changes occurring in PM muscle. Some reports have indicated that the activity of μ-calpain, as measured by caseinolytic activity of supernatants of muscle extracts, declines significantly within the first 24 to 72 h PM (Koohmaraie et al., 1987). In contrast, Geesink and Goll (1995) have reported recently that as PM aging time increases, μ-calpain becomes associated with the myofibrillar component of skeletal muscle tissue samples. These authors suggested that PM activity of μ-calpain may be sustained over a longer period of time than was originally indicated by μ-calpain activity in muscle extracts (Geesink and Goll, 1995). Additional research is needed to determine the factors that govern the μ-calpain activity and interactions with substrates within the skeletal muscle cell PM in order to develop additonal methods whereby tenderness can be effectively altered or predicted.

Implications

Postmortem degradation of five myofibrillar and other cytoskeletal proteins (titin, nebulin, filamin, desmin, and troponin-T) in bovine skeletal muscle was similar to that observed using μ-calpain under postmortem-like conditions to degrade purified myofibrils, consistent with the hypothesis that μ-calpain is responsible for some significant changes occurring in postmortem aged beef. The rate of postmortem degradation of these five proteins was greater in myofibrils from samples with lower shear force values than in samples with higher shear force values, suggesting that degradation of the proteins is involved in the tenderization process. Further studies on properties controlling μ-calpain degradation of key muscle proteins may lead to more efficient methods of producing consistently tender beef.

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