

Postmortem protein degradation is a key contributor to fresh pork loin tenderness^{1,2,3}

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ABSTRACT: The objective of this study was to determine factors that influence tenderness independent of variation in pH, color, or marbling. To achieve the objective, 2 sample groups were chosen from a population of 159 pork loins aged 11 to 16 d. Predetermined ranges (ultimate pH, 5.54 to 5.86; marbling score, 1.0 to 3.0; percent total lipid, 1.61 to 3.37%) were defined for inclusion of individual loins in the study. The pork loins with the greatest ($n = 12$) and least ($n = 12$) Instron star probe values were assigned to 2 classification groups. The high star probe group had an average star probe that was 2.8 kg greater than the low star probe group (7.75 vs. 4.95 kg). Pork quality and sensory characteristics of pH, subjective and instrumental color values, cook loss, sensory tenderness, chewiness, juiciness, pork flavor, and off flavor were determined on fresh, never frozen pork chops. Lipid content, sarcomere length, myosin heavy-chain profile, and calpain autolysis were determined. Degradation of troponin-T, desmin, filamin, and

titin were evaluated on the protein extracts from each sample. Pork loin pH, subjective color scores, Minolta L values, sarcomere length, and myosin heavy-chain composition were not different across groups. Chops from the low star probe group had a significantly greater marbling score (2.3 vs. 1.9) and lipid content (2.61 vs. 2.23%). Calpain-1 was completely autolyzed in both high and low star probe samples, demonstrating that calpain-1 potentially had been active in all samples. Low star probe whole-muscle protein extracts had more troponin-T ($P < 0.01$), desmin ($P < 0.01$), and filamin degradation ($P < 0.01$) than high star probe samples. Both classification groups showed degradation of titin. Remarkably, some high star probe samples still had observable intact bands of titin on SDS-PAGE gels. These results demonstrate that significant variation in instrumental tenderness is observed within a moderate pH range. Lipid content and proteolysis both appear to contribute to this variation.

Key words: pork, proteolysis, sarcomere length, tenderness

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INTRODUCTION

Tenderness is a fundamental component related to the quality of meat products (Becker, 2000; Mennecke et al., 2007; Moeller et al., 2010). Importantly, pork flavor and tenderness have been identified as the primary determinants of fresh pork quality by international customers of U.S. fresh pork (Murphy et al., 2015). The consumer desire for tenderness demonstrates a need for methods to determine pork quality. Identifying biomarkers to classify tough and tender pork products could be a way to distinguish high-quality pork products for consumers.

Meat tenderness can be influenced by factors such as pH (Huff-Lonergan et al., 2002; Moeller et al., 2010)

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and lipid content (Lonergan et al., 2007). Additionally, pork color has been shown to be associated with differences in pork tenderness (Norman et al., 2003). In many cases, these relationships are dictated by an obvious contribution of high and low ultimate pH and/or lipid content. However, undefined factors—or a combination of factors—create uncertainty about criteria to predict fresh pork quality. Arkfeld et al. (2016) very recently reported that color and marbling scores were not correlated to sensory tenderness or chewiness in a population of loins destined for a quality focused market. Notable variation in tenderness is often observed in the intermediate range of these traits (pH, 5.55 to 5.85; lipid content, 1.5 to 3.5%); therefore, factors other than ultimate pH and lipid content must influence tenderness within these intermediate ranges (Sosnicki, 2015). These could include aging time and protein degradation. The objective of this project was to identify other factors associated with aged pork loin tenderness when key features, such as pH and lipid content, are constrained.

MATERIALS AND METHODS

Collection of Pork Loins and Sensory Data

Loins (0.32-cm trim, strap-off) were collected at 1 d postmortem from 159 carcasses from Duroc-sired crossbred commercial pigs (market weight, approximately 122 kg) that were humanely harvested at a commercial facility under the supervision of the USDA Food Safety Inspection Service. The experiments described here were conducted only on the loins and, therefore, no animal care protocol is necessary. Loins were vacuum packaged and transported to Iowa State University on ice. Aging time varied to avoid freezing any samples before sensory analysis. After aging 11 to 16 d at 4°C, the packaged loins were weighed and then removed from the vacuum bags. Loins were cut into 2.54-cm-thick LM chops and trimmed of external fat. Loin pH was measured from center and sirloin chops using a Hanna HI 9025 pH meter (Hanna Instruments, Woonsocket, RI). The pH meter was calibrated before the first measurement on each testing day using pH 4 and 7 buffers. Maintenance of the calibration was monitored between each sample (within range of pH 6.95 to 7.05). Average pH was calculated using both center and sirloin pH of the LM. Chops were cut and allowed to bloom for 20 min. Visual color scores were assigned using a 6-point scale (1 = pale pinkish gray/white; 6 = dark purplish red; National Pork Board, 2000), and marbling scores were assigned using a 10-point scale (1 = 1% intramuscular fat; 10 = 10% intramuscular fat; National Pork Board, 2000) to chops from the 10th to 12th rib section from each loin by a trained technician using standard pictures. Hunter L, a,

and b were measured on 1 chop from the 10th to 12th rib section of each loin using a Minolta Chroma Meter with a D65 light source, 50-mm aperture, and 0° observer. Two chops from the center portion of each loin (approximately 11 cm from the sirloin end) were used to collect sensory and chop cook loss data by cooking loin chops to an internal temperature of 68°C on clamshell grills (Hobart, Troy, OH). Cooked chops were cut, and 1.5-cm cube samples were immediately delivered to a panel for evaluation. A trained panel ($n = 4$; Institutional Review Board identification number: 14-553) evaluated tenderness, juiciness, chewiness, pork flavor, and off flavor using a 10-point category scale (low values indicate lower degrees of characteristics and high values indicate higher degrees of characteristics). Eight samples were evaluated per session. Cook loss was determined with the following equation: $[(\text{raw weight} - \text{cooked weight})/\text{raw weight}] \times 100$. An Instron (Instron Industrial Products, Grove City, PA) fitted with a 5-point star probe attachment was used to measure instrumental tenderness on a cooked chop from the center portion of the loin. A star probe was used in this study because it measures the force (kg) to puncture, shear, and compress meat, similar to the nature of chewing (Huff-Lonergan et al., 2002; Anderson et al., 2012).

Total Lipid Extraction

Loin chops were minced finely with a knife, individually snap frozen in liquid nitrogen, and powdered using a blender (Waring Commercial, New Hartford, CT). Total lipid in the samples was extracted in triplicate as described by Folch et al. (1957).

Final Sample Selection

The 159 pork samples were sorted based on aged chop star probe values. Samples that fell within the top and bottom 20% of high and low star probe force were chosen to obtain a sample set with extreme differences in star probe values. Marbling scores and pH criteria were applied for further refinement of the experimental groups. Previous research has demonstrated that pH influences the ultimate quality of pork LM samples (pork samples with pH >5.80 have superior sensory scores and instrumental tenderness values compared to pork samples with pH <5.50), regardless of lipid content (Lonergan et al., 2007). Results from that same study demonstrated that a greater lipid content in pork loins with an intermediate pH range (5.50 to 5.80) tends to result in more tender pork (Lonergan et al., 2007). These results demonstrate that pH and percent lipid play a role in ultimate pork quality (Lonergan et al., 2007). Therefore, because of the well-documented contributions of extreme variations in lipid

and pH, additional criteria were used to define the experimental samples for the current study. Total lipid content and sensory scores (tenderness, chewiness, and juiciness) were used as criteria for final definition of the experimental groups. Sensory scores were evaluated for consistency with star probe data, and any deviating samples were not selected for the experimental groups. Samples within the ranges of 1.61 to 3.37% total lipid content, 1.0 to 3.0 marbling score, and pH 5.56 to 5.86 (post-aging center and sirloin portion) were selected to obtain a balanced experimental design of $n = 12$ for both low and high star probe groups (approximately the top and bottom 7.5% of the original 159 pork loins sorted by star probe values). The range of star probe values for the high star probe group was 6.96 to 9.05 kg. The range of the star probe values for the low star probe group was 4.49 to 5.26 kg.

Sample Preparation

Whole-Muscle Protein Preparation. Approximately 200 g of LM from each of the selected samples for the experiment were homogenized and powdered in liquid nitrogen. Whole-muscle protein extracts of the selected pork LM were prepared in 10 mM sodium phosphate, pH 7.0, and 2% SDS (wt/vol) as described (Huff-Lonergan et al., 1996b). Protein concentrations were determined using the detergent-compatible (DC) protein assay (Bio-Rad, Hercules, CA), and samples were then diluted to 6.4 mg/mL using whole-muscle solubilizing buffer. Samples were diluted to a final protein concentration of 4 mg/mL with 0.5 vol of gel buffer (3 mM EDTA, 3% [wt/vol] SDS, 30% [vol/vol] glycerol, 0.001% pyronin Y [wt/vol], 30 mM Tris-HCl, pH 8.0) and 0.1 vol of 2-mercaptoethanol. Samples were heated for 15 min at approximately 50°C and stored at -80°C.

Myofibrillar Protein Preparation. All steps were conducted at 4°C. Myofibrillar proteins were separated from sarcoplasmic proteins as described (Anderson et al., 2014). Briefly, powdered muscle samples (2 g) were mixed with 4.5 mL cold sarcoplasmic extraction buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.0; Cruzen et al., 2015) and homogenized using a Polytron PT 3100 (Polytron, Lucerne, Switzerland) until the solution was mixed thoroughly (approximately 20 to 40 s). Each sample was then centrifuged at 40,000 × *g* for 20 min, and the resulting supernatant was filtered through cheesecloth and collected for sarcoplasmic sample preparation. Myofibrillar protein samples were prepared from the remaining pellet from the sarcoplasmic extraction. The myofibrillar pellet (1 g) was mixed with 20 mL of standard salt solution (100 mM KCl, 20 mM potassium phosphate [pH 6.85 to 6.9], 2 mM MgCl₂, 1 mM EDTA, and 1 mM NaN₃) by breaking apart the pellets with a spatula and vortex mixing for 10 s. This solution was then cen-

trifuged at 1,000 × *g* at 4°C for 10 min, and the supernatant was discarded. Samples were washed in the standard salt solution and centrifuged 3 times total. Pellets were washed 2 times with 20 mL of Tris wash buffer (5 mM Tris-HCl, pH 8.0) and broken apart by vortex mixing for 10 s. The sample solution was then centrifuged at 3,020 × *g* for 10 min, and the supernatant was discarded. Myofibrillar proteins were extracted from the washed pellets with 5 vol (of the initial weight of the pellet) of myofibrillar extraction buffer (8.3 M urea, 2 M thiourea, 2% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), pH 8.5, with 2 M Tris). Each pellet was broken apart in the solution with a spatula, vortexed for 15 to 20 s, and gently mixed at 4°C for 30 min. Samples were centrifuged at 10,000 × *g* at 20°C for 30 min, and the protein concentration of the supernatant was determined using premade reagents (Bradford Quickstart; Bio-Rad). The protein concentration was adjusted to 6.4 mg/mL with myofibrillar extraction buffer and adjusted to 4 mg/mL as described for preparation of whole-muscle samples.

SDS-PAGE and Western Blotting

Gel Systems. One-dimensional SDS-PAGE was performed to examine the extent of protein degradation in structural proteins. To quantify the extent of troponin-T degradation and desmin degradation/abundance, 15% polyacrylamide separating gels (10 by 10 cm; acrylamide:N,N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.05% [vol/vol] tetramethylethylenediamine [TEMED], 0.05% [wt/vol] ammonium persulfate [AMPER], 0.5 M Tris-HCl, pH 8.8) were used. The extent of calpain-1 autolysis was determined using 8% polyacrylamide separating gels. A 5% stacking gel (10 by 10 cm; acrylamide:N,N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.125% TEMED, 0.075% [wt/vol] AMPER, 0.125 M Tris-HCl, pH 6.8) with 10 lanes was used for all of the gels described. SE260 Hoefer Mighty Small II electrophoresis units (Hoefer, Inc., Holliston, MA) were used to run the 15% and 8% gels. The running buffer was 25 mM Tris, 192 mM glycine, 2 mM EDTA, and 0.1% (wt/vol) SDS. Troponin-T degradation was determined by using 20 µg of whole-muscle protein samples run for approximately 330 V·h. Gels to determine desmin degradation were loaded with 40 µg of whole-muscle protein. Whole-muscle protein extracts were run for approximately 360 V·h. Gels for calpain-1 immunoblots were loaded with 40 µg of whole-muscle protein samples and run for approximately 300 V·h.

The identification of titin degradation was achieved with 5% acrylamide continuous gels (18 by 16 cm; acrylamide:N,N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.067% TEMED,

0.1% [wt/vol] AMPER, 2 mM EDTA, 200 mM Tris-HCl, pH 8.0). Myofibrillar protein samples (40 µg) were used to analyze titin degradation. The same running buffer was used as described above, with the addition of 5 mM dithiothreitol. Gels were run on SE600 Hoefer electrophoresis units (Hoefer, Inc.) at a rate of 10 mA for approximately 1,600 total V·h. After electrophoresis, gels were fixed and stained using the Silver Stain Plus kit (161-0449; Bio-Rad) to determine the amount of titin degradation. Samples were run with 2 myofibrillar protein references with varying degrees of titin degradation. Reference samples consisted of 1) myofibrillar protein extract prepared from a pork chop aged for 1 d, and 2) myofibrillar protein extract prepared from an aged (7 to 10 d) pork loin chop that was not part of this study. Samples were evaluated for the presence of T1 (intact) and/or T2 (degraded) bands to determine the extent of titin degradation (Wang et al., 1979). One gel (whole-muscle samples) was stained with Sypro Ruby protein gel stain (Thermo Fisher Scientific Inc.) and visualized according to the manufacturer's recommendations.

Filamin degradation was measured by running 3 to 12% precast TEA-tricine gradient gels (10 by 10 cm; PCG2003; Sigma-Aldrich, St. Louis, MO) at a constant rate of 20 V for a total of approximately 360 V·h. The running buffer used for these gels consisted of 60 mM triethanolamine, 40 mM tricine, and 0.1% (wt/vol) SDS, with the addition of 5 mM DTT. Gels were run on SE260 Hoefer Mighty Small II electrophoresis units.

Transfer Conditions. Following electrophoresis, gels for troponin-T, desmin, calpain-1, and filamin were transferred to polyvinylidene difluoride (PVDF) membranes (0.2-µm pore size). Gels were transferred onto membranes using TE-22 Mighty Small Transphor units (Hoefer, Inc.), running for a constant voltage of 90 V for 1.5 h at approximately 4°C. The transfer buffer contained 25 mM Tris, 192 mM glycine, 2 mM EDTA, and 15% (vol/vol) methanol.

Western Blotting. After transfer, gels were discarded and membranes were blocked in a PBS-Tween solution (80 mM Na₂HPO₄, anhydrous, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% [vol/vol] polyoxyethylene sorbitan monolaurate [Tween 20]) containing 5% nonfat dry milk (NFDM). Gels were blocked for approximately 1 h at room temperature. Primary antibodies, diluted in PBS-Tween, were added to the blots immediately after blocking. The primary antibody dilutions contained troponin-T (1:80,000) using monoclonal mouse anti-troponin-T (T6277, JLT-12; Sigma-Aldrich), desmin (1:40,000) using polyclonal rabbit anti-desmin antibody produced at Iowa State University (Huff-Lonergan et al., 1996a), calpain-1 (1:10,000) using monoclonal mouse anti-calpain-1 (MA3-940; Thermo Scientific, Rockford, IL), and filamin (1:1,000) using polyclonal rabbit an-

tibody made at Iowa State University (Huff-Lonergan et al., 1996a). The primary antibody solution for filamin was diluted in a solution of PBS-Tween and 5% NFDM. All blots were incubated in primary antibodies overnight (approximately 16 to 20 h) at 4°C. After primary incubation, blots (with the exception of filamin) were washed in PBS-Tween 3 times for 10-min intervals. Filamin blots were washed with PBS-Tween containing 5% NFDM for the same period. Blots were then incubated for 1 h at room temperature with secondary antibodies diluted in PBS-Tween. The dilutions for the secondary antibodies contained troponin-T (1:10,000) goat anti-mouse-horseradish peroxidase (HRP) antibody (A2554; Sigma-Aldrich), desmin (1:20,000) goat anti-rabbit-HRP antibody (31460; Thermo Scientific), calpain-1 (1:10,000) goat anti-mouse-HRP antibody (A2554; Sigma-Aldrich), and filamin (1:20,000) goat anti-rabbit-HRP (31460; Thermo Scientific). The secondary antibody for filamin was diluted in 5% NFDM and PBS-Tween. After secondary incubation, all blots were washed with PBS-Tween 3 times for 10-min intervals. Proteins were detected using a chemiluminescent detection kit (ECL Prime; GE Healthcare, Piscataway, NJ), and images of blots were obtained and analyzed using a ChemiImager 5500 (Alpha Innotech, San Leandro, CA) and Alpha Ease FC software (version 3.03; Alpha Innotech). Densitometry was used to quantify the protein bands, and comparisons were made by taking the ratio of the measured protein band to the internal reference used for that protein. The 37-kDa intact band, 30-kDa degradation band, and 27- to 30-kDa degradation product of troponin-T (whole-muscle protein extract) were all measured and compared to corresponding bands of a 7-d aged LM whole-muscle pork reference sample. The 55-kDa intact band and 38-kDa degradation bands of desmin (whole-muscle fraction) were measured and compared to the corresponding bands of a 0/7-d aged mixed pork LM whole-muscle sample. Autolysis of the catalytic subunit of calpain-1 was determined using the whole-muscle protein extract. The appearance of the intact 80-kDa catalytic subunit and the autolysis products was compared to a 0/7-d aged mixed pork LM whole-muscle protein reference sample. The 290-kDa (approximate molecular weight) of filamin (whole-muscle fraction) was compared to a 7-d aged postmortem pork LM whole-muscle sample. Samples were run in duplicate for each protein measured.

Muscle Fiber Type Analysis

To determine the predominant fiber type of each sample, myosin heavy-chain (MHC) isoforms were determined (types IIA+IIX, and type IIB). Muscle fiber types were determined as described by Melody et al. (2004)

using a modification of the procedure described by Talmadge and Roy (1993). A 6% separating acrylamide gel (acrylamide:N,N'-bis-methylene acrylamide = 50:1 [wt/wt], 0.4% [wt/vol] SDS, 0.05% [vol/vol] TEMED, 0.1% [wt/vol] AMPER, 30% [vol/vol] glycerol, 100 mM glycine, and 200 mM Tris, pH 8.8) and a 4% stacking acrylamide gel (acrylamide:N,N'-bis-methylene acrylamide = 50:1 [wt/wt], 0.4% [wt/vol] SDS, 0.05% [vol/vol] TEMED, 0.1% [wt/vol] APS, 30% [vol/vol] glycerol, 4 mM EDTA, and 200 mM Tris-HCl, pH 6.7) were used for determination of MHC isoforms.

After electrophoresis, gels were immediately stained with Colloidal Coomassie Blue stain (1.7% ammonium sulfate, 30% methanol, 3% phosphoric acid, and 0.1% Coomassie G-250) for 20 to 24 h. Gels were then destained using double-distilled water (ddH₂O), and proteins were detected using a ChemImager 5500 (Alpha Innotech) and Alpha Ease FC software (version 3.03; Alpha Innotech). Densitometry was used to determine abundance of MHC type IIa+x and type IIb bands. Results were reported as a percentage of the total type II fibers detected. All gels for MHC determination were performed in duplicate.

Sarcomere Length

A small amount of powdered muscle tissue (approximately 0.02 g) was placed on a glass microscope slide and immersed in approximately 50 µL of sucrose solution (0.2 M sucrose, 0.1 M NaHPO₄, pH 7.2). The beam of a helium-neon laser ($\gamma = 0.6328$ nm; Spectra Physics Inc., Eugene, OR) was passed through the powdered tissue, and the distance between first-order diffraction bands was recorded. Six diffraction bands were measured on each spot of powdered tissue, and 6 spots were used for each chop. Thus, a total of 36 diffraction bands were measured for each chop. Sarcomere length was calculated using the equation prescribed by Cross et al. (1981).

Confirmation of Identity of Filamin

One-dimensional SDS-PAGE gels were prepared to confirm the identity of the high-molecular-weight protein filamin. Preparative, continuous gels (8%, 18 by 16 cm, 1.5 mm thick; filtered reagents) were loaded with 200 and 225 µg of protein and run for 2,200 V·h. Gels were stained using filtered Colloidal Coomassie Blue stain (1.7% ammonium sulfate, 30% methanol, 3% phosphoric acid, and 0.1% Coomassie G-250) and destained with filtered ddH₂O.

Protein bands (an intact band and large degradation product of filamin) were excised from the gels and sent to the Iowa State University Protein Facility. Spots were digested with trypsin using Genomic Solutions Investigator

Table 1. Summary of the fresh pork loin quality and sensory traits from the initial sample of 159 fresh, never frozen aged commercial pork loins

Item	Range	Mean	SD
Average pH ¹	5.40–6.18	5.64	0.12
Marbling score ²	1.0–4.0	2.2	0.71
Color score ³	2.0–4.5	3.1	0.57
L value ⁴	42.5–55.5	49.7	2.23
a value ⁴	12.5–16.2	14.2	0.69
b value ⁴	1.91–4.23	3.0	0.51
Tenderness ⁵	1.0–10.0	6.6	1.72
Chewiness ⁵	1.0–10.0	4.3	2.04
Juiciness ⁵	3.0–10.0	6.8	1.27
Pork flavor ⁵	1.0–7.0	3.5	1.05
Off flavor ⁵	1.0–6.0	1.6	1.02
Percent cook loss ⁶	13.1–28.4	21.7	3.23
Star probe force, kg ⁷	4.16–10.1	6.17	1.08

¹Average pH taken from center and sirloin portion of the *Longissimus dorsi*.

²National Pork Board standards, 10-point scale (1 = 1% intramuscular fat; 10 = 10% intramuscular fat).

³National Pork Board standards, 6-point scale (1 = pale pinkish gray/white; 6 = dark purplish red).

⁴Hunter L, a, and b determined with Minolta Chroma Meter with D65 light source, 50-mm aperture, and 0° observer.

⁵As determined by a trained panel ($n = 4$) using a 10-point category scale; low values indicate lower degrees of characteristics and high values indicate higher degrees of characteristics.

⁶Chops were cooked to an internal temperature of 68°C on clamshell grills. Percent cook loss = [(raw weight – cooked weight)/raw weight] × 100.

⁷Chops cooked to an internal temperature of 68°C on clamshell grills. Force necessary to compress chop to 20% of cooked chop thickness was recorded (Huff-Lonergan et al., 2002).

ProGest automated digester (Chelmsford, MA), separated by liquid chromatography, and analyzed by tandem mass spectrometry (MS/MS) using a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific). Resulting raw peptide fragment patterns were compared to the database search program MASCOT database search (Matrix Science, London, UK) using UniProt to identify proteins. The peptides identified were compared to the peptide sequence of full-length filamin (*Homo sapiens*; accession number Q14315).

Statistical Analysis

All pH, visual color, marbling, sensory, cook loss, total lipid, Western blot, sarcomere length, and MHC data were analyzed using the MIXED procedure in SAS (v.9.4; SAS Inst. Inc., Cary, NC). The model included the fixed effect of force (high or low star probe force) and the random effect of aging time. The analyses for troponin-T, filamin, and desmin included an additional random effects of gel in the model. Least squares means and standard errors were reported for all measured attributes, and the significance level was determined at $P \leq 0.05$.

Table 2. Summary of the fresh pork loin quality and sensory characteristics from samples selected for low and high star probe groups

Item	Low star probe (n = 12)		High star probe (n = 12)		P-value
	Least squares mean	SE	Least squares mean	SE	
Average pH ¹	5.71	0.03	5.66	0.03	0.19
Marbling score ²	2.3	0.14	1.9	0.14	0.03
Total lipid, % ³	2.61	0.13	2.23	0.13	0.05
Color score ⁴	3.3	0.16	3.1	0.17	0.52
Sarcomere length, μm	1.89	0.02	1.97	0.02	<0.05
Myosin heavy-chain type IIa+IIx, % ⁵	22.3	1.74	22.3	1.75	0.99
Myosin heavy-chain type IIb, % ⁵	77.7	1.84	77.7	1.85	0.99
L value ⁶	48.9	0.61	49.6	0.61	0.41
a value ⁶	14.5	0.19	13.89	0.19	0.04
b value ⁶	2.91	0.13	2.86	0.13	0.79
Tenderness ⁷	8.1	0.32	3.9	0.32	<0.01
Chewiness ⁷	2.3	0.31	7.9	0.31	<0.01
Juiciness ⁷	7.8	0.29	6.3	0.29	<0.01
Pork flavor ⁷	4.3	0.29	2.9	0.29	<0.01
Off flavor ⁷	1.3	0.32	1.9	0.32	0.22
Percent cook loss ⁸	19.3	0.93	23.0	0.93	0.01

¹Average pH taken after aging period from the center and sirloin portion of the *Longissimus dorsi*.

²National Pork Board standards, 10-point scale (1 = 1% intramuscular fat; 10 = 10% intramuscular fat).

³Total lipid extracted as described by Folch et al. (1957; with slight modifications).

⁴National Pork Board standards, 6-point scale (1 = pale pinkish gray/white; 6 = dark purplish red).

⁵Proportion of myosin heavy chain detected as type IIa+IIx or IIb based on SDS-PAGE migration.

⁶Hunter L, a, and b determined with Minolta Chroma Meter with D65 light source, 50-mm aperture, and 0° observer.

⁷As determined by a trained panel (n = 4) using a 10-point category scale; low values indicate lower degrees of characteristics, and high values indicate higher degrees of characteristics.

⁸Chops were cooked to an internal temperature of 68°C on clamshell grills. Percent cook loss = [(raw weight – cooked weight)/raw weight] × 100.

Table 3. Proteolysis of troponin-T and desmin in aged pork LM whole-muscle samples

Item	Low star probe (n = 12)		High star probe (n = 12)		P-value
	Least squares mean	SE	Least squares mean	SE	
Troponin-T					
37-kDa intact band ¹	0.94	0.05	1.27	0.05	<0.01
30-kDa degradation band ²	1.37	0.07	0.65	0.07	<0.01
Degradation products (27- to 30-kDa) ³	1.25	0.06	0.60	0.06	<0.01
Desmin, whole muscle					
55-kDa intact band ⁴	0.28	0.06	0.68	0.06	<0.01
38-kDa degradation band ⁵	1.10	0.10	0.31	0.10	<0.01
Filamin					
290-kDa intact band ⁶	0.92	0.08	1.37	0.08	<0.01

¹Ratio of the densitometry units of the 37-kDa intact band of the sample compared to the 37-kDa intact band of the reference sample.

²Ratio of the densitometry units of the 30-kDa degradation band of the sample compared to the 30-kDa degradation band of the reference sample.

³Ratio of the densitometry units of the degradation products (27- to 30-kDa) of the sample compared to the degradation products (27- to 30-kDa) of the reference sample.

⁴Ratio of the densitometry units of the intact 55-kDa band of the sample compared to the 55-kDa band of the reference sample.

⁵Ratio of the densitometry units of the 38-kDa degradation band of the sample compared to the 38-kDa degradation band of the reference sample.

⁶Ratio of the densitometry units of the 290-kDa intact band of the sample compared to the 290-kDa intact band of the reference sample.

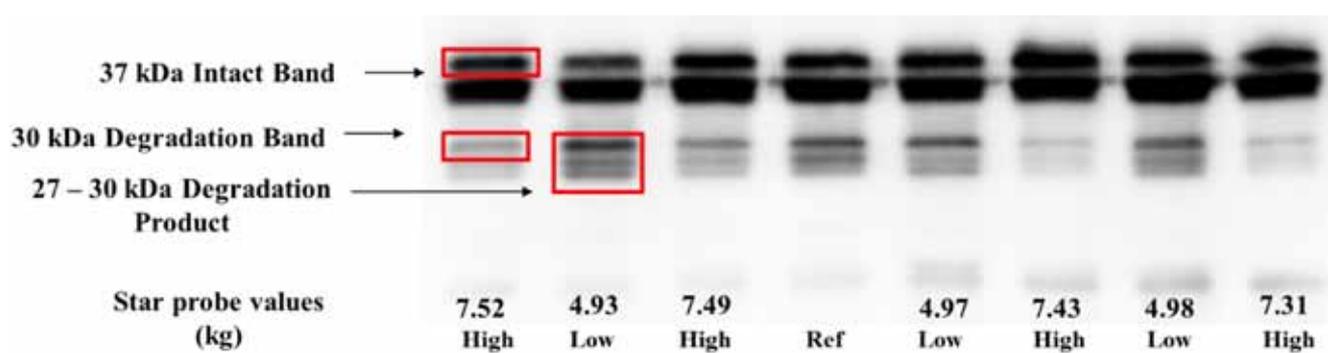


Figure 1. Representative Western blot of intact and degraded troponin-T in aged pork LM whole-muscle samples. Intact bands (37 kDa), degradation bands (30 kDa), and degradation products (27 to 30 kDa) were compared to corresponding bands of a 7-d aged pork LM sample (Ref). This same reference sample was used on all Western blots. Star probe values (kg) are provided for the samples, and samples are labeled high/low depending on star probe force.

RESULTS

Fresh Meat Characteristics and Sensory Analysis

The means, ranges, and standard deviations of the measured attributes from the original 159 loins are found in Table 1. The summary quality characteristics of the loins from the high and low star probe categories are detailed in Table 2. There was a 2.80 kg difference between the average force for the low star probe group (4.95 ± 0.25 kg) and the average for the high star probe group (7.75 ± 0.74 kg). The average aging time for each group was 13 d, and the median aging time for each group was 14 d. There was not a remarkable difference in the average pH, marbling, or color score of either group compared to the entire sample of 159 loins (summarized in Table 1). There was no difference in average ultimate pH (center and sirloin portion) of the LM between the low star probe group and the high star probe group ($P = 0.19$). The low star probe group had higher marbling scores ($P = 0.03$) as well as greater percent total lipid content ($P = 0.05$). Visual color scores were not different between the 2 sample groups ($P = 0.52$). Hunter color measurements showed no difference in L and b values, whereas samples in the low star probe group had greater HunterLab a values than samples in the high star probe group ($P = 0.04$). The sensory results showed low star probe samples were more tender ($P < 0.01$), less chewy ($P < 0.01$), more juicy ($P < 0.01$), and had more pork flavor ($P < 0.01$) than high star probe samples, while there was no difference in off flavor between the 2 groups ($P = 0.22$). Chops in the high star probe group did have approximately 4% units more cook loss than samples in the low star probe group ($P = 0.01$). Finally, the low star probe group did have shorter sarcomeres than the high star probe group ($P < 0.01$).

Proteolysis

Protein bands for troponin-T Western blots were measured using densitometry by analyzing the 37-kDa

intact band, the 30-kDa degraded band, and the 27- to 30-kDa degraded product in the whole-muscle protein fraction of samples in low and high star probe groups (Fig. 1). Intact troponin-T was 26% less abundant in low star probe samples than in the high star probe samples ($P < 0.01$; Table 3). Consequently, the abundance of the 30-kDa degradation band and 27- to 30-kDa degradation product of troponin-T were increased by over 100% in the low star probe samples ($P < 0.01$; Table 3). Desmin degradation was analyzed using densitometry to measure the abundance of the 55-kDa intact band and the 38-kDa degradation band in the whole-muscle protein fraction of samples (Fig. 2). Similar to troponin-T results, abundance of intact desmin was 59% less in the low star probe samples ($P < 0.01$; Table 3). A degradation product corresponding to the 38-kDa band was 255% more abundant in low star probe samples in comparison to high star probe samples ($P < 0.01$; Table 3).

The intact band of filamin (290 kDa) was measured using densitometry (Fig. 3). The low star probe group samples had 33% less abundant intact filamin compared to high star probe samples ($P < 0.01$; Table 3). Intact and degraded protein bands of filamin were successfully identified using mass spectrometry. Peptides were dispersed evenly throughout the entire protein of intact filamin. Identified peptides from the degraded band included peptides from the entire protein, excluding AA at the carboxy-terminal (AA 2,616 to 2,725).

Calpain-1 autolysis was examined using Western blots to identify the appearance of an intact 80-kDa band, as well as 78-kDa and 76-kDa autolysis products in whole-muscle samples (Fig. 4). In all of the samples, the only band identified was the 76-kDa band, indicating calpain-1 was completely autolyzed in both low and high star probe groups.

Titin degradation was determined using stained SDS-PAGE gels to identify the presence of intact (T1, approximately 3,000 kDa) and degraded (T2, approximately 2,400 kDa) titin in myofibrillar samples (Fig. 5). Remarkably, some samples in the high star

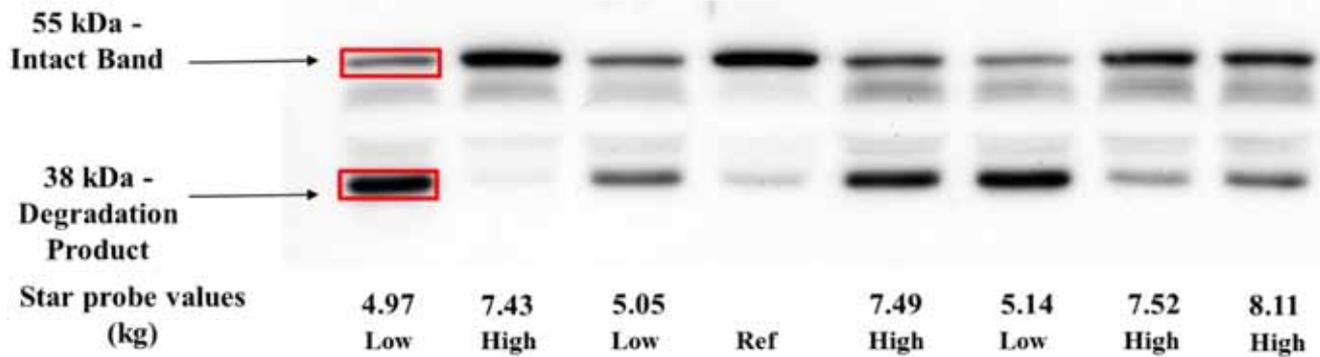


Figure 2. Representative Western blot of intact and degraded desmin in aged pork LM whole-muscle samples. Intact bands (55 kDa) and degradation bands (38 kDa) were compared to corresponding bands of a mixed 0/7-d aged pork LM sample (Ref). This same reference sample was used on all western blots. Star probe values (kg) are provided for the samples, and samples are labeled high/low depending on star probe force.

probe group did exhibit a small amount of T1, indicating some intact titin was still present in samples. Intact titin (T1) was not present in any samples from the low star probe group. Degraded titin (T2) was present in all samples from both classification groups.

Myosin Heavy Chain

Type IIa+IIx and IIb MHC isoforms were analyzed using Colloidal Coomassie Blue stained gels to measure the percentage of each isoform in whole-muscle protein samples (Fig. 6). There were no differences in the proportion of type IIa+IIx and type IIb MHC isoform percentages across the star probe groups (Table 2).

DISCUSSION

The summary data confirm that this original sample of 159 loins is similar to other samples of commercial pork in the literature. For example, Arkfeld et al. (2015a) reported similar color scores (3.2) but slightly lower marbling (1.57) and pH (5.57) values in a population of 151 loins. Moeller et al. (2010) reported similar color scores (3.1) and slightly higher marbling (2.5) and pH (5.76) in a population of 679 loins selected at commercial processing plants.

The key feature of this study is a comparison of pork loin chops that were similar in pH and color but different in star probe values. The trained panel sensory analysis revealed the low star probe group was significantly more tender, juicy, and less chewy than the high star probe group. The amount of marbling and total lipid were significantly different between low and high star probe groups. This observation is consistent with a previous report of significant negative correlations of lipid content and star probe values when ultimate pH is between 5.5 and 5.8 (Lonergan et al., 2007). However, a review of the literature reveals mixed results regarding the contributions of marbling

and lipid content to pork tenderness (Rincker et al., 2008; Arkfeld et al., 2015b).

Cook loss was notably greater in the high star probe group. This is consistent with Dilger et al. (2010) and with the observed greater juiciness scores in the low star probe group. Based on the small yet significant difference in the HunterLab *a* values, the low star probe loins were more red than the high star probe loins. This was not detected in the color scores assigned by trained personnel, as there was no difference in visual color scores between the classification groups.

There were no significant differences between the low and high star probe groups for pH and color (*L* and *b* values). These results show that there was little association with these measured attributes to tenderness in this specialized comparison when pH, marbling, and lipid content were constrained. This suggests that the extreme differences in tenderness in these samples were due to other characteristics or to events that occur postmortem. The lack of detectable differences between the groups for color scores and *L* values suggests that there were no dramatic differences in pH decline between these groups that might manifest a large difference in lightness. However, the rate of pH decline was not documented in these samples.

Degradation of proteins influences muscle structure and can play a role in meat tenderness (Taylor et al., 1995; Wheeler et al., 2000; Melody et al., 2004). The calpain system, specifically calpain-1, plays a large role in the proteolysis of muscle proteins (Huff-Lonergan et al., 1996a; Lametsch et al., 2004; Geesink et al., 2006). In this study, the appearance of the 76-kDa autolysis product of calpain-1 demonstrates that calpain-1 was completely autolyzed in samples from both classification groups. Activation of calpain-1 is closely coupled with autolysis (Baki et al., 1996). Autolysis lowers the amount of calcium needed for activation of calpain-1 (Suzuki et al., 1981). Calpain-1 in muscle degrades the proteins desmin, troponin-T, and titin during

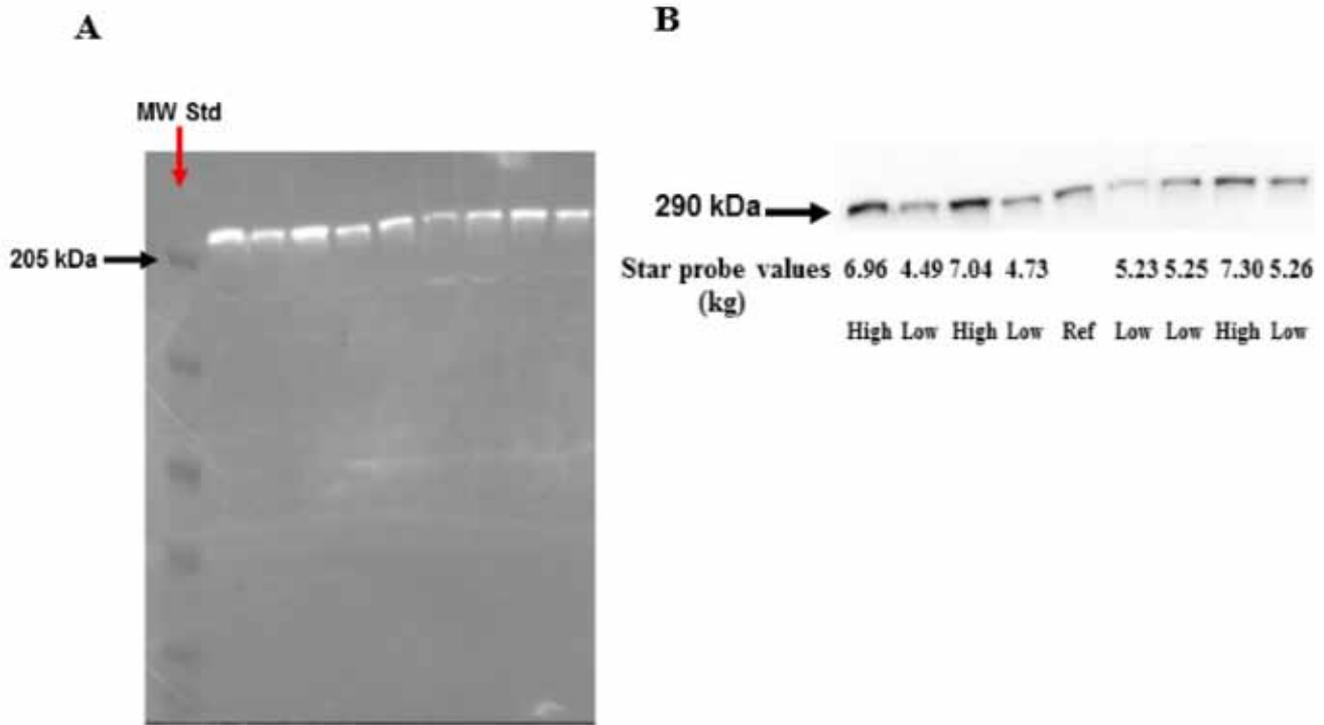


Figure 3. Representative Western blots of intact filamin in aged pork LM from low and high star probe samples. A MW Std was used to identify the approximate molecular weight of the protein bands (A). Samples were compared to a 7-d aged LM (Ref; used on each western blot) to identify abundance of intact filamin (B).

postmortem aging in beef (Huff-Lonergan et al., 1996a) and pork (Lonergan et al., 2001; Melody et al., 2004). The rate of activation and autolysis of calpain-1 could impact the amount of protein degradation by calpain-1. Calpain-1 activity is inhibited as pH declines from pH 7 to pH 6 and below (Carlin et al., 2006; Bee et al., 2007) or by oxidative conditions (Rowe et al., 2004a; Chen et al., 2014). Postmortem pH decline data are not available for these loins, but if differences in pH decline actually existed, they could potentially explain the differences in proteolysis exhibited between classification groups in this study. Moving forward, it would be beneficial to measure the amount of protein oxidation in these samples to determine if there was a difference in cellular environment between sample groups that could help explain the proteolytic differences.

Degradation of proteins occurs throughout the entire postmortem aging period (Huff-Lonergan et al., 1996b; Lametsch et al., 2004). The samples used in this study were all aged for 11 to 16 d, and results showed degradation of the proteins troponin-T, desmin, filamin, and titin in both classification groups. The low star probe group exhibited more degradation products of troponin-T and desmin than the high star probe groups. These results are consistent with observations that degradation of these proteins is associated with differences in measured instrumental tenderness values in pork muscles. Chops from LM had more deg-

radation of troponin-T, less intact desmin, and lower Warner-Bratzler shear force values (kg) than tougher semimembranosus muscles (Melody et al., 2004).

Troponin-T is a key regulatory protein involved with the actomyosin complex formed during contraction. Degradation of this protein could demonstrate that there is a weakening of this actomyosin complex, ultimately influencing tenderness (Huff Lonergan et al., 2010). Degradation of troponin-T could also just be an indicator of overall skeletal muscle protein degradation. Desmin is an intermediate protein that is an integral part of myofibril structure that aids in connecting adjacent myofibrils to each other and with other muscle fiber components (Clark et al., 2002). It stands to reason that the degradation of this protein as seen in this study could ultimately alter the integrity of the alignment of myofibrils to each other and to the sarcolemma.

Filamin is a large protein located in the Z-line. Filamin interacts with F-actin and functions to aid in the stabilization of the muscle cytoskeleton and helps with transmitting chemical signals at the Z-line (Wang and Singer, 1977; Clark et al., 2002). The dimerization of filamin is essential for the interaction of filamin with F-actin (Pudas et al., 2005). Extreme proteolytic differences in the degradation of filamin were observed between classification groups where low star probe samples had significantly less intact filamin. Myofibrils isolated from low and high Warner-Bratzler shear force beef samples had

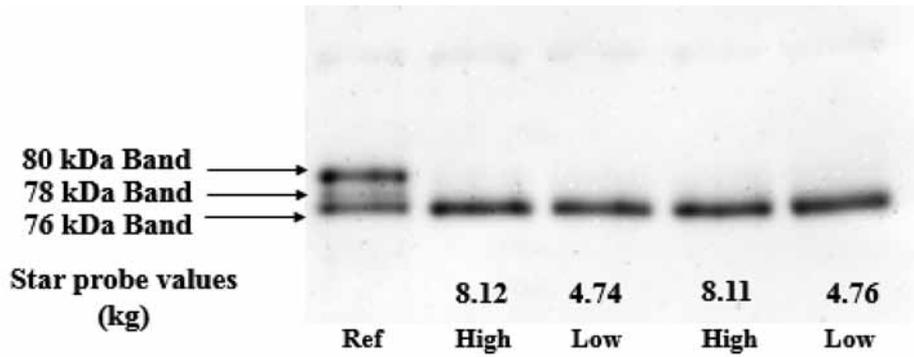


Figure 4. Representative Western blot of calpain-1 autolysis in aged LM whole-muscle pork samples. Samples in low and high star probe groups were compared to a mixed sample containing both 0- and 7-d aged LM in a whole-muscle sample (Ref; used on each western blot) to identify the presence of intact calpain-1 (80 kDa) and autolyzed (78 kDa and 76 kDa) protein bands.

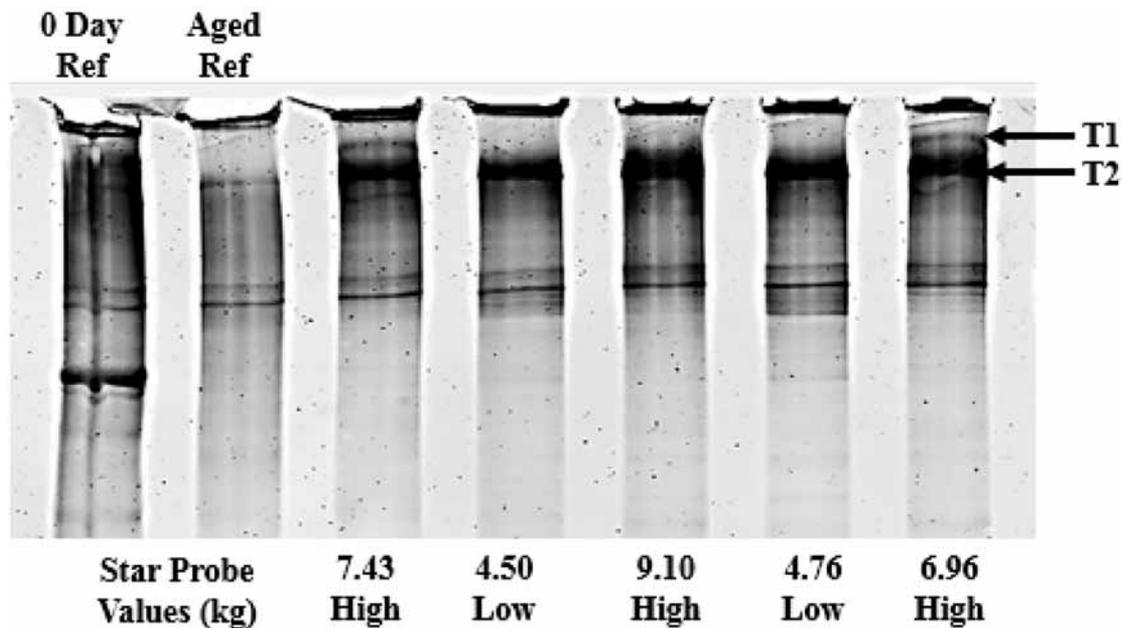


Figure 5. Representative SDS-PAGE Sypro Ruby stained gel showing titin degradation in aged LM whole-muscle pork samples. Samples in low and high star probe groups were evaluated for the presence of the intact 3,000 kDa titin band (T1) and the degraded 2,400 kDa titin band (T2). A 0-d aged whole-muscle LM sample and a 7-d aged LM whole-muscle sample (from outside of this experiment) were run on the gel to provide references for T1 and T2 bands.

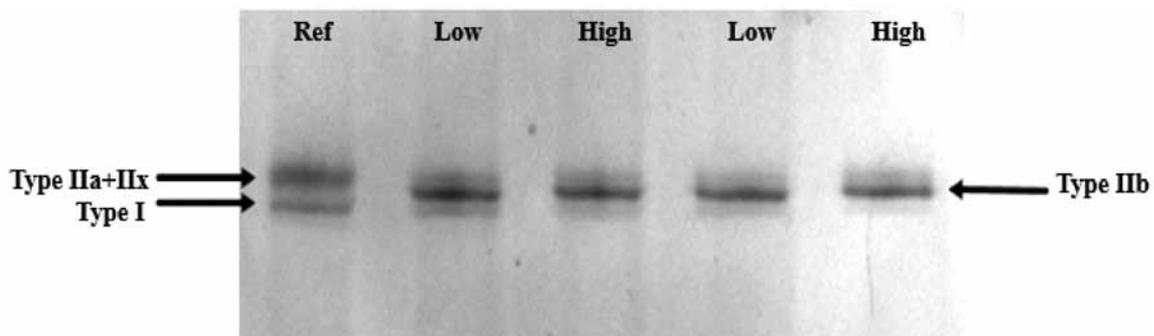


Figure 6. Representative SDS-PAGE colloidal coomassie blue stained gel used to evaluate myosin heavy-chain isoforms. A diaphragm sample was used as a reference (Ref) to identify location of type IIa and type I myosin heavy-chain isoforms. Samples (low and high) were analyzed for percentage of abundance of type IIa+IIx and type IIb isoforms.

different rates of filamin degradation during aging (Huff-Lonergan et al., 1996a). In that study, low-shear-force samples degraded earlier postmortem than high-shear-force samples (Huff-Lonergan et al., 1996a).

Intact (290 kDa) and degraded (278 kDa) filamin were successfully identified in high and low star probe samples from the current study. These results demonstrate that the removal of the 109 AA from the carboxy-terminal end in degraded filamin could be the first cleavage of intact filamin observed in postmortem muscle. The carboxy-terminal end of filamin is responsible for dimerization and interaction with other cellular components such as β integrins, androgen receptors, and portions of potassium channels (Stossel et al., 2001; Feng and Walsh, 2004; Pudas et al., 2005; Nakamura et al., 2011). Therefore, the loss of this carboxy-terminal end causes the loss of ability for this protein to form dimers and impairs anchoring to the Z-line and could impair the structural integrity of the internal architecture of the muscle cell.

Degradation products of titin (2,400 kDa) were also found in samples from both classification groups (Fig. 5). Some high star probe samples did exhibit presence of intact titin (T1 band, 3,000 kDa; lanes 3 and 5 in Fig. 5). The presence of intact titin in aged samples is a novel result because titin is typically degraded earlier postmortem by calpain-1 (Huff-Lonergan et al., 1995; Taylor et al., 1995; Melody et al., 2004; Rowe et al., 2004b). The rate of titin degradation is known to be slower in beef that exhibits high-shear-force values regardless of animal age or gender (Huff-Lonergan et al., 1995). Low star probe samples from the current study may have exhibited a faster rate of postmortem titin degradation due to differences in calpain-1 activity. If calpain-1 became inactive at an earlier time postmortem, protein degradation could be limited in samples (Li et al., 2004; Bee et al., 2007). This could potentially explain the proteolytic differences in troponin-T, desmin, filamin, and titin between low and high star probe samples in the current study. Results from this study demonstrate that proteolysis played a major role in the differences in tenderness exhibited between the classification groups.

There were no differences in proportion of MHC isoforms identified from the 2 classification groups. Myosin heavy-chain type IIb was the predominant isoform in samples from both classification groups. These results are consistent with other observations that showed that the predominant MHC isoform in pork LM samples 45 min postmortem was type IIb (Melody et al., 2004). Muscle fibers that contain predominately type IIb MHC isoforms are fast-twitch fibers that rely primarily on glycolytic metabolism (Reggiani et al., 2000). Myosin heavy-chain isoforms (I, IIa, IIx, IIb) differ in their ability to migrate on SDS-PAGE gels (Schiaffino et al., 1989).

Type IIa/IIx isoforms tend to comigrate and migrate the least, type IIb isoforms migrate a little further down the gel, and type I isoforms migrate the most with gel electrophoresis (Schiaffino et al., 1989). During aging, MHC can be degraded in pork LM (Lametsch et al., 2003). A 56-kDa fragment of the globular head domain of MHC was identified to increase in abundance during postmortem aging in whole-muscle pork samples (Lametsch et al., 2003). Pork LM with low total protein solubility and low pH at 45 min postmortem also resulted in degradation of MHC (Choi et al., 2010). Degradation of MHC during postmortem aging could influence the migration pattern of MHC isoforms. Since the samples used were from aged pork loins, degradation of MHC might influence the results of the MHC fractionation using the SDS-PAGE approach. Therefore, examination of fiber types through other methods that are based on other phenotypic traits might be warranted in future studies.

Although there was no difference in distribution of MHC type II isoforms in the classification groups, there still may be a difference in fiber type that could be measured with other analyses. Desmin degradation occurs at a faster rate and to a greater extent in type IIb fibers compared to type I fibers in pork LM (Muroya et al., 2010). The authors speculated that differences in desmin degradation may be due to fiber type differences influencing calpain activity (Muroya et al., 2010). Cellular environment can also impact the activity of proteases to degrade desmin in muscle. Oxidation of desmin changes the secondary structure of desmin, which increases susceptibility to proteolysis by caspases (Chen et al., 2014). Oxidation is also known to decrease the activity of calpain-1, ultimately reducing desmin degradation by calpain-1 (Rowe et al., 2004b; Carlin et al., 2006).

Conclusions

Tenderness in commodity pork is influenced by a variety of factors. This complicates the mission to generate biomarkers for fresh pork tenderness. The sample libraries (high and low star probe) in the current experiment were assembled to constrain pH and lipid content. Variation in tenderness in aged pork LM from this study was attributed to lipid content and differences in myofibrillar and cytoskeletal protein degradation. Samples with low star probe values exhibited substantially more degradation of troponin-T, desmin, filamin, and titin. The results demonstrate that tenderness can vary a great deal in reasonably moderate pH, color, and marbling levels. Importantly, this variation cannot be attributed to sarcomere length but is markedly associated with the degradation of myofibrillar and cytoskeletal proteins. These results imply that methods to rapidly measure lipid content and protein

degradation should be developed in an effort to measure pork quality and especially tenderness.

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