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Early postmortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles

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ABSTRACT: The objective of this study was to determine whether differences in pork tenderness and water-holding capacity could be explained by factors influencing calpain activity and proteolysis. Halothane-negative (HAL-1843 normal) Duroc pigs (n = 16) were slaughtered, and temperature and pH of the longissimus dorsi (LD), semimembranosus (SM), and psoas major (PM) were measured at 30 and 45 min and 1, 6, 12, and 24 h postmortem. Calpastatin activity; \( \mu \)-calpain activity; and autolysis and proteolysis of titin, nebulin, desmin, and troponin-T were determined on muscle samples from the LD, SM, and PM at early times postmortem. Myofibrils from each muscle were purified to assess myofibril-bound \( \mu \)-calpain. Percentage drip loss was determined, and Warner-Bratzler shear (WBS) force was analyzed. Myosin heavy-chain (MHC) isoforms were examined using SDS-PAGE. The pH of PM was lower \((P < 0.01)\) than the pH of LD and SM at 30 and 45 min and 1 h postmortem. The PM had a higher \((P < 0.01)\) percentage of the MHC type IIa/IIx isoforms than the LD. The LD had the greatest proportion of \((P < 0.01)\) MHC IIb isoforms of any of the muscles. The PM had the lowest \((P < 0.01)\) percentage of MHC IIb isoforms and a greater \((P < 0.05)\) percentage of type I MHC isoforms than the LD and SM. The PM had less \((P < 0.01)\) drip loss after 96 h of storage than the SM and LD. The PM had more desmin degradation \((P < 0.01)\) than the LD and SM at 45 min and 6 h postmortem. Degradation of titin occurred earlier in the PM than the LD and SM. At 45 min postmortem, the PM consistently had some autolysis of \( \mu \)-calpain, whereas the LD and SM did not. At 6 h postmortem, some autolysis of \( \mu \)-calpain (80-kDa subunit) was observed in all three muscles. The rapid pH decline and increased rate of autolysis in the PM paralleled an earlier appearance of myofibril-bound \( \mu \)-calpain. The SM had higher calpastatin activity \((P < 0.05)\) at 45 min, 6 h, and 24 h and had higher WBS values at 48 h \((P < 0.01)\) and 120 h \((P < 0.05)\) postmortem than the LD. At 48 and 120 h postmortem, more degradation of desmin, titin, and nebulin were observed in the LD than in the SM. These results show that \( \mu \)-calpain activity, \( \mu \)-calpain autolysis, and protein degradation are associated with differences in pork tenderness and water-holding capacity observed in different muscles.

Key Words: Calpain, Calpastatin, Pork, Proteolysis, Tenderness, Water-Holding Capacity

Introduction

Water-holding capacity influences the profitability of fresh pork products by affecting processing yields and palatability. Product losses due to purge or drip loss can range from 2 to 10% when meat is cut into chops (Offer and Knight, 1988). This represents an economic loss to both processors and retailers. A major factor in determining consumer satisfaction with meat is tenderness. Although the process of postmortem tenderization is more rapid in pork than in beef (Koohmaraie et al., 1991), there is still significant variability in pork tenderness (DeVol et al., 1988).

Degradation of the muscle proteins (Huff-Lonergan et al., 1995, 1996a; Taylor et al., 1995) in postmortem muscle is associated with indices of meat tenderness. Muscle protein degradation may also be associated with drip loss. Moreover, research has suggested that reduced degradation of proteins that tie the myofibril to the cell membrane (such as desmin) may allow shrinkage of the myofibril to result in shrinkage of the muscle cell. This shrinkage opens drip channels and results in increased drip loss (Morrison et al., 1998; Kristensen...
and Purslow, 2001; Rowe et al., 2001b). Therefore, increased degradation of proteins (like desmin) could prevent myofibril shrinkage from being effectively transmitted to the entire cell and would allow more moisture to reside in the tissue. Many proteins involved in tenderization and drip loss are substrates of the enzyme \( \mu \)-calpain (Huff-Lonergan et al., 1996a). Differences in \( \mu \)-calpain, m-calpain, and calpastatin activity (Ilian et al., 2001) may ultimately influence tenderness and water-holding capacity by impacting the rate and extent of proteolysis. Therefore, the hypothesis guiding this study was that differences in water-holding capacity and tenderness between muscles (longissimus dorsi, semimembranosus, and psoas major) may be partially explained by variations in factors that influence calpain activity and protein proteolysis in postmortem muscle.

**Materials and Methods**

**Animals**

Sixteen halothane-negative (HAL-1843 normal) purebred Duroc barrows of similar genetics, age, and weight (Table 1) from the Iowa State University Swine Teaching Farm were used. Animals were pen-fed and finished under standard commercial practices. The pigs were harvested over a 5-wk period (2 pigs/d). Pigs were slaughtered at the Iowa State University Meat Laboratory using approved humane procedures. Carcasses were chilled in a forced-air cooler at \(-5^\circ\text{C}\) for 24 h. At 30 and 45 min and 1, 6, 12, and 24 h postmortem, temperature (Barnant Thermocouple Thermometer Dual J-T-E-K, Model No. 600-1040; Barnant Co., Barrington, IL) and pH measurements (pH-Star S; SFK Technology, Inc., Herlev, Denmark) were taken at the last rib region of the longissimus dorsi (LD), and the center portions of the semimembranosus (SM) and the psoas major (PM) from the left side of each carcass. Samples (10 g) for calpastatin activity assays, \( \mu \)-calpain activity, and autolysis, and purification of myofibrils were also collected from the left side of each carcass at 45 min and 6 and 24 h postmortem. Chops (2.54 cm thick) for drip loss and Warner-Bratzler shear force (WBSF) analysis were collected at 24 h postmortem from the LD, SM, and PM. At 24 h postmortem, the initial weight was recorded on another four 2.54-cm-thick chops from each muscle from each carcass for determination of drip loss. Chops were stored in a sealed plastic bag at \(4^\circ\text{C}\). After 24 h (48 h postmortem) and 96 h (120 h postmortem) of storage, two chops for each time point from each muscle were blotted, and reweighed (final weight) for determination of drip loss. Drip loss percentage was calculated by the following equation: 

\[
\text{Drip Loss} = \left( \frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} \right) \times 100.
\]

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\[
\text{Drip Loss} = \left( \frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} \right) \times 100.
\]

Frozen 2.54-cm-thick chops were thawed at \(2^\circ\text{C}\) and used for WBSF determination. Chops were broiled in an electric broiler (Model 6850; General Electric, Chicago Heights, IL) 15 cm away from the heat source. Chops were broiled to an internal temperature of \(30^\circ\text{C}\), turned, and broiled to a final temperature of \(70^\circ\text{C}\). Temperature was monitored using and Electro-therm digital probe (Model No. TM99A; Cooper Instrument Corp., Middlefield, CT). Steaks were covered with Saran wrap and allowed to chill overnight at \(4^\circ\text{C}\). Warner-Bratzler shear force was measured in accordance with AMSA (1995) guidelines. Chops were equilibrated to room temperature (approximately 1 to 2 h), and four 1.27-cm cores were taken from each chop parallel to the muscle fiber orientation. Peak force for each core was measured using the Texture Analyzer TA-XT2i (Texture Technologies, Scarsdale, NY) with the Warner-Bratzler Probe and Guillotine Set (TA-7B USDA; Texture Technologies) and the penetration speed of the blade was 3.3 mm/s. All data were collected using Texture Expert software Version 1.22 (Texture Technologies).

**Sarcoplasmic Protein Extraction**

The initial sarcoplasmic protein extraction procedure was conducted according to the procedures of Shackelford et al. (1994), with modifications as noted in the following description. Finely minced, 10-g tissue samples from the LD, SM, and PM were homogenized in 3 vol (wt/vol) of an extraction buffer containing 10 mM EDTA, 0.1% (vol/vol) \( \beta \)-mercaptoethanol (MCE), 100 mg/L trypsin inhibitor, 2 \( \mu \)M E-64, 2 mM phenylmethylsulfonylfluoride, and 100 mM Tris-HCl, pH 8.3. The homogenate was centrifuged at 25,000 \times g for 30 min, and the supernatant was filtered through cheesecloth. Protein content of each sample was determined following the procedure outlined by Bradford (1976). Supernatant samples were collected to determine \( \mu \)-calpain autolysis using immunoblotting and for measurement of relative calpain activity using casein zymography.

**Calpastatin Activity**

Supernatants from the sarcoplasmic protein extraction (45 min and 6 and 24 h postmortem) were placed
in dialysis buffer (40 vol; 1 mM EDTA and 80 mM Tris, pH 7.40) for a minimum of 6 h at 4°C. Dialysates were heated in a preheated water bath (98°C) for 15 min and subsequently cooled in an ice water bath. Coagulated protein was separated by centrifugation at 25,000 × g for 10 min at 4°C. Calpastatin activity was determined as described by Koochmaraie et al. (1995). One unit of calpastatin activity was defined as the ability to inhibit 1 unit of porcine lung m-calpain activity (Koochmaraie, 1990).

**Casein Zymography**

Relative differences in m-calpain activity were determined in samples at each time point using casein zymography. The casein zymography procedure described by Raser et al. (1995) was used with slight modifications. One volume of supernatant (from the extracted tissue sample) was combined with 1 vol of tracking dye solution (20% [vol/vol] glycerol, 0.75% [vol/vol] MCE, 0.1% [wt/vol] bromophenol blue, and 150 mM Tris-HCl, pH 6.8). Samples were loaded immediately onto non-denaturing 12.5% PAGE casein gels (separating gel = acrylamide:N,N′-bis-methylene acrylamide = 100:1 [wt/wt], 0.05% [vol/vol] N,N′N′-tetramethylethylenediamine [TEMED], 0.05% [wt/vol] ammonium persulfate [APS], casein [2.1 mg/mL], and 375 mM Tris-HCl, pH 8.8; stacking gel = acrylamide:N,N′-bis-methylene acrylamide = 100:1 [wt/wt], 0.125% [vol/vol] TEMED, 0.075% [wt/vol] APS, and 125 mM Tris-HCl, pH 6.8). Gels (10 cm wide × 8 cm tall) were run on SE 260 Hoefer Mighty Small II electrophoresis units (Hoefer Scientific Instruments, San Francisco, CA). Gels were pre-run at 100 V for 15 min. The running buffer used contained 192 mM glycine, 1 mM EDTA, 0.5% [vol/vol] MCE, and 25 mM Tris, pH 8.3. Gels were run at a constant voltage (100 V) for approximately 22 h, and then incubated in three changes (20 min each) of 5 mM CaCl₂, 0.1% [vol/vol] MCE, and 50 mM Tris-HCl, pH 7.5. Gels were then incubated overnight in 5 mM CaCl₂, 0.1% [vol/vol] MCE, and 50 mM Tris-HCl, pH 7.5. The following day, gels were stained in a solution containing 0.1% [wt/vol] Coomassie brilliant blue R-250, 40% [vol/vol] methanol, and 7% [vol/vol] glacial acetic acid for approximately 1 h. Gels were destained using an excess of destain (40% [vol/vol] methanol and 7% [vol/vol] glacial acetic acid). m-Calpain activity was indicated by clear zones in the stained gels.

**Purification of Myofibrils**

Highly purified myofibrils from samples collected at 45 min and 1, 6, and 24 h were made to examine the relative amount of m-calpain bound to the myofibrils from each of the muscles. Two grams of the pellet from the initial extraction was used for the extraction of highly purified myofibrils. Purification of myofibrils followed the procedures of Goll et al. (1974) using the modifications of Huff-Lonergan et al. (1996a,b). Purified myofibrils were stored at −20°C in 20 vol of 50% (vol/vol) 100 mM KCl-HCl, pH 6.8, and 50% (vol/vol) glycerol.

**Indirect Immunofluorescence Localization of μ-Calpain**

Indirect immunofluorescence localization of m-calpain on myofibrils was conducted according to the procedure of Tan et al. (1993) on positively charged glass slides (Fisher Superfrost Plus; Fisher Scientific, Pittsburgh, PA). A monoclonal anti-m-calpain primary antibody (MA3-940; Affinity BioReagents, Golden, CO) diluted 1:100 with PBS, and Alexa-Fluor 346 goat anti-mouse IgG secondary antibody (Molecular Probes, Inc., Eugene OR) at a 1:100 dilution were used. Slides were examined using epifluorescence or phase-contrast imaging on a Zeiss Photomicroscope III with a 40x Neofluor objective (Carl Zeiss, Oberkochen, Germany), and images were recorded digitally with a SPOT RT camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

**Whole-Muscle Sample Preparation and SDS-PAGE Gel Sample Preparation**

Samples were made from muscle (0.5 g) taken at 45 min and 6, 24, 48, and 120 h from the LD, SM, and PM for SDS-PAGE analysis of titin and nebulin and for Western blotting of troponin-T and desmin. Whole-muscle protein extraction and SDS-PAGE gel sample preparation was conducted according to Lonergan et al. (2001a,b). Protein concentration was determined as described by Lowry et al. (1951) using premixed reagents (Bio-Rad, Hercules, CA). Gel samples were frozen at −80°C until analysis.

**SDS-PAGE and Western Blotting**

A 10% polyacrylamide separating gel (acrylamide:N,N′-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.05% [vol/vol] TEMED, 0.05% [wt/vol] APS, and 0.5 mM Tris-HCl, pH 8.8) was used for determination of m-calpain autolysis (sarcoplasmic fraction, myofibrillar fraction) and desmin (whole-muscle samples). A 15% polyacrylamide separating gel was used for determination of troponin-T (whole-muscle samples). A 5% polyacrylamide gel (acrylamide:N,N′-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.125% [vol/vol] TEMED, 0.075% [vol/vol] APS, and 0.125 mM Tris-HCl, pH 6.8) was used for the stacking gel. A 5% polyacrylamide continuous gel (acrylamide:N,N′-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.067% TEMED, 0.1% [wt/vol] APS, 2 mM EDTA, and 200 mM Tris-HCl, pH 8.0) was used for determination of titin and nebulin (whole-muscle samples; Huff-Lonergan et al., 1996b).

**Running Conditions.** Gels (10 cm wide × 8 cm tall) for analysis of desmin degradation were run on SE 260 Hoefer Mighty Small II electrophoresis units (Hoefer Scientific Instruments). Gels (10 cm wide × 12 cm tall) for analysis of m-calpain autolysis and troponin-T deg-
radiation were run on SE 280 Hoefer Tall Mighty Small electrophoresis units. The running buffer contained 25 mM Tris, 192 mM glycine, 2 mM EDTA, and 0.1% [wt/vol] SDS. Gels were loaded with 30 μg per lane of total protein for desmin (whole-muscle samples), 60 μg per lane of total protein for troponin-T (whole-muscle samples), or 60 μg per lane of total protein for μ-calpain (sarcoplasmic fraction) and run at a constant voltage of 120 V. Gels for myofibril-bound μ-calpain were loaded with 80 μg of purified myofibrillar protein per lane and run at a constant 20 V. Gels (18 cm wide × 16 cm tall) for titin and nebulin were run on SE 400 Hoefer units (Hoefer Scientific Instruments). The same running buffer was used as described previously with the addition of 0.1% (vol/vol) MCE. Gels for titin and nebulin analysis were loaded with 80 μg of protein per lane and run at 10 mA for approximately 24 h. Following electrophoresis, gels for titin and nebulin were stained with 0.1% (vol/vol) Coomassie brilliant blue R-250, 40% (vol/vol) methanol, and 7% (vol/vol) glacial acetic acid. Gels were destained using an excess of 40% (vol/vol) methanol, and 7% (vol/vol) glacial acetic acid.

Transfer Conditions. Gels for desmin, troponin-T, and μ-calpain (sarcoplasmic and myofibrill-associated) were transferred to polyvinylidene difluoride (PVDF) membranes (Schleicher and Schuell, Inc., Keene, NH) using a TE22 Mighty Small Transphor electrophoresis unit (Hoefer Scientific Instruments) at a constant voltage of 90 V for 1.5 h. The transfer buffer consisted of 25 mM Tris, 192 mM glycine, 2 mM EDTA, and 15% (vol/vol) methanol. The temperature of the transfer buffer was maintained between 4° and 8°C using a refrigerated circulating water bath (Ecoline RE106; Lauda Brinkmann, Westbury, NY).

Western Blots. Western blotting and chemiluminescent detection were done as described by Huff-Lonergan et al. (1996a). Primary antibodies included mouse anti-troponin-T (JLT-12; Sigma, St. Louis, MO; diluted 1:30,000), polyclonal rabbit anti-desmin (No. V2022; Biomed, Foster City, CA; diluted 1:30,000), and anti-μ-calpain (Clone 9A4H803, against domain III, amino acids 465-520; ABR, Golden, CO; diluted 1:10,000). Secondary antibodies included goat anti-mouse-HRP (No. A2554 Sigma; diluted 1:20,000 for troponin-T or 1:10,000 for μ-calpain) and goat anti-rabbit-HRP (No. A9169; Sigma; diluted 1:20,000). Densities of immunoreactive bands were quantified by densitometry using ChemiImager 5500 (Alpha Innotech, San Leandro, CA) and AlphaEaseFC (v. 2.03; Alpha Innotech). Troponin-T degradation was indicated by an increase of the 30-kDa band, desmin degradation was indicated by a decrease in intensity of an approximately 55-kDa band, and desmin degradation product was indicated by an increase in intensity of an approximately 38-kDa band. intact desmin degradation ratio was calculated as the intensity of each immunoreactive desmin band over the intensity of the immunoreactive desmin band in a reference sample (24-h postmortem porcine muscle) that was loaded on each gel. Troponin-T degradation and desmin degradation product ratios were calculated as the intensity of each band of interest over the intensity of the corresponding bands in the reference samples (7-d postmortem porcine muscle).

**Muscle Fiber Type Analysis**

Forty-five minute postmortem whole-muscle SDS-PAGE gel samples (described above) were diluted with distilled, deionized water to 0.256 mg/mL. One volume of each sample was combined with 1 vol of tracking dye solution (50% [vol/vol] glycerol, 2% [wt/vol] SDS, 0.1% [wt/vol] bromophenol blue, and 60 mM Tris-HCl, pH 6.8) and 0.05 vol of MCE for a final protein concentration of 0.125 mg/mL. Gel samples were frozen at −80°C for further analysis.

Myosin heavy-chain isoforms were determined 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] APS, 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 myosin heavy-chain (MHC) isoform. Gels (18 cm wide × 16 cm tall) for MHC were run on SE 400 Hoefer units (Hoefer Scientific Instruments). The running buffer for the upper chamber contained 200 mM Tris, 300 mM glycine, 0.2% [wt/vol] SDS, and 0.1% [vol/vol] MCE and the running buffer for the lower chamber contained 100 mM Tris, 150 mM glycine, 0.1% [wt/vol] SDS, and 0.1% [vol/vol] MCE. Gels for MHC were loaded with 2 μg of protein per lane, and run at a constant voltage of 100 V for approximately 48 h. The FAST-Silver kit (Geno Technology, Inc., St. Louis, MO) was used to develop and fix the gel to examine MHC. The density of type I, type IIA/IIX, and type IIB myosin heavy-chain bands were quantified by using ChemiImager 5500 (Alpha Innotech, San Leandro, CA) and AlphaEaseFC (version 2.03, Alpha Innotech, San Leandro, CA). Within each sample, percentages for each MHC (type I, type IIA/IIX, type IIB) isoform were calculated as a percentage of the total MHC isoforms in each lane.

**Statistical Analyses**

Data were analyzed using the GLM procedure of SAS (Version 8.01; SAS Inst., Inc., Cary, NC) and least squares means for all traits of interest were computed. Each pig (n = 16) served as a replicate in the experiment with slaughter date as the block and muscle (LD, SM, and PM) as the individual unit. At each time point, the main effect was muscle (LD, SM, and PM).
Table 2. Effect of muscle on temperature and pH decline during the first 24 h postmortem in longissimus dorsi (LD), semimembranosus (SM), and psoas major (PM)

<table>
<thead>
<tr>
<th>Item</th>
<th>LD</th>
<th>SM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle temperature, °C</td>
<td>35.89 ± 0.171</td>
<td>36.37 ± 0.781</td>
<td>35.83 ± 0.381</td>
</tr>
<tr>
<td>30 min</td>
<td>35.89 ± 0.171</td>
<td>36.37 ± 0.781</td>
<td>35.83 ± 0.381</td>
</tr>
<tr>
<td>45 min</td>
<td>33.65 ± 0.431</td>
<td>34.93 ± 0.510</td>
<td>32.22 ± 0.574</td>
</tr>
<tr>
<td>1 h</td>
<td>29.94 ± 0.475</td>
<td>32.30 ± 0.199</td>
<td>26.34 ± 0.503</td>
</tr>
<tr>
<td>6 h</td>
<td>8.89 ± 0.473</td>
<td>15.30 ± 0.497</td>
<td>8.03 ± 0.350</td>
</tr>
<tr>
<td>12 h</td>
<td>3.31 ± 0.356</td>
<td>7.28 ± 0.439</td>
<td>3.41 ± 0.433</td>
</tr>
<tr>
<td>24 h</td>
<td>1.94 ± 0.161</td>
<td>2.42 ± 0.193</td>
<td>1.85 ± 0.178</td>
</tr>
<tr>
<td>Muscle pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>6.07 ± 0.064</td>
<td>6.22 ± 0.251</td>
<td>5.67 ± 0.044</td>
</tr>
<tr>
<td>45 min</td>
<td>5.96 ± 0.074</td>
<td>6.06 ± 0.222</td>
<td>5.49 ± 0.047</td>
</tr>
<tr>
<td>1 h</td>
<td>5.84 ± 0.074</td>
<td>5.89 ± 0.261</td>
<td>5.49 ± 0.063</td>
</tr>
<tr>
<td>6 h</td>
<td>5.68 ± 0.051</td>
<td>5.71 ± 0.150</td>
<td>5.63 ± 0.024</td>
</tr>
<tr>
<td>12 h</td>
<td>5.67 ± 0.040</td>
<td>5.69 ± 0.038</td>
<td>5.67 ± 0.027</td>
</tr>
<tr>
<td>24 h</td>
<td>5.67 ± 0.034</td>
<td>5.69 ± 0.108</td>
<td>5.69 ± 0.021</td>
</tr>
</tbody>
</table>

a,b,cWithin a row, means without a common superscript letter differ (P < 0.01).

Results and Discussion

Average live weight, carcass data, and instrumental measures of color of the LD, SM, and PM muscles at 24 h postmortem are included in Table 1. The PM had a lower (P < 0.01) L*, and higher (P < 0.01) a* and b* values than the SM and LD. The LD had lower (P < 0.05) a* and lower (P < 0.01) b* than the SM and PM. These results indicate that the PM is a darker, redder, more yellow muscle than both the SM and LD.

Temperature, pH Decline, and Myosin Heavy-Chain Isoforms

There were no (P > 0.05) differences in temperature among the LD, SM, and PM at 30 min postmortem (Table 2). Beginning at 45 min, however, the temperature of the SM was the highest of all three muscles at each time point through 24 h postmortem (P < 0.05 at 45 min; P < 0.01 at 1 h; and P < 0.05 at 6, 12, and 24 h). At both 45 min (P < 0.05) and 1 h (P < 0.01) postmortem, the PM had the lowest temperature of all three muscles.

The PM had a more rapid pH decline within the first hour postmortem than the LD and SM (Table 2) as evidenced by lower (P < 0.01) pH at 30, 45, and 60 min postmortem. However, by 6, 12, and 24 h postmortem, no differences (P > 0.05) in pH were observed among the three muscles.

Myosin heavy-chain isoforms (types I, IIa, IIb, and IIX) were examined using electrophoresis (Figure 1). Type IIa and IIX were not separated in the analysis due to the difficulty in differentiating between the two bands using densitometry; therefore, percentages of type I, type IIa/IIX, and type IIb were analyzed in each muscle (Table 3). The PM had a greater (P < 0.01) percentage of MHC type IIa/IIX isoforms than the LD but was similar (P > 0.05) to the SM. The LD had the highest (P < 0.01) percentage of MHC isoform IIb followed by the SM. The PM had the lowest (P < 0.01) percentage of MHC isoform IIb, but had the greatest (P < 0.05) percentage of MHC isoform type I compared to the LD and the SM.

In bovine muscle, Koohmaraie et al. (1988) also observed that bovine PM had a lower pH than the LD at 1, 3, and 6 h postmortem but not at 9, 12, and 24 h postmortem. Similarly, Ilian et al. (2001) observed in ovine muscle that the PM had a lower pH at 1, 3, and 6 h postmortem than the LD. The exact reason for the more rapid pH decline in the PM in the current study and in others is not known. In the current study, there was a greater percentage of MHC type I isoform (P < 0.05) in the PM than in the other two muscles; thus, indicating a higher percentage of slow fibers in the PM than in the other two muscles (Table 3 and Figure 1). This fact, combined with the faster rate of temperature decline in the PM than the LD and SM at 45 min and 1 h, evidenced by lower (P < 0.01) pH at 30, 45, and 60 min postmortem, supports the findings of the current study.

Figure 1. Coomassie-stained 6% polyacrylamide gel showing differences in myosin heavy-chain isoforms at 45 min postmortem of the longissimus dorsi (LD), semimembranosus (SM), and psoas major (PM). Lanes 1, 2, and 3 correspond to the LD, SM, and PM, respectively, of a typical animal in the study. The top band is the type IIa/IIX isoforms, the second band is the type IIb and IIX isoforms, and the bottom band is the type I myosin heavy-chain isoforms. All lanes were loaded with 2 μg of protein.
The increased rate of autolysis is significant because autolysis of \( \mu \)-calpain reduces the \( \text{Ca}^{2+} \) requirement for calpain activity from approximately 3 to 50 \( \mu \text{M} \) (preautolysis) to 0.5 to 2.0 \( \mu \text{M} \) (Goll et al., 1995), bringing the \( \text{Ca}^{2+} \) requirement closer to the physiological range (Zimmerman and Schlaepfer, 1991). Autolysis has also been associated with terminal activation in postmortem muscle (Geesink and Koohmaraie, 1999). Rowe et al. (2001a) found that porcine LD, with low 2-h \( \text{pH} \), had a greater rate of autolysis of \( \mu \)-calpain and earlier degradation of specific myofibrillar proteins. Therefore, as suggested by others, earlier autolysis may be associated with earlier activation of \( \mu \)-calpain and protein degradation in the PM in this study. Indeed, in the current study, the degradation of titin and desmin occurred at an earlier time postmortem in the PM than in the other two muscles (Figure 3). At 45 min, the T2 band (degradation product of titin) and another degradation product of titin migrating at approximately 1,200 kDa (Huff-Lonergan et al., 1996a) were more pronounced in the PM sample than in the other two muscles. By 6 h postmortem, intact titin (T1) was not evident in the PM sample but was still visible in the LD and SM. Likewise, desmin degradation was more rapid in the PM (desmin degradation product; Table 4) as indicated by a more evident desmin degradation product in the PM sample at 45 min and 6 h postmortem than in the LD or SM (Figure 4).

In addition to having a more rapid \( \text{pH} \) decline, earlier autolysis of \( \mu \)-calpain, and earlier degradation of some key proteins, the PM also had a faster rate of appearance of myofibrill-bound \( \mu \)-calpain (Figure 2A, myofibril). The earlier appearance of myofibrill-bound \( \mu \)-calpain in the PM might allow \( \mu \)-calpain to come into proximity to substrates like titin and nebulin. In fact,
Delgado et al. (2001) suggested that myofibril-bound calpain might degrade desmin, troponin-T, titin, and nebulin. It is possible, therefore, that pH decline may affect not only the rate of autolysis and possibly activation of calpain in the supernatant, but also the rate of its association with the myofibril (Figure 2).

It has been shown that μ-calpain becomes tightly associated with myofibrils isolated from postmortem muscle (Boehm et al., 1998; Delgado et al., 2001). However, what has not been made clear is whether μ-calpain precipitates non-specifically onto the myofibrils or if it binds to certain regions of the myofibril. To answer this question, we examined myofibrils from the samples in this study using immunofluorescent microscopy. The micrographs clearly showed that monoclonal antibodies against μ-calpain localized specifically at the A-band and at the Z-line as early as 45 min and 6 h postmortem (Figure 5), possibly putting this enzyme in closer to potential substrates like titin.

**Warner-Bratzler Shear Force**

At 24 h postmortem, the PM had lower \( P < 0.01 \) WBSF than the LD (Table 3). However, shear force values for the LD and PM were not \( P > 0.05 \) different at 48 and 120 h postmortem. The LD had a greater change in WBSF between 24 \( (P < 0.01) \) and 120 h \( (P < 0.05) \) than the PM (Table 3). Numerous factors (collagen content/cross-linking and sarcomere length) contribute to the tenderness of the PM. However, these may not be the only factors (Wheeler and Koohmaraie, 1999; Wheeler et al., 2000); variation in the rate of tenderization between the LD and PM might also be influenced by μ-calpain activity and subsequent autolysis. Casein zymography in the current study showed that the PM had less μ-calpain activity in the sarcoplasmic fraction by 45 min postmortem than the LD and SM. By 6 h postmortem, the PM had lost most μ-calpain activity in the sarcoplasmic fraction (Figure 6A). This parallels the increased autolysis of μ-calpain that was noted at 45 min and 6 h postmortem (Figure 2). This is also consistent with previous observations in bovine studies (Koohmaraie et al., 1988; Ilian et al., 2001). Muscle pH may affect the differences observed in μ-calpain activity. It has been hypothesized that more rapid pH decline, accompanied with a higher Ca\(^{2+}\) concentration, resulted in increased μ-calpain autolysis, and possibly earlier activation (Claeys et al. 2001) and subsequently, lower μ-calpain activity at later times postmortem. In the present study, the increased rate of autolysis may indicate early activation and earlier loss of μ-calpain activity.
Table 4. Drip loss percentage and desmin degradation product of the longissimus dorsi (LD), semimembranosus (SM), and psoas major (PM)

<table>
<thead>
<tr>
<th>Item</th>
<th>LD</th>
<th>SM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drip loss, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h of storage</td>
<td>1.57 ± 0.384</td>
<td>1.46 ± 0.373</td>
<td>1.06 ± 0.250</td>
</tr>
<tr>
<td>96 h of storage</td>
<td>2.96 ± 0.623</td>
<td>2.78 ± 0.551</td>
<td>1.10 ± 0.214</td>
</tr>
<tr>
<td>Desmin degradation producta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45 min postmortem</td>
<td>0.126 ± 0.027</td>
<td>0.177 ± 0.036</td>
<td>0.286 ± 0.039</td>
</tr>
<tr>
<td>6 h postmortem</td>
<td>0.165 ± 0.106</td>
<td>0.188 ± 0.056</td>
<td>0.415 ± 0.097</td>
</tr>
<tr>
<td>24 h postmortem</td>
<td>0.306 ± 0.081</td>
<td>0.233 ± 0.071</td>
<td>0.391 ± 0.057</td>
</tr>
</tbody>
</table>

aRatios were calculated as the intensity of the desmin degradation product band in each sample over the intensity of the intact desmin degradation product band in the internal designated densitometry standard.

b,cWithin a row, means without a common superscript letter differ (P < 0.01).

x,yWithin a row, means without a common superscript letter differ (P < 0.05).

activity in the PM compared to the other muscles; therefore, proteolysis was complete at an earlier time postmortem, and the subsequent rate of tenderization was minimized after loss of activity.

O’Halloran et al. (1997) showed that LD with a rapid rate of glycolysis and accelerated pH decline had less μ-calpain activity at 3 h postmortem, was more tender, and had greater proteolysis of skeletal muscle proteins at earlier times postmortem than did LD with slower rates of glycolysis. This observation supports the results of the current study and further indicates that the rate of early postmortem pH decline may play a pivotal role in regulating the rate of postmortem tenderization.

Warner-Bratzler Shear Force Between the LD and SM. The SM had higher WBSF values at 48 (P < 0.01) and 120 h (P < 0.05) postmortem than the LD (Table 3). However, the LD and SM had similar μ-calpain activity at 6 h postmortem (Figure 6B), whereas, at 24 h postmortem, both the LD and SM had minimal μ-calpain activities in the sarcoplasmic fraction as noted by a lack of a clearing zone in those samples (data not shown). The decline in μ-calpain activity appears to be similar between the LD and SM; therefore, other factors, such as calpastatin activity, may have a significant role in determining WBSF differences between the LD and SM.

In the present study, the SM had higher (P < 0.05) calpastatin activity than the LD at 45 min, 6 h, and 24 h postmortem, and the SM had higher (P < 0.05) WBSF values (Table 3). Calpastatin inhibits the rate and extent of postmortem proteolysis (Geesink and Koohmaraie, 1999) and thus the development of tenderness (Lonergan et al., 2001b).

Proteolysis has an important role in determining ultimate tenderness (Wheeler and Koohmaraie, 1994). Degradation of several proteins, including titin, nebulin, desmin (Taylor et al., 1995; Huff-Lonergan et al., 1996a), and troponin-T (Huff-Lonergan et al., 1996a; Lonergan et al., 2001b), has been shown to be related to increases in tenderness. In this study, the degradation of nebulin in the LD was more evident than in the SM at 48 and 120 h postmortem (Figure 3). Additionally, at 120 h, the LD had less intact titin (Figure 3, A)}
Biochemical factors influencing pork quality

Figure 5. Immunofluorescent and phase-contrast micrographs of myofibrils isolated from A) 45 min and B) 6 h postmortem psoas major muscles. Bright bands in the immunofluorescent images indicate where μ-calpain monoclonal antibodies were located.

Water-Holding Capacity

The PM had lower ($P < 0.05$) average drip loss after 24 h of storage than the LD. After 96 h of storage, the PM had lower ($P < 0.01$) drip loss percentages than both the SM and LD (Table 3). At 45 min and 6 h postmortem, the PM had more ($P < 0.01$) desmin degradation product than the LD and the SM (Figure 4A and Table 4). However, by 24 h postmortem, the PM was only different ($P < 0.05$) from the SM. The variation in water-holding capacity among muscles may be due to differences in postmortem degradation of intermediate filament proteins like desmin (Kristensen and Purslow, 2001; Rowe...
A Calpain activity 45 min postmortem
LD SM PM Std
μ-calpain
m-calpain

B Calpain activity 6 h postmortem
LD SM PM Std
μ-calpain
m-calpain

Figure 6. Casein zymography gels depicting μ-calpain activity in sarcoplasmic extracts of the longissimus dorsi (LD), semimembranosus (SM), and psoas major (PM) at A) 45 min and B) 6 h postmortem. Lanes 1, 2, and 3 correspond to the LD, SM, and PM, respectively, of a typical pig in the study. The internal standard (Std) corresponds to purified m-calpain (0.35 unit/mL). Lanes 1, 2, and 3 were loaded with 140 μg of protein. Lane 5 was loaded with 0.0035 unit of purified porcine m-calpain activity. The uppermost clear zone indicates μ-calpain activity and the bottom clear zone indicates m-calpain activity.

In the current study, the PM had more rapid degradation of the intermediate filament protein desmin (Figure 4 and Table 4). This increased degradation may compensate for some of the shrinkage of the muscle cell due to the drop in pH. Kristensen and Purslow (2001) hypothesized that more water could be retained in the muscle cell early postmortem when rapid degradation of intermyofibril linkages (desmin) occurs, thus creating more “space” for water to reside. Therefore, less water would be lost initially, and ultimately, would result in a greater water-holding capacity.

Implications

Differences in the pork quality traits of tenderness and water-holding capacity that exist between muscles may be related to differences in myosin heavy-chain isoform content, proteolysis, and the rate of activation/autolysis of μ-calpain. Rate of postmortem pH decline within the first 6 h after exsanguination influences the rate of μ-calpain activity and autolysis and may play a pivotal role in regulating early postmortem proteolysis and ultimately drip loss and the rate of postmortem tenderization. Decreasing the variation in these traits could lead to the production of pork that is more uniform in quality.

Literature Cited

Huff-Lonergan, E., T. Mitsuhashi, F. C. Parrish, Jr., D. G. Olson, and R. M. Robson. 1996b. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting comparisons of purified...
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Erratum

It has come to our attention that Table 1 of Early postmortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles (J. Anim. Sci. 2004. 82:1195–1205) was omitted. The table appears below.

Table 1. Description of carcasses (n = 16)

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live weight, kg</td>
<td>120.28</td>
<td>1.66</td>
</tr>
<tr>
<td>Hot carcass weight, kg</td>
<td>94.39</td>
<td>1.36</td>
</tr>
<tr>
<td>Last-rib fat thickness, cm</td>
<td>2.17</td>
<td>0.12</td>
</tr>
<tr>
<td>(L^*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Longissimus dorsi</td>
<td>46.36</td>
<td>1.17</td>
</tr>
<tr>
<td>Semimembranosus</td>
<td>46.53</td>
<td>1.10</td>
</tr>
<tr>
<td>Psoas major</td>
<td>43.04</td>
<td>0.87</td>
</tr>
<tr>
<td>(a^*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Longissimus dorsi</td>
<td>3.70</td>
<td>1.03</td>
</tr>
<tr>
<td>Semimembranosus</td>
<td>6.33</td>
<td>1.01</td>
</tr>
<tr>
<td>Psoas major</td>
<td>10.99</td>
<td>1.20</td>
</tr>
<tr>
<td>(b^*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Longissimus dorsi</td>
<td>10.32</td>
<td>0.51</td>
</tr>
<tr>
<td>Semimembranosus</td>
<td>12.61</td>
<td>0.43</td>
</tr>
<tr>
<td>Psoas major</td>
<td>15.01</td>
<td>0.34</td>
</tr>
</tbody>
</table>

\(L^*\) = a measure of lightness to darkness (larger value indicates a lighter color); \(a^*\) = a measure of redness (larger value indicates a redder color); \(b^*\) = a measure of yellowness (larger value indicates a more yellow color).

Within a trait, means for each variable without a common superscript letter differ \((P < 0.01)\).