Calpain-1 activity in bovine muscle is primarily influenced by temperature, not pH decline

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*J ANIM SCI* 2014, 92:1261-1270.
doi: 10.2527/jas.2013-7270 originally published online February 3, 2014

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Calpain-1 activity in bovine muscle is primarily influenced by temperature, not pH decline

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ABSTRACT: The objectives of this study were to 1) determine the conditions (temperature and pH) that exist in early postmortem muscle of normally chilled and delay chilled beef carcasses to provide a model for in vitro work and 2) determine the mechanism by which early postmortem temperature/pH conditions found in beef muscle influence the enzymes that regulate the aging process in vitro. For objective 1, 7 finished beef animals (HCW 385 ± 8 kg) were harvested with the right sides subjected to normal chilling (2.3°C) approximately 1.25 h postmortem and the left sides subjected to ambient temperature (delay chilling; 22.6°C) for an additional 4.75 h postmortem and then allowed to chill at 2.3°C. Delay chilled carcasses had a more rapid pH decline (P < 0.05) and a slower rate of carcass cooling (P < 0.05). No differences were observed between normally chilled and delay chilled samples for sarcomere length or postmortem proteolysis of troponin T (TnT; P > 0.10). Warner-Bratzler shear force (WBSF) was reduced in steaks from normally chilled carcasses at 14 d (P < 0.05), while results indicated a strong, positive correlation between 14-d WBSF and 3-h longissimus dorsi muscle (LM) temperature (r = 0.67, P < 0.01) as well as a strong, negative correlation between 14-d WBSF and 6-h LM pH (r = −0.65, P < 0.02). These results were used to design the methodology for objective 2, where isolated myofibrils were subjected to μ-calpain digestion at 4 or 22°C with either a fast or slow initial pH decline. As expected, digestions with a fast initial pH decline had lower pH values in the early time points of the incubation (P < 0.05). No differences were detected in μ-calpain activity or in the degradation of intact TnT between the fast and slow pH decline treatments (P > 0.10); however, warmer digestions resulted in a tendency for increased activation of μ-calpain (P < 0.10) and a significant reduction in intact TnT (P < 0.05). Additionally, a temperature × time interaction was revealed in μ-calpain activity and in the degradation of intact TnT (P < 0.05). Specifically, assayed calpain activity was lower at 0.17, 0.33, 1, and 3 h and greater at 72 h in warmer digestions, while intact TnT disappearance was greater as both time and digestion temperature increased. Meat aging and μ-calpain activity are influenced by both temperature and pH, but more research is necessary to fully realize their relationships.

Key words: beef, chilling, μ-calpain, proteolysis


INTRODUCTION

Postmortem proteolysis of key myofibrillar proteins is one of the primary outcomes of postmortem aging of meat, resulting in the improvement in meat tenderness over time. While this process is important in providing the consumer with a tender product, the mechanisms involved in postmortem proteolysis remain under debate. Numerous studies (Koohmaraie, 1988; Huff-Lonergan et al., 1996a; Geesink et al., 2006; Mohrhauser et al., 2011) provide compelling evidence that postmortem tenderization of beef is primarily modulated by the calpain protease system, specifically μ-calpain. These abundant enzymes have access to their substrates postmortem, require no ATP for activation, and have the ability to reproduce postmortem changes in vitro (Koohmaraie, 1988; Huff-Lonergan et al., 1996a). Despite this convincing
evidence, it has been argued that calpains may lack the ability to function under normal postmortem temperature and pH conditions. In particular, in vitro proteolytic assays show the activity of μ-calpain decreases rapidly during storage (Boehm et al., 1998; Camou et al., 2007). Therefore, altering muscle temperature and pH has been studied using a delayed chilling model. Delayed chilling is a nontraditional method where carcasses are held in an unchilled environment for a period of time before chilling (Smulders et al., 1992; Savell et al., 2005), resulting in the potential for improvements in beef tenderness (Fields et al., 1976; Yu et al., 2008). Therefore, the objective of this study was 2-fold: 1) determine the conditions (temperature and pH) that exist in early postmortem muscle of normally chilled and delay chilled beef carcasses to provide a model for in vitro work and 2) determine the mechanism by which specific early postmortem temperature/pH conditions found in normally chilled and delay chilled muscle influence the enzymes that regulate the aging process in vitro.

MATERIALS AND METHODS

Animal care and experimental protocols were not submitted for approval by the Animal Care and Use Committee as samples were collected from the state-inspected South Dakota State University Meat Lab.

Experiment 1

Sample Collection. Seven market weight (HCW 385 ± 8 kg), Limousin-based crossbred animals managed as a common group were purchased from a local producer and harvested at the South Dakota State University Meat Lab using standard harvesting procedures. After final inspection (approximately 1.25 h postexsanguination), sides were divided into different treatments: the right side of each carcass was placed into a cooler (normal chilling; 2.3°C) while each left side was subjected to ambient temperature (delay chilling; 22.6°C) for an additional 4.75 h postmortem and then allowed to chill at 2.3°C. From each side (starting at the 13th rib and moving posterior), an approximately 1-cm thick, 30-g, cross-section sample of the LM was removed at 0.75, 1.5, 3, 6, 9, 12, 24, 48, 72, 168, and 336 h. This sample was used for different measurements; the medial portion was used for determination of pH and the central portion was used for the evaluation of proteolysis of troponin T (TnT). Samples for proteolysis analysis were vacuum packaged and frozen at –20°C while pH samples were evaluated immediately. Additionally, at 24 h postmortem, a 0.635-cm thick sample from the LM was removed and frozen at –20°C for later analysis of sarcomere length. To compare the treatment effect on aging, three 2.54-cm steaks were removed from the 9th through the 12th rib section of the LM for Warner-Bratzler Shear Force (WBSF) analysis and aged for 1, 7, and 14 d. Steaks for WBSF were vacuum packaged, stored at 4°C until completion of the aging periods, and then frozen at –20°C until further analysis.

Temperature and pH Measurements. Temperature decline was monitored using a Temprecord Multitrip temperature recorder (Sensitech Inc., Beverly, MA) placed in the center of the LM between the 12th and 13th ribs before the imposed chilling conditions for each side. Muscle temperature was logged every minute and downloaded to software for retrieval and further analysis. For pH analysis, muscle (5 g) was knife-minced and added to 50 mL of a 5 mM sodium iodoacetate and 150 mM potassium chloride solution (Bendall, 1973). Samples were homogenized using an Ultra-Turrax T25 homogenizer (Janke & Kunkel GmbH & Co., KG., Staufen, Germany) at medium speed and pH was measured using a ThermoOrion PerpHect pH Meter, model 330 (Orion Research, Inc., Boston, MA).

Warner-Bratzler Shear Force. Steaks frozen for WBSF analysis were thawed for 24 h at 4°C. Steaks were cooked on an electric clam shell grill (George Forman Indoor/Outdoor Grill, Model GGR62; Lake Forest, IL) to 71°C. Peak internal cooked temperature measurements were recorded for each steak using a hand held thermometer (model 39658-K; Atkins Technical, Gainesville, FL). Cooked steaks were cooled for 24 h at 4°C before removing 6 cores (1.27 cm in diameter) parallel to the muscle fiber orientation (AMSA, 1995). A single, peak shear force measurement was obtained for each core using a Warner-Bratzler shear machine (G-R Electric Manufacturing Company, Manhattan, KS). Measurements of the peak shear force were recorded and averaged to obtain a single shear force value for each steak.

Myofibril Preparation and Sarcomere Length Determination. Myofibrils from each sample were purified according to Weaver et al. (2008). Sarcomere length was determined using a procedure described by Mohrhauser et al. (2011). The average of 5 sarcomeres was calculated for each myofibril and was determined across 30 myofibrils per sample.

Protein Degradation Analysis. Longissimus dorsi muscle samples collected at all time points were extracted for sarcoplasmic and myofibrillar protein as described by Huff-Lonergan et al. (1996b) with slight modifications. Briefly, 0.2 g of muscle were minced and added to 25 volumes of homogenizing solution. Samples were homogenized using a motor driven Dounce homogenizer and clarified by centrifugation at 1,500 × g for 15 min at 4°C. Protein concentration was determined using the Pierce BCA Protein assay (Thermo Fisher Scientific Inc., Rockford, IL) and samples were diluted with water to a
Temperature and pH effects on μ-calpain activity

for 90 min at 4°C. Following electrophoresis, gels were washed 4 times in PBST and bands were visualized using a LI-COR Odyssey scanner. Immunoreactive TnT and actin was identified and the disappearance of intact TnT was quantified using LI-COR Odyssey scanner. The density of bands was then normalized to the relative intensity of bands was expressed relative to the intensity of protein bands. A 5-by-10 cm template (3M, St. Paul, MN) was used to ensure similar sampling areas and each location was swabbed using a Spongicle Sponge-Stick (3M). Swabs were then shipped on ice to Minnesota Valley Testing Laboratories, Inc. (New Ulm, MN), for the evaluation of total aerobic plate counts.

**Microbiology.** At 1 and 8 d postmortem, each carcase side was swabbed once at each of 3 locations including the rump, flank, and brisket for the analysis of microbial counts. A 5-by-10 cm template (3M, St. Paul, MN) was used to ensure similar sampling areas and each location was swabbed using a Spongicle Sponge-Stick (3M). Swabs were then shipped on ice to Minnesota Valley Testing Laboratories, Inc. (New Ulm, MN), for the evaluation of total aerobic plate counts.

**Statistical Analysis.** Least squares means for LM temperature and pH, WBSF, sarcomere length, and microbiological analyses were computed using Mixed Model (PROC MIXED) procedures of SAS (SAS Inst. Inc., Cary, NC), using animal as a random variable and chilling method as the main effect. Means were separated using the least significant differences (PDIF option in SAS) and tested to a predetermined significance level of 0.05 and trends at a predetermined significance level of 0.10.

Proteolysis data were analyzed using repeated measure (PROC MIXED) procedures of SAS, where animal was a random variable. The model tested the main effects of chilling method and time as well as their interaction. Time was the repeated variable while an autoregressive (AR(1)) covariance structure was used in the model. Means were separated using the least significant differences (PDIF) and tested to a predetermined significance level of 0.05 and trends at a predetermined significance level of 0.10. Correlation analysis was performed using the PROC CORR function of SAS, while linear, quadratic, and logarithmic effects of temperature and pH on 14-d WBSF were analyzed using the PROC REG function of SAS.

**Experiment 2**

**Digestion of Myofibrils with μ-Calpain.** The results from Exp. 1 were used to design Exp. 2 with the aim of evaluating μ-calpain activity during the digestion of already purified myofibrils with differing pH declines to mimic conditions found in muscle during the conversion of muscle to meat. Digestions occurred at either 4 or 22°C with either a fast or slow pH decline during the first hour of the reaction. The desired 1-h pH for digestions subjected to a fast pH decline was 5.6 to 5.8 while the desired 1-h pH for the slow pH decline was 6.2 to 6.4. For each temperature and pH decline combination, 2 myofibril populations were used. The pH decline of the digestions was monitored using a ThermoOrion PerpHect pH Meter, model 330 (Orion Research, Inc., Boston, MA), with pH measurements taken at 0, 0.17, 0.33, 0.5, 1, 3, 24, and 72 h. Myofibrils were digested by μ-calpain according to a modified procedure of Huff-Lonergan et al. (1996a). Eight milliliters of glycerinated myofibrils were centrifuged at 3,100 × g for 6 min at 4°C and washed with 4 mL of 5 mM Tris-HCl (pH 8.0). The samples were then centrifuged at 3,100 × g for 6 min at 4°C and washed with 4 mL of 50 mM 2-[N-Morpholino] ethanesulfonic acid (MES)-Tris (pH 6.8). Samples were again centrifuged at 1,100 × g for 6 min at 4°C and washed with 4 mL of 50 mM MES-Tris (pH 6.8). Finally, samples were centrifuged at 1,100 × g for 6 min at 4°C and resuspended with 4 mL of 50 mM MES-Tris (pH 6.8). Protein concentration was then determined using the Biiuret procedure and 10 mL of myofibrils were placed in SnakeSkin dialysis tubing (10K MWCO; Thermo Fisher Scientific, Rockford, IL) with a protein concentration of 4 mg/mL by adding 50 mM MES-Tris (pH 6.8). One hundred micromolar CaCl₂ and 15 mM 2-mercaptoethanol were added to each reaction tube. The digestion reaction was started by adding μ-calpain to myofibrils (0.45 units/mL of myofibrils; μ-calpain [48.2 U/mg protein] purified according to
the procedure of Thompson and Goll [2000] with minor modifications as described by Maddock et al. [2005]). Aliquots were removed at 0, 0.17, 0.33, 0.5, 1, 3, 24, and 72 h and placed into 0.5 volumes and 0.1 volumes of pyronin Y sample buffer and 2-mercaptoethanol, respectively, as described in Exp. 1. Samples were denatured at 100°C for 5 min, cooled, and stored at −20°C. Samples were then analyzed for TnT degradation using the gel electrophoresis, transfer conditions, and western blotting techniques described previously. The digestion pH was adjusted by placing the dialysis tubing containing the reaction into a 50 mM MES, 100 μM CaCl₂, and 15 mM 2-mercaptoethanol buffer containing either lactic acid or Tris base.

Calpain Activity. Calpain activity was also evaluated in this study using a procedure from Koohmaraie (1990) by measuring the release of trichloroacetic acid-soluble polypeptides resulting from the digestion of casein by the μ-calpain. Results are based on the change of absorbance as 1 unit of activity is equal to the amount of μ-calpain necessary to increase the absorbance by 1 in a 60-min incubation. Three hundred microliters of digestion components were added to 1 mL of 1x Tris-EDTA buffer (40 mM Tris and 0.5 mM EDTA, pH 7.35), 1 mL of casein assay buffer (7 mg/mL casein in 100 mM Tris-acetate, pH 7.5), 100 μL of 100 mM CaCl₂, and 4 μL of 2-mercaptoethanol. Blanks (containing no sample) and samples were incubated at 25°C for 60 min and stopped by the addition of 2 mL 5% trichloroacetic acid. Samples were then centrifuged at 1,500 × g for 20 min at 22°C and the absorbance was read at 278 nm using a Shimadzu UV-2101PC spectrophotometer (International Equipment Trading Ltd., Vernon Hills, IL). Calpain activity was calculated by subtracting the absorbance of the blank from the absorbance of the sample and multiplying the difference by the inverse of the sample inclusion (1/0.3 in this case).

Statistical Analysis. Least squares means for pH data were computed using General Linear Model (PROC GLM) procedures of SAS (SAS Inst. Inc., Cary, NC). Main effects consisted of rate of pH decline and temperature, while their interaction was also tested. Means were separated using the least significant differences (PDIF) and tested to a predetermined significance level of 0.05 and trends at a predetermined significance level of 0.10. Proteolysis and μ-calpain activity data were analyzed using repeated measure (PROC MIXED) procedures of SAS. Main effects consisted of rate of pH decline, temperature, and time, while all possible interactions were also tested. Time was the repeated variable while a compound symmetry (CS) covariance structure was used in the model. Means were separated using the least significant differences (PDIF) and tested to a predetermined significance level of 0.05 and trends at a predetermined significance level of 0.10.

RESULTS AND DISCUSSION

Experiment 1

During the conversion of muscle to meat, circulatory system function is lost, resulting in the lack of transport of nutrients to skeletal muscle and the loss of waste removal from skeletal muscle, ultimately causing the depletion of ATP and a buildup of lactic acid within muscle. During this process, muscle is converted into the rigor state with formation of actomyosin bonds that will not be released due to the lack of ATP. These rigor bonds are formed with the complete exhaustion of ATP, resulting in maximum muscle tension (Aberle et al., 2001; Savell et al., 2005). However, during storage, endogenous enzymes are able to decrease muscle tension as proteins are cleaved, weakening the structural integrity of muscle to improve meat tenderness in a process known as postmortem proteolysis (Aberle et al., 2001). This process is thought to primarily involve the calpain enzyme system (Koohmaraie, 1988; Huff-Lonergan et al., 1996a; Geesink et al., 2006; Mohrhauser et al., 2011); however, because prerigor postmortem temperature and pH conditions within muscle are not constant, optimal conditions for μ-calpain activity (25°C and pH of 6.5) are not always present within postmortem muscle (Boehm et al., 1998; Maddock et al., 2005). Additionally, it has been demonstrated that both pH and temperature can have dramatic effects on μ-calpain autolysis and activity. Of note, Koohmaraie (1992) demonstrated that either increased temperature or decreased pH can result in the acceleration of μ-calpain autolysis, while also causing a more rapid decline in μ-calpain activity. Further research by Maddock et al. (2005) has also shown the importance of pH on μ-calpain activity and autolysis, reporting that samples incubated at a pH of 6.5 had the slowest autolysis and greatest μ-calpain activity remaining at 30 and 60 min when compared to samples incubated at a pH of 6.0 and 7.5. Therefore, muscle pH and temperature appear to be an important factor in μ-calpain activity, and it is plausible that alterations in muscle temperature and pH through delayed chilling may have the potential to influence μ-calpain activity and meat aging.

In Exp. 1 of this study, carcass sides were subjected to normal chilling or delayed chilling conditions to alter postmortem muscle pH and temperature. As expected, carcass sides subjected to delayed chilling showed a slower temperature decline (Fig. 1a) as delay chilled carcasses had higher LM temperatures at 3, 6, 9, and 12 h (P < 0.05). However, at 24 h postexsanguination, muscle temperature did not differ between the 2 treatments (P > 0.10). As a result of the different chilling methods, differences in pH decline also occurred (Fig. 1b). Delay chilled carcasses demonstrated a faster pH decline resulting in lower pH values at 6, 12, and 24 h (P < 0.05).
Temperature and pH effects on μ-calpain activity

Previous research has proven that delayed chilling of carcasses results in faster pH declines of LM (Marsh et al., 1988; Whipple et al., 1990; Yu et al., 2008). Whipple et al. (1990) reported a significantly lower pH at 6, 9, and 12 h postmortem in the LM of carcasses held at 22°C for 6 h compared to carcasses immediately chilled at −1°C; however, unlike the present study, Whipple et al. (1990) found no differences in ultimate pH at 24 h.

Some researchers have indicated that delaying postmortem chilling improves tenderness. Fields et al. (1976) showed no differences in WBSF of LM steaks from steer carcasses at 2 d postmortem, while LM steaks from sides held at 14 to 19°C for 12 h were more tender at 7 d when compared to steaks from sides chilled at 0 to 2°C. Dutson et al. (1977) evaluated meat tenderness in LM steaks from carcasses chilled at 1°C and from carcasses held at 22°C for 4 h, then held at 16°C for 8 h, and then chilled at 1°C and found an improvement in WBSF in the delay chilled samples. Whipple et al. (1990) reported an improvement in WBSF in delay chilled LM steaks from Bos indicus carcasses at 1 d postmortem, while no differences were found at 14 d postmortem. Most recently, Yu et al. (2008) observed reduced WBSF values at 1, 4, and 6 d postmortem in LM steaks from carcasses chilled for 4 h at 2°C, 4 h at 12°C, and then 16 h at 2°C when compared to LM steaks from carcasses chilled at 2°C for 24 h. In contrast, the present study found that steaks aged for 1 and 7 d showed no differences in WBSF as a result of chilling method (P > 0.10; Table 1); however, when aged for 14 d, steaks from normally chilled carcasses required less force to shear, indicating that they were more tender than steaks from delay chilled carcasses (P < 0.05).

As part of the explanation for the improvements in tenderness observed with delayed chilling, many researchers have evaluated alterations in sarcomere length. Sarcomere length has been proven to be greatly affected by chilling rates in relation to rigor onset (Locker and Hagyard, 1963). In particular, cold shortening is a phenomenon that occurs when carcasses are chilled too rapidly, causing muscle temperature to drop less than 14°C before the onset phase of rigor mortis (Locker and Hagyard, 1963). Rapid cooling of carcasses results in the dysfunction of the sarcoplasmic reticulum, causing an excess of calcium due to problems with calcium binding in the sarcoplasmic reticulum. Excess calcium in addition to ATP still present before rigor onset allows the muscle to contract at a maximum level, causing severe shortening and a reduction in tenderness (Aberle et al., 2001; Savell et al., 2005). Much of the research described previously (Fields et al., 1976; Dutson et al., 1977; Yu et al., 2008) that has indicated tenderness improvements due to delayed chilling have also reported longer sarcomeres due to delayed chilling. Therefore, improvements in tenderness in these studies may be attributed to the prevention of cold shortening. In contrast, no differences in sarcomere length were discovered in this study (P > 0.10; Table 1). The lack of sarcomere length differences due to chilling rate in this study is likely due to the use of cattle that are more representative of today’s beef industry in hot carcass weight (385 ± 8 kg) and 12th rib backfat (1.45 ± 0.97 cm), potentially resulting in adequate insulation of the muscle and the prevention of cold shortening characteristics of the LM.

While previous research has indicated that delayed chilling of carcasses may appear to improve meat tenderness primarily through the prevention of cold shortening, other aspects impacting meat tenderness may be

### Table 1. Least squares means of Warner-Bratzler Shear Force (WBSF) and sarcomere length of longissimus dorsi from normal and delay chilled carcasses

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal (n = 7)</th>
<th>Delayed (n = 7)</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-d WBSF, kg</td>
<td>6.92</td>
<td>6.34</td>
<td>0.45</td>
<td>0.1869</td>
</tr>
<tr>
<td>7-d WBSF, kg</td>
<td>4.36</td>
<td>4.76</td>
<td>0.50</td>
<td>0.2659</td>
</tr>
<tr>
<td>14-d WBSF, kg</td>
<td>3.79</td>
<td>4.56</td>
<td>0.31</td>
<td>0.0144</td>
</tr>
<tr>
<td>Sarcomere length, μm</td>
<td>1.77</td>
<td>1.82</td>
<td>0.04</td>
<td>0.3336</td>
</tr>
</tbody>
</table>

Figure 1. a) Postmortem longissimus dorsi temperature declines from carcasses normally (n = 7) and delayed chilled (n = 7). Error bars reflect the SEM. Temperatures were significantly different at 3, 6, 9, and 12 h (P < 0.05).

b) Postmortem pH decline of normally chilled (n = 7) and delay chilled beef carcasses (n = 7). Error bars reflect the SEM. Measurements of pH were significantly different at 6, 12, and 24 h (P < 0.05).
influenced during delayed chilling practices. Research by Marsh et al. (1987, 1988) demonstrated that tenderness is related to the rate of decline of both muscle pH and temperature. Using various cooling and glycolytic rates (3-h muscle temperature range of 19 to 37°C; 3-h muscle pH range of 5.4 to 7.0) produced from electrical stimulation, Marsh et al. (1987, 1988) found a quadratic relationship between 3-h pH and WBSF, where LM steaks were most tender when attaining a 3-h pH of 6.0 to 6.2 while steaks with 3-h pH values outside of that range were tougher. Similarly, in the present study, correlation analysis (data not presented in tabular form) revealed strong relationships of both LM temperature at 3 h postmortem ($r = 0.67$, $P < 0.01$) and pH at 6 h postmortem ($r = -0.65$, $P < 0.02$) with 14-d WBSF. Meanwhile, the data showed that 6-h pH and 3-h temperature were highly correlated ($r = -0.67$, $P < 0.01$), indicating that 6-h pH was undoubtedly dependent on 3-h temperature and, therefore, 6-h pH should not be included with 3-h temperature in a regression model to explain 14-d WBSF variability. Further analysis of the data revealed a quadratic relationship between 3-h LM temperature and 14-d WBSF (Fig. 2; $P < 0.05$, $r^2 = 0.66$). Using the equation garnered from this relationship, 14-d WBSF was lowest when 3-h LM temperature was around 32°C. Because 14-d WBSF is influenced by 3-h LM temperature and was not influenced by sarcomere length in this study, it is apparent that the rate of cooling and pH decline has an influence on meat tenderness, which is not due to cold shortening.

Other aspects of meat tenderization must be influenced by temperature and pH, particularly postmortem proteolysis. As mentioned previously, postmortem proteolysis via proteases plays a key role in the tenderization of meat, and, again, the aging process is thought to primarily involve the calpain enzyme system. However, it has been shown that both pH and temperature can have dramatic effects on μ-calpain autolysis and activity (Koohmaraie, 1992; Camou et al., 2007), while Ca$^{2+}$ concentrations (needed for calpain activation) within postmortem muscle are greater at elevated temperatures (Goll et al., 2003). Additionally, calpastatin activity has been shown to decrease at elevated temperatures (Geesink et al., 2000; Pomponio and Ernborg, 2012), while its inhibition of μ-calpain appears to not be influenced by pH (Geesink and Koohmaraie, 1999; Maddock et al., 2005). Therefore, alterations in muscle temperature and resulting pH in this study may have the potential to influence μ-calpain activity and meat aging; however, limited research has been performed evaluating μ-calpain activity and subsequent postmortem proteolysis under differing early postmortem conditions. To evaluate protease activity alterations due to different chilling and pH declines, Whipple et al. (1990) subjected both Bos taurus and Bos indicus carcasses to normal chilling (–1°C) or delayed chilling treatments (22°C for 6 h and then at –1°C). In that study, greater μ-calpain activity was assayed at 6 and 24 h postmortem in carcasses chilled at –1°C when compared to carcasses held at 22°C for 6 h and then chilled at –1°C. These findings led the authors to conclude that the decrease in μ-calpain activity in the delay chilled carcasses could explain the improvements in 1 d WBSF and myofibrillar fragmentation index at 3, 7, and 14 d postmortem found due to delayed chilling.

Activity of both μ-calpain and calpastatin was not directly evaluated in the present experiment; however, proteolysis of TnT was analyzed because its degradation appears to be an excellent indicator of the extent of meat aging resulting from proteolytic degradation (Koohmaraie, 1994). Despite the relationship of 14-d WBSF and 3-h LM temperature discovered in this study, proteolysis of TnT was not different between treatments at all time points (Fig. 3; $P > 0.10$). Although the breakdown of TnT is generally regarded as a good indicator of protease activity and postmortem proteolysis, it remains uncertain whether breakdown of this regulatory protein aids in the improvement of tenderness. Therefore, it remains as a possibility that
Table 2. Least squares means of total aerobic microbial counts from swabs of rump, flank, and brisket locations at 1 and 8 d from normal and delay chilled carcasses

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal (n = 7)</th>
<th>Delayed (n = 7)</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-d rump, cfu</td>
<td>1,741</td>
<td>1,000</td>
<td>439</td>
<td>0.0890</td>
</tr>
<tr>
<td>8-d rump, cfu</td>
<td>1,109</td>
<td>30,394</td>
<td>21,173</td>
<td>0.3658</td>
</tr>
<tr>
<td>1-d flank, cfu</td>
<td>1,227</td>
<td>1,264</td>
<td>489</td>
<td>0.9585</td>
</tr>
<tr>
<td>8-d flank, cfu</td>
<td>3,637</td>
<td>241,140</td>
<td>149,300</td>
<td>0.3036</td>
</tr>
<tr>
<td>1-d brisket, cfu</td>
<td>2,297</td>
<td>2,486</td>
<td>622</td>
<td>0.8120</td>
</tr>
<tr>
<td>8-d brisket, cfu</td>
<td>44,536</td>
<td>138,811</td>
<td>72,530</td>
<td>0.3935</td>
</tr>
</tbody>
</table>

Figure 4. pH of in vitro digestions of purified myofibrils subjected to different pH declines at 4 and 22°C. Each temperature and pH decline combination consisted of n = 2 and the error bars reflect the SEM. In digestions occurring at 4°C, pH was significantly different at 0.17, 0.33, and 1 h (P < 0.05). In digestions occurring at 22°C, pH was significantly different at 0.5 and 1 h (P < 0.05) and tended to be different at 0.17, 0.33, and 3 h (P < 0.10).

Experiment 2

Although it has been repeatedly reported that in vitro μ-calpain digestion of purified myofibrils can produce similar myofibrillar protein degradation patterns found in normal postmortem skeletal muscle (Koohmaraie et al., 1986; Koohmaraie, 1992; Huff-Lonergan et al., 1996a; Mohrhauser et al., 2011), conditions of these digestions have not mimicked the early temperature and pH conditions found in postmortem muscle due to many limitations. Therefore, most of the in vitro myofibrillar digestions by μ-calpain have occurred under constant pH and temperature conditions (Koohmaraie et al., 1986; Huff-Lonergan et al., 1996a; Weaver et al., 2009; Mohrhauser et al., 2011). For example, Koohmaraie et al. (1986) compared myofibrillar digestions occurring at either 5 or 25°C and at pH values of 5.5, 5.8, 6.2, or 7.5. Although these are biologically relevant conditions in postmortem muscle, the development of an in vitro procedure that could more closely mimic postmortem temperature/pH combinations could help further our understanding of postmortem proteolysis. Therefore, using the temperature and pH conditions gathered from the delayed versus normal chilling model in Exp. 1, Exp. 2 was designed to evaluate alterations in μ-calpain activity due to different temperature/pH combinations. Due to limitations in creating differing temperature declines, temperatures (either 4 or 22°C) remained constant throughout each digestion. Meanwhile, the pH declines of the digestions were altered by placing dialysis tubing containing the digestion ingredients into solutions with different pH values. This experimental setup allowed for desired changes in pH decline (fast initial pH decline or slow initial pH decline) without altering the concentration of the digestion ingredients. These fast and slow initial pH declines can be seen in Fig. 4. As designed, digestion conditions resulted in differing pH at various time points, regardless of reaction temperature. In digestions occurring at 4°C, digestion reactions subjected to a fast initial pH decline had significantly lower pH values at 0.17, 0.33, and 1 h (P < 0.05) when compared to the incubations subjected to a slow initial pH decline, while pH values were not different at 3, 24, and 72 h (P > 0.10). Similarly, the fast and slow initial pH declines of the digestions performed at 22°C were different. Digestion reactions subjected to a fast initial pH decline had significantly lower pH values at 0.5 and 1 h (P < 0.05), while pH values tended to be lower at 0.17, 0.33, and 3 h (P < 0.10).

Remaining μ-calpain activity as a percentage of activity at the start of the digestion is shown in Fig. 5 for digestions performed at 4 and 22°C. Assayed activity of μ-calpain decreased over time as expected (P < 0.0001), while the initial rate of pH decline had no effect on μ-calpain activity (P > 0.10). Additionally, results indicated a temperature × time interaction (P < 0.01), while assayed activity of μ-calpain tended to be lower in incubations at 22°C than 4°C (P < 0.10). Activity of μ-calpain was lower in myofibril digestions performed at 22°C compared to digestions performed at 4°C at
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0.17, 0.33, 1, and 3 h ($P < 0.05$) and tended to be lower at 0.5 h ($P < 0.10$). However, at 72 h, μ-calpain activity was lower in digestions performed at 4°C compared to those at 22°C ($P < 0.05$). The influence of temperature on μ-calpain activity in this study was expected as room temperature (22°C) is a more optimal condition for proteolytic μ-calpain activity (Koohmaraie, 1992; Boehm et al., 1998; Camou et al., 2007). At this more ideal temperature, μ-calpain has been shown to be more active, yet our results indicate lower activities in this study, possibly due to the acceleration of autolysis, resulting in more self-destruction of the enzyme.

Just as in Exp. 1, degradation of the myofibrillar protein TnT was evaluated in Exp. 2 to determine if different combinations of temperature and rates of pH decline affected protein degradation. Figure 6 illustrates the breakdown of intact TnT as a result of different pH declines at 4 and 22°C. Representative western blot images for TnT disappearance due to differing pH declines at 4 and 22°C are found in Fig. 7 and 8, respectively. Similar to the μ-calpain activity results, the rate of initial pH decline had no significant impact on the degradation of intact TnT ($P > 0.10$). As expected, intact TnT decreased over time ($P < 0.0001$), while warmer temperatures resulted in a greater disappearance of intact TnT of isolated myofibrils incubated with μ-calpain ($P < 0.05$). Additionally, a temperature × time interaction was revealed in the proteolysis of TnT ($P < 0.01$). Less intact TnT was found in myofibrils digested at 22°C compared to digestions at 4°C at 0.33, 0.5, 1, 3, 24, and 72 h ($P < 0.05$). Although the in vitro digestions lacked temperature decline, calpastatin, and changes in ionic strength, intact TnT degradation patterns revealed during the in vitro digestions by μ-calpain appear similar to degradation patterns for TnT that was seen in postmortem muscle during Exp. 1, regardless of pH decline and temperature of the digestion. Additionally, muscle tempera-

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Figure 7. Representative western blots, prepared from a 15% resolving gel transferred to a polyvinylidene difluoride membrane, of isolated myofibrils from bovine semitendinosus muscle digested with μ-calpain at 4°C. Digestions were subjected to an initial fast pH or slow pH decline and sampled after 0, 0.17, 0.33, 0.5, 1, 3, 24, and 72 h of incubation. Blots were labeled with monoclonal anti-troponin T (JLT-12; Sigma, St. Louis, MO). REF = reference sample used across gels; Intact = location of intact troponin-T.
ture appeared to be the most influential factor in meat tenderization during Exp. 1, while temperature was also more influential to TnT degradation in the in vitro diges-
tions of myofibrils as rate of pH decline was not influen-
tial. These results would suggest that early postmortem 
muscle temperature, while influencing pH, may provide 
the largest impact on meat tenderization. Still, a better 
understanding of the relationship of temperature and pH 
with meat aging and μ-calpain activity is needed.

Implications

This study indicates that delayed chilling of beef 
carcasses does not improve meat tenderness or show im-
provements in postmortem protein degradation. There-
fore, delayed chilling is most likely not a good method 
to improve tenderness of beef carcasses. Overall, this 
research indicates that meat tenderization is strongly as-
associated with muscle temperature. However, more re-
search is necessary to fully understand the relationships 
of temperature, pH, and meat aging to more consistently 
provide a tender product for the consumer.

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