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Effect of pH and ionic strength on μ- and m-calpain inhibition by calpastatin[1]

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ABSTRACT: The objectives of this study were to determine the extent to which pH and ionic strength influence μ- and m-calpain activity and the inhibition of calpains by calpastatin. Calpastatin, μ-calpain, and m-calpain were purified from at-death porcine semimembranosus. μ-Calpain or m-calpain (0.45 U) were incubated with the calpain substrate Suc-Leu-Leu-Val-Tyr-7-amino-4-methyl coumarin in the presence of calpastatin (0, 0.15, or 0.30 U of calpain inhibitory activity) under the following pH and ionic strength conditions: pH 7.5 and 165 m*M* NaCl or 295 m*M* NaCl; pH 6.5 and 165 m*M* NaCl or 295 m*M* NaCl; and pH 6.0 and 165 m*M* NaCl or 295 m*M* NaCl. The reactions were initiated with addition of 100 μM (μ-calpain) or 1 m*M* $CaCl_2$ (m-calpain), and calpain activity was recorded at 30 and 60 min. μ-Calpain had the greatest ($P < 0.01$) activity at pH 6.5 at each ionic strength. Higher ionic strength decreased μ-calpain activity ($P < 0.01$) at all pH conditions. Inhibition percent of μ-calpain by calpastatin was not affected by pH; however, it was influenced by ionic strength. Inhibition of μ-calpain by calpastatin was higher ($P < 0.01$) at 295 m*M* NaCl than at 165 m*M* NaCl when 0.3 units of calpastatin were included in the assay. Activity of m-calpain was greater ($P < 0.01$) at pH 7.5 than at pH 6.5. m-Calpain activity was not detected at pH 6.0. Inhibition of m-calpain was greater ($P < 0.01$) when 0.15 and 0.3 U calpastatin were added at pH 6.5 than 7.5 at 165 m*M* NaCl, whereas percentage inhibition of m-calpain was greater ($P < 0.01$) at 295 m*M* than 165 m*M* NaCl at pH 7.5 and 6.5. These observations provide new evidence that defines further the influence of pH decline and increased ionic strength on μ-calpain, m-calpain, and calpastatin activity, thereby helping to more accurately define a role for these enzymes in the process of postmortem tenderization.

Key Words: Calpain, Calpastatin, Ionic Strength, Proteolysis, pH

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Introduction

Changes in the muscle intracellular environment occurring early postmortem are known to influence meat quality. One of the most pronounced changes is the pH decline from near neutral pH in living muscle to approximately 5.6 in meat. Another change that occurs is the increase in ionic strength from an approximate equivalent of 165 m*M* NaCl in living muscle to an approximate equivalent of 295 m*M* NaCl in meat (Winger and Pope, 1980-1981).

The calcium-activated proteinases, calpains, are responsible for much of the postmortem proteolysis of myofibrillar and cytoskeletal proteins. This proteolysis is responsible for the increase in tenderness observed in meat during postmortem storage (Goll et al., 1992; Koohmaraie, 1992b; Koohmaraie et al., 2002). Calpain activity is influenced by intracellular environmental factors, including calcium concentration, pH, ionic strength, and calpastatin (the endogenous inhibitor specific for calpains). Calpains have a pH optimum of 7.5 (Edmunds et al., 1991; Wang and Jiang, 1991). An increase in ionic strength has been shown to decrease μ-calpain activity by decreasing the stability of autolyzed μ-calpain (Geesink and Koohmaraie, 2000; Li et al., 2004). The effects of the combination of pH and ionic strength on calpain activity in the presence of calpastatin have not been elucidated. Therefore, the hypothesis of this study was that postmortem pH and ionic strength affect the activity of μ- and m-calpain and the inhibition of calpains by calpastatin. For this reason, the first objective of this study was to determine the extent to which pH and ionic strength influence μ-and m-calpain activity. The second objective was to determine the extent to which pH and ionic strength influence the ability of calpastatin to inhibit the activity of μ- or m-calpain.

Materials and Methods

Purification of Calpastatin, μ-Calpain, and m-Calpain

Calpastatin, μ-calpain, and m-calpain were purified from porcine skeletal muscle based on the procedures

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outlined by Thompson and Goll (2000), with minor modifications. A 2-kg porcine semimembranosus sample was taken from a market barrow approximately 25 min after exsanguination. The muscle was ground immediately and homogenized in six volumes of ice-cold buffer containing 10 m*M* EDTA, 0.1% (vol/vol) β-mercaptoethanol, and 100 m*M* Tris-HCl, pH 8.3. This buffer also included protease inhibitors (2.5 μ*M* trans-epoxysuccunyl-L-leucylamido-[4-guanidino] butane, 0.1 mg/mL of ovomucoid trypsin inhibitor, and 0.2 m*M* phenylmethylsulfonylfluoride). The homogenate was centrifuged at 9,750 × *g* at 4°C for 30 min. The supernatant fraction was filtered through cheesecloth, and proteins were salted out at between 0 and 45% ammonium sulfate saturation. Proteins were collected at 9,750 × g at 4°C for 30 min, resuspended in 1 m*M* EDTA, 0.1 % (vol/vol) β-mercaptoethanol, 40 m*M* Tris-HCl, pH 7.4 (**TEM**), stirred overnight at 4°C, and dialyzed against TEM. The sample was loaded on a Q-Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ) anion-exchange column (900 mL) that had been previously equilibrated in TEM. Using a gradient of 0 to 500 m*M* KCl in TEM (total volume 4,500 mL), porcine calpastatin, μ-calpain, and m-calpain were eluted in three separate peaks from the column (calpastatin eluted between 90 and 150 m*M* KCl, μ-calpain between 160 and 190 m*M* KCl, and m-calpain between 250 and 280 m*M* KCl).

Purification of Calpastatin

Fractions containing calpastatin activity were pooled and further purified using methods described by Thompson and Goll (2000), with modifications from Geesink and Koohmaraie (1998). The sample was heated at 100°C for 20 min, chilled on ice, and centrifuged at 9,750 × *g* at 4°C for 30 min. Calpastatin was further purified using successive chromatography over Phenyl Sepharose 6 Fast Flow (Amersham Biosciences), Blue Sepharose 6 Fast Flow (Amersham Biosciences), and EMD TMAE 650 S (EM Science, Gibbstown, NJ). Purified calpastatin consisted only of a 68-kDa band when analyzed by SDS-PAGE and had a specific activity of 365 U/mg of protein. One unit of calpastatin activity was defined as the ability to inhibit one unit of m-calpain caseinolytic activity (Koohmaraie et al., 1995).

Purification of μ- and m-Calpain

μ-Calpain and m-calpain were purified according to the methods of Thompson and Goll (2000), with minor modifications. Fractions from the Q-sepharose column containing μ-calpain activity were pooled. Pooled μ-calpain was purified using successive chromatography over a Phenyl Sepharose 6 Fast Flow (Amersham Biosciences), Butyl Sepharose 4 Fast Flow (Amersham Biosciences), EMD TMAE 650 S (EM Science), and DEAE-TSK Toyopearl (Supelco, Bellefonte, PA). Purified μ-calpain had a specific activity of 75 U/mg of protein. One unit of calpain was defined as the amount of calpain required to increase the absorbance at 278 nm of the supernatant fraction by one unit due to the release of trichloroacetic acid-soluble polypeptides resulting from the digestion of casein (Koohmaraie, 1990). Fractions from the Q-sepharose column containing m-calpain activity were pooled and purified using successive chromatography over a Phenyl Sepharose 6 Fast Flow (Amersham Biosciences), Reactive Red 120 (Sigma, St. Louis, MO), and DEAE-TSK Toyopearl (Supleco). Purified m-calpain had a specific activity of 186.4 U/mg of protein. The purified μ-calpain, m-calpain, and calpastatin were stored in TEM with the addition of 1 m*M* sodium azide at 4°C.

Calpain Activity Assays

The technique used in this experiment is a sensitive activity assay using a calpain substrate, Suc-Leu-Leu-Val-Tyr-7-amino-4-methyl coumarin (Bachem, Torrence, CA; 10 mg/mL in dimethyl sulfoxide; Sasaki et al., 1984). Although the use of this specific peptide as a substrate for μ-calpain and m-calpain allows for highly controlled experiments, it does not represent the diversity of all the potential calpain substrates. It should be noted that the substrate concentration used is quite close to the K_m for μ-calpain (Saski et al., 1984). Therefore, the reaction was occurring under first-order conditions, rather than zero-order conditions that occur with protein substrates. The K_m of protein substrates for calpains tends to be in the low micromolar ranges (Goll et al., 2003). Highly purified calpains (μ- or m-calpain; 0.45 U of caseinolytic activity) were incubated with 170 μ*M* Suc-Leu-Leu-Val-Tyr-7-amino-4-methyl coumarin in the presence of either highly purified calpastatin (0.15 or 0.30 U measured against 0.45 U of m-calpain caseinolytic activity) or 0 U of calpastatin under the following conditions: 1) pH 7.5, 165 m*M* NaCl; 2) pH 7.5, 295 m*M* NaCl; 3) pH 6.5, 165 m*M* NaCl; 4) pH 6.5, 295 m*M* NaCl; 5) pH 6.0, 165 m*M* NaCl; or 6) pH 6.0, 295 m*M* NaCl. The buffers were either 50 m*M* HEPES (pH 7.5 and 6.5) or 50 m*M* 2-(4-morpholino)-ethane sulfonic acid (pH 6.0). Dithiothreitol (1 m*M* final concentration) was added before the addition of $CaCl_2$ to ensure fully reduced conditions. Reactions were initiated with the addition of 100 μ*M* $CaCl_2$ (μ-calpain) or 1 m*M* $CaCl_2$ (m-calpain). Three replications of each pH, ionic strength, and calpastatin combination were conducted. Calpain activity and inhibition percent by calpastatin (percentage of inhibition = 1 – [calpain activity without calpastatin/calpain activity with calpastatin] × 100) were measured at regular intervals from 0 (immediately before the addition of $CaCl_2$) to 60 min in a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Standard curves were generated for each experiment using 7-amino-4-methyl coumarin of known concentrations. A control with $CaCl_2$ (without the addition of calpain) for each pH and ionic strength

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Table 1. Least squares means (±SE) of μ-calpain activity at 30 and 60 min after addition of $CaCl_2$

	Activity[a]			
	165 m*M* NaCl		295 m*M* NaCl	
Treatment	30 min	60 min	30 min	60 min
pH 7.5				
0 U calpastatin	94.21 ± 2.50[cx]	152.53 ± 1.54[cw]	55.55 ± 2.51[dz]	86.74 ± 1.54[dy]
0.15 U calpastatin	62.67 ± 1.05[dx]	113.79 ± 4.61[dw]	38.05 ± 1.96[ez]	58.40 ± 4.61[fy]
0.30 U calpastatin	44.99 ± 1.84[ex]	79.83 ± 1.65[ew]	21.00 ± 0.63[fz]	35.50 ± 1.65[gy]
pH 6.5				
0 U calpastatin	131.65 ± 6.79[by]	208.18 ± 8.87[bw]	100.62 ± 3.79[bz]	166.19 ± 7.20[bx]
0.15 U calpastatin	87.01 ± 5.69[cy]	146.95 ± 7.51[cw]	69.19 ± 6.03[cz]	114.12 ± 7.11[cx]
0.30 U calpastatin	60.44 ± 1.87[dy]	112.38 ± 4.16[dw]	33.29 ± 2.33[ez]	72.59 ± 2.96[ex]
pH 6.0				
0 U calpastatin	46.47 ± 3.20[ex]	68.12 ± 4.48[fw]	18.35 ± 1.63[fz]	27.79 ± 1.63[hy]
0.15 U calpastatin	29.91 ± 2.50[fx]	49.39 ± 2.79[gw]	12.28 ± 0.92[gz]	19.54 ± 0.92[iy]
0.30 U calpastatin	24.08 ± 0.39[gx]	38.68 ± 0.61[hw]	6.91 ± 1.34[hz]	12.30 ± 1.34[jy]

[a]Activity = fluorescence units with $CaCl_2$ minus fluorescence units with EDTA (n = 3).
[b,c,d,e,f,g,h,i,j]Within a column, least squares means that do not have a common superscript differ, $P < 0.01$.
[w,x,y,z]Within a row, least squares means that do not have a common superscript differ, $P < 0.05$.

condition was conducted to determine whether $CaCl_2$ affected the fluorescence of the peptide. An EDTA control (20 m*M* EDTA final concentration added before addition of calpain and $CaCl_2$) was included for each pH and ionic strength condition and used for the baseline.

To ensure that pH and ionic strength did not directly influence the peptide, assays were also conducted with the enzyme carboxypeptidase Y (Calbiochem, La Jolla, CA). Carboxypeptidase Y has a pH optimum of pH 5.5 to 6.5, stability up to pH 8.0, and Suc-Leu-Leu-Val-Tyr-7-amino-4-methyl coumarin is a substrate for this enzyme (Stennicke et al., 1994). In these assays, carboxypeptidase Y was added in place of μ- or m-calpain at 0.5 μL (0.0045 μM final concentration). Comparison of activity measured in the assays at pH 7.5, 6.5, and 6.0, as well as ionic strength of 165 m*M* NaCl and 295 m*M* NaCl, was done to determine whether these environmental conditions changed the susceptibility of the peptide to proteolysis.

SDS-PAGE Gel System and Western Blotting

Samples used for Western blotting of μ-calpain autolysis were prepared by removing 20 μL from the fluorescence assay (pH 7.5, 6.5, and 6.0; 165 m*M* NaCl; 0 units calpastatin) at 5-min increments for 25 min. This experiment included two replications. Each aliquot was added to 10 μL of sample buffer tracking dye solution (3 m*M* EDTA, 3% SDS, 20% glycerol, 0.003% pyronin Y, and 30 m*M* Tris-HCl, pH 8.0 (Wang, 1982), and 0.1% β-mercaptoethanol. Gel samples were heated at 50°C for 20 min, and subsequently frozen to –80°C until analysis. Samples were fractionated using 9% polyacrylamide separating gels and transferred to a membrane (Melody et al., 2004). A sensitive chemiluminescent system (ECL Plus kit, Amersham Biosciences) was used to detect labeled protein bands using a charged coupled device camera (FluorChem 8800, Alpha Innotech Corp., San Leandro, CA) and FluorChem IS-800 software (Alpha Innotech Corp.).

Statistical Analyses

Data were analyzed using a split-split-plot design. The whole plot was pH 7.5, 6.5, or 6.0. The split plot was ionic strength (165 or 295 m*M* NaCl). The split-split plot was calpastatin (0, 0.15, or 0.30 units). The experimental unit was each cuvette placed in the fluorometer. Calpain activity at 30 and 60 min was analyzed using PROC MIXED of SAS (SAS Inst., Inc., Cary, NC). Least squares means were separated using tests of effect slices, and significance was defined as $P < 0.05$.

Results and Discussion

Carboxypeptidase Y Activity Control

Ionic strength and pH conditions used in these experiments did not alter susceptibility of the calpain substrate to proteolysis. Fluorescence at pH 7.5 and 165 m*M* NaCl was defined as 100% of carboxypeptidase Y activity. Fluorescence at pH 6.5 and 165 m*M* NaCl was 97.2% of activity; pH 6.0 and 165 m*M* NaCl was 99.2%; pH 7.5 and 295 m*M* NaCl was 100.3%; pH 6.5 and 295 m*M* NaCl was 97.3%; and pH 6.0 and 295 m*M* NaCl was 99.7% after 60 min. It was concluded that the calpain substrate was not affected ($P > 0.50$) by the different pH and ionic strength conditions and was appropriate to use for our study.

μ-Calpain Activity

At both 165 and 295 m*M* NaCl, μ-calpain activity was greater ($P < 0.05$) at pH 6.5 than at pH 7.5 or 6.0 (Table 1). The slope of the rate of hydrolysis of the

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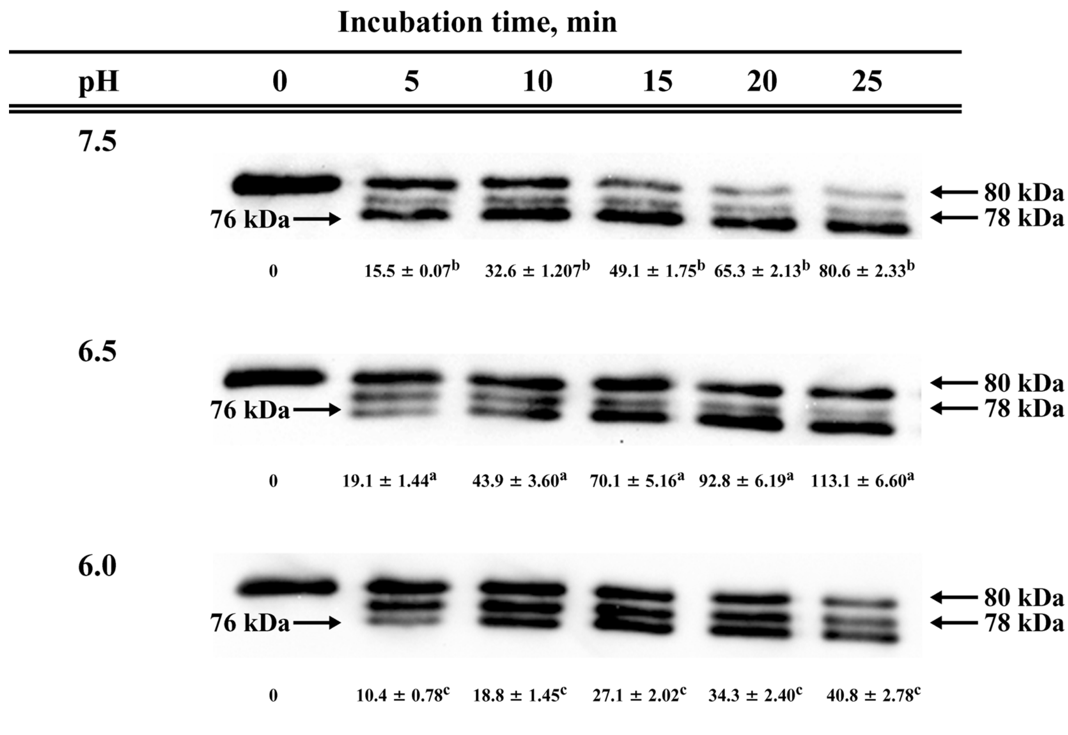

Figure 1. Western blots of purified μ-calpain using monoclonal mouse anti-μ-calpain antibody (catalog No. MA3-940; Affinity BioReagents, Golden, CO). Samples were acquired from activity assays in pH conditions of 7.5, 6.5, and 6.0 at 165 m*M* NaCl. Lane 1 shows μ-calpain before the addition of 100 μ*M* $CaCl_2$ indicated by incubation time = 0 min. Lanes 2 through 5 show samples acquired from the fluorescent assays at 5-min intervals after the addition of $CaCl_2$ from 5 to 25 min. The uppermost band is the unautolyzed 80-kDa subunit, the second band is the autolyzed 78-kDa subunit, and the third band is the autolyzed 76-kDa subunit. To compare autolysis with activity, the number under each lane is the least squares mean (n = 3) of measurements taken from the activity assays at each time point. [a,b,c]Within a column, least squares mean that do not have a common superscript differ, $P < 0.05$.

peptide by μ-calpain during the first 5 min indicates that, at pH 6.5, μ-calpain was more active than at pH 7.5. This is consistent with the results of Geesink and Koohmaraie (2000), who reported that autolyzed porcine skeletal μ-calpain activity was more stable (indicated by greater activity) at pH values of 6.2 to 6.4 when evaluating pH ranges from 7.6 to 5.6. To understand the mechanism underlying the greater activity of μ-calpain at pH 6.5, μ-calpain autolysis was examined using Western blots. Appearance of the 78- and 76-kDa immunoreactive bands (Figure 1) indicated that autolysis occurred in all pH conditions; however, the rate of autolysis within the first 5 min was slowest at pH 6.5. μ-Calpain was almost completely autolyzed by 20 min, as shown by the less intense 80-kDa band at pH 7.5 than in samples incubated at pH 6.5. In contrast, the intact 80-kDa band of μ-calpain in the pH 6.5 incubation was still relatively more intense at 25 min, indicating less autolysis had occurred during the entire 25-min incubation. The more limited autolysis at pH 6.5 most likely contributed to the greater μ-calpain activity remaining at 30 and 60 min compared with the other pH incubations. This is important because autolysis of μ-calpain has been shown to lead to a loss of activity (Edmunds et al., 1991; Koohmaraie, 1992a). Geesink and Koohmaraie (2000) determined that μ-calpain had approximately 70% of initial activity after incubation for 30 min at pH 6.2 and approximately 22% of initial activity remaining at pH 7.5. In the current study, μ-calpain autolyzed more rapidly at pH 7.5, which resulted in less active μ-calpain present in the assay to degrade the peptide at the later time points.

Autolysis observed at pH 6.0 shows greater intensity of the 78-kDa autolysis product. Very little 78-kDa autolysis product was detected at pH 7.5 or 6.5 at all time points. This contrast indicates that pH affects the accumulation of the 78-kDa autolysis product. Koohmaraie et al. (1986) observed that, under conditions of pH 5.5 to 5.8 at 5°C, μ-calpain had 24 to 28% of its activity after 90 min at pH 7.5 at 25°C, indicating less proteolytic activity at a lower pH. A rapid pH decline in postmortem muscle also decreases μ-calpain activity (Claeys et al., 2001) and has been shown to decrease the degradation of troponin-T (Lonergan et al., 2001). Melody et al. (2004) observed that porcine muscles with low early postmortem pH (psoas major) had earlier μ-calpain autolysis and inactivation and earlier degradation of desmin than muscles with higher early postmortem pH (LM and semimembranosus). Additionally, moderate rates of postmortem pH decline (pH of 5.8 to

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Table 2. Least squares means (±SE) of m-calpain activity taken at 30 and 60 min after addition of $CaCl_2$

Treatment	Activity[a] 165 m*M* NaCl 30 min	165 m*M* NaCl 60 min	295 m*M* NaCl 30 min	295 m*M* NaCl 60 min
pH 7.5				
0 U calpastatin	98.12 ± 2.98[bx]	120.74 ± 6.85[bw]	15.95 ± 0.50[bz]	19.63 ± 1.05[by]
0.15 U calpastatin	72.92 ± 2.64[cy]	88.71 ± 3.19[cx]	8.87 ± 0.14[cz]	10.23 ± 0.23[cz]
0.30 U calpastatin	50.11 ± 1.01[dy]	61.32 ± 1.74[dx]	4.90 ± 0.64[dz]	5.07 ± 0.76[dz]
pH 6.5				
0 U calpastatin	36.37 ± 0.52[ex]	58.45 ± 2.15[dw]	5.10 ± 0.25[dz]	7.66 ± 0.41[ey]
0.15 U calpastatin	22.92 ± 1.39[fy]	37.59 ± 2.81[ex]	1.97 ± 0.24[ez]	2.84 ± 0.06[fz]
0.30 U calpastatin	10.56 ± 1.96[gy]	20.89 ± 1.66[fx]	1.25 ± 0.08[ey]	1.85 ± 0.07[fy]

[a]Activity= fluorescence units with $CaCl_2$ minus fluorescence units with EDTA (n = 3).
[b,c,d,e,f,g]Within a column, least squares means that do not have a common superscript differ, $P < 0.01$.
[w,x,y,z]Within a row, least squares means that do not have a common superscript differ, $P < 0.05$.

6.2 at 3 h) have been shown to produce the most tender beef loin steaks, whereas rapid rates (pH of 5.5 at 3 h) and slow rates (pH 6.8 at 3 h) of postmortem glycolysis produced less tender meat (Marsh et al., 1987). The novel observations in the current study indicated greater μ-calpain activity at the intermediate pH (6.5) than pH 7.5 and 6.0 could help explain the tenderization effects reported by Marsh et al. (1987). If the pH decline is rapid, μ-calpain activity is diminished due to the lower pH. If postmortem glycolysis is slow and the pH does not decrease as rapidly, μ-calpain may autolyze earlier postmortem, thereby losing proteolytic activity earlier and not allowing for maximal proteolysis. Thus, intermediate pH decline allows more proteolysis and slower completion of autolysis, thereby ultimately allowing for greater postmortem protein degradation and increased tenderization.

μ-Calpain was more active ($P < 0.01$) at 165 m*M* NaCl than at 295 m*M* NaCl under all experimental conditions. Thus, it is expected that μ-calpain activity will be decreased as intracellular ionic strength increases in postmortem muscle. Geesink and Koohmaraie (2000) also demonstrated that increased ionic strength decreased the activity and stability at concentrations of NaCl as low as 100 m*M*. Likewise, Li et al. (2004) observed irreversible loss of autolyzed μ-calpain activity at salt concentrations as low as 100 m*M* KCl at pH 7.5. They determined that ionic strengths of 300 m*M* or greater decreased autolyzed μ-calpain activity by 50 to 55% within 5 min. The rate of activity loss was slower at lower ionic strengths (Li et al., 2004).

m-Calpain Activity

In contrast to μ-calpain, m-calpain activity was greater ($P < 0.05$) at pH 7.5 than pH 6.5 (Table 2). Dayton et al. (1976) and Edmunds et al. (1991) determined the optimum pH of porcine muscle m-calpain to be 7.5. In the current study, no measurable activity of m-calpain was observed at pH 6.0 at either ionic strength. m-Calpain was more active ($P < 0.01$) at 165 m*M* NaCl than 295 m*M* NaCl (Table 2). This ionic strength effect was greater than the differences observed for μ-calpain. At pH 7.5, the increase in ionic strength decreased μ- and m-calpain activity by 43.1 and 83.7%, respectively. Li et al. (2004) observed similar effects of ionic strength on autolyzed m-calpain, and they hypothesized an ionic strength equal to 100 m*M* KCl or greater caused disassociation of the two m-calpain subunits, allowing for inactivation of the proteinase due to the formation of dimers and trimers of the large subunit.

The activities of μ- and m-calpain observed with regard to pH may be evidence for a difference in the roles of μ- and m-calpain in postmortem muscle. It has been hypothesized that μ-calpain is the protease primarily responsible for the postmortem degradation of myofibrillar and cytoskeletal proteins resulting in tenderization of meat, whereas the role of m-calpain may be limited based on its high calcium requirement. The results from this study also indicate a more important role for μ-calpain in postmortem muscle, as μ-calpain activity was measured at pH 6.0, whereas no m-calpain activity was detected. Additionally, although the higher ionic strength decreased μ-calpain activity, m-calpain activity was diminished to a greater extent by the increase in ionic strength.

μ-Calpain Inhibition by Calpastatin

In porcine muscle, the ratio of calpastatin activity to μ-calpain activity is approximately 1.5:1 (Ouali and Talmant, 1990). In the current study, the ratio of calpastatin to calpain added to the assays was 1:3 and 2:3 to evaluate the effect of calpastatin on calpain without the occurrence of complete inhibition. Calpastatin inhibited ($P < 0.001$) μ-calpain activity (Table 3), but inhibition percent was not altered by pH. This finding is consistent with results observed by Geesink and Koohmaraie (1999). Inhibition percent of μ-calpain by calpastatin was greater at 295 than at 165 m*M* NaCl at pH 7.5, 6.5, and 6.0 when the higher level of calpas-

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Table 3. Least squares means (±SE) of percent inhibition of μ-calpain by 0.15 and 0.3 units of calpastatin calculated from μ-calpain activity measured at 30 and 60 min after the addition of $CaCl_2$[a]

	Percent inhibition			
	165 m*M* NaCl		295 m*M* NaCl	
Treatment	30 min	60 min	30 min	60 min
pH 7.5				
0.15 U calpastatin	33.48 ± 1.12[cy]	25.39 ± 1.59[cz]	40.93 ± 3.53[dy]	32.67 ± 5.31[cz]
0.30 U calpastatin	52.25 ± 1.96[by]	47.66 ± 2.96[bz]	75.58 ± 1.14[bw]	58.98 ± 1.91[bx]
pH 6.5				
0.15 U calpastatin	33.90 ± 4.32[cy]	29.41 ± 3.61[cy]	31.24 ± 6.00[cy]	31.33 ± 4.27[cy]
0.30 U calpastatin	54.09 ± 1.42[bx]	46.02 ± 2.00[by]	66.92 ± 2.32[bw]	56.32 ± 1.77[bx]
pH 6.0				
0.15 U calpastatin	35.63 ± 5.37[cx]	27.49 ± 4.10[cy]	33.11 ± 2.87[cx]	29.70 ± 3.32[cy]
0.30 U calpastatin	48.19 ± 1.41[bz]	43.21 ± 0.89[bz]	62.35 ± 0.81[bx]	55.74 ± 4.83[by]

[a]Inhibition was calculated as the percent inhibition = 1 - (calpain activity without calpastatin/calpain activity with calpastatin); n = 3.
[b,c]Within a column, least squares means that do not have a common superscript differ, $P < 0.01$.
[x,y,z]Within a row, least squares means that do not have a common superscript differ, $P < 0.05$.

tatin (0.3 U) was added to the assay. An increase in ionic strength in postmortem muscle was predicted to increase the inhibitory efficiency of calpastatin; however, there were no significant differences in inhibition percent between 165 and 295 m*M* NaCl when 0.15 units of calpastatin were added to the assay. In general, the inhibition percent is numerically greater at 295 than 165 m*M* NaCl when the lower level of calpastatin is present. The lack of significance could be due to the higher variance of inhibition percent between replications in the assays. The observation that calpastatin is a substrate for calpain (Mellgren et al., 1986; Doumit and Koohmarie, 1999) may contribute to this variation.

m-Calpain Inhibition by Calpastatin

Inhibition of m-calpain by calpastatin was greater ($P < 0.01$) at pH 6.5 than 7.5 at both 165 and 295 m*M* NaCl (Table 4). Otsuka and Goll (1987) determined that calpastatin had a broad optimal pH, and concluded that inhibition of m-calpain by calpastatin was not affected by pH when casein was a substrate. Differences in results between these two studies could be due to differences in substrates used. As mentioned previously, the proteolytic susceptibility of the peptide used as a substrate in this study was not affected by pH or ionic strength, whereas pH may affect the solubility of casein used to determine calpain activity because the isoelectric point of casein is 4.6. In addition, the lowest ionic strength condition used in the current study was 165 m*M* NaCl. In the Otsuka and Goll (1987) study, the ionic strength conditions used were much lower. In the current study, inhibition of m-calpain was greater at 295 than at 165 m*M* NaCl at both 30 and 60 min and with the addition of both 0.15 and 0.3 U of calpastatin. The high inhibition percent of m-calpain by calpastatin at the lower pH and higher ionic strength provides further evidence against a role for m-calpain in postmor-

Table 4. Least squares means (±SE) of percent inhibition of m-calpain by 0.15 and 0.3 units of calpastatin calculated from m-calpain activity at 30 and 60 min after the addition of $CaCl_2$[a]

	Percent inhibition			
	165 m*M* NaCl		295 m*M* NaCl	
Treatment	30 min	60 min	30 min	60 min
pH 7.5				
0.15 U calpastatin	25.68 ± 2.69[cz]	26.53 ± 2.63[ez]	44.36 ± 0.89[ey]	47.89 ± 1.17[dx]
0.30 U calpastatin	48.92 ± 1.78[fz]	49.21 ± 1.44[cz]	69.29 ± 4.07[cy]	74.17 ± 3.88[bx]
pH 6.5				
0.15 U calpastatin	36.98 ± 3.83[ez]	35.70 ± 4.81[dz]	61.37 ± 4.65[dy]	62.92 ± 0.78[cy]
0.30 U calpastatin	70.92 ± 5.36[by]	64.26 ± 2.83[bz]	75.49 ± 1.52[bw]	75.85 ± 0.92[bw]

[a]Inhibition was calculated as the percent inhibition = 1 - (calpain activity without calpastatin/calpain activity with calpastatin); n = 3.
[b,c,d,e]Within a column, least squares means that do not have a common superscript differ, $P < 0.01$.
[x,y,z]Within a row, least squares means that do not have a common superscript differ, $P < 0.05$.

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tem muscle. The observations made for μ-calpain inhibition were different, in that μ-calpain inhibition was not as great as m-calpain inhibition at 295 m*M* NaCl and μ-calpain was not affected by pH, indicating that μ-calpain could be more active at conditions found in postmortem muscle.

In early postmortem muscle, μ-calpain inactivation, in response to either a rapid pH decline or by rapid autolysis, has the potential to decrease proteolysis of myofibrillar proteins and subsequent postmortem tenderization. However, intermediate pH decline allows for proteolytic activity of μ-calpain, but a slower rate of autolysis could explain a portion of the variation in meat tenderness. A major role for m-calpain in postmortem proteolysis is not supported because the high ionic strength and low pH conditions, similar to conditions observed in postmortem muscle, seem to limit m-calpain activity. Additionally, the inhibition of μ-calpain and m-calpain by calpastatin was greater at the higher ionic strength, indicating that calpastatin inhibits calpains to a greater extent in conditions similar to meat than in conditions found in living muscle.

Implications

Rate of pH decline in postmortem muscle has the potential to affect the rates of activation and subsequent autolytic inactivation of μ-calpain. In addition, an increase in ionic strength in postmortem muscle may allow for more efficient inhibition of calpains by calpastatin. Therefore, rates of pH decline and increases in ionic strength are important variables to be considered when examining the variations in calpain-induced proteolysis of meat proteins in postmortem muscle. Studies centered on the question of how intracellular environmental factors affect μ- and m-calpain will lead to a greater understanding of the role of calpain and calpastatin in the conversion of muscle to meat.

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