

6-2005

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

K. R. Maddock
Iowa State University

Elisabeth J. Huff-Lonergan
Iowa State University, elonerga@iastate.edu

L. J. Rowe
Iowa State University

Steven M. Lonergan
Iowa State University, slonerga@iastate.edu

Follow this and additional works at: http://lib.dr.iastate.edu/ans_pubs



Part of the [Agriculture Commons](#), and the [Meat Science Commons](#)

The complete bibliographic information for this item can be found at http://lib.dr.iastate.edu/ans_pubs/25. For information on how to cite this item, please visit <http://lib.dr.iastate.edu/howtocite.html>.

This Article is brought to you for free and open access by the Animal Science at Iowa State University Digital Repository. It has been accepted for inclusion in Animal Science Publications by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

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

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 mM NaCl or 295 mM NaCl; pH 6.5 and 165 mM NaCl or 295 mM NaCl; and pH 6.0 and 165 mM NaCl or 295 mM NaCl. The reactions were initiated with addition of 100 μ M (μ -calpain) or 1 mM 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 mM NaCl than at 165 mM 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 mM NaCl, whereas percentage inhibition of m-calpain was greater ($P < 0.01$) at 295 mM than 165 mM 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.

Keywords

calpain, calpastatin, ionic strength, proteolysis, pH

Disciplines

Agriculture | Animal Sciences | Meat Science

Comments

This article is from *Journal of Animal Science* 83 (2005): 1370–1376. Posted with permission.

JOURNAL OF ANIMAL SCIENCE

The Premier Journal and Leading Source of New Knowledge and Perspective in Animal Science

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

K. R. Maddock, E. Huff-Lonergan, L. J. Rowe and S. M. Lonergan

J ANIM SCI 2005, 83:1370-1376.

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://www.journalofanimalscience.org/content/83/6/1370>



American Society of Animal Science

www.asas.org

Effect of pH and ionic strength on μ - and m-calpain inhibition by calpastatin¹

K. R. Maddock, E. Huff-Lonerger, L. J. Rowe, and S. M. Lonergan²

Department of Animal Science, Iowa State University, Ames 50011

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 mM NaCl or 295 mM NaCl; pH 6.5 and 165 mM NaCl or 295 mM NaCl; and pH 6.0 and 165 mM NaCl or 295 mM NaCl. The reactions were initiated with addition of 100 μ M (μ -calpain) or 1 mM CaCl₂ (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 mM NaCl than at 165 mM 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 mM NaCl, whereas percentage inhibition of m-calpain was greater ($P < 0.01$) at 295 mM than 165 mM 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

©2005 American Society of Animal Science. All rights reserved.

J. Anim. Sci. 2005. 83:1370–1376

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 mM NaCl in living muscle to an approximate equivalent of 295 mM 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

¹This journal paper of the Iowa Agric. and Home Econ. Exp. Stn., Ames, Project No. 3700, was supported by Hatch Act and State of Iowa funds and USDA National Research Initiative Competitive Grants Program Project No. 2000-01705 and 2003-01484.

²Correspondence: 2372 Kildee Hall (phone: 515-294-9126; fax: 515-294-9143; e-mail: slonerga@iastate.edu).

Received May 20, 2004.

Accepted March 18, 2005.

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 mM EDTA, 0.1% (vol/vol) β -mercaptoethanol, and 100 mM Tris-HCl, pH 8.3. This buffer also included protease inhibitors (2.5 μ M trans-epoxysuccinyl-L-leucylamido-[4-guanidino] butane, 0.1 mg/mL of ovomucoid trypsin inhibitor, and 0.2 mM phenylmethylsulfonylfluoride). The homogenate was centrifuged at $9,750 \times g$ 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 $9,750 \times g$ at 4°C for 30 min, resuspended in 1 mM EDTA, 0.1% (vol/vol) β -mercaptoethanol, 40 mM 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 mM 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 mM KCl, μ -calpain between 160 and 190 mM KCl, and m-calpain between 250 and 280 mM 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 $9,750 \times g$ 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 (Supelco). 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 mM 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, Torrance, 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 (Sasaki 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 mM NaCl; 2) pH 7.5, 295 mM NaCl; 3) pH 6.5, 165 mM NaCl; 4) pH 6.5, 295 mM NaCl; 5) pH 6.0, 165 mM NaCl; or 6) pH 6.0, 295 mM NaCl. The buffers were either 50 mM HEPES (pH 7.5 and 6.5) or 50 mM 2-(4-morpholino)ethane sulfonic acid (pH 6.0). Dithiothreitol (1 mM 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 mM 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 = $1 - [\text{calpain activity without calpastatin} / \text{calpain activity with calpastatin}] \times 100$) 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

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

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

^aActivity = 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 mM 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 mM NaCl and 295 mM 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 mM 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 mM EDTA, 3% SDS, 20% glycerol, 0.003% pyronin Y, and 30 mM 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 mM 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 mM NaCl was defined as 100% of carboxypeptidase Y activity. Fluorescence at pH 6.5 and 165 mM NaCl was 97.2% of activity; pH 6.0 and 165 mM NaCl was 99.2%; pH 7.5 and 295 mM NaCl was 100.3%; pH 6.5 and 295 mM NaCl was 97.3%; and pH 6.0 and 295 mM 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 mM 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

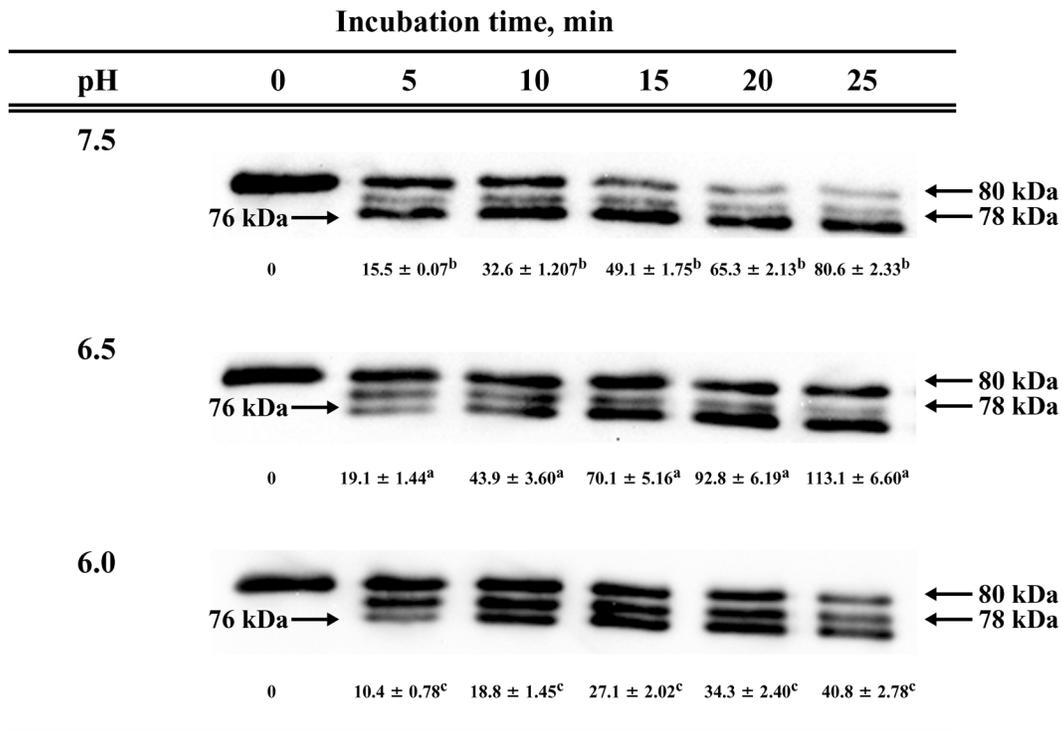


Figure 1. Western blots of purified μ -calpain using monoclonal mouse anti- μ -calpain antibody (catalog No. MA3-940; Affinity BioReagents, Golden, CO). Samples were acquired from activity assays in pH conditions of 7.5, 6.5, and 6.0 at 165 mM NaCl. Lane 1 shows μ -calpain before the addition of 100 μ M CaCl₂ 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 CaCl₂ 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

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

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

^aActivity= 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 mM NaCl than at 295 mM 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 Koohmariaie (2000) also demonstrated that increased ionic strength decreased the activity and stability at concentrations of NaCl as low as 100 mM. Likewise, Li et al. (2004) observed irreversible loss of autolyzed μ -calpain activity at salt concentrations as low as 100 mM KCl at pH 7.5. They determined that ionic strengths of 300 mM 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

mM NaCl than 295 mM 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 mM 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 Koohmariaie (1999). Inhibition percent of μ -calpain by calpastatin was greater at 295 than at 165 mM NaCl at pH 7.5, 6.5, and 6.0 when the higher level of calpas-

Table 3. Least squares means (\pm 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

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

^aInhibition 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 mM NaCl when 0.15 units of calpastatin were added to the assay. In general, the inhibition percent is numerically greater at 295 than 165 mM 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 mM 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 mM 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 mM 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 (\pm 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

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

^aInhibition 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, $P < 0.05$.

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 mM 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.

Literature Cited

- Claeys, E., S. DeSmet, D. Demeyer, R. Geers, and N. Buys. 2001. Effect of rate of pH decline on muscle enzyme activities in two pig lines. *Meat Sci.* 57:257–263.
- Dayton, W. R., D. E. Goll, M. G. Zeece, R. M. Robson, and W. J. Reville. 1976. A Ca^{2+} protease possibly involved in myofibrillar protein turnover. Purification from porcine muscle. *Biochemistry* 15:2159–2167.
- Doumit, M. E., and M. Koohmaraie. 1999. Immunoblot analysis of calpastatin degradation: Evidence for cleavage by calpain in postmortem muscle. *J. Anim. Sci.* 77:1467–1473.
- Edmunds, T., P. A. Nagainis, S. K. Sathe, V. F. Thompson, and D. E. Goll. 1991. Comparison of the autolyzed and unautolyzed forms of μ - and m-calpain from bovine skeletal muscle. *Biochim. Biophys. Acta* 1077:197–208.
- Geesink, G. H., and M. Koohmaraie. 1998. An improved purification protocol for heart and skeletal muscle calpastatin reveals two isoforms resulting from alternative splicing. *Arch. Biochem. Biophys.* 356:19–24.
- Geesink, G. H., and M. Koohmaraie. 1999. Effect of calpastatin on degradation of myofibrillar proteins by μ -calpain under postmortem conditions. *J. Anim. Sci.* 77:2685–2692.
- Geesink, G. H., and M. Koohmaraie. 2000. Ionic strength-induced inactivation of μ -calpain in postmortem muscle. *J. Anim. Sci.* 78:2336–2343.
- Goll, D. E., R. G. Taylor, J. A. Christiansen, and V. F. Thompson. 1992. Role of proteinases and protein turnover in muscle growth and meat quality. Pages 25–36 in *Proc. 44th Annu. Recip. Meat Conf. Natl. Livest. and Meat Board, Chicago, IL.*
- Goll, D. E., V. F. Thompson, H. Li, W. Wei, and J. Cong. 2003. The calpain system. *Physiol. Rev.* 83:731–801.
- Koohmaraie, M. 1990. Quantification of Ca^{2+} -dependent protease activities by hydrophobic and ion-exchange chromatography. *J. Anim. Sci.* 68:659–665.
- Koohmaraie, M. 1992a. Effect of pH, temperature, and inhibitors on autolysis and catalytic activity of bovine skeletal muscle μ -calpain. *J. Anim. Sci.* 70:3071–3080.
- Koohmaraie, M. 1992b. The role of Ca^{2+} -dependent proteases (calpains) in postmortem proteolysis and meat tenderness. *Biochimie* 74:239–245.
- Koohmaraie, M., M. P. Kent, S. D. Shackelford, E. Veiseth, and T. L. Wheeler. 2002. Meat tenderness and muscle growth: Is there any relationship? *Meat Sci.* 62:345–352.
- Koohmaraie, M., J. E. Schollmeyer, and T. R. Dutson. 1986. Effect of low calcium requiring calcium activated factor on myofibrils under varying pH and temperature. *J. Food Sci.* 51:28–32.
- Koohmaraie, M., S. D. Shackelford, T. L. Wheeler, S. M. Lonergan, and M. E. Doumit. 1995. A muscle hypertrophy condition in lamb (callipyge): Characterization of effects on muscle growth and meat quality traits. *J. Anim. Sci.* 73:3596–3607.
- Li, H., V. F. Thompson, and D. E. Goll. 2004. Effects of autolysis on properties of μ - and m-calpain. *Biochim. Biophys. Acta* 1691:91–103.
- Lonergan, S. M., E. Huff-Lonergan, L. J. Rowe, D. L. Kuhlers, and S. B. Jungst. 2001. Selection for lean growth efficiency in Duroc pigs influences pork quality. *J. Anim. Sci.* 79:2075–2085.
- Marsh, B. B., T. P. Ringkob, R. L. Russell, D. R. Schwartz, and L. A. Pagel. 1987. Effects of early post-mortem glycolytic rate on beef tenderness. *Meat Sci.* 21:241–248.
- Mellgren, R. L., M. T. Mericle, and R. D. Lane. 1986. Proteolysis of calcium-dependent protease inhibitor by myocardial calcium-dependent protease. *Arch. Biochem. Biophys.* 246:233–239.
- Melody, J. L., S. M. Lonergan, L. J. Rowe, T. W. Huiatt, M. S. Mayes, and E. Huff-Lonergan. 2004. Early postmortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles. *J. Anim. Sci.* 82:1195–1205.
- Otsuka, Y., and D. E. Goll. 1987. Purification of the Ca^{2+} -dependent proteinases inhibitor from bovine cardiac muscle and its interaction the millimolar Ca^{2+} -dependent proteinases. *J. Biol. Chem.* 262:5839–5851.
- Ouali, A., and A. Talmant. 1990. Calpains and calpastatin distribution in bovine, porcine, and ovine skeletal muscle. *Meat Sci.* 28:331–348.
- Sasaki, T., T. Kikuchi, N. Yumoto, N. Yoshimura, and T. Murachi. 1984. Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. *J. Biol. Chem.* 259:12489–12494.
- Stennicke, H. R., U. H. Mortensen, U. Christensen, S. J. Remington, and K. Breddam. 1994. Effects of introduced aspartic and glutamic acid residues on the P_i substrate specificity, pH dependence, and stability of carboxypeptidase Y. *Protein Eng.* 7:911–916.
- Thompson, V. F., and D. E. Goll. 2000. Purification of μ -calpain, m-calpain, and calpastatin from animal tissues. *Methods Mol. Biol.* 144:3–16.
- Wang, J. H., and S. T. Jiang. 1991. Properties of calpain II from *Tilapia* muscle (*Tilapia nilotica* \times *Tilapia aurea*). *Agric. Biol. Chem.* 55:339–345.
- Winger, R. J., and C. G. Pope. 1980–81. Osmotic properties of post-rigor beef muscle. *Meat Sci.* 5:355–369.

References

This article cites 26 articles, 12 of which you can access for free at:
<http://www.journalofanimalscience.org/content/83/6/1370#BIBL>

Citations

This article has been cited by 9 HighWire-hosted articles:
<http://www.journalofanimalscience.org/content/83/6/1370#otherarticles>