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# Effect of extended aging on calpain-1 and -2 activity in beef *longissimus lumborum* and *semimembranosus* muscles

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ABSTRACT

Our objectives were to (Exp. 1) determine the effect of postmortem aging (2, 3, 4, 14, 28, and 42 days) on calpain-1 and -2 activity in beef *longissimus lumborum* (LL) and *semimembranosus* (SM) steaks and (Exp. 2) determine the effect of postmortem aging for two extended periods (63 and 84 days) on calpain-2 activity of beef SM steaks. Calpain-1 was not active in either muscle following 14 days of aging. Native calpain-2 activity decreased ($P < 0.001$) with longer aging periods for both the LL and SM in Exp. 1 and for the SM in Exp. 2. Autolyzed calpain-2 activity increased ($P < 0.001$) with longer aging for the LL and SM in Exp. 1 and for the SM in Exp. 2. Our results indicate that both calpain-1 and calpain-2 may contribute to the postmortem improvement of beef tenderness, with calpain-1 being responsible for the tenderness improvement early postmortem and calpain-2 responsible for additional tenderization during extended aging.

## 1. Introduction

The 2010/2011 National Beef Tenderness Survey revealed that post-fabrication aging times for subprimal cuts in cold storage facilities ranged from 1 to 358 days and 9 to 67 days for retail and foodservice subprimals, respectively (Guelker et al., 2013). We recently found that consumer perception of *semimembranosus* (SM) tenderness was greater after 42 days of aging than after 14 days (Colle et al., 2016), while *longissimus lumborum* (LL) tenderness did not improve after 14 days of aging (Colle et al., 2015). Since the calpain system is believed to be involved in postmortem tenderness improvement (Goll, Thompson, Taylor, & Ouali, 1998; Koohmaraie, 1988, 1992; Pringle, Williams, Lamb, Johnson, & West, 1997), proteolysis of myofibrillar proteins during extended aging by calpain-2 may explain the improved tenderness of SM steaks.

Calpain proteases are enzymes that breakdown myofibrillar proteins. The calcium requirements for half maximal activity of calpain-1 and -2 are 3–50 μM and 400–800 μM, respectively (Goll, Thompson, Li, Wei, & Cong, 2003). Both calpain-1 and -2 undergo autolysis which reduces the calcium requirement for half maximal activity of calpain-1 and -2 to 0.5–2.0 μM and 50–150 μM, respectively (Goll et al., 2003). Autolysis of calpains coincides with their activation, degradation of myofibrillar proteins, and subsequent improvement in tenderness (Geesink, Kuchay, Chishti, & Koohmaraie, 2006; Huff-Lonergan, Zhang, & Lonergan, 2010). Calpain-1 is believed to be largely if not solely responsible for the postmortem tenderization of beef (Geesink et al., 2006; Koohmaraie & Geesink, 2006; Koohmaraie, Seideman, Schollmeyer, Dutson, & Crouse, 1987). However, Camou, Marchello, Thompson, Mares, and Goll (2007) found that by day 2 minimal calpain-1 activity was detected in beef LL and SM muscles and Boehm, Kendal, Thompson, and Goll (1998) found that on day 7 less than 4% of beef SM calpain-1 activity was present. Koohmaraie et al. (1987) did find that 20% of calpain-1 activity was present in beef LL on day 14.

Several studies have found that postmortem free calcium levels are too low to activate calpain-2 (Geesink & Koohmaraie, 1999; Ji & Takahashi, 2006; Veiseth, Shackelford, Wheeler, & Koohmaraie, 2001). Greaser, Cassens, Hoekstra, and Briskey (1969) did note that calcium concentrations increase initially postmortem. However, Parrish, Selvig, Culler, and Zeece (1981) found that in tough and tender beef *longissimus* muscles aged for 10 to 14 days, free calcium concentrations ranged from 638.0 to 970.6 μM. These concentrations would be high enough to activate calpain-2.

Our objectives were to (Exp. 1) determine the effect of postmortem aging (2, 3, 4, 14, 28, and 42 days) on calpain-1 and -2 activity in beef *longissimus lumborum* (LL) and *semimembranosus* (SM) steaks and (Exp. 2) determine the effect of postmortem aging for two extended periods (63 and 84 days) on calpain-2 activity of beef SM steaks.

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## 2. Materials and methods

### 2.1. Product procurement

At 48 h postmortem, strip loin [Institutional Meat Purchase Specifications (IMPS) 180; NAMP, 2011] and top (inside) round (IMPS 168; NAMP, 2011) from USDA Choice carcasses (n = 12) were purchased from AB Foods (Toppenish, WA) and transported to the University of Idaho Meat Science Laboratory.

### 2.2. Preparation of product

The LL and SM were removed from their respective wholesale cuts and samples from the anterior portion of the LL and proximal portion of the SM were removed and vacuum packaged. Subsamples were removed and frozen in liquid nitrogen and stored at −75 °C for calpain analysis on days 2, 3, 4, 14, 28, and 42 postmortem (Exp. 1). In Exp. 2 samples from the SM were frozen in liquid nitrogen and stored at −75 °C for calpain analysis on days 2, 3, 4, 14, 28, 42, 63, and 84 days postmortem.

### 2.3. Calpain extraction

Calpain extraction was completed following the procedures in Raser, Posner, and Wang (1995), Pomponio et al. (2008) and Pomponio and Ertbjerg (2012) with minor modifications. One gram of each sample was placed in extraction buffer (3 mL) (100 mM Tris, 10 mM EDTA, 10 mM DTT, pH 8.3) and homogenized (POLYTRON® PT 10–35 GT; PT-DA 12/2EC-B154) at 12,000 rpm on ice 3 times for 15 s with 15 s cooling between bursts. The homogenate was pipetted into two 2 mL microcentrifuge tubes. Samples were then centrifuged for 30 min at 8800 × *g* at 4 °C. The supernatant fluid was aliquoted and stored at −75 °C until calpain analysis.

### 2.4. Casein zymography

Calpain-1 and -2 activity was determined utilizing casein zymography as described by Pomponio et al. (2008) with minor modifications. A day 0 sample from the *sternocephalicus* was collected 10 min postmortem from a steer harvested at the University of Idaho Meat Science Lab. This sample served as the reference standard on each gel. Polyacrylamide gels (12.5%; 75:1 acrylamide to bisacrylamide) containing 0.2% casein were poured and overlaid with stacking gel (4%; 75:1 acrylamide to bisacrylamide) the day the gels were run. Gels (8 × 10 × 0.1 cm) were pre-run with running buffer (25 mM Tris, 1 mM DTT, 192 mM glycine, 1 mM EDTA, pH 8.3) at 100 V for 15 min in an ice bath before loading samples. Sample buffer (10 μL) (150 mM Tris, 20% glycerol, 10 mM DTT, 0.02% bromophenol blue, pH 6.8) was added to the supernatant fluid containing calpain extract (40 μL). Samples (20 μL) were loaded and the gels were run at 100 V for 6 h in an ice water bath. Gels were then placed in incubation buffer (~60 mL; 50 mM Tris, 10 mM DTT, 4 mM calcium chloride, pH 7.5) at room temperature with slow shaking for 17 h. Buffer was changed (~60 mL) at 30 min and (~130 mL) 60 min. Gels were stained in Coomassie Blue R-250 for 1 h and destained in Coomassie Blue R-250 destaining solution for 3 h. The clear bands indicating calpain activity were quantified by inverting the image and then comparing the density of each band to the reference standard on each gel utilizing a ChemiDoc MP™ System (BioRad). Autolysis was used as an indicator of calpain activation (Geesink et al., 2006).

### 2.5. Statistical analysis

Before analysis, calpain band densities were taken as a percentage of a day 0 reference standard included on each gel. Data were analyzed using the Mixed Model procedure of the Statistical Analysis System (SAS Institute, Inc., Cary, NC). The LL and SM removed from the strip loins and top rounds, respectively, served as the experimental units (n = 12 of each subprimal). Day of aging was analyzed as a repeated measure. Differences in least squares means (LSM) were compared by the DIFF option. *P*-values of ≤ 0.05 were considered statistically significant. Calpain activity is reported as a percentage of the internal reference standard on each gel and therefore may be > 100%.

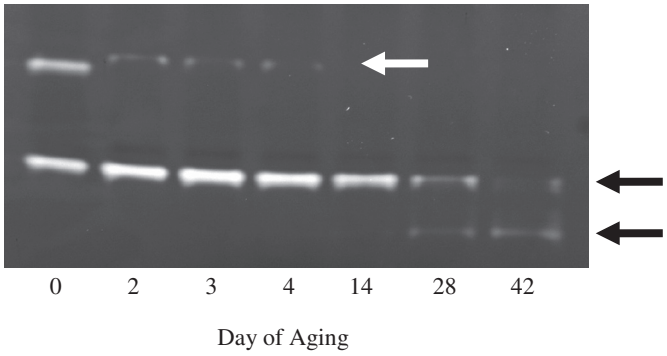


**Fig. 1.** Casein zymogram showing native calpain-1 activity (top arrow), native calpain-2 activity (middle arrow), and autolyzed calpain-2 activity (bottom arrow) in the *semimembranosus* aged 2 to 42 days. The day 0 sample (left lane) was from the *sternocephalicus* of a different animal. This sample was frozen 10 min postmortem and was included on all gels as a standard. Samples were aged for up to 42 days postmortem, frozen in liquid nitrogen, and stored at −75 °C until the completion of all aging periods.

## 3. Results

Calpain activity was measured in beef LL and SM using the casein zymography method. An example of a casein zymogram from the SM is shown in Fig. 1. The top row of bands is calpain-1, the middle row of bands is native calpain-2, and the bottom row of bands is autolyzed calpain-2 (Pomponio et al., 2008).

By day 2 of aging only 5.4% and 2.0% of the calpain activity present in the zero hour reference standard was detected in the SM and LL, respectively. Aging longer than 2 days led to decreased ($P < 0.05$) SM calpain-1 activity but did not affect LL calpain-1 activity, which was already low (Table 1). Calpain-1 activity in the SM decreased from day 2 to 3 and by day 14 there was no calpain-1 activity detected in any of the SM samples. Calpain-1 activity of the LL did not significantly decrease past 2 days of aging but like the SM by day 14 of aging no calpain-1 activity was detected in any of the samples.

Native calpain-2 activity decreased ($P < 0.001$) with longer aging periods for both the LL and SM in Exp. 1 (Table 2) and the SM in Exp. 2 (Table 3). Activity decreased from days 2 to 4, 4 to 14, and 14 to 28 of aging in the LL, while native calpain-2 activity in the SM remained constant from day 2 to 4 then decreased from days 4 to 14 and 14 to 28 of aging (Exp. 1). Similar results were observed for the SM in Exp. 2, and native calpain-2 activity also decreased from day 42 to 63 of aging (Table 3).

Autolyzed calpain-2 activity increased ($P < 0.001$) with longer

**Table 1**
Calpain-1 activity of extended aged beef (Exp. 1; n = 12 per muscle).

| | Day of aging | | | | | | SEM |
|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 14 | 28 | 42 | |
| *Longissimus lumborum* | | | | | | | |
| Calpain-1 activity[1] | 2.0 | 1.5 | 0.8 | NA[2] | NA | NA | 0.7 |
| *Semimembranosus* | | | | | | | |
| Calpain-1 activity | 5.4[a] | 1.2[b] | 0.9[b] | NA | NA | NA | 1.4 |

[ab]Within a row, means without a common letter differ ($P < 0.05$).
[1] Percentage of day 0 calpain-1 activity.
[2] No activity detected.