PROTEOLYSIS IN VITRO REVEALS CALPAIN-1 ACTIVITY DURING THE BEEF MATURATION PROCESS

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I. **Introduction** - Significant efforts have been made to unravel the mechanisms underlying meat maturation and tenderization (Voges et al., 2007). While data supporting calpain-1 (CALPN1)-mediated proteolysis are robust, specific mechanisms responsible for controlling CALPN1 autolysis and its activity postmortem remain sketchy. Some debate regarding how long the protease remains active postmortem exist. Koohmaraie et al. (1988) suggest approximately 50% of the aging response or protease activity is achieved within 24 hrs postmortem in beef carcasses. While Boehm, Kendall, Thompson, & Goll (1998) found minimal degradation of desmin and titin within 24 hrs postmortem suggesting the bulk of proteolysis occurs after 24 hrs. To unravel these inconsistencies, we utilized an *in vitro* proteolysis system to address these outstanding questions and understand better CALPN1 activation during meat maturation.

II. **Materials and Methods** - Cross-bred yearling steers (n = 3) were harvested at the Virginia Tech Meat Science Research and Teaching Center using standard industry protocols. Bovine *longissimus thoracis* et *lumborum* (LTL) and the *extensor carpi radialis* (ER) samples were collected within 5 min post-exsanguination, deemed 0 d, and at 1, 2, 7, and 14 d postmortem. Myofibrils were purified from bovine *semitendinosus* (ST) 24 hrs post-exsanguination according to Weaver et al. (2007) and diluted to 50% with glycerin plus 0.1 MM phenylmethylsulfonyl fluoride for storage at -20°C. Glycerinated myofibrils were washed according to Huff-Lonergan et al. (1996) and Weaver et al. (2009). Protein concentration of preparations was determined and 4 mg/mL of isolated myofibrils were added to each digestion along with various treatments. To ensure the quality control of the myofibril preparation, three controls were utilized for each digestion. Control 1 contained only myofibrils; control 2 included myofibrils with 15 mM mercaptoethanol and 100 µM $CaCl_2$; and control 3 contained myofibrils with 15 mM mercaptoethanol, 100 µM $CaCl_2$, and CALPN1 (calpain-1, porcine erythrocyte, EC 3.4.22.17, Calbiochem, LaJolla, CA). Aliquots of all digestions were collected at 0, 2, 120, 480, and 1440 min. Control samples (0 min) were removed before addition of any treatments. Two min samples were taken immediately after the addition of treatments. Treatments included the addition of powdered LTL and ER muscles collected from carcasses at 0, 1, 2, 7, and 14 d. To stop reactions, aliquots of solubilization buffer (1:1) containing 8 M urea, 2 M thiourea, 3% (w/v) SDS, 75 mM dithiothreitol, 0.05 M Tris-HCl (pH 6.8) were added and used for subsequent SDS-PAGE and Western blotting protocols. Statistical analyses were performed using SAS Software (SAS 9.4, SAS Institute INC, Cary, NC, USA). Prior to data analyses, normality of residuals and homogeneity of variances were tested using the Shapiro-Wilk and Levene tests, respectively. Data were analyzed as a

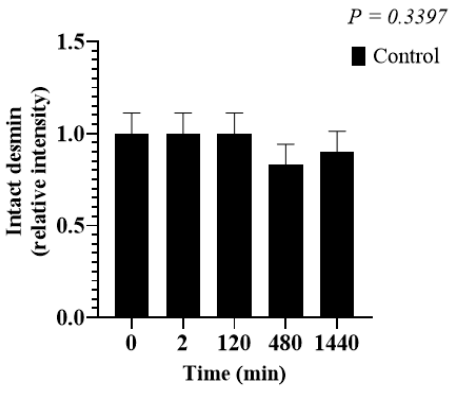

Figure 1: Mean percentage of desmin degradation.

completely randomized design, considering treatments as fixed effects and animals as random effects. Digestion data were analyzed as repeated measures. Differences were considered statistically significant when $P \leq 0.05$, or unless otherwise stated.

III. **Results and Discussion** - To ensure the validity and specificity of the model, we first showed isolated myofibrils were free of contaminating protease because myofibrils were used as a surrogate for assessing the amount and activity of endogenous proteases present in skeletal muscle. Figure 1 demonstrates that myofibrils experienced little desmin degradation after 1440 min in digestion buffer at room temperature. Next, we incorporated LTL muscle into myofibril digestions from carcasses aged 0, 1, 2, 7, and 14 d. Figure 2 shows that 0 d LTL and 1 d LTL had the least amount of desmin degradation, while 2, 7, and 14 d LTL had the greatest proteolysis *in vitro*. Because isolated myofibrils did not degrade in this system (Figure 1), any proteolysis came from muscle tissues derived from carcasses. To validate further our approach, we used two muscles known to differ in proteolysis postmortem. Specifically, we used the LTL, a muscle that undergoes significant proteolysis postmortem, and the ER that experiences negligible levels of proteolysis postmortem. Figure 3 shows minimal desmin degradation was observed when powdered ER was added to purified myofibrils regardless of aging time. These data argue the *in vitro* model containing purified myofibrils is a valuable approach to studying temporal changes in proteolysis as muscles age in beef carcasses.

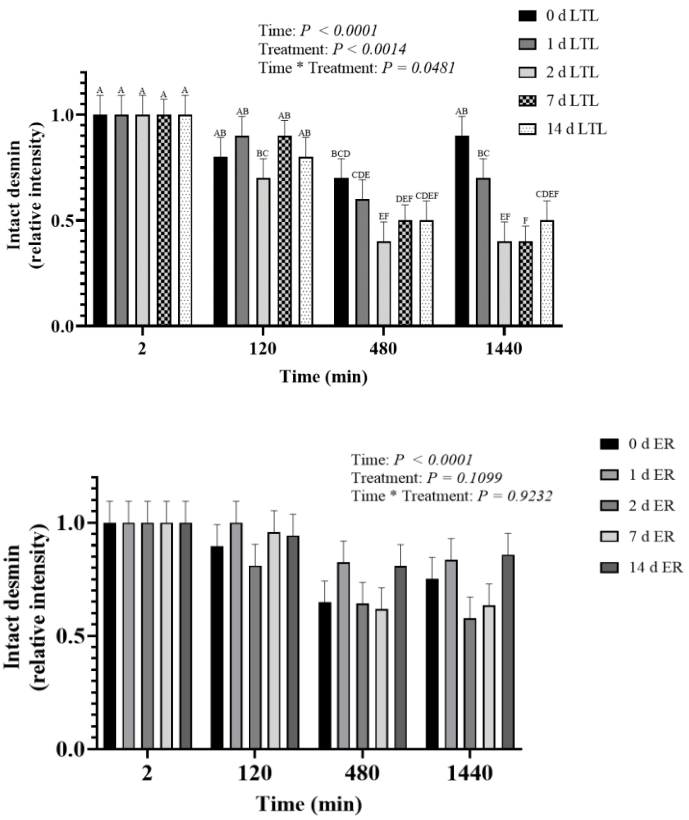

Figure 2: Mean percentage of desmin degradation

IV. **Conclusions** - These data show little 'active' protease exists in beef muscle prior to 2 d aging and argue this approach is a viable tool for studying how proteolysis is regulated postmortem.

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