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Factors regulating lamb *longissimus* tenderness are affected by age at slaughter ☆

E. Veiseth [a], S.D. Shackelford [b], T.L. Wheeler [b], M. Koohmaraie [b,*]

[a] *Department of Chemistry and Biotechnology, Agricultural University of Norway, 1432 Aas, Norway*
[b] *Roman L. Hruska US Meat Animal Research Center, ARS, USDA, P.O. Box, 166, Spur 18-D, Clay Center, NE 68933-0166, USA*

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Abstract

The objective of this experiment was to determine age-related changes in collagen concentration, sarcomere length, calpain (μ- and m-) and calpastatin activities, postmortem proteolysis and Warner–Bratzler shear force (WBSF) in ovine *longissimus thoracis et lumborum*. Rambouillet lambs were slaughtered at 2, 4, 6, 8 and 10 months of age and samples of *longissimus* were collected at 0, 2 and 10 days postmortem. Collagen concentration and sarcomere lengths were determined from the cores used for WBSF measurements and reflected changes in the background toughness. *Longissimus* collagen concentration did not change ($P > 0.05$) due to lamb age. Sarcomere lengths also showed age-related changes, increasing ($P < 0.05$) from 1.35 μm at 6 months to 1.48 and 1.55 μm at 8 and 10 months, respectively. The extent of calpain mediated proteolysis determines the improvement in meat tenderness with postmortem storage. The most notable change in the calpain proteolytic system was the decline ($P < 0.05$) in calpastatin activity from 4.18 to 1.91 U/g muscle between 2 and 10 months. The activity of μ-calpain showed a 16% increase ($P < 0.05$) from 4 to 6 months, before it dropped again at 8 and 10 months. There was a gradual decline ($P < 0.05$) in m-calpain activity with age, and by 10 months m-calpain activity had reduced to 80% of 2 months levels. The ratio of μ-calpain to calpastatin activities increased ($P < 0.05$) from 2 to 6 months (from 0.31 to 0.56) with no further changes ($P > 0.05$) at 8 or 10 months. There were no age-related changes ($P > 0.05$) in desmin degradation at day 2, however, examination of day 10 samples showed increased ($P < 0.05$) degradation from 2 to 6 months. Thus, the changes observed in the ratio of μ-calpain to calpastatin activities are reflected in the extent of postmortem proteolysis. Meat tenderness was measured using WBSF at 2 and 10 days postmortem. Because little proteolysis had taken place at 2 days postmortem, the decline in day 2 WBSF from 6 to 8 months could be explained by changes in sarcomere length. However, at 10 days postmortem, where WBSF was shown to decrease from 2 to 8 months, the improvement in tenderness could be explained by the amount of postmortem proteolysis. The data presented in this paper show evidence that sarcomere length is the main determinant of background toughness in ovine *longissimus*, and that postmortem proteolysis, resulting from μ-calpain activity regulated by calpastatin, is the main determinant of ovine *longissimus* tenderization during aging. Thus, lamb *longissimus* tenderness after refrigerated storage is determined by postmortem proteolysis and its interaction with sarcomere length.
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Keywords: Calpains; Calpastatin; Collagen; Proteolysis; Sarcomere; WBSF

1. Introduction

Variation in tenderness is a major concern for the US meat industry, and effort has been put into understanding the causes for this variation. In a study to determine the sources of variation in meat tenderness, Wheeler and Koohmaraie (1994) concluded that sarcomere shortening during rigor development was the cause of toughening of lamb *longissimus thoracis et lumborum* from 0 to 24 h postmortem, referred to as the tough-

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

* Corresponding author. Tel.: +1-402-762-4221; fax: +1-402-762-4149.
E-mail address: koohmaraie@email.marc.usda.gov (M. Koohmaraie).

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ening phase. Further, Koohmaraie, Doumit, and Wheeler (1996) showed that if *longissimus* is prevented from shortening during the period of rigor development, shear force values do not increase. While the toughening phase is similar in all carcasses, the tenderization process is highly variable. Evidence has shown that the calpain proteolytic system is responsible for postmortem tenderization during cooler storage (for reviews, see Goll, Taylor, Christiansen, & Thompson, 1991; Koohmaraie, 1994, 1996); specifically, μ-calpain degrades key myofibrillar and associated proteins, leading to weakening of myofibrils and, thus, tenderization. The tenderness variation in *longissimus* after postmortem storage is caused by variation in the rate and extent of postmortem proteolysis by the calpain system.

Several studies have investigated the effect of animal age on meat tenderness, and produced contradicting conclusions. While some groups reported a decrease in meat tenderness with increasing animal age (Hiner & Hankins, 1950; Reagan, Carpenter, & Smith, 1976), others have found an increase (Duckett, Snowder, & Cockett, 2000) or no effect (Dikeman et al., 1986; Weller, Galgan, & Jacobson, 1962). Further, it has been shown that activity levels of the calpain proteolytic system, particularly calpastatin, change with animal age (Northcutt, Pringle, Dickens, Buhr, & Young, 1998; Ou, Meyer, & Forsberg, 1991; Whipple & Koohmaraie, 1992). The objective of this paper was to examine the effect of age on the calpain proteolytic system, postmortem proteolysis and meat tenderness of ovine *longissimus*.

2. Materials and methods

2.1. Animals

The Roman L. Hruska US Meat Animal Research Center (MARC) Animal Care and Use Committee approved the use of animals for this study. Fifty-nine Rambouillet lambs were slaughtered at 2, 4, 6, 8 and 10 months of age ($n = 12$ per age group, except at 10 months where $n = 11$). Within 20 min of exsanguination, *longissimus thoracis et lumborum* from one side of the carcass was removed for analysis of calpain and calpastatin activities, diced muscle was also snap frozen in liquid nitrogen for immunological analysis. The carcasses were stored at 1 °C until 48 h postmortem, when the whole *longissimus* from the other side of the carcass was dissected out, trimmed of subcutaneous fat and weighed. Starting at the posterior end of the *longissimus*, samples were taken in the following order: day 2 immunological analyses (snap frozen), day 2 Warner–Bratzler Shear Force (WBSF), day 10 WBSF and day 10 immunological analyses (snap frozen). The portions for day 10 analyses were vacuum packed as one portion and stored at 1 °C until 10 days postmortem. Due to limited sample size, WBSF analyses at 2 days postmortem were not performed for 2-month old lambs.

2.2. Calpains and calpastatin

Longissimus used for analysis of the proteolytic activities of μ-calpain and m-calpain and the inhibitory activity of calpastatin was trimmed of visible fat and connective tissue and finely diced. The muscle extracts were prepared by homogenizing 50 g of muscle in 3 volumes of prerigor extraction buffer (50 mM Tris base, 10 mM EDTA, 0.05% 2-mercaptoethanol [MCE], 2 mM phenylmethylsulfonyl fluoride [PMSF], 100 mg/L ovomucoid, 16 mg/L leupeptin, adjusted with HCl to pH 8.3). Homogenization was performed using a Waring blender (Dynamics Co. of America, New Hartford, CT) 3×30 s on high speed interspersed with 30 s cooling periods. The homogenate was centrifuged at $16{,}000g_{max}$ for 2 h and the supernatant was dialyzed against dialysis buffer (40 mM Tris base, 5 mM EDTA, 0.05% MCE, pH 7.35) overnight. The following day, samples were clarified by centrifugation at $28{,}000g_{max}$ for 1 h and filtered over glass wool, before loading by gravity onto 212-mL DEAE-Sephacel columns equilibrated with elution buffer (40 mM Tris base, 0.5 mM EDTA, 0.05% MCE, pH 7.35). Columns were washed with elution buffer until the A_{278} of the outflow was less than 0.1. Bound proteins were eluted with a gradient from 25 to 500 mM NaCl (375 mL of elution buffer containing 25 and 500 mM NaCl; 40 mL/h), and 140 fractions of 5 mL were collected. The proteolytic activities of μ-calpain and m-calpain and the inhibitory activity of calpastatin in these fractions were determined using a standard casein assay as described by Koohmaraie (1990).

2.3. Warner–Bratzler shear force

The portions of *longissimus* assigned for WBSF determination were cut into 2.54 cm thick chops and cooked on a belt grill (Model TBG-60 Magigrill, MagiKitch'n Inc., Quakertown, PA). Belt grill settings necessary to achieve a final temperature of 71 °C for 2.54 cm thick lamb chops were determined: top and bottom heat = 163 °C, preheat = 149 °C, height (gap between platens) = 2.16 cm, cook time = 5.3 min. Warner–Bratzler shear force was determined as described by Wheeler, Shackelford, and Koohmaraie (1998).

2.4. Sarcomere length

Sarcomere lengths were determined on the cooked cores, after determination of WBSF, as described by Wheeler, Shackelford, and Koohmaraie (2002) for cooked samples. One cube of tissue was removed from each core and fixed as described by Koolmees, Korteknie,

and Smulders (1986). Six fibers were pulled out of each cube (total of 36 fibers per observation), and sarcomere lengths were determined by helium neon laser diffraction (Model 05-LHR-021, Melles Griot, Carlsbad, CA) according to Cross, West, and Dutson (1981).

2.5. Collagen concentration

Collagen concentration was estimated from cores (Wheeler et al., 2002), after determination of WBSF, for hydroxyproline quantification according to Avery, Sims, Warkup, and Bailey (1996) with modifications by Wheeler et al. (2002). Data were expressed as milligrams of collagen per gram of cooked muscle.

2.6. Immunoblotting

Protein extraction, electrophoresis, Western blotting and quantification of desmin were conducted as described by Wheeler et al. (2002). At-death reference samples for each animal were used to calculate percentage desmin degradation at 2 and 10 days postmortem.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance using the GLM procedures of SAS (SAS Inst. Inc., Cary, NC), where age at slaughter was treated as a class (not continuous) variable with five levels (2, 4, 6, 8 and 10 months). When the effect of age at slaughter was significant, means were separated using the PDIFF procedure, a pair-wise *t*-test.

3. Results and discussion

Not surprisingly, hot carcass weight (HCW) and *longissimus* weight increased ($P < 0.05$) with age (Table 1). Both these weights approximately quadrupled between 2 and 10 months. The concurrent changes in HCW and *longissimus* weight were reflected in a correlation of 0.96 between these two traits (data not shown).

Several studies have explored the relationship between meat tenderness and animal age, and reports exist which show no relationship (Dikeman et al., 1986; Weller et al., 1962), and both positive (Duckett et al., 2000) and negative (Hiner & Hankins, 1950; Reagan et al., 1976) relationships. As shown in Table 1, there was no difference ($P > 0.05$) in day 2 WBSF between 4- and 6-month or between 8- and 10-month old lambs. However, a decline ($P < 0.05$) in day 2 WBSF was detected between 6 and 8 months. Day 10 WBSF showed a gradual decline ($P < 0.05$) from 2 to 6 months, and then a large decrease ($P < 0.05$) between 6 and 8 months. Overall, the day 10 WBSF values more than halved

Table 1
Effect of lamb age on hot carcass weight (HCW), and *longissimus* (LD) weight, Warner–Bratzler shear force (WBSF), collagen content, sarcomere length, activities of the calpain proteolytic system and desmin degradation

Lamb slaughter age (months)	HCW (kg)	LD (g)	WBSF (N)		Collagen concentration[a]	Sarcomere length (μm)	Calpastatin [b]	μ-Calpain[b]	m-Calpain[b]	Desmin degradation[c]		Ratio of μ-calpain to calpastatin
			Day 2	Day 10						Day 2	Day 10	
2	8.8^{h}	218.7^{h}	NM	79.1^{d}	3.88	1.33^{f}	4.18^{d}	1.28^{e}	1.55^{d}	3.8	16.0^{f}	0.31^{f}
4	16.9^{g}	436.2^{g}	80.8^{d}	69.0^{de}	3.72	1.35^{f}	3.20^{e}	1.24^{e}	1.44^{de}	3.6	44.7^{e}	0.40^{e}
6	22.4^{f}	539.4^{f}	86.2^{cd}	64.8^{e}	3.86	1.35^{f}	2.69^{f}	1.48^{d}	1.55^{d}	7.2	76.7^{d}	0.56^{d}
8	26.7^{e}	635.6^{e}	60.7^{e}	36.4^{f}	3.69	1.48^{e}	2.25^{fg}	1.34^{de}	1.37^{ef}	8.3	74.7^{d}	0.62^{d}
10	34.9^{d}	785.9^{d}	59.3^{e}	34.9^{f}	3.62	1.55^{d}	1.91^{g}	1.21^{e}	1.25^{f}	9.1	78.3^{d}	0.64^{d}
SEM	1.01	21.39	5.0	4.8	0.19	0.02	0.17	0.05	0.06	2.51	5.65	0.03

[a] Reported as mg collagen/g cooked muscle.
[b] Activity reported as U/g muscle.
[c] Percentage loss of desmin from at-death levels.
[d–h] Within a column, means without a common superscript letter differ ($P < 0.05$).

between 2 and 10 months. Similar results have been reported from lambs ranging from 100 to 246 days of age (Duckett et al., 2000). To determine the source of age-related variation in WBSF, traits known to influence meat tenderness were measured.

For the purposes of this study, we have differentiated between tenderness at the completion of rigor (background toughness) and improvements in tenderness with postmortem storage (tenderization). Background toughness is attributed to collagen concentration and sarcomere length. While there are no changes in collagen concentration during postmortem storage (Koohmaraie, Seideman, Schollmeyer, Dutson, & Crouse, 1987), sarcomeres shorten during rigor development (Wheeler & Koohmaraie, 1994) and may (Gothard, Mullens, Boulware, & Hansard, 1966; Stromer, Goll, & Roth, 1967; Wheeler & Koohmaraie, 1994) or may not (Wheeler & Koohmaraie, 1999), then lengthen slightly with extended storage. Tenderization is primarily dependent on postmortem proteolysis mediated by the calpain proteolytic system (Koohmaraie, 1994, 1996). Thus, ultimate tenderness after postmortem storage of meat is determined by both background toughness and tenderization. Wheeler, Shackelford, and Koohmaraie (2000) examined the variation in tenderness at 1 day postmortem in five different pork muscles, and found that the relative contribution of collagen content, sarcomere length and postmortem proteolysis to meat tenderness variation was muscle-specific. Because Wheeler et al. (2002) showed that analyses of biochemical traits, including collagen content and sarcomere length, on the cooked cores used for WBSF determination could explain more of the variation of tenderness measurements than analyses performed on raw samples, we chose to perform collagen content and sarcomere length analysis on the cooked cores used for day 10 WBSF determinations.

As shown in Table 1, collagen concentration per gram cooked muscle was not affected ($P > 0.05$) by lamb age.

No change in sarcomere length occurred during the first 6 months, but sarcomeres were less contracted ($P < 0.05$) at 8 and again at 10 months (Table 1). Smith, Dutson, Hostetler, and Carpenter (1976) showed that lamb carcasses with increased quantities of fat chilled more slowly and had less shortening of sarcomeres. Thus, increasing amounts of subcutaneous fat and increasing *longissimus* size with increasing age, leading to slower temperature decline, is a possible explanation for the reduced shortening of sarcomeres at 8 and 10 months in this study. Previously, it has been shown that there is a strong positive relationship between sarcomere length and meat tenderness when the sarcomeres are shorter than 2.0 μm (Bouton, Harris, Shorthose, & Baxter, 1973; Herring, Cassens, Suess, Brungardt, & Briskey, 1967; Wheeler et al., 2000). Since all sarcomere lengths in this study were shorter than 2.0 μm, it is likely that the changes in sarcomere length would affect the background toughness of *longissimus*. However, the relative significance of sarcomere length on ultimate tenderness is dependent upon the extent of tenderization resulting from the third factor, proteolysis (Wheeler et al., 2000).

Several groups have examined the effect of animal age on the calpain proteolytic system. Typically, studies indicate that calpain and calpastatin activities decline with increasing animal age (Northcutt et al., 1998; Ou & Forsberg, 1991; Ou et al., 1991; Shackelford, Wheeler, & Koohmaraie, 1995). As shown in Table 1, there was a steady decline ($P < 0.05$) in calpastatin activity with increasing age in this study, and at 10 months calpastatin activity had dropped to less than one-half of the activity measured at 2 months. Duckett et al. (2000) also found a comparable decline in calpastatin activity between 19- and 246-days old lambs. The changes detected in μ-calpain and m-calpain activities were not as dramatic (Table 1). There was a 16% increase ($P < 0.05$) in μ-calpain activity between 2 and 6 months, however, in 8-month lambs, μ-calpain activity had returned to the levels found at 2 months. No changes ($P > 0.05$) were detected in m-calpain activity during the first 6 months, but a decrease ($P < 0.05$) was detected at 8 and 10 months. There was approximately 20% less m-calpain activity detected at 10 months compared to activities measured during the first 6 months.

The impact of changes in calpain and calpastatin activities upon proteolysis was examined by quantifying loss of native desmin during postmortem storage of *longissimus*. Previously, we have shown that it was possible to detect loss of desmin as early as 9 h postmortem and that 31% of desmin was lost by 24 h postmortem in ovine *longissimus* (Veiseth, Shackelford, Wheeler, & Koohmaraie, 2004). However, in this study, relatively little desmin proteolysis (<9.1%) had occurred at 2 days postmortem, and the extent of proteolysis at day 2 was not influenced ($P > 0.05$) by animal age (Table 1). At 10 days postmortem, age related differences became apparent. Desmin degradation detected at 10 days postmortem increased ($P < 0.05$) from 2 to 6 months, but no further increases ($P > 0.05$) in desmin degradation were detected from 6 to 10 months. The result that younger lambs had less desmin proteolysis in the *longissimus* than older lambs agrees with Whipple and Koohmaraie (1992), who found that 8-week old lambs had less postmortem proteolysis than 26-week old lambs.

Because m-calpain is not active under normal postmortem conditions (Koohmaraie et al., 1987; Veiseth, Shackelford, Wheeler, & Koohmaraie, 2001), the reduction detected in measurable m-calpain activity with increasing lamb age in this study is not expected to have an effect on postmortem proteolysis. Changes in postmortem proteolysis are likely to be due to changes in the

relative activities of μ-calpain and calpastatin. Previously, elevated calpastatin activities have been shown to be responsible for reduced μ-calpain to calpastatin ratios, and have resulted in a reduced rate and extent of postmortem proteolysis. Examples of this include in vitro models, such as myofibril incubations (Geesink & Koohmaraie, 1999a), and in vivo models, such as callipyge lambs (Geesink & Koohmaraie, 1999b; Koohmaraie, Shackelford, Wheeler, Lonergan, & Doumit, 1995) and β-adrenergic agonist administration (Koohmaraie, Shackelford, Muggli-Cockett, & Stone, 1991; Kretchmar, Hathaway, Epley, & Dayton, 1990). Therefore, the reduced calpastatin activity detected from 2 to 6 months in this study may be responsible for the significant increases in desmin degradation detected at 10 days postmortem over the same period. Although calpastatin activity showed an additional decline from 6 to 10 months, no further increases in desmin degradation were detected from 6 to 10 months. The lack of further improvements in desmin degradation during this period can be explained, however, by considering the changes in calpastatin and μ-calpain activities simultaneously. While calpastatin showed a gradual decline throughout the increasing age groups, μ-calpain showed an increase at 6 months before it declined again at 10 months. Cumulatively, these changes increased ($P < 0.05$) the ratio of μ-calpain to calpastatin activities from 2 to 6 months, but no further changes were detected up to 10 months (Table 1). Thus, the increase in the ratio of μ-calpain to calpastatin activities from 2 to 6 months coincided with increased postmortem proteolysis. Additionally, when the ratio of μ-calpain to calpastatin activities did not change ($P > 0.05$) from 6 to 10 months, the extent of proteolysis did not change either.

By examining collagen concentration, sarcomere length and proteolysis, it is possible to assess the impact of these traits on meat tenderness at different ages. In this study, collagen concentration did not contribute to differences in *longissimus* WBSF among lamb ages. Thus, the age-related changes in WBSF at 2 and 10 days postmortem must be related to changes in either sarcomere length, postmortem proteolysis, or both. Since the level of desmin degradation at day 2 did not change with age, WBSF differences at 2 days postmortem are more likely to be related to changes in background toughness. Indeed, the decrease in day 2 WBSF at 8 and 10 months coincided with a reduced shortening of sarcomeres at the same time (Table 1). However, the gradual decrease in WBSF at 10 days postmortem was a result of increasing postmortem proteolysis, which was a consequence of decreasing calpastatin activity and an increasing μ-calpain to calpastatin ratio. Importantly, even at 10 days postmortem the effect of increasing sarcomere length on WBSF was detectable, emphasizing the importance of sarcomere length on the background toughness of *longissimus*. The results in this study demonstrated that increasing lamb age had a positive effect on desmin proteolysis and sarcomere length and a neutral effect on collagen concentration. Cumulatively, these changes result in improvement of ultimate *longissimus* tenderness as slaughter age of lambs increases from 2 to 10 months.

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