CALPASTATIN ACTIVITY AND MUSCLE PROTEIN DEGRADATION IN WEANLING CALVES AND CULL COWS

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Abstract – The objective of this paper was to determine the effect of age and muscle type on calpastatin activity and protein degradation in beef cattle. Four cull cows/weanling calf pairs were slaughtered in 4 different days and had longissimus dorsi (LD), semimembranosus (SM) and triceps brachii (TB) muscle samples taken 90 min after exsanguination. Calpastatin activity was measured after 0, 1 or 6 days of aging, using the heated protocol. Titin and troponin T degradation was measured on day 6 muscle samples. Data were analyzed in SAS using PROC MIXED with a split plot design with age group as the whole plot and muscle as the split plot. Calpastatin activity at 6 days post-mortem was higher (P < 0.05) in the cows when compared to the calves. TB had higher calpastatin activity (P < 0.05) than LD and SM. Troponin T and titin degradation products at 6d were more abundant in muscles from calves (P < 0.05) compared to cow muscles and decreased in TB (P < 0.05) across age groups compared to LD or SM. These data suggest that old animals have a more stable calpastatin, which remains active for a longer period, which may be linked to lower protein turn-over and tougher meat.

Key Words – beef cattle; titin; troponin-T

I. INTRODUCTION

It is well known that calpastatin is the main inhibitor of the calpain proteases, which are responsible for the tenderization process that takes place during the conversion of muscle to meat [1]. The calpastatin activity, however, is influenced by a number of factors, including animal breed, nutritional status, gender, age, muscle type, and use of exogenous compounds, like β-agonists, etc. It is well established that as the animal becomes older, the amount of insoluble collagen in the muscle increases, leading to tougher meat [2]. However, calpastatin activity could also play a role in the toughness of beef from old animals. However, only few studies have assessed how calpastatin activity changes as the animal becomes older [3], and very few have looked at calpastatin stability throughout the aging process. Thus, the objective in this paper was to assess calpastatin activity and protein muscle degradation pattern in young calves and cull beef cows, and how it could change among different muscles.

II. MATERIALS AND METHODS

A – Sample preparation

Four cow/calf pairs were slaughtered at Iowa State Meat Laboratory at 4 different days, following animal husbandry guidelines. At slaughter the cows and calves were 3,330 ± 626 and 222 ± 7.35 days old, respectively, weighing 585.51 ± 38.21 and 248.30 ± 10.32 kg of body weight. Longissimus dorsi (LD), semitendinosus (SM) and triceps brachii (TB) muscle samples were taken within 90 minutes postmortem, placed on ice and immediately transported to the laboratory. Each muscle sample was processed into 3 portions that were submitted to 0, 1 and 6 days of aging at 4 ^{o}C. Following each aging time, samples were taken from each muscle and used for calpastatin activity determination. Samples taken after 1 and 6 days of aging were used for μ-calpain autolysis and protein degradation (titin and troponin-T) respectively and were minced, frozen in liquid nitrogen, pulverized and stored at -80 ^{o}C until used for the biochemical analyses. Whole muscle extracts were then prepared from the powdered samples, following the procedure of Lonergan et al. [4]. The extracts were used for Western blotting and SDS PAGE assays to determine μ-calpain autolysis, and troponin T and titin degradation using procedures described by Kim et al. [5].

B – Calpastatin activity determination

The calpastatin activity protocol used was adapted from Shackelford et al. [6]. Following each aging time, fresh, unfrozen muscle samples were minced (5 g) and homogenized in 15 mL cold extraction buffer (100 mM Tris, 10 mM EDTA, 2 µM E-64; pH 8.3; 4 ^{o}C) using a polytron PT 3100 (3-30 s bursts). The homogenate was centrifuged at 18,000 rpm for 20 min at 4 ^{o}C, and the supernatant was dialyzed overnight against 40 vol of 40 mM Tris-HCl ph 7.4, 1 mM EDTA. The dialyzed sample was transferred to 50 mL tubes and heated in a water bath (preheated to 95 ^{o}C) for 30 min. Following heating, samples were chilled in an ice water bath for 15 min and the coagulated protein was dissembled by vortexing the tube. The samples were transferred to 30 mL tubes and centrifuged again at 18,000 rpm for 20 min at 4 ^{o}C. Following centrifugation the samples were filtered through cheesecloth and had their volume determined and recorded for subsequent calculation of calpastatin activity. Calpastatin calculation was determined according to Koohmaraie [7].

C – Protein degradation and µ-calpain autolysis

After polyacrylamide gel electrophoresis, polyacrylamide gels (10% for troponin-T, 5 % for titin, and 8 % for µ-calpain autolysis) were transferred to polyvinylidene difluoride transfer membrane. Then, each designated primary (troponin-T = 1:40,000 dilution with PBS-Tween, monoclonal anti-troponin-T antibody; µ-calpain = 1:10,000 dilution with PBS-Tween, monoclonal anti µ-calpain antibody) and secondary antibody (µ-calpain and troponin-T = 1:10,000 with PBS-Tween, goat anti-mouse-horseradish peroxidase) were applied. Membranes were thoroughly washed with PBS-Tween between primary and secondary incubations as well as after the sedondary incubation to remove non-bound antibodies. Protein bands were detected using a chemiluminescent detection kit. The density of the immunoreactive bands were quantified by densitometry using ChemiImager 5500 (Alpha Innotech, San Leandro, CA) and Alpha Ease FC software (v. 2.03; Alpha Innotec). A reference sample was used on each blot to standardize densitometry data to compare differences between blots. The ratio of the intensity of the degraded band in a sample to the intensity of the degraded band in the reference sampe (beef longissimus dorsi, aged 7 days postmortem at 4^{o} C) which appeared on all blots, was used to normalize comparisons across all blots. The resulting ratio was used to analyse differences among cow x calf muscles in the intensity of the degraded troponin T and titin bands.

D – Statistical analysis

Data were analyzed in SAS v.9.2 using PROC MIXED with a split plot design with age group (cow x calf) as the whole plot and muscle as the split plot. For calpastatin activity, data were analyzed as repeated measures to accommodate the effects of aging time.

III. RESULTS AND DISCUSSION

Age group x time interaction was significant ($P<0.05$) for calpastatin activity, as it did not change between cow and calf until day 1 post-mortem (Table 1). However, at day 6, calpastatin activity remained higher in the cow muscles when compared to the calf (Table 1). At day 6 of aging, only 49.53% of the calpastatin activity detected at day 0 was still active in the calf, while in the cow 81.95% of day 0 calpastatin activity was detected. Although it is well defined that older animals produce tougher meat than young animals as a result of a higher concentration of insoluble collagen in the meat, less post-mortem proteolysis could be another factor, as the calpastatin seems to remain active for a longer period in the cow when compared to the calf. Duarte et al. [8] detected lower myofibrillar fragmentation index in beef bulls containing 8 permanent incisors when compared to younger animals (2 permanent incisors), which corroborates the findings of calpastatin activity in the current work.

Table 1 – Calpastatin activity (units of activity per g of muscle tissue) in young calves and cull cows in longissimus dorsi (LD), semimembranonus (SM), and triceps brachii (TB) at days 0, 1 and 6 of aging

Calpastatin activity	Days of aging			SEM
	0	1	6	
Calf	2.12	1.84	1.05	0.10
Cow	2.05	1.80	1.68	0.07
	Muscle			
LD		1.69b		0.09
SM		1.45b		0.10
TB		1.98a		0.11

Within a column, means lacking a common letter differ (P< 0.05)

Calpastatin activity in TB muscle was greater (P < 0.05) than LD or SM across age groups (Table 1). Calpastatin activity has been positively correlated to the slow myosin heavy chain content of the different muscles of the ovine carcass [9]. In another study including different muscles in cattle, pigs, and sheep, it was reported that the calpain/calpastatin ratio was higher in fast-twitch glycolytic than in slow-twitch oxidative muscles [10]. According to Kirchofer et al. [11], SM and LD are classified as white muscles, whilst TB is considered an intermediate muscle, as it contains less white muscle fibers (fast-twitch). So, the higher calpastatin activity detected on TB muscle could be related to its higher content of slow myosin heavy chain compared to LD and SM.

Corroborating the calpastatin activity data, TB muscle had less troponin T degradation (P < 0.05) than LD and SM (Table 3).

The intensity of day 6 degraded troponin T and titin bands were lower in the cow muscles (P < 0.05) compared to calf muscle (Table 2). This result agrees with the calpastatin activity values discussed earlier. As both troponin T and titin are degraded by calpains, higher calpastatin activity means more inhibition of the calcium dependent proteases, leading to less protein degradation in the muscle. Figure 1 shows clearly the difference between cows and calves troponin T degradation at day 6 post mortem.

Troponin T and titin are myofibrillar proteins prone to degradation by the calpains, and the their lower degradation shown in this paper reinforces the finding that as the animal becomes older, the calpastatin remains active for a longer period post mortem when compared to young animals.

Table 2 – Ratio of band corresponding to degraded troponin T (30 kDa band) and titin (T2) in young calves and cull cows muscles to reference

Protein	Age group		SEM
	Cow	Calf	
Troponin T degraded band	0.328b	0.994a	0.101
Titin degraded band	0.897b	1.168a	0.046

Within a row, means lacking a common letter differ (P<0.05)

Table 3 – Ratio of band corresponding to degraded troponin T (30 kDa) and titin (T2) in longissimus dorsi (LD), semimembranonus (SM), and triceps brachii (TB) muscles to reference

Protein	Muscle			SEM
	LD	SM	TB	
Troponin T degraded band	0.805a	0.818a	0.360b	0.101
Titin degraded band	1.109a	1.052ab	0.937b	0.046

Within a row, means lacking a common letter differ (P<0.05)

Titin degradation was greater in LD (P < 0.05) compared to TB, and SM was intermediate on the relative intensity of the titin degradation product (Table 3). As stated earlier, the troponin T and titin degradation data mirror the calpastatin activity measured on day 6.

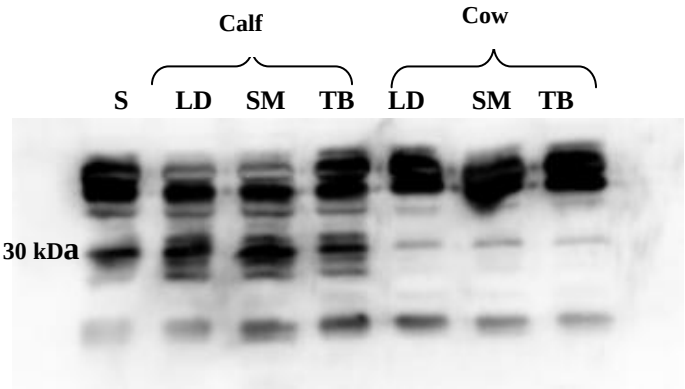

Figure 1. Western Blot of troponin T degradation in beef cull cows and weanling calves LD, SM and TB muscles

The extent of µ-calpain autolysis was greater in the calves' muscles ($P < 0.05$) compared to the muscles of the cows (Table 4), as the percentage of the intact 80 kDa large subunit was 77.17 in the cows and only 47.13 in the calves on day 1 muscle samples. On the other hand, the aulolyzed 78- and 76 kDa products were less abundant in the cows muscles compared to the calves.

Table 4 – Percentage of total detected µ-calpain large subunit present as intact (80 kDa) and the autolysis products (78- and 76 kDa) in muscles from cull cows and weanling calves

Item	Cow	Calf	SEM
80 – kDa	77.17a	47.13b	5.02
78 – kDa	16.39b	25.92a	1.83
76 - kDa	6.44b	26.95a	3.68

Within a row, means lacking a common letter differ ($P<0.05$)

The µ-calpain autolysis results are consistent with the observation that there was less degraded troponin T and titin in the muscle of the cows (Table 2). Coupled with the calpastatin activity data (Table 1), the results show that muscle protein degradation is less in cull cows compared to young calves, suggesting lower protein turn-over and energy expenditure.

IV. CONCLUSION

Calpastatin activity remains higher in muscle of older animals as the meat ages compared to young animals, which can impact meat tenderness. Triceps brachii has lower protein degradation compared to longissimus and semimembranosus, showing how differences in muscle fiber types can impact post-mortem proteolysis.

ACKNOWLEDGEMENTS

The first author thanks CNPq and FAPEMIG for providing scholarship and funding to conduct this project at Iowa State University and to participate at the 58th ICOMST.

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