# ACTIVITY OF ENZYMES AND EXPRESSION OF GENES INVOLVED IN BEEF TENDERIZATION IN TWO BOVINE SKELETAL MUSCLES

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**Abstract – The objective of this trial was to determine the early *post-mortem* activity of enzymes and expression of some genes involved in tenderization process of beef in two muscle types. Samples of *Longissimus dorsi* (LD) and *Infraspinatus* (IS) muscles were collected within 20min from slaughtering of 17 Italian Simmental young bulls. By SDS-PAGE the αII spectrin and its degradation products were analyzed to assess μ-calpain (CAPN1) and Caspase 3 (CASP3) activities. The mRNA abundances of CAPN1, m-Calpain (CAPN2), CASP3, CASP9, Heat Shock Protein 70 (HSP70), HSP27 and HSP40 were detected by RT-PCR. Also $pH_{48h}$, and WBSF after 7 days of ageing were measured. LD showed lower WBSF and higher CAPN1 activity than IS, while CASP3 activity was similar between muscles. Within each muscle CAPN1 activity was higher than that of CASP3. LD had lower HSP27 and HSP70 mRNA abundances than IS. No differences between muscles were found in the expression of the other genes. Even if some interesting tendencies were highlighted concerning CAPN1 and CASP9 expression at slaughter, the differences in WBSF between muscles seem to be mainly linked to CAPN1 activity and HSPs early *post-mortem* expression.**

**Key Words – Calpain, Caspase, Heat Shock Proteins, Tenderness, αII spectrin degradation products**

## I. INTRODUCTION

From the consumer point of view, tenderness is one of most important factors able to modify its buying decision process for beef [1]. Tenderness is affected by many factors such as: breed, amount and solubility of collagen, intramuscular fat content, pH decline, fiber type characteristics and consequently type of muscle [2], and *post-mortem* proteolysis which have a key role [3]. Koohmaraie [4] explained that 70% of tenderness was explained by environmental and 30% by genetic factors. It is widely recognized that proteolytic enzymes such as calpain and caspase directly contribute to beef tenderness. Moreover Hocquette *et al.* [5] found that the expression of some genes, such as heat shock proteins, are related to beef tenderness in Charolais young bulls, however the same authors highlighted the difficult to extent these results outside the reference population. Aim of this study is to determine different activities of enzymes and expression of some genes involved in tenderization process of beef in two muscle types in Italian Simmental young bulls.

## II. MATERIALS AND METHODS

Seventeen Italian Simmental young bulls were randomly chosen from 1 farm, fed corn silage-based diets and slaughtered at 694±11.6kg (mean±se) of weight. At slaughter, samples of *Longissimus dorsi* muscle (LD) and *Infraspinatus* muscle (IS) were obtained within 20min of exsanguination, frozen in $N_2$, and stored at -80ºC until transcriptomic and proteomic analysis. After chilling at 4ºC for 48h, samples of LD ($6^{th}$-$7^{th}$ ribs) and IS were collected, pH measured by a pH-meter (Crison 52-32 electrode), and then aged at 4°C for 7d. Shear force was measured on raw sample, using a Warner-Bratzler device (WBSF), with a triangular hole in the shear blade, mounted on a Lloyd TAPlus texture analyser (ELIS, IT). Measurement was recorded as the peak yield force (N), required to shear, at a 100 mm/min crosshead speed, perpendicular to the direction of fibres, 7 cylindrical cross-section replicates, (15mm diameter×30mm length).

RNA was extracted from 40 mg muscle using RNeasy Fibrous Tissue Mini Kit (Qiagen, DE). Concentration and purity of RNA were assessed using spectrophotometer NanoDrop 2000c

(Thermo Fisher Scientific, MA). To obtain cDNA, the iScript cDNA Synthesis kit (BioRad, IT) was used. Each 20 µl of reaction contained 4 µl of 5X iScript reaction mix, 1µl of RNase H+ MMLV-derived reverse transcriptase, 10µg of RNA, 5µg of nuclease-free water. The mixture was held 5min at 25°C, 1h at 42°C, and 5min at 85°C before being cooled at 16°C. The cDNA was quantified using NanoDrop 2000c. A qualitative PCR was carried out to verify primers (Table 1). The quantitative PCR was performed using the Bio-Rad CFX96 system (BioRad, CA), on a reaction volume of 20µL, containing 0.3µL of each forward and reverse primer (0.3µM), 10µL of iQ SYBR Green Supermix (BioRad, CA), 8.4µL of sterile water, 1µL of cDNA. Amplification conditions included 1 cycle of 3min at 95°C, 40 PCR cycles of 15s at 95°C, 30s at 60°C, and 30s at 72°C, then 1min at 95°C followed to a melt curve of 55-95°C with 0.5°C increments every 5s. Each sample was analysed in triplicate and relative gene expression was calculated according to the efficiency-corrected method [6]. β-actin, cyclophilin, glyceraldehyde-3-P dehydrogenas, and ribosomial protein large P0, were treated as reference genes and used for normalization of RT-qPCR data (M<0.10). Data were presented in fold-change ratio having as reference IS; primer efficiency was calculated and verified using the standard curve obtained by serial dilution of the pooled cDNA [6].

CAPN1 and CASP3 activities were assessed through αII spectrin cleavage products. Total protein was extracted from 100mg of each muscle. Sample were homogenized 3 time for 30s with Ultra-Turrax® T25 in extraction buffer (0,05M dtt, 2% sds, 60mM tris-HCl, 0,2% antiproteases), boiling for 2min at 98°C, mixed 30min at room temperature and centrifuged 10min at 11000rpm. Protein was determinate by the Near UV Absorbance (280nm) method [7]. Equivalent quantity of protein of each sample were subject to 8% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [8]. Proteins separated via SDS-PAGE were transferred to 0.2µm pore size nitrocellulose membranes using a semi-dry blotting apparatus (TE 22 transfer unit, Amersham Biosciences) for 1h in a transfer buffer. After staining with Ponceau S. solution (ATX Poceaus S red staining solution Fluka), to verify transfer efficiency, the nitrocellulose sheets were saturated with 3% (w/v) non-fat dry milk in PBS buffer plus 0.1%, Tween 20 for 1h at room temperature and then incubated overnight with the antibodies spectrin α II (C-3; Santa Cruz Biotechnology, inc.). Blots were then rinsed 3 times with PBS buffer plus Tween 20 and incubated for 1.30h with anti-mouse-IgG (1/8000) at room temperature. Protein bands were acquired using the instrument SF Launcher and processed using ImageQuant TL; data were expressed in percentage on total protein detected. Normality of data distribution was tested by Kolmogorov-Smirnov test. Differences between muscles were carry out using paired samples t-test procedure. Pearson coefficients were used to determine associations between variables.

Table 1. Primer sequences (5' to 3') and amplification products

| Gene | Primers Forward-Reverse, Amplicon (A; bp), Accession Number (AN), Efficiency (E), $R^2$ |
|---|---|
| CAPN1 | F:AACCGGATCCGGAATTACCTGTCCATCTTC<br>R:GTAACCACTTAAACAAGTCAAAGGTCACCA<br>A:282; AN:NM_174259; E:0.98; $R^2$: 0.99 |
| CAPN2 | F:CGAGGACATGCACACCATTGGCTTCG<br>R:TCCTCGCTGATGTCAATCTCGTCAATGTTG<br>A:314; AN: NM_001103086.1; E:1.00; $R^2$: 0.99 |
| CASP3 | F:AGAACTGGACTGTGGTATTGAGA<br>R:CACAAAGAGCCTGGATGAAC<br>A:167; AN: NM_001077840.1; E: 1.00; $R^2$: 0.99 |
| CASP9 | F:CCTGTGGTGGAGAGCAGAAAG<br>R:CATCTGGCTCGTCAATGGAA<br>A:134; AN: NM_001205504.1; E: 0.99; $R^2$: 0.99 |
| HSP70 | F:AACAAGATCACCATCACCAACG<br>R:TCCTTCTCCGCCAAGGTGTTG<br>A:274; AN: NM_00174550; E: 0.98; $R^2$: 0.99 |
| HSP27 | F:CGTTGCTTCACTCGCAAATA<br>R:TACTTGTTTCCGGCTGTTCG<br>A:210; AN: NM_001025569.1; E: 0.92; $R^2$: 0.99 |
| HSP40 | F:GGACTGACCATTGCTGCTG<br>R:CAAACCCACCTCTGTAATAGC<br>A:138; AN: NM_001034458.1; E: 1.00; $R^2$: 0.99 |
| Cycloph | F: GGATTTATGTGCCAGGGTGGTGA<br>R: CAAGATGCCAGGACCTGTATG<br>A: 119; AN: NM _00178320; E: 1.00: $R^2$: 0.99 |
| β-actin | F: CTCTTCCAGCCTTCCTTCCT<br>R: GGGCAGTGATCTCTTTCTGC<br>A:177; AN: NM_00173979; E: 0.96; $R^2$: 0.99 |
| GAPDH | F: TCATCCCTGCTTCTACTGGC<br>R: CCTGCTTCACCACCTTCTTG<br>A:177; AN:NM_001034034; E:0.93; $R^2$: 0.99 |
| RPLP0 | F: CAACCCTGAAGTGCTTGACAT<br>R: AGGCAGATGGATCAGCCA<br>A: 226; AN: NM_001012682; E: 0.93; $R^2$: 0.99 |

## III. RESULTS AND DISCUSSION

IS showed higher $pH_{48}$ (5.72 *vs.* 5.52; P<0.01) and WBSF (30.27 *vs.* 20.20 N; P<0.01; data not tabulated) than LD on raw samples; highlighting a probable different proteolysis between muscles. αII Spectrin is a muscle structural protein that is degraded during proteolysis, its full length is 250 kDa (SBDP250). SBDP250 level was higher in IS than LD (P<0.01; Figure 1), it means that, at slaughter, αII spectrin was less degraded in IS than LD, consistent with WBSF results.

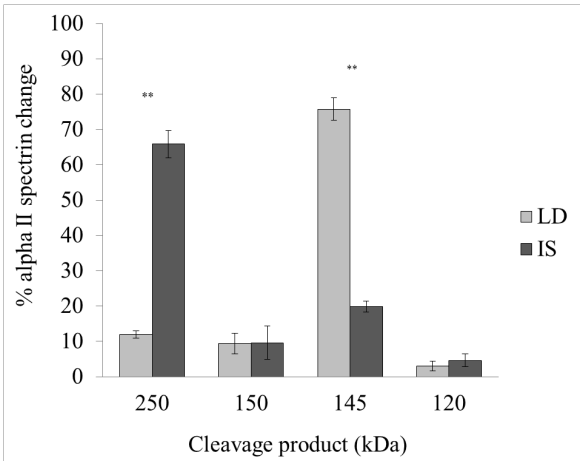


Figure 1. Cleavage products of αII spectrin in *Longissimus dorsi* (LD), *Infraspinatus* (IS) muscle at slaughter. Data expressed in percentage on the total protein detected.**P<0.01.

μ-Calpain (CAPN1) and m-calpain (CAPN2) are responsible for *post-mortem* tenderization being involved in muscle proteolysis in presence of calcium. CAPN1 mRNA abundance was slightly higher in LD than IS (P=0.08), while CAPN2 expression was similar between muscles (P>0.05; Table 2). 150 kDa peptide (SBDP150) and 145 kDa peptide (SBDP145) derived from αII spectrin cleavage. However SBDP145 is an indirect indicator of CAPN1 activity, while SBDP150 can derive both by CAPN1 and caspase activity. As reported in Figure 1, SBDP150 level was similar between muscles (P>0.05), while SBDP145 was higher in LD than IS (P<0.01), suggesting that CAPN1 was more active in LD than in IS, that is consistent with the transcriptomic and WBSF analysis. According to the fiber-type composition, IS is classified as a red muscle (rich in type I fiber), while LD as white muscle (rich in type IIB fiber). Ouali *et al.* [9] showed higher CAPN2 content in red muscle, Muroya *et al.* [10], considering both red and white muscles, had not find differences in CAPN1 and CAPN2 mRNA level. Nowak [11] reviewed that CAPN1 plays a role in the beginning of meat tenderization, while CAPN2 contribute later, and are regulated by calpastatin. Caspases favors death cell process. CASP9 is activated in response of hypoxia and ischemia related to physiological post-slaughter condition and actives CASP3, which is considered an effector caspase. CASP3 and CASP9 mRNA abundance was similar between muscles (P>0.05; Table 2). From αII spectrin cleavage, 120kDa degradation product (SBDP120) is an indirect indicator of CASP3 activity. Differences in SBDP120 level between muscles were not found (P>0.05; Figure 1). Conversely to Kemp *et al.* [12], correlation between WBSF and SBDP120 was not found in LD neither in IS (P>0.05). Taking into account our results, CASP3 *post-mortem* activity seems not influence the WBSF variability between and within muscles. Underwood *et al.* [13] claimed that CASP3 is not involved in the *post-mortem* tenderization of beef. Positive correlations were found between CAPN1 and CASP9 mRNA abundance both in IS (r=0.680; P=0.03) and LD (r=0.507; P=0.04), confirming the hypothesis that these enzymes are involved in the early *post-mortem* proteolysis [12]. From western blotting analysis, SBDP145 level was higher than SBDP120 both in LD (P<0.01) and IS (P<0.01; Figure 1), it means that the activity of CAPN1 was higher than that of CASP3 in both muscles. In our study significant correlations between SBDP120 level and CASP3 mRNA abundance was not found, however a slightly positive correlation between SBDP145 level and CAPN1 mRNA abundance was highlighted (r=0.355; P=0.08).

Heat Shock Proteins (HSPs) are released by cell in response to hypoxia and ischemia conditions with the aim to preserve the cell functionality inhibiting directly apoptosis. LD showed lower level of HSP27 (P<0.01) and HSP70 (P<0.05), but similar (P>0.05) mRNA level of HSP40, a co-chaperone of HSP70, than IS. Our results are not in agreement with a previous study carried out in pig [14], where white muscles showed higher expression of HSP27 than red ones, but

without statistical significance. However, considering the anti-apoptotic activity of HSPs, the lower HSP27 and HSP70 abundances found in LD than IS, seem consistent with the WBSF results. In literature controversial results concerning relationship between HSPs, HSP27 in particular, and shear force were reported [5,15]. A recent study [5] reported that HSP27 could be positively or negatively correlated to shear force depending on breed.

Table 2 Fold change ratio in relative RNA expression of genes of *Longissimus Dorsi* (LD) and *Infraspinatus* muscle (IS).

| Gene | IS | LD | SEM |
|---|---|---|---|
| CAPN1 | 1.00 | 1.33 | 0.190 |
| CAPN2 | 1.00 | 1.05 | 0.138 |
| CASP3 | 1.00 | 1.21 | 0.161 |
| CASP9 | 1.00 | 0.94 | 0.086 |
| HSP70 | $1.00^{a}$ | $0.68^{b}$ | 0.129 |
| HSP27 | $1.00^{A}$ | $0.53^{B}$ | 0.074 |
| HSP40 | 1.00 | 1.02 | 0.097 |

$^{A,B:}$ $P<0.01$; $^{a,b:}$ $P<0.05$

## IV. CONCLUSION

Tenderization process of beef is complex. Taking into account the results of the present study, the differences in WBSF between muscles seem to be mainly linked to CAPN1 activity and to HSPs early *post-mortem* expression even if some interesting tendencies were highlighted concerning CAPN1 and CASP9 expression at slaughter.

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