Immunological detection of m- and μ-calpains in the skeletal muscle of Marchigiana cattle

E. Varricchio,[1] M.G. Russolillo,[2] L. Maruccio,[3] S. Velotto,[4] G. Campanile,[3] M. Paolucci,[1] F. Russo[1]

[1]Department of Biological, Geological and Environmental Sciences, University of Sannio, Benevento; [2]"Centro Studi" Province of Benevento, University of Sannio, Benevento; [3]Department of Veterinary Medicine and Animal Productions, University Federico II of Naples; [4]Department of Soil, Plant, Environment and Animal Production, University Federico II of Naples, Italy

Abstract

Calpains are Ca^{2+}-dependent proteases able to cleave a large number of proteins involved in many biological functions. Particularly, in skeletal muscle they are involved in meat tenderizing during post mortem storage. In this report we analyzed the presence and expression of μ- and m-calpains in two skeletal muscles of the Marchigiana cattle soon after slaughter, using immunocytochemical and immunohistochemical techniques, Western blotting analysis and Casein Zymography. Therefore, the presence and the activity of these proteases was investigated until 15th day post mortem during normal process of meat tenderizing. The results showed m- and μ-calpain immunosignals in the cytoplasm both along the Z disk/I band regions and in the form of intracellular stores. Moreover, the expression level of μ-calpain but not m-calpain decreased after 10 days of storage. Such a decrease in μ-calpain was accompanied by a gradual reduction of activity. On the contrary, m-calpain activity persisted up to 15 days of post mortem storage. Such data indicate that expression and activity of both μ-calpain and m-calpain analyzed in the Marchigiana cattle persist longer than reported in literature for other bovines and may be related to both the type of muscle and breed examined.

Introduction

Calpains are a large family of intracellular cysteine proteases. To date, 14 members have been identified, which are expressed in an ubiquitous or tissue-specific manner.[1,2] In skeletal muscle, the calpain system consists of three proteases, ubiquitously expressed isoforms μ-calpain, m-calpain, and p94 (or calpain 3). The terms μ-calpain and m-calpain refer to the micromolar Ca^{2+}-requiring (μ-calpain) and millimolar Ca^{2+}-requiring (m-calpain) proteases, respectively.[3,4] Both proteases are heterodimers, each one composed of a 80 kDa catalytic subunit and a 28 kDa regulatory subunit. The 28 kDa subunit is identical in both the μ- and m-calpains. Both the 80 kDa and the 28 kDa subunits undergo auto-proteolysis from the N-terminus resulting in the conversion of the 80 kDa subunit into a 76 kDa form through a 78 kDa intermediate.[5] In both μ- and m-calpains the 80 kDa subunit is divided into four domains based on the amino acid sequence: domain I or NH_2-terminal domain; domain II; domain III that contains two potential EF-hand Ca^{2+} binding sequences, one at the domain II/III boundary and one at the domain III/IV boundary; and domain IV which contains four sets of sequences that predict EF-hand Ca^{2+} binding sites.[2] Associated with the calpain proteolytic enzyme family is the calpain-specific endogenous inhibitor, calpastatin.[1,6] Calpastatin contains 4 inhibitory domains, each one capable of inhibiting calpain activity.

Recently, some research groups have identified calpastatin genes polymorphism and have shown that some of them are predictive of carcass quality in cattle and in pigs.[7-9] Additionally, there are markers within the calpastatin and μ-calpain genes that are able to identifying beef cattle with the genetic potential to produce tender meat.[10,11] The calpain-calpastatin system has different yet crucial roles in the cell. During embryo development, the knockout of both μ- and m-calpain is lethal.[11,12] Such proteases are able to cleave a large number of proteins both *in vitro* and *in vivo*. They act on cytoskeletal proteins, especially those involved in cytoskeletal/plasma membrane interactions,[13-17] on some transcription factors[18-21] and so they are involved in the cell cycle,[22-24] regulation of gene expression[25-27] and apoptosis.[28-31] Particularly, the calpains-calpastatin system brings about cytoskeletal damage and membrane disruption in muscle cells. In this manner, it is involved in the pathogenetic mechanism of muscular dystrophy[32,33] in living animals and in meat tenderness.[34]

In striated muscles, after death, the calpains rapidly cleave titin and nebulin at sites near the Z-disk, thereby severing their attachment to the proteins in the Z-disk. In addition, the calpains cleave the intermediate filament protein desmin that attaches the Z-disk to the sarcolemma; hence, the proteins constituting Z-disk, including α-actinin, are released, and the Z-disk disappears leaving a space in the myofibril.[1,35,36] The calpains also rapidly cleave T and I troponins and tropomyosin, and C-protein,[35-37] which contribute to the stability of the thin and thick filaments, respectively. Calpains cleave myosin and actin, the two major proteins in striated muscle, very slowly, if at all.[38] Some ultrastructural analyses reveal μ-calpain immunoreactivity in the bovine skeletal muscle within sarcomeres, essentially at the center of the I-band and at the periphery of Z-lines,[39] where it co-localize with myotilin, an alpha-actinin, gamma filamin binding protein found in the Z-band edges. In the bovine skeletal muscle induced by conditioning and high-pressure treatment, the calpain localization was two times greater in both the I-band/Z-disk and A-band in the muscle immediately after thawing than after 7 days.[40] In light of the crucial role played by calpains on meat tenderness, the aim of this study was to analyze the presence and expression of μ- and m- calpains in the skeletal muscle of the Marchigiana cattle soon after slaughter and during aging, using immunocytochemical and immunohistochemical techniques and Western blotting analysis. Moreover, Casein Zymography was performed to consider the proteolitic activity of μ- and m-calpain during aging in the skeletal muscle of this cattle breed.

For this study, we have chosen the Marchigiana cattle for its commercial interest and meat quality. The meat of this animal is a PGI (Protected Geographical Indication) product and the name of the trade mark is *Vitellone Bianco dell'Appennino Centrale*.

Correspondence: Dr. Finizia Russo, Department of Biological, Geological and Environmental Sciences, University of Sannio, via Port'Arsa 11, 82100 Benvento, Italy.
Tel. +39.0824.305163 - Fax: +39.0824.23013.
E-mail: finiziarusso@libero.it

Key words: m-calpain, μ-calpain, skeletal muscle, Marchigiana cattle, immunohistochemistry, Electron Microscopy.

Received for publication: 17 January 2012.
Accepted for publication: 14 November 2012.

European Journal of Histochemistry 2013; 57:e2
doi:10.4081/ejh.2013.e2

Materials and Methods

Animals and tissue preparation

Samples of masseter and diaphragm muscles were collected from ten males 24-month-old Marchigiana cattle obtained from a local slaughterhouse (Campolattaro, Benevento, Italy). The animals were born and farmed in

the province of Benevento (Italy) and their meat was intended for human consumption. Sampling was made immediately after death. Cubic fragments of 1 cm of length underwent different treatments for immunohistochemistry and scanning electron microscopy (SEM) analysis. For immunohistochemistry, muscle samples were submerged in 2-metilbutane, extra pure (Acros Organics, NJ, USA) for 5 seconds and then frozen in liquid nitrogen before being cut at the cryostat. For immunogold-labeling SEM analysis, specimens were submerged in PBS for 1 h at room temperature (RT). Additional muscle samples were stored at 4°C, collected and frozen (-80°C) at 0, 5, 10 and 15 days *post-mortem* for Western blotting analysis and Casein Zymography.

Immunohistochemistry

Frozen samples of masseter and diaphragm muscles were serially cut at a cryostat in transversal and longitudinal sections of 10 μm. After blocking endogenous peroxidase activity with 0,3% hydrogen peroxide for 20 min at RT, the sections were rinsed in 0.01 M phosphate buffered saline (PBS), pH 7.4, for 15 min. Primary antibodies were monoclonal antibodies raised in mouse against the domain III/IV of m-calpain (C-268; Sigma, Sant Louis, MO, USA) and polyclonal antibodies raised in rabbit against domain IV of μ-calpain (C-5611; Sigma). Primary antibodies were diluted 1:50 and applied on the sections overnight in a moist dark chamber at 4°C. The other components of the immunological reaction were contained in the Envision Dako (K4006, DakoCytomation, Glostrup, Denmark) employed with mouse antibodies and Vectastain Elite ABC Kit (PK-6101; Vector Laboratories Inc., Burlingame, CA, USA) employed with rabbit antibodies. The final staining was performed using a solution of 3-3' diaminobenzidine tetrahydrocloride (DAB; Sigma) of 10 mg in 15 mL 0.5M Tris buffer, pH 7.6, containing 0.03% hydrogen peroxide. The images of the immunostainings were acquired and photographed using the microscope Leica DMRA2 (Leica, Wetzlar, Germany) equipped with a DC300F digital camera.

Negative controls were obtained substituting the primary antisera with PBS or normal serum in the specific step, or alternatively, by absorbing each primary antiserum with an excess of the relative peptide (100 μg of peptide/mL of diluted antiserum).

Immunogold-labeling SEM analysis

Samples were incubated for 2 h in a solution containing normal goat serum (900.077; Aurion, Wageningen, The Netherlands) diluted 1:10 in PBS, and then incubated with primary monoclonal antibodies raised in mouse against the domain III/IV of m-calpain (C-268; Sigma) and primary polyclonal antibodies raised in rabbit against the domain IV of μ-calpain (C-5611; Sigma), diluted 1:50 in PBS, overnight at 4°C. After washing in PBS, the samples were incubated with gold-conjugated goat anti-mouse IgG (806.022, Aurion) and goat anti rabbit IgG (106.011, Aurion) diluted 1:200 in PBS for 1 h at RT. The secondary antibody was conjugated with gold particles of different sizes (5 and 15 nm). After washings in PBS, samples were fixed in 2.5% glutaraldehyde in 0.1 M Cacodylate buffer, at pH 7.2, for 30 min. After washings with distilled water, samples were subjected to silver enhancement (500.055, Aurion). The silver enhancement process enables the use of antibodies conjugated with small (6 nm) gold particles allowing fast penetration and high labeling efficiency.[41] Samples were then dehydrated through an ethanol series and dried to the critical point. The specimens, mounted on stubs, were examined under a LEO 435 VP scanning electron microscope at variable pressure (80-120 Pa) in the backscattered electron mode, which allows the detection of gold particles associated with cells even if they are located intracellularly.[42]

Since the samples were not coated by gold, only conjugated gold deriving from immunocytochemical reaction was observed by SEM and photographed.

Western blot analysis

Proteins from masseter and diaphragm muscle samples were extracted with Lysis buffer (220 mM D-Mannitol, 70 mM Saccharose, 1 mM EDTA, 20 mM Tris pH 7.4, containing protein inhibitors 2 mM PMSF, 1 mM pepstatin A, 2 mM trypsin inhibitor from chicken egg white). Muscle samples were homogenized with ultra-turrax T25 (IKA-labortechnik, Staufen, Germany) for three times at 500 rpm, 800 rpm and 14,000 rpm for 10 min/each. The supernatants were collected and underwent protein determination with the Bio-Rad dye protein assay (Bio-Rad laboratories Inc., UK). Samples were boiled at 98°C for 10 min in loading buffer (50 mM trisHCl pH 6.8, 100 mM β-mercaptaethanol, 2% SDS, 0,1% blue bromophenol, 10% glycerol). The proteins were separated on a 8% SDS-polyacrylamide gel electrophoresis with 4% stacking gel in 1% Tris-glycine buffer (0.025 M Tris, 0.192 M glycine, and 0.1% SDS pH 8.3) in a miniprotean cell (Bio-Rad) at 130 volts for 2 h. The separated proteins were electro transferred onto a nitrocellulose membrane with transfer buffer (39 mM Tris base, 0.2 M glycine, and 20% methanol pH 8.5) in a minitransfer cell (Bio- Rad) at 100 volts at 4°C for 2 h. Membranes were incubated at 4°C for 1 h in blocking buffer containing 1%PBS, 0.05% Tween 20 and 5% dried non-fat milk and with monoclonal antibodies raised in mouse against domain III/IV of m-calpain (C-268; Sigma) and monoclonal antibodies against β-actin (A5441, Sigma) as an internal marker, and with polyclonal antibodies raised in rabbit against domain IV of μ-calpain (C-5611; Sigma) and polyclonal antibodies against β-actin (A5060, Sigma) as an internal marker over night at 4°C. Primary antibodies were diluted 1:1000. The incubation with secondary anti-mouse and anti-rabbit IgG (1:5000) was carried out for 1h at RT. Signals were detected by chemiluminescence with the Immobilan Western Chemiluminescent HRP substrate Kit (Millipore, Billerica, MA, USA) with Chemidoc (Bio-Rad). A prestained molecular-weight ladder (Novex Sharp protein standard, LC5800, Invitrogen, Hilden, Germany) was used to determine protein size. Western blotting bands were quantified by Quantity One (Bio-Rad) software. Rat skeletal muscle was used as a positive control.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Turkey's test. The analysis were carried out with the Statistica version 7.0 statistical package (Statsoft inc., Tulsa, OK, USA). Data are expressed as mean ± SEM.

Casein zymography method

To determine activity of μ- and m- calpain the casein zymography method based on the protocol described by Raser, Posner, and Wang[43] was used. One gram of each sample was homogenized with ultra-turrax T25 (13.500 rpm) in 6 mL of extraction buffer (50 mM Tris; 5 mM EDTA; 10 mM Monothioglycerol; one tablet per 50 mL of Protease Inhibitor Cocktail Tablet COMPLETE, RAS Roche Applied Science, Mannheim, Germany; pH 8.0) and centrifuged for 30 min a 4°C and 15,000g. Each sample was run using 12,5% casein precast gel (Bio-Rad Laboratories, Hercules, CA) twenty-five μL of sample buffer (300 mM Tris, 40% glycerol, 0,02% bromophenol blue, 100 mM DTT, pH 6.8) were added to 75 μL of the supernatant and 15 μL sample were loaded into each well of the gel. Before loading the samples, the gel was prerun for 15 min at 80 V (running buffer: 25 mM Tris, 192 mM glycine, 1 mM EDTA, pH 8.3).

Electrophoresis was carried out at 80 V for 3 h at 4°C. Gels were then removed, rinsed with deionized H_2O, and incubated with shaking at RT in 100 mL of incubation buffer (50 mM Tris, 4 mM $CaCl_2$, 10 mM monothioglycerol, pH 7.5) for 1 h; gels were rinsed twice. The calpain activity was stopped by washing the gel overnight with shaking, using 20 mM Tris, 10 mM EDTA, pH 7.0. Gels were stained for 6 h with colloidal Comassie Brilliant Blue G[44] and destained overnight with deionized H_2O. Signals were detected by UV transillumination with Chemidoc (Bio-Rad).

Results

Since the results obtained for both masseter and diaphragm muscles were similar, we will refer to them as the *skeletal muscles* of Marchigiana cattle.

Immunohistochemistry

In the longitudinal sections of the skeletal muscle of Marchigiana cattle, both m- and μ- calpain immunopositivity could be seen (Figure 1a,b). The positivity was present for both calpains along Z disk/I band regions. In the A-band area the positivity was absent (Figure 1a,b).

Immunogold-labeling SEM analysis

Both m- and μ-calpain immunoreactive gold particles were detected in the cytoplasm (Figure 2). In particular, immunopositivity was localized along the Z disk/I band regions (Figure 2a,b) and in the intracellular stores (Figure 2c,d).

Western blot analysis

The espression of m- (Figure 3a) and μ-calpains (Figure 3b) was detected at 0, 5, 10 and 15 days of storage at 4°C in the masseter (Figure 3a) and diaphragm (Figure 3b) muscles. Both m- and μ-calpains showed a molecular mass of about 80 kDa. A significant decrease in the level of expression for μ-calpain was detected at the 10th and the 15th day (Figure 3b); m-calpain showed a significant decrease at the 15th day (Figure 3b). Densitometric analysis of the immunoreactive bands was performed and β-actin (molecular mass of about 42 kDa), as an internal marker, was used to normalize the optical density.

Casein zimography

Four bands of enzymatic activity were identified on the gels. Two of these bands were located on the top of the gels, where μ-calpain normally is positioned; the other two migrated further into gels, where m-calpain runs.[43,45] We assumed that the two bands located on top of the gels corresponded to native and autolyzed μ-calpain activities (Figure 4, thin arrow), and the other two bands represent native and autolyzed m-calpain activities (Figure 4, thick arrow). The autolyzed forms of both μ- and m-calpain migrated slightly faster than the native forms.

Therefore, autolyzed μ-calpain migrated at a position in between native μ-calpain and native m-calpain.[46] During the *post-mortem* storage we observed a gradual reduction of native and autolyzed μ-calpain activities while native and autolyzed m-calpain activities persisted up to 15 days (Figure 4).

Controls

Negative controls did not show specific immunostaining. Moreover, the incubation of m-and μ-calpain antiserum preincubated with its homologous antigen showed no immunoreactivity. In Western blotting analysis, rat skeletal muscle, used as a positive control, showed a band of about 80 kDa when antibodies against m- and μ-calpains were employed (Figure 3a,b).

Discussion

In this study, we report on the presence, level of expression and enzymatic activity of m- and μ-calpain in the masseter and diaphragm muscle of Marchigiana cattle.

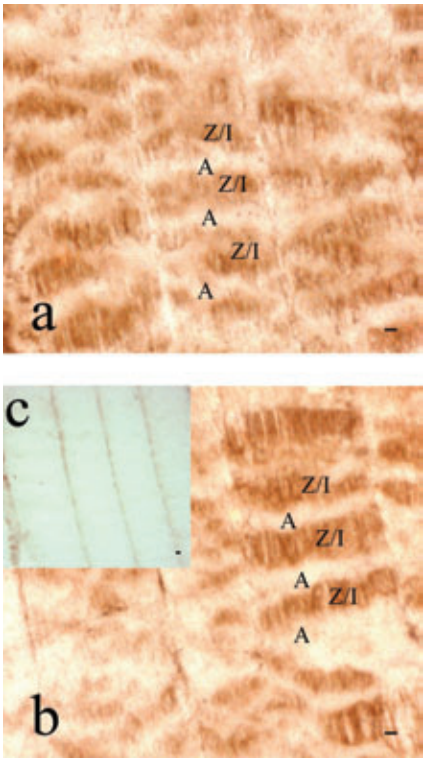

Figure 1. Immunohistochemistry: longitudinal sections of Marchigiana cattle messeter (a) and diaphragm (b) muscles. m- (a) and μ-(b) calpain immunopositivity was identified in the Z disk/I band region (Z/I), while the positivity was absent in the A-band area (A). Negative control (c). Scale bars: 10 μm.

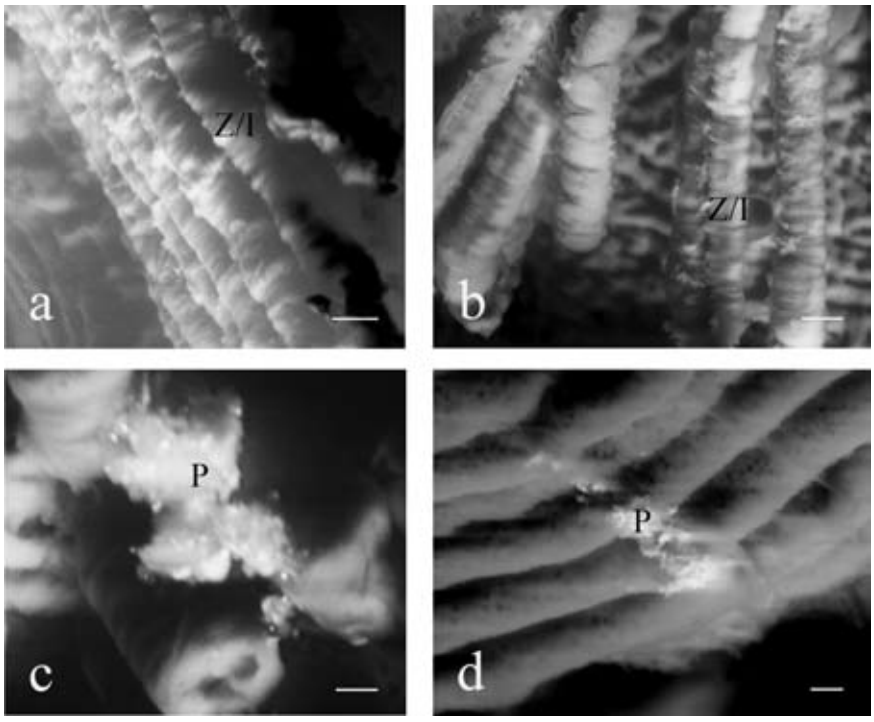

Figure 2. Immunogold labeling/SEM analysis: m- (a,c) and μ- (b,d) calpain immunoreactive gold particles localized in Z disk/I band region (Z/I) (a,b) and in the intracellular stores (P) (c,d) in the masseter muscle. Scale bars a,b: 30 μm; scale bars c,d: 10 μm.

Immunohistrochemistry and immunogold-labeling SEM analysis revealed the presence of calpains both along the Z disk/I band areas and in intracellular stores. These results are in agreement with the current literature. In fact, in normal skeletal muscle the majority of calpains are located on or next to the Z-disk with few in correspondence to the I-band and very few in correspondence to the A-band, as well as in vesicles and subcellular organelles.[36,47-50] Particularly, during the first few hours post-mortem, m- and µ-calpains are localized in subcellular organelles in the inner sarcoplasm, and then spread along Z disk/I-band areas of myofibrils beginning the transformation of muscle into meat.

The presence of m-and µ-calpains in correspondence to the Z disk/I band areas of the diaphragm and masseter muscles of Marchigiana cattle suggests that in these muscles the proteolytic action of calpains occurs on those proteins that are involved in keeping miofilaments attached to the myofibril. In fact, calpains rapidly cleave titin and nebulin at the point where these 2 polypeptides enter the Z disk.[2] Titin and nebulin cleavage, together with that of desmin and filamin, release α-actinin,[51] the principal Z disk protein, from the myofibril. Calpains do not degrade at all or degrade very slowly, actin and myosin,[2,35,51,50] the two major proteins in skeletal muscle myofibrils, implying that calpains had a limited and very specific subsite specificity.[35] Calpains also degrade M proteins, tropomyosin and troponin, albeit at slower rates than titin and nebulin.[50,52] In general, calpains cleave proteins at a limited number of sites and produce large polypeptide fragments rather than small peptides or amino acids.[50,52,53] Because of the limited specificity of the calpains, further degradation of myofibril proteins in aminoacids requires the participation of other proteases. It seems likely that the proteasome plays a major role in the degradation and release of actin, myosin and the other myofibrillar protein fragments.[54-56] The proteosome, on the other hand, cannot degrade intact myofibrils[57] or cytoskeletal complexes, likely because the entrance to the central cavity of the proteosome containing the active sites is only 19-13 Å in diameter and is much too narrow to allow entry of myofibrils that range from 10 to 100 µm in diameter. Therefore, the calpains begin the process of muscle transformation in meat that in turn, requires other proteases to be completed. Among these, the caspase system could be active *post-mortem* and contribute to tenderization[9,58,59] throughout an interaction with the calpain system. In fact, caspases may contribute to decrease the calpastatin level in the muscle aging and this, in turn, could results in the activation of calpains and thus reducing toughness.[9,60]

In the skeletal muscle of Marchigiana cattle the level of expression of m- and µ-calpains

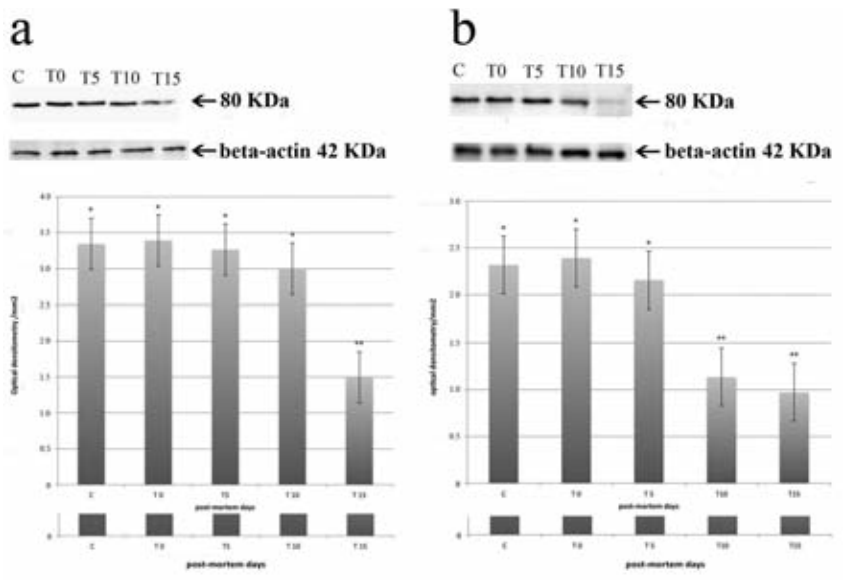

Figure 3. Upper: Western blotting analysis carried out on masseter (blot-a) and diaphragm (blot-b) muscles. The detected m- (blot-a) and µ- (blot-b) calpains showed a molecular mass of 80 kDa. β-actin showed a molecular mass of 42 kDa (blot a,b). C=control (rat skeletal muscle); T0= soon after animals slaughter; T5=5th day post-mortem (p.m.) storage; T10=10th day post mortem storage; T15= 15th day *post-mortem* storage. Down: densitometric analysis of the immunoreactive bands. Each value represents the mean ±SEM of ten independent experiments. Asterisks indicate statistically significant differences (P<0.05).

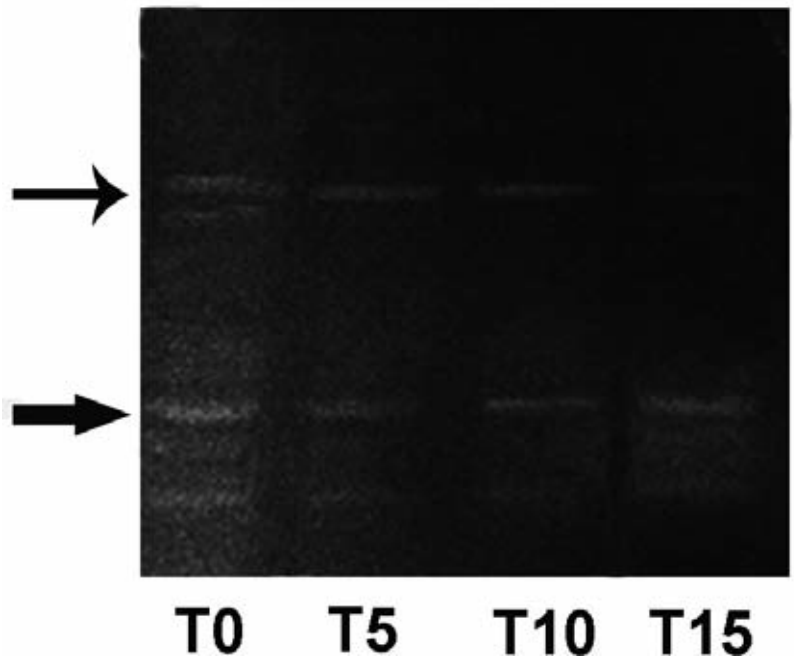

Figure 4. Top: native and autolyzed µ-calpain activities (thin arrow). Down: native and autolyzed m-calpain activities (thick arrow). T0=soon after animals slaughter; T5=5th day *post-mortem* (p.m.) storage; T10=10th day *post-mortem* storage; T15=15th day post-mortem storage.

and their enzymatic activity was detected up to 15 days *post-mortem*. Our results indicate a decrease in the level of expression of µ-calpain after 10 days of storage, while m-calpain expression persisted up to 15 days of *post-mortem* storage. The trend of activity of both µ- and m-calpain overlap with their expression pattern. It is well known that post mortem activation of m-calpain is due to the increasing concentration of Ca^{2+}.[28] The activity of µ- and m-calpain is synergistic: µ-calpain contributes to early post-mortem proteolysis, while m-calpain is partially activated and contributes to tenderization during prolonged ageing.[34,61-63] In the bovine skeletal muscles (*longissimus dorsi*, *semimembranosus*, *triceps brachii* and *psoas major*), the proteolytic activity of µ-calpain decreases rapidly during post mortem storage and very little activity can be detected after 48 h *post-mortem* storage at 4°C, so that only 10 to 20% of m-calpain activity remained after 144 h *post-mortem*.[46,64] The proteolitic activity of calpains depends on the Ca^{2+} concentration and pH of the muscle during the *post mortem* storage.[46,65,66] We choose, as muscle samples, the diaphragm and the masseter muscle because, in live animals, they are striated muscles subjected to mechanical and functional stresses during breathing (diaphragm muscle) and chewing (masseter muscle). By histoenzymatic staining, these muscles show a prevalence of oxidative fibers rather than glycolityc ones (*data not shown*) that could lead a slower lowering of the pH in the post-mortem storage and explain the slower trend of activity and expression of both µ- and m-calpain reported here.

Our results are in agreement with the picture emerging from previous researches, although we reveal a greater persistence of expression and enzymatic activity of m- and µ-calpains in Marchigiana cattle skeletal muscle. Such disagreement could be ascribed to the type of muscle chosen or to the breed considered for this study. Certainly, further investigations, involving, also, ultrastructural analysis, may help to study the role of calpains in meat tenderness in the Marchigiana cattle.

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