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Original Articles

Involvement of Calpain 3 in Postmortem Talin 2 Degradation Influencing Meat Quality

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Summary : The present experiment was conducted to determine which calpain isoforms contribute to the postmortem degradation of the costamere protein talin 2. The chicken *pectoralis major* (PM) and *biceps femoris* (BF) muscles, gizzard (G) and heart (H) were stored for 4 days at 4°C. Talin 2 in PM and BF were degraded during early postmortem time, but that in G and H were not degraded. While, desmin in PM was degraded faster than the others. The ratio of calpain 1 mRNA level to calpastatin was seemed to correspond to the postmortem degradation of desmin. The ratio of calpain 2 to calpastatin and the ratio of calpain 3 to ribosomal protein large P1 were seemed to correspond to the postmortem degradation of talin 2. Desmin in the isolated myofibrils of the skeletal muscles was degraded with 5 mM Ca^{2+} and not degraded with leupeptin, the ubiquitous calpain inhibitor. However, talin 2 was degraded with 5 mM Ca^{2+} and leupeptin. Desmin and talin 2 in the heart were not influenced by 5 mM Ca^{2+}. Furthermore, talin 2 was degraded with 1 μM Ca^{2+} at 4°C, but desmin was not. Therefore, we propose that talin 2 is degraded by calpain 3 during postmortem storage.

Key words : Calpain, Meat quality, Postmortem proteolysis, Talin.

Introduction

In skeletal muscle fibers, talin is localized in costameres and myotendinous junctions (MTJs) (Tidball *et al.* 1986). Talin is a large protein (235-270 kDa) with binding sites for integral membrane proteins (integrins β1A, β1D, β2 and β3), cytoskeletal proteins (F-actin, vinculin, and α-actinin) and focal adhesion kinase (FAK). It contains hydrophobic domains that anchor the protein to the cell membrane. The polypeptide chain consists of two domains: a globular N-terminal head domain (45-50 kDa) and a C-terminal rod domain (190-220 kDa). These two domains are generated by cleavage with calpain. The head domain has a globular conformation and is responsible for interactions with membrane phospholipids, F-actin, FAK and integrins. The rod domain assumes an extended conformation and couples F-actin to vinculin (Brzóska *et al.* 2004; Muguruma *et al.* 1990; Muguruma *et al.* 1992; Muguruma *et al.* 1995).

Postmortem changes in muscle proteins contribute to fresh meat quality. Meat tenderness is determined by the amount and solubility of connective tissue, sarcomere shortening during rigor development, and postmortem proteolysis of myofibrillar and myofibrillar-associated proteins. Given the effect of postmortem proteolysis on the muscle ultrastructure, titin and desmin are likely key substrates that determine meat tenderness (Koohmaraie & Geesink 2006). Degradation of costameres including desmin, talin and vinculin may also significantly weaken muscle structure (Taylor *et al.* 1995). A current hypothesis pro-

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poses that proteolysis of key muscle proteins, including the intermediate filament protein desmin and the costameric proteins vinculin and talin, minimizes the loss of water-holding capacity (Bee *et al.* 2007) caused by lateral shrinkage of myofibrils in postmortem muscle (Diesbourg *et al.* 1988). The proteolysis of muscle proteins contributing to these characteristics of fresh meat is usually attributed to the calcium-dependent protease calpain 1 (μ-calpain) (Koohmaraie 1992). Activation of calpain 1 might therefore be expected to predict the water-holding capacity and tenderness of fresh meat.

Vertebrates have two separate talin genes, *TLN1* and *TLN2*, which are found in all available vertebrate genomes. Several studies have suggested that talin 1 and talin 2 play non-redundant roles in vertebrates (Senetar *et al.* 2007).

Calpain 1 (μ-calpain) is known to degrade intermediate filament proteins and costameric proteins (including desmin, vinculin and talin) in postmortem muscle (Barbut *et al.* 2008). Chicken gizzard talin is cleaved into an N-terminal 47 kDa head domain and a C-terminal 190 kDa tail domain with calpain 2 (m-calpain) (Nuckolls *et al.* 1990). Furthermore, mouse skeletal muscle talin is a potential substrate for calpain 3 (p94) (Taveau *et al.* 2003). However, this study did not distinguish between talin1 and talin 2. Recently, it was reported that talin 2, but not talin 1 was localized in costameres in mouse skeletal and cardiac muscle and that, unlike talin 1, talin 2 may not be cleaved by calpain 2 (Senetar *et al.* 2007). We previously found that talin 2 was expressed in chicken skeletal muscle, and proposed that the degradation of talin 2 may influence the quality of chicken meat (Tomisaka *et al.* 2011). In this study, we aim to demonstrate which enzymes degrade talin 2 in chicken skeletal muscle during postmortem storage.

Materials and Methods

Meat samples

Chicken *pectoralis major* and *biceps femoris* muscles, gizzard, and heart were purchased from a meat shop. All meat was stored around 4°C for approximately 1 day (1d) after slaughter. Each sample was then stored at 4°C for an additional 1 (2d), 2 (3d), 3 days (4d).

Sample preparation

Samples (0.5 g) at each storage time point were added to 5 mL of rigor buffer (RB ; 75 mmol/L KCl, 10 mmol/L KH_2PO_4, 2 mmol/L $MgCl_2$, 2 mmol/L EGTA, pH 7.0) and homogenized using a Polytron (Kinematica, Littau, Switzerland) on the lowest setting, using 3-4-s bursts. The homogenate was centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant was discarded. Then 5 mL of fresh RB was added to the pellet and the homogenization was repeated. This process was repeated three times to obtain the final precipitate, which was added to 10 mL of RB. The protein concentration was measured using the biuret method and adjusted to 6 mg/mL. Samples were mixed 1 : 1 (final concentration of protein 3 mg/mL) with standard sample buffer (8 mol/L urea, 2 mol/L thiourea, 3 % (w/v) sodium dodecyl sulfate (SDS), 75 mmol/L Dl-dithiothreitol, 25 mmol/L Tris-HCl, pH 6.8), heated at 95°C for 5 min in a dry bath heater, cooled, and applied to an electrophoresis gel.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Polyacrylamide separating gels of 7.5 and 10 % were used for the determination of talin and desmin, respectively. Plate gels (120 mm wide × 102 mm long × 1 mm thick) were run on an AE-6400 electrophoresis unit (Atto, Tokyo, Japan) at a constant current of 20 mA. Gels were transferred to polyvinylidene difluoride membranes using a semidry transfer unit model BE-300 (Bio Craft, Tokyo, Japan) at a constant current of 144 mA for 40 min. EzBlot (Atto) reagents were used for the transfer buffer. After transfer, the membranes were blocked overnight at room temperature in EzBlock (Atto) in TTBS (20 mmol/L Tris, 500 mmol/L NaCl, 0.05 % (v/v) Tween20) and incubated for 1 h at room temperature with the primary antibody. The primary antibodies used in the western blotting procedure were anti-desmin (clone DE-U-10, catalog number D8281, Sigma-Aldrich, Saint Louis, MO, USA) and anti-talin 2 (catalog number AF4738, R & D Systems, Minneapolis, MN), diluted 1 : 1000 in TTBS. The membranes were then rinsed thoroughly with TTBS and incubated in a 1 : 5000 dilution of the secondary antibody in TTBS for 1 h at room temperature. The secondary antibodies for anti-desmin and anti-talin 2 were alkaline phosphatase (AP)-conjugated anti mouse IgG (catalog number AP1293, Sigma-Aldrich) and AP-conjugated anti goat IgG (catalog number AP106A, Chemicon International Temecula, CA), respectively. After thorough rinsing in TBS, the protein bands were visualized using the AP Conjugate Substrate Kit (Bio-Rad Laboratories, Hercules, CA, USA).

Real-time polymerase chain reaction (PCR)

RNA was extracted from 1d samples using

Table 1. Primers

Accession No.	Name		5'-sequence-3'
NM_205303	calpain 1 (μ-calpain)	Forward	CTGGATAAATCAGGCACCATGAG
		Reverse	TGATGCAGCTTGTTATTCAGCTTG
NM_205080	calpain 2 (m-calpain)	Forward	AATTGACGTGGATCGATCTGGAA
		Reverse	ACCGAGCCACAATGATCTGATGTA
NM_001004405	calpain 3 (p94)	Forward	TTCCGACACCTCTGGGACAAG
		Reverse	TGACTGCATTGCGCATCTCATA
NM_001137650	calpastatin	Forward	AGCCTTCAGCTCCTGCATCTACA
		Reverse	TGCTTCATCAGCTACGTGACCA
NM_205322	Ribosomal protein large P1 (RPLP1)	Forward	TCTCCACGACGACGAAGTCAC
		Reverse	TGCAGATGAGGCTTCCGATG

RNeasy Fibrous Tissue Mini kit (Qiagen, UK) according to the manufacturer's instructions. The RNA yield was quantified by a Qubit™ fluorometer using the Quant-iT RNA assay kit (Invitrogen, Carlsbad, CA, USA). A total of 1 μg of RNA from each sample was reverse transcribed in a 20 μL reaction using the PrimeScript® RT reagent Kit (Perfect Real Time) (Takara Bio Co., Shiga, Japan), according to the manufacturer's instructions. The primers and ribosomal protein large P1 (RPLP1) primers used as a reference gene for real-time PCR analysis were purchased from Takara Bio. These primers are shown in Table 1. Real-time PCR was performed using SYBR® Premix Ex Taq™ (Perfect Real Time) (Takara Bio) on a Smart Cycler® II System (Cepheid, Sunnyvale, CA). Reactions contained 2 μL of cDNA and 0.2 μmol/L forward and reverse primer sets in a total volume of 25 μL. Reactions were carried out as follows: 95°C for 30 seconds, followed by 45 cycles of 95°C for 5 seconds, and 60°C for 20 seconds. Each run was completed with a melting curve analysis to confirm the specificity of amplification and the absence of primer dimers. The amplification of genomic DNA was prevented by DNase treatment of extracted RNA. The ratios of calpains 1, 2, 3 and calpastatin mRNAs were normalized against ribosomal protein large P1 (RPLP1). The ratios of calpains 1 and 2 mRNAs were also normalized against calpastatin.

Myofibril isolation

Myofibrils were isolated from chicken *pectoralis major, biceps femoris* muscles and heart by a modification of the procedure of Goll *et al.* (1974) and Kendall *et al.* (1993). The *pectoralis major, biceps femoris* muscles and heart were obtained from a broiler chicken within 30 min after exsanguinations at a slaughter house. Eight grams of each muscle was homogenized in 80 mL of standard salt solution (SSS; 20 mM potassium phosphate, 2 mM $MgCl_2$, 2 mM ethylene glycol tetraacetic acid (EGTA), 1 mM NaN_3, 100 mM KCl, 2 mM phenylmethylsulfonyl fluoride (PMSF), pH 6.8) and centrifuged for 10 min at 1,000 × g at 4°C. The pellet was washed with 48 mL of SSS and centrifuged as before. The pellet was homogenized in 64 mL of SSS and strained through a strainer before centrifugation. This step was performed twice to facilitate removal of connective tissue. The pellet was washed successively with 64 mL of SSS plus 1 % Triton X-100 (twice), 64 mL of 100 mM KCl (six times), and 64 mL of 100 mM NaCl (three times). Isolated myofibrils were stored in a solution of 100 mM NaCl and 1 mM NaN_3 at 4°C until they were used (≤24 h). The myofibrils were washed three times with 100 mM Tris (pH 7.0) and 1 mM NaN_3 to remove NaCl. The biuret protein assay was performed on the preparation to determine myofibrillar protein concentration (Gornall *et al.* 1949).

Assays of myofibril-bound calpain

The effects of incubating isolated myofibrils in the presence or absence of Ca^{2+} on the integrity of myofibrillar proteins were assayed to determine if talin 2 and desmin are cleaved by myofibril-bound calpain during this incubation according to Boehm *et al.* (1998). The isolated myofibrils were incubated with or without 5 mM EDTA, 5 mM Ca^{2+}, 20 μM leupeptin and 5 mM Ca^{2+} + 20 μM leupeptin. The incubation buffer consisted of 2.4 mg myofibrillar protein/mL, 100 mM KCl, 20 mM Tris-HCl (pH7.5), 0.1% 2-mercaptoethanol and 1mM NaN_3. Samples were incubated for 24 h at 25°C. The isolated myofibrils were also incubated with 1 μM, 50 μM or 5 mM Ca^{2+} for 1, 3 and 7 d at 4°C in the same incubation buffer. The reactions were stopped by adding an equal volume of the SDS-PAGE sample buffer and heating

in a heating block for 5 min at 95°C. The samples were subjected to SDS-PAGE and western blotting as described above. Before western blotting, 7.5 and 10 % polyacrylamide separating gels were used for the determination of talin and desmin, respectively.

After the incubation for 24 h at 25 °C, the *pectoralis major* and *biceps femoris* samples were subjected to SDS-PAGE using 10% polyacrylamide separating gel and the gel was stained with Silver Stain Kit (Bio-Rad).

Statistical analysis

Real-time PCR results are expressed as the percentage of the mean for the highest expressing muscle. Values are reported as the means ± SEM for five samples of each muscle. Data were analyzed by one-way ANOVA followed by post-hoc analysis using the Tukey test.

Results and Discussion

Postmortem proteolysis of talin 2

Figure 1 shows the western blotting results of talin labeled by the anti-talin 2 antibody and desmin in the *pectoralis major* and *biceps femoris* muscles, gizzard and heart stored at 4 °C for 1 to 4 days postmortem. In our previous study, it was suggested that talin labeled by the anti-talin 2 antibody represents the chicken talin 2 isoform (Tomisaka *et al.* 2011). Talin 2 bands in the *pectoralis major* and *biceps femoris* muscles were reduced even at 1 and 2 days postmortem, and band 'a' around 200 kDa was strongly labeled in both muscles (Figures 1(A) and (C)). However, the talin 2 bands in the gizzard and heart were not reduced, and band 'a' was weaker than in skeletal muscles during 4 days storage (Figures 1(E) and (G)). As described above, talin consists of a head (45-50 kDa) and a rod domain (190-200 kDa), which are generated by cleavage with

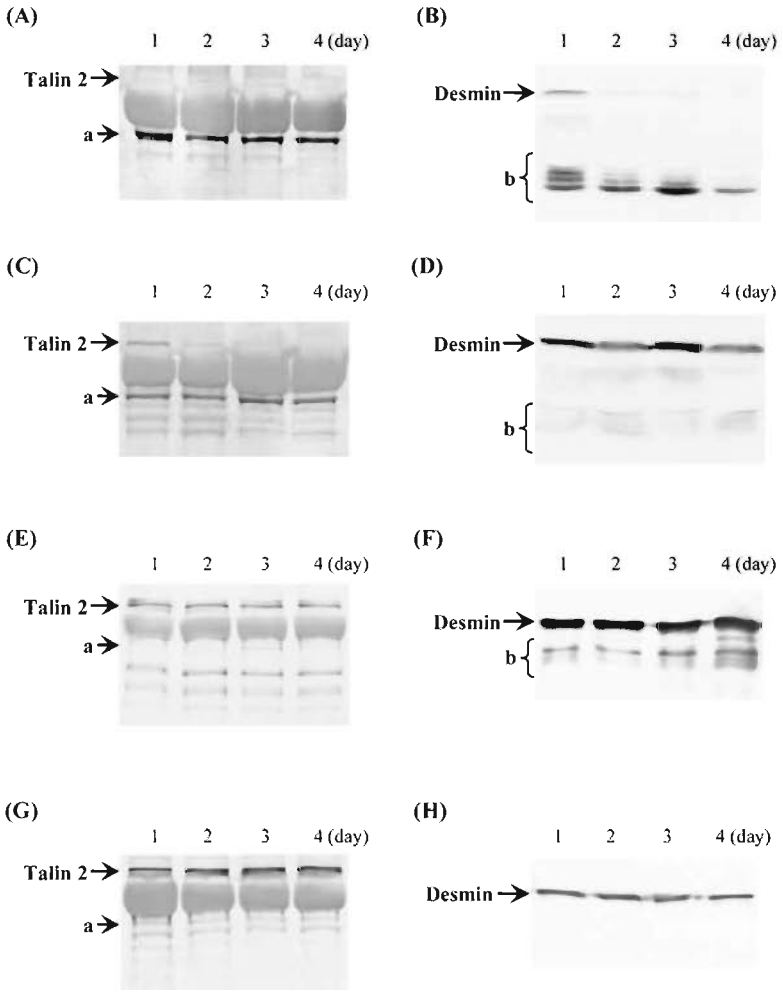

Fig. 1. Western blotting of talin and desmin in the *pectoralis major* and *biceps femoris* muscle, gizzard and heart stored at 4 °C for 1-4 days. (A), (B) : *pectoralis major*. (C), (D) : *biceps femoris*. (E), (F) : gizzard. (G), (H) : heart. (A), (C), (E), (G) : talin labeled by anti-Talin2. (B), (D), (F), (H) : desmin.

calpain. Therefore, band 'a' is likely to be the degradation product of the talin 2 rod domain. While, a number of talin 2 variants may be exist in skeletal muscle, because it was reported that not the only promoter might be used in skeletal muscle (Debrand *et al.* 2009). Therefore, the band 'a' may be a talin 2 variant. However, we suggest that talin 2 was degraded during early postmortem storage in the skeletal muscles, although talin 2 degradation in the gizzard and heart was considerably slower than in the skeletal muscles. Desmin in the *pectoralis major* muscle was reduced early and bands 'b' were strongly labeled. However, bands 'b' were less intense in the *biceps femoris* muscle and gizzard, and were not present in the heart during postmortem storage. Desmin (molecular mass 52 kDa) is degraded by calpain 1, and provides 35 and 38 kDa degradation products (Huff-Lonergan *et al.* 1996). These results suggest that desmin was degraded earlier during the postmortem stage in the *pectoralis major* muscle than in the *biceps femoris* muscle and gizzard, and desmin degradation in the heart did not occur during 4 days storage. Consequently, the degradation of talin 2 and desmin suggests that the activities of the proteases for talin 2 in skeletal muscles differ from those in the gizzard and heart, and that the protease for talin 2 differs from that for desmin during postmortem storage.

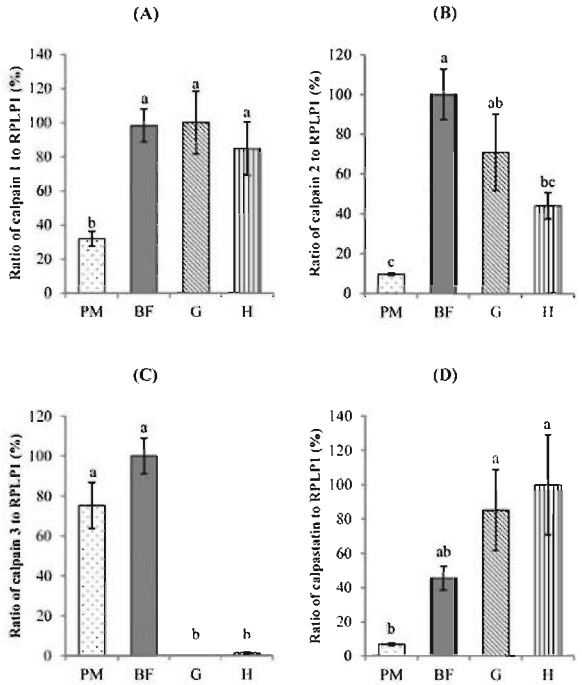

Fig. 2. Ratio of calpain 1, 2, 3 and calpastatin to ribosomal protein large P1 (RPLP1) mRNA in the *pectoralis major*, *biceps femoris*, gizzard and heart. (A) : calpain 1. (B) : calpain 2. (C) : calpain 3. (D) : calpastatin. PM : *pectoralis major*. BF : *biceps femoris*. G : gizzard. H : heart. All results are expressed as the mean ± SEM. Different letters are used to identify significant differences between means. Statistical significance was established as $P < 0.05$.

Expressions of calpains and calpastatin mRNA

Next, we measured expression of calpain 1, calpain 2, their inhibitor calpastatin, and calpain 3 mRNA levels in *pectoralis major* and *biceps femoris* muscles, gizzard and heart. The ratios of them were normalized against house-keeping gene RPLP1. The expressions of calpains 1 and 2 mRNA levels in *pectoralis major* muscle were the lowest in the samples (Figures 2(A) and (B), respectively). These results could not explain the postmortem degradation of both talin 2 and desmin, because the rate of degradation of talin 2 in the *pectoralis major* was faster than the gizzard and heart, and that of desmin was the fastest in the samples (Figure 1). While, the expressions of calpain 3 in the *pectoralis major* and *biceps femoris* muscles were substantially higher than in the gizzard and heart (Figure 2(C)). This is not surprising, as calpain 3 is a muscle-specific protease (Sorimachi *et al.* 1989). This result could explain the postmortem degradation of talin 2, because the degradation of talin 2 in *pectoralis major* and *biceps femoris* muscles were faster than the gizzard and heart.

The expression of calpastatin mRNA in *pectoralis major* which had a greater degradation of desmin during postmortem storage was the lowest and that in heart which had no degradation was the highest (Figure 2(D)). Whipple *et al.* (1990) reported that post-rigor calpastatin activity has explained a high proportion of the variation of meat tenderness. Melody *et al.* (2004) documented differences in calpastatin activity between muscles measured 6 and 24 h postmortem in *semimembranosus* (highest), *longissimus dorsi* (intermediate), and *psoas major* (lowest). These differences corresponded to significant differences in desmin degradation between the *psoas major* and *semimembranosus*. These findings may provide evidence that variation in calpastatin activity may provide a partial explanation for variation in observed proteolysis. Similaly, a higher calpastatin activity in slow oxidative muscles (type I) relative to fast glycolytic muscles (type II) has been reported in lambs (Singh *et al.* 1997). It is further supported by observations that fast glycolytic muscles in cattle have a lower calpastatin activity and are rated more tender than slow muscles, both by sensory and shear force analysis (O'Halloran *et al.* 1997). One interpretation

could be that where there are more slow glycolytic fibers, tenderization is likely to be impaired due to increased calpastatin inhibition of calpain-mediated proteolysis (Sazilli *et al.* 2005).

The ratio of calpain to calpastatin in cells is physiologically important because this ratio greatly influences the ability of Ca^{2+} to activate calpain. That is, an increased calpain-to-calpastatin ratio would favor calpain activation, whereas a decreased calpain-to-calpastatin ratio would favor calpain inhibition (French *et al.* 2006). Therefore, we calculated the ratios of calpains 1 and 2 mRNA levels to calpastatin as a normalizer. The ratio of calpain 1 to calpastatin was the highest in *pectoralis major* (Figure 3(A)), although the ratio of calpain 1 to RPLP1 was lowest in *pectoralis major* (Figure 2(A)). The chicken thigh muscle contains both slow (type I) and fast (types IIa and IIb) contracting fibers (Suzuki *et al.* 1985), while the *pectoralis major* muscle contains only fast contracting (type IIb) myofibers (Remignon *et al.* 1996). Muroya *et al.* (2010) reported that desmin and troponin T are degraded faster in type IIb muscle fibers than in type I fibers during postmortem aging of porcine muscle. Therefore, it is hypothesized that activity of protease in the chicken *pectoralis major* muscle is higher than in the *biceps femoris* muscle. This hypothesis is supported by the ratio of calpain 1 to calpastatin in this study. In other words, our current results suggest that the ratio of calpain 1 to calpastatin mRNA expression, but not the absolute expression of calpain 1, reflects the degree of protease activity and postmortem proteolysis of desmin in the muscles. The ratio of calpain 2 to calpastatin mRNA expression in the *pectoralis major* and *biceps femoris* muscles were higher than in the gizzard and heart (Figure 3(B)).

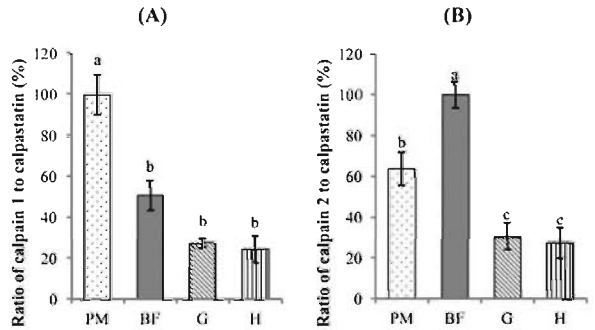

Fig. 3. Ratio of calpain 1 and 2 to calpastatin mRNA in the *pectoralis major*, *biceps femoris*, gizzard and heart. (A) : calpain 1. (B) : calpain 2. PM : *pectoralis major*. BF : *biceps femoris*. G : gizzard. H : heart. All results are expressed as the mean ± SEM. Different letters are used to identify significant differences between means. Statistical significance was established as $P < 0.05$.

This finding may explain the postmortem degradation of talin 2 which had the faster degradation in *pectoralis major* and *biceps femoris* muscles than the gizzard and heart.

Taking together, the results on the expression of ubiquitous and muscle-specific calpain isoforms mRNA levels and the ratios of ubiquitous calpains to calpastatin with the degree of proteolysis in desmin and talin 2 during postmortem storage, it appears that desmin is degraded by calpain 1 and talin 2 is degraded by calpain 2 or calpain 3.

Myofibril-bound calpain activity

We isolated myofibrils from the *pectoralis major* and *biceps femoris* muscles (skeletal muscles), and heart (cardiac muscle). Talin 2 levels are similar in the *pectoralis major* and *biceps femoris* as in the heart, as shown by similar expression of talin 2 mRNA levels and band patterns in western blotting between the skeletal muscles and heart (Tomisaka *et al.* 2011). The isolated myofibrils were incubated with EDTA, Ca^{2+} or leupeptin to assay calpain activity in the skeletal (myofibril-bound) and cardiac muscle. Figure 4 shows western blotting of desmin and talin 2 in the incubated samples. Desmin was degraded in the myofibrils incubated with Ca^{2+}, and was not changed after incubation with EDTA and leupeptin (Figures 4(A) and (C)). Like desmin, talin 2 was degraded in the myofibril incubated with Ca^{2+}, and was not changed after incubation with EDTA. However, talin 2 was not degraded after incubation with leupeptin (+Leu, Figures 4(B) and (D)), but was degraded with leupeptin and Ca^{2+} (+Ca+Leu, Figures 4(B) and (D)). Desmin and talin 2 in the heart were not degraded under any incubation conditions (Figures 4(E) and (F)). Calpains 1 and 2 are Ca^{2+}-dependent proteases, and are inhibited by leupeptin (Sorimachi *et al.* 1997). Calpain 3 is also Ca^{2+}-dependent, but is not inhibited by leupeptin (Murphy 2010). Furthermore, our results suggested that desmin and talin 2 in the heart were not degraded due to the significantly lower ratios of calpains 1 and 2 to calpastatin (Figures 3(A) and (B)) and the significantly lower expression levels of calpain 3 mRNA (Figure 2(C)), nor due to the disappearance of calpains during the isolation process. It was also suggested that Ca^{2+} does not influence the degradation of desmin and talin 2 directly, although Tatsumi and Takahashi (2003) reported that calcium ions at 0.1 mM reacted with titin and nebulin filaments and induced their severance. Therefore, in this study we propose that desmin was degraded by ubiquitous calpains and talin 2 was degraded by calpain 3.

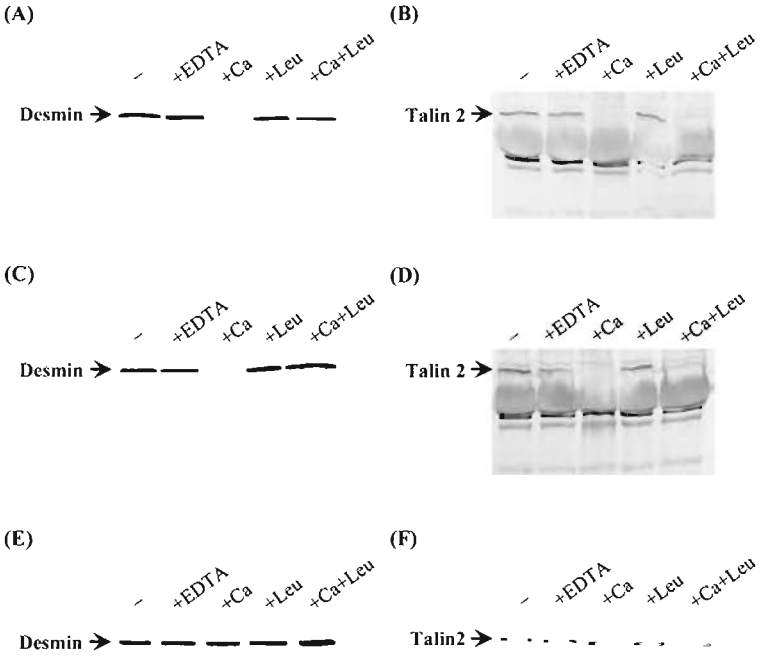

Fig. 4. Western blotting of isolated myofibrils obtained from the *pectoralis major* ((A), (B)) and *biceps femoris* ((C), (D)) muscles, and heart ((E), (F)) with Ca^{2+} or leupeptin. The isolated myofibrils were incubated with 5mM EDTA (+EDTA), 5 mM Ca^{2+} (+Ca), 20 μM leupeptin (+Leu), 5 mM Ca^{2+} + 20 μM leupeptin (+Ca+Leu) or without them (–). The blot was labeled with anti-desmin ((A), (C), (E)) or anti-talin2 ((B), (D), (F)) antibody.

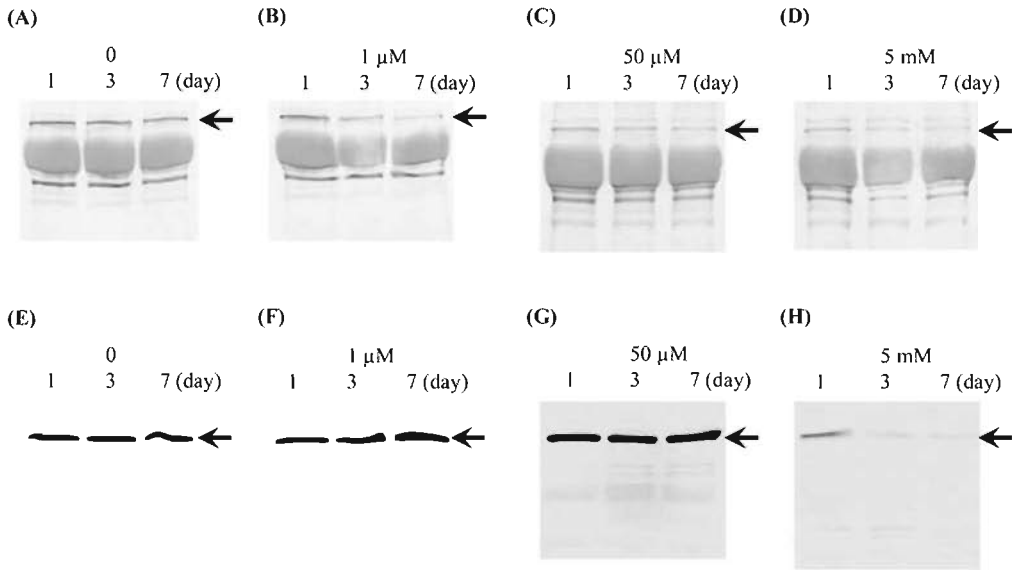

Fig. 5. Talin 2 and desmin in the isolated myofibril incubated with 0, 1 μM, 50 μM and 50 mM Ca^{2+} for 1, 3 and 7 d at 4°C. Arrows in (A), (B), (C), (D) : talin 2. Arrows in (E), (F), (G), (H) : desmin. (A), (E) : without Ca^{2+}. (B), (F) : with 1 μM Ca^{2+}. (C), (G) : with 50 μM Ca^{2+}. (D), (H) : with 5 mM Ca^{2+}.

The degradations of desmin and talin 2 with the lower concentration of Ca^{2+} at lower temperature were researched. The degradation of desmin was observed with 5 mM Ca^{2+} even at 4°C (Figure 5(H)) and that was small with 50 μM (Figure 5(G)). The degradation of talin 2 was also observed at 4°C as desmin.

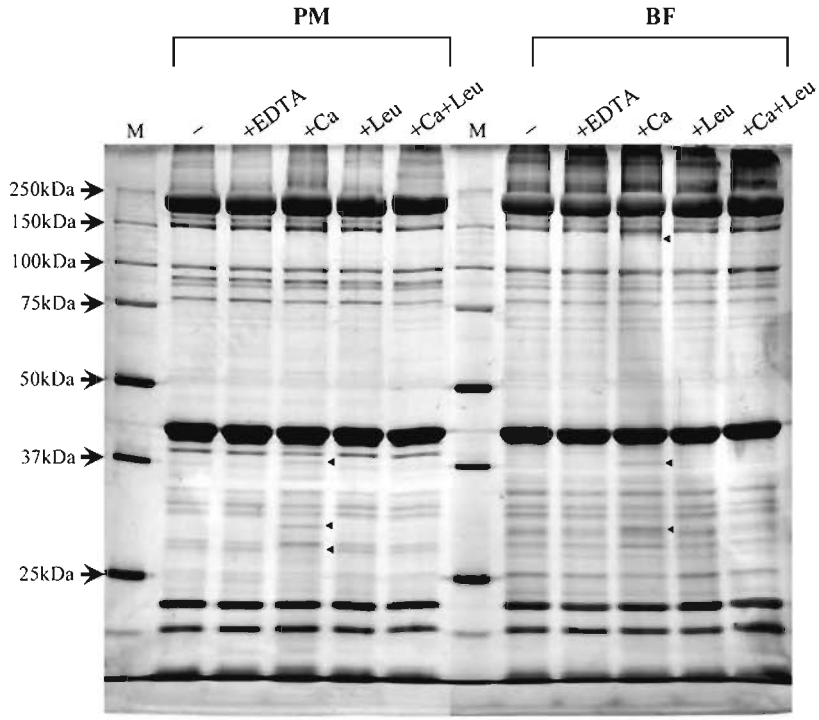

Fig. 6. SDS-PAGE of isolated myofibrils obtained from the *pectoralis major* (PM) and *biceps femoris* (BF) muscles with Ca^{2+} or leupeptin. The isolated myofibrils were incubated with 5mM EDTA (+EDTA), 5 mM Ca^{2+} (+Ca), 20 μM leupeptin (+Leu), 5 mM Ca^{2+} + 20 μM leupeptin (+Ca+Leu) or without them (–). M : molecular weight marker. Arrow heads shows bands appeared or diappeared.

Remarkably, talin 2 degraded even with 1 μM Ca^{2+} (Figure 5(B)). Calpain 1 requires between 5 and 65 μM Ca^{2+} for half-maximal activity (Goll *et al.* 1992), while 200 nM Ca^{2+} resulted in approximately 20% autolysis of calpain 3 (Murphy 2010). Therefore, again, our observation in this study may suggest that talin 2 is degraded by calpain 3 during postmortem storage.

Figure 6 shows an SDS-PAGE gel of myofibrils incubated in the presence or absence of Ca^{2+}. It shows that there are several bands that appeared or disappeared in the myofibrils of the *pectoralis major* and *biceps femoris* muscles after incubation with Ca^{2+} (Figure 6, lane +Ca). These changes were not apparent in the myofibrils incubated with both Ca^{2+} and leupeptin (Figure 6, lane +Ca+Leu). In sheep, variations in calpain 3 mRNA and protein levels have been reported to strongly correlate with variations in tenderness (Ilian *et al.* 2001) and to be involved in myofibrillar protein degradation in the ovine *longissimus dorsi* (LD) muscle (Ilian *et al* 2004). Although Geesink *et al.* (2005) reported that postmortem proteolysis occurred in a similar fashion in calpain 3 knockout mice and in control wild type mice, with no differences detected in desmin, nebulin, troponin-T or vinculin degradation. Therefore, they suggested that calpain 3 is not involved in meat tenderization. Similar to these findings, it appeared that desmin was not degraded by calpain 3 in our study. However, it is likely that talin 2 is degraded by calpain 3. Therefore, our results suggest that the extent of postmortem protein degradation with calpain 3 is restricted in comparison with calpains 1 and 2.

For the most part, the two ubiquitous calpains cleave the same substrates, including cytoskeletal proteins, such as the troponin complex (TnC, TnI, TnT, tropomyosin), α-actinin, titin, the Z-disk proteins fodrin and desmin and the sarcolemmal-associated spectrin complex of proteins *in vitro* (Goll *et al.* 2003; Murphy 2010). Titin and desmin are likely to be key substrates that determine meat tenderness (Koomaraie & Geesink 2006). Therefore, experimental investigations have tried to determine which isoform is primarily involved in postmortem proteolysis. Calpain 1 is activated in early postmortem (within 3 days of slaughter), during the period when postmortem proteolysis of key myofibrillar proteins is known to take place (Taylor *et al.* 1995). Sensky *et al.* (1996) have reported that calpain 2 persists longer than the less stable calpain 1 in aging muscle from the porcine *longissimus*, suggesting that it is not activated early postmortem. Additionally, the Ca^{2+} concentrations that

exist in postmortem muscle are less than those required for activation of calpain 2 (Boehm *et al.* 1998). Recently, the evidence for a significant role of calpain 1 in postmortem proteolysis has further been strengthened from observations made in calpain 1 knockout mice (Geesink *et al.* 2005).

Calpain 3 binds to titin at the N_2 line (Keira *et al.* 2003 ; Sorimachi *et al.* 1995), a site where proteolysis has been linked to meat tenderization (Taylor *et al.* 1995), and colocalizes with talin at costameres in mouse skeletal muscle (Taveau *et al.* 2003). Both titin and talin are substrates of calpain 3 (Taveau *et al.* 2003). Conti *et al.* (2008) and Senetar *et al.* (2007) have suggested that mouse skeletal muscle talin at costameres is talin 2, but not talin 1. Like calpains 1 and 2, calpain 3 is a Ca^{2+}-dependent protease, but the activation of calpain 3 needs a small but sustained increase in Ca^{2+}. Both calpains 1 and 3 must autolyse to become active in a Ca^{2+}-dependent manner (Murphy 2010). Therefore, it is likely that lower concentrations of Ca^{2+} during early postmortem time cause earlier activation of calpain 3 than of calpain 1. It has been shown that calpain 3 is tightly bound to titin, and is actually quite stable in skeletal muscle (Murphy *et al.* 2006). However, using western blotting it has been established that calpain 3 does autolyze in postmortem muscle (Koohmaraie & Geesink 2006). Therefore, it is highly likely that calpain 3 is activated and degrades talin 2 in chicken skeletal muscle during the early postmortem stages.

Recently, a model has been proposed that suggests that the protease family of caspases could be active postmortem and contribute to tenderization. It has been hypothesized that the process of slaughter and exsanguination initiates the apoptotic pathways and caspase activity may contribute to early postmortem proteolysis (Kemp *et al.* 2010). Calpain inhibition through over-expression of calpastatin has been shown to increase caspase 3 activity and apoptosis. Additionally the endogenous calpain inhibitor calpastatin is also cleaved by caspase 1, 3 and 7, generating distinct degradation patterns. Therefore, if caspases are active in the postmortem muscle they may influence meat quality by proteolysis of calpastatin (Kemp *et al.* 2010). Calpain 3 may also influence meat quality in a similar way as the caspases, because calpastatin is cleaved by calpain 3 (Ono *et al.* 2004). Furthermore, the other myofibrillar proteins, including filamin C (Taveau *et al.* 2003), vinexin, ezrin and myosin light chain 1 (Cohen *et al.* 2006), are also substrates of calpain 3.

Thus, calpain 3 may degrade myofibrillar proteins during postmortem storage, which influences meat quality. However, it appears that the extent of protein degradation with calpain 3 is restricted in comparison with calpains 1 and 2. Consequently, we propose that talin 2 is degraded by calpain 3 in skeletal muscle during the early postmortem stages, and that not only calpain 1 but also calpain 3, in part, participate in postmortem proteolysis in chicken skeletal muscle. This may result in development of tenderness and improving the water-holding capacity of chicken meat.

Conclusion

Postmortem degradation of costamere proteins influences meat qualities such as tenderness and water-holding capacity. Talin 2 is localized in the costameres and its degradation may influence meat quality. In this study, we investigated if the ubiquitous calpains, calpains 1 (μ-calpain) and 2 (m-calpain), and muscle-specific calpain, calpain 3 (p94), are responsible for postmortem degradation of talin 2. Talin 2 in the *pectoralis major* and *biceps femoris* muscles were reduced during early postmortem time. However, talin 2 in the gizzard and heart did not diminish during 4 days of storage. Desmin in the *pectoralis major* muscle decreased early. However, the degradation in the *biceps femoris* muscle and gizzard was low, and there was no degradation in the heart during postmortem storage. The ratio of calpain 1 to calpastatin mRNA levels correlated with postmortem degradation of desmin, and the expression of calpain 3 mRNA levels and the ratio of calpain 2 to calpastatin mRNA levels correlated with degradation of talin 2. Desmin degradation was inhibited by leupeptin, but that of talin 2 was not. It is known that leupeptin inhibits calpains 1 and 2, but not calpain 3. Therefore, we propose that talin 2 is degraded by calpain 3 during early postmortem storage.

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肉質に影響するタリン２の死後分解におけるカルパイン３の関与

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要 約

本研究は，コスタメアタンパク質タリン２の死後分解に関わるカルパインアイソフォームを決定するために実施された．ニワトリの胸肉，腿肉，砂のう筋および心筋を４℃で４日間貯蔵した．胸肉と腿肉のタリン２は死後早期に分解したが，砂のう筋と心筋では分解しなかった．一方，胸肉のデスミンは他の組織よりも早期に分解した．カルパスタチンに対するカルパイン１の相対的な発現量は，デスミンの死後分解の速度と一致しているように見えた．カルパスタチンに対するカルパイン２の相対的な発現量およびリボゾーマルプロテインラージP1に対するカルパイン３の相対的な発現量は，タリン２の死後分解の速度と一致しているように見えた．骨格筋から抽出された筋原線維におけるデスミンは，５mMのカルシウムイオンの添加によって分解したが，それはロイペプチンの添加によって阻害された．しかしながら，タリン２は，デスミン同様，５mMのカルシウムイオンの添加で分解したが，ロイペプチンの添加で阻害されなかった．心筋におけるデスミンとタリン２はともに，５mMのカルシウムイオンに影響を受けなかった．さらに，タリン２は４℃で１μMのカルシウムイオンの添加によっても分解したが，デスミンは分解しなかった．よって，我々は，タリン２が死後の熟成期間中にカルパイン３によって分解されることを提案する．

キーワード：カルパイン，肉質，死後分解，
タリン