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Influence of Calpain on Lamb Myofibrillar Proteins Degradation during *in vitro* Culture

Mukaddas MUHTAR, Ma Yi QIAO, Batur ABLIKIM, Arzugul ABDUWALI and Yusup SULAYMAN*

College of Food Science and Pharmacy, Xinjiang Agricultural University, Wulumuqi, P.R. China

**Corresponding author: batur6805@126.com*

Abstract

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Tenderness is an important indicator of meat quality, degradation plays important role during the maturation, therefore improving meat quality and tenderness. Understanding the process of myofibrillar and which enzymes affect degradation is more needed to prove. In this study we used longest muscle of Bashibai sheep of Xinjiang as the experiment material. The isolated myofibrillar protein and μ-calpains are incubated at 4°C about 1, 3, 7, 14, and 21 days using *in vitro* culture method and analysed by SDS-PAGE, Western blotting. Results showed that μ-calpain can improve the degradation of some myofibrillar proteins. Desmin was degraded by the 3rd, troponin–T was completely degraded by the 7th day.

Keywords: lamb; myofibrillar; protein; tenderness; μ-calpain

Providing high-quality proteins for human consumption is an important responsibility of meat researchers (Pereira & Vicente 2013). From a sensory point of view, tenderness is the one of the most important quality. Meat tenderness increases the consumer's interest for buying (Ristic & Miscevic 2012) and is dependent on genotype, age, sex, management factors as diet, pre slaughter handling, post-mortem mature, and type of muscle (Maltin *et al*. 2003; Koohmaraie & Geesink 2006; Marino *et al*. 2015). It has been reported that apply some methods such as electrical stimulation and stretching can improve meat tenderness (Locker 1960; Davey *et al*. 1967; Herring *et al*. 1967; Macfarlane *et al*. 1974; Devine *et al*. 2002; Nollet & Toldra 2006). Myofibril as basis for composition of muscle fibre plays an important role to improve meat tenderness (Hopkins & Thompson 2002). Myofibril composed of various myofibrillar proteins, during maturation Desmin degrade rapidly, (Lonergan *et al*. 2010), degradation of muscle protein (Desmin) and troponin-T will destroy the original structure of myofibrils to reduce shear force, therefore improve the meat tenderness (Taylor *et al*. 1995; Koohmaraie 1996). In essence, there are two explanation for myofibrillar degradation: one is calcium ions directly affected myofibrillar protein degradation, this interpretation is also known as calcium ion theory (Takahashi 1999). Another explanation is that myofibrillar protein degraded by endogenous enzymes, and more scholar think calcium ions only make affect for this process, it cannot degrade myofibrillar protein without endogenous enzyme (Huang *et al*. 2004).

There are many endogenous enzymes in the muscle, for example lysosomal tissue proteins, proteasome, apoptotic enzymes, and calcium-activating enzymes (Costelli *et al*. 2005). In such a complex biochemical system, the absence of other endogenous enzyme, calpain can promote myofibrillar protein degradation. Which kind of calpain plays an important role, needs to prove. This experiment, utilized *in vitro* culture

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mode, studied the effect of calpain on the degradation of myofibrillar protein to provide a theoretical basis for the improvement of mutton tenderness.

MATERIAL AND METHODS

Five Bashibai sheep was slaughtered. The *longissimus dorsi* muscle used as the experimental material, myofibrils were extracted and purified according to the procedure reported by (Etilnger & Fischman 1976; Smuder *et al*. 2010) with small modification, minced muscle was homogenised in approximately 7.5 volumes of pyrophosphate relaxing buffer (PRB) (100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 1m M NaN_3, 2 mM $Na_4P_2O_7$, 10 mM Tris-maleate, pH 6.8), using a polytron at a speed of 15 000 rpm for 10 s, three times, with a 15 s interval cooling period between bursts, and then the homogenate was centrifuged at 1 000 *g* for 10 minutes. The pellet was subsequently washed eight times, each wash with 10 volumes of low salt buffer (same as PRB except for pyrophosphate being omitted). In the last step, the myofibrils were suspended in incubation buffer (5 mM Hepes, 100 mM NaCl, 10 mM DTT, 0.1% Chaps, and 5 mM NaN_3, pH 7.4). Protein concentration was determined with a BCA Protein Assay Kit, following the standard protocol recommended by the manufacturer (Pierce).

In vitro culture of calpain. Calpain was added in myofibrillar protein, in the condition that calpain was the only active substance in this system, reference used (Xue *et al*. 2012) method and slightly modified. After separated and purified, concentration of the myofibrillar protein was diluted 10 mg/ml, 60 µl myofibrillar protein, added 15 µl of a 0.3 g/l calcium chloride and mixed, and 5 µl of calpain was added to the experimental group and the control group were added 5 l incubation buffer, without DTT. The volume of 80 µl, the 500 mol/l samples storaged in 4°C, incubated for 0, 1, 3, 7, 14, and 21 days and added sample treatment solution of 70 ml (125 mmol/l Tris, 2% SDS, 0.8% β-mercaptoethanol, 20% glycerinum, 0.02% bromophenol blue, pH 6.8). The samples were well mixed and heated in a 50°C water bath for 20 min, then stored at –20°C for subsequent SDS-PAGE and Western blotting.

SDS-PAGE electrophoresis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 5% stacking gel and 12.5% separating gel (acrylamide and methyl acrylamide 37.5 : 1 [w/w]), were run at a constant voltage of 80 V for 30 min, and then a constant voltage of 130 V for nearly 2 hours. After electrophoresis, a portion of the gel was stained with (0.1% CBB coomassie brilliant blue, 50% methyl alcohol, 10% glacial acetic acid) for 4 h, placed in a bleaching solution (25% methyl alcohol, 7% glacial acetic acid) overnight, analysis of observation, another part of the gel for protein immunoblot analysis.

Western Blot. SDS-PAGE Western Blot Simultaneously, the proteins and peptides in the other gel were transferred onto polyvinylidine fluoride (PVDF) membranes (Milli-pore) in transfer buffer containing 25 mM Tris-Base, PVDF membrane containing 5% non-fat dry milk powder in TBS-Tween (TTBS: 0.05% Tween-20, 20 mM Tris-Base, 137 mM NaCl, and 5 mM KCl). Kept at room temperature 2 h, rinsing 1time end reacted with diluted 500-fold desmin, troponin-T protein monoclonal antibody 2 h in 4°C and dipped three times with TTBS 15 min added Peroxidase- labeled anti-mouse secondary antibody reacted 90 min at room temperature used TTBS rinsing 3 times, express colour.

RESULTS AND DISCUSSION

Analysis of myofibrillar protein degradation. The Figure 1 shows SDS-PAGE analysis of myofibrillar proteins without endogenous enzyme. Calpain (CAPN) Ca^{2+}dependent cysteine proteases. They divided in µ-calpains and m-calpains, which require respectively micro and millimolar concentrations of

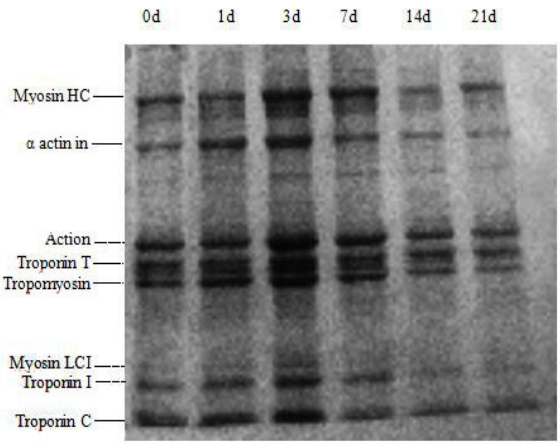

Figure 1. SDS-PAGE separation patterns of myofibrillar protein extract from longissimus dorsi muscle after removed endogenous enzyme from raw meat, kept at 4°C for 21days, with calcium ion

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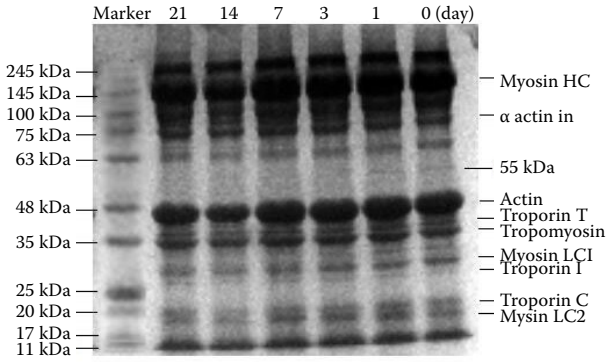

Figure 2. SDS-PAGE separation patterns of myofibrillar protein extract from lam *longissimus dorsi* muscle after removed endogenous enzyme and calcium ion to insure calpain is the only influenceable factor of this system, kept at 4°C for 21 days, just as *in vitro* culture of lamb myofibrillar proteins with calpain

Ca^{2+} for their activity; a numerical classification is established on the basis of the domain compositions. The skeletal muscle comprises CAPN1 (μ-type) and CAPN2 (m-type) (ubiquitous), CAPN3 (skeletal muscle-specific) and the endogenous inhibitor calpastatin (CAST) (Goll *et al*. 2003). From (Figure 1) we can learn that in the absence of endogenous enzymes, myofibrillar proteins did not degrade significantly under the action of calcium alone, and no bands appear or disappear with the incubation time.

We can affirm from (Figure 2) that in the absence of other endogenous enzymes, calpain was incubated with single factor of myofibrils, the day 1 compared to day 0, 55 kDa myofibrillar proteins fragment has become blurred. And after incubated 3 days, this fragment disappeared. Near the proteins fragment of 35 kDa, in the day 0 and day 1 is also clearly visible when it incubated 3 days it was significantly unclear and by the time of the first 7 days, this electrophoretic band was completely disappeared.

Desmin degradation analysis. Desmin, with molecular weight of approximately 53 kDa, desmin is the main intermediate filament protein of all muscle cells, which links individual myofibrils at the level of the Z-disk and connects them to the sarcolemma and mitochondria (Paulin & Li 2004). During the ageing of mammalian skeletal muscle, desmin is one of the earliest marker proteins for muscle as it participates in the early establishment of sarcomere structure. Owing to these important structure-maintaining functions, desmin degradation is believed to be essential for the tenderization of meat during postmortem ageing (Taylor *et al*. 1995).

In the present study, muscle proteins were apparently degraded during incubation (Figure 3). The immunoprecipitated zone still complete band on 1 day but compared with the day 0, 1 day ban is little faded and desmin was degraded at day 3, at the 7th day of incubation, the band had completely disappeared desmin had completely degraded. This result same as (Muroya *et al*. 2010; Kemp & Parr 2012) who found that desmin degradation was evident at 3 days postmortem ageing and was almost completely degraded after 7 days but they did not provide the evidence of the enzyme that affects degradation. Desmin is believed to have a significant impact on meat tenderization by distinctively affecting meat shear force and water-holding capacity (Taylor *et al*. 1995; Kristensen & Purslow 2001; Lonergan *et al*. 2010).

The band at 35 kDa was the degradation product of muscle intermolecular protein, 35 kDa was more vague at 0 day and 1 day after incubation, and the clearest band appeared when incubated 3 days. With the prolongation of time, the band was also degraded and eventually disappeared, this observation is in accord with the findings of (Kristensen & Purslow 2001) who analysed on porcine myofibrillar protein by Western Blot and summarized that in the mature process of degradation, desmin has conversed small molecular protein which molecular weight was 35 kDa, this conclusion is consistent with the experimental results.

Degradation analysis of troponin-T. The molecular weight of troponin-T is about 35 kDa, and is

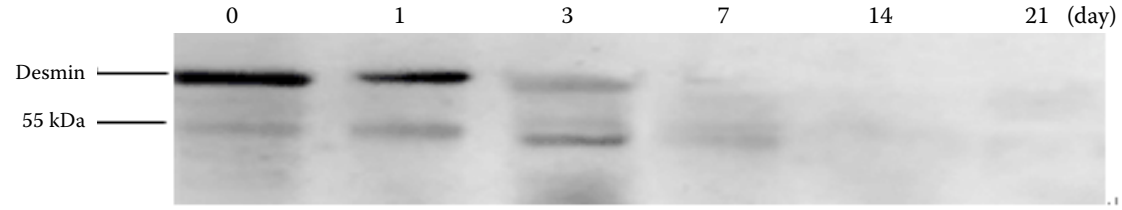

Figure 3. SDS-PAGE separation patterns of desmin during *in vitro* culture, extract from lam *longissimus dorsi* muscle (after removed endogenous enzyme and calcium ion to insure calpain is the only influenceable factor of this system), kept at 4°C for 21 days

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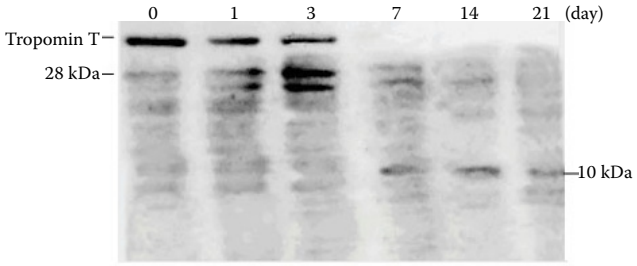

Figure 4. SDS-PAGE separation patterns of troponin-T during *in vitro* culture, extract from lam *longissimus dorsi* muscle (after removed endogenous enzyme and calcium ion to insure calpain is the only influenceable factor of this system), kept at 4°C for 21 days

the tropomyosin-binding component of the troponin complex, which is involved in the calcium-dependent regulation of skeletal muscle contraction (Harris *et al*. 2001). Degradation of troponin-T has been proved be highly related to the post-mortem tenderization of meat (Anderson *et al*. 2012; Cruzen *et al*. 2014).

In this study, troponin-T had a relatively complete immunoprecipitation on the first day of incubation, and the troponin-T band disappeared after 7 days of incubation, indicating that troponin-T was completely degraded during incubation (Figure 4). At 28 kDa and 10 kDa, the bands were appeared, 28 kDa was significantly increased at day 1. However, the bands at 28 kDa gradually disappeared with the increase of incubation time, (Harris *et al*. 2001) who reported that during postmortem storage of meat, proteolysis of troponin-T results in two apparently stable fragments with molecular weights of 28 kDa and 30 kDa, amounts increased with extended storage time, after incubation for 3 days, troponin-T produced a second degradation, the 28 kDa protein fragment degrades to a smaller protein fragment with a molecular weight of 10 kDa, while troponin-T has been completely degraded, protein band disappears, (Huang *et al*. 2004) who showed that myofibrillar protein of mature beef was degraded by endogenous enzymes and would produce two kinds of protein fragments that molecular weights was 30 kDa and10 kDa.

CONCLUSIONS

Experiment utilized *in vitro* culture, study on the effect of single factor calpain myofibrillar protein degradation which incubated *in vitro* with calpain and verifed the role of calpain in the maturation of mutton. The experimental results showed that the control group (Figure 1) was no obvious effect to degradation of myofibrillar protein, which provided with the extension of time, myofibrillar protein was not occurrence degradation. During incubation with calpain (Figure 2) the original part of the protein band had been disappeared the corresponding molecular degrade band arisen.

Process of *in vitro* culture myofibrillar protein was directly degraded by calpain compared with the control group, which without calpain, we can learn that myofibrillar protein of this group was maintained the original structure, does not appeared any degradation. By this way we can summarize degradation of myofibrillar protein is mainly attributed to the role of calcium activated enzyme. And that calpain can promote the degradation of protein in a way to ultimately improve the tenderness of mutton. (Li *et al*. 2017) who found the degradation of MHC, desmin, actin and troponin-T were performed by western blot.

Compared with the control group we can affirm that after incubation with μ-calpain myofibrillar protein was obviously degraded, which illustrated that calpain can promote the degradation of myofibrillar protein.

Under the condition of *in vitro* culture, desmin and troponin-T were degraded too (Figure 3 and 4) and the degradation of desmin and troponin-T is mainly due to the effect of μ-calpain. Further, μ-calpain can improve the tenderness of mutton.

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