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Calpastatin inhibits the activity of phosphorylated μ-calpain *in vitro*

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ABSTRACT

The objective of this study was to investigate the effect of phosphorylation on the sensitivity of μ-calpain to the inhibition induced by calpastatin. Purified μ-calpain was incubated with alkaline phosphatase (AP) or protein kinase A (PKA) to modulate the phosphorylation level of μ-calpain. Accurately 25, 50, 100 and 150 units of AP/PKA-treated μ-calpain were mixed with the same amounts of heat stable proteins and incubated at 4 °C. In the calpastatin-free system, AP and PKA-treated μ-calpain had higher proteolytic activity compared to the control. Intact AP-treated μ-calpain degraded fastest in the 50, 100 and 150 unit μ-calpain incubation systems. However, the degradation rate of μ-calpain in control and PKA group was non-significant in 100 and 150 unit μ-calpain systems. Our results demonstrated that, compared to dephosphorylated and control μ-calpain, calpastatin presents greater inhibition to PKA phosphorylated μ-calpain. This study increases understanding of the mechanism of μ-calpain activity regulated by phosphorylation.

1. Introduction

The calpain system, which includes μ-calpain, m-calpain and calpastatin, is believed to be the most important contributor to proteolytic tenderization of meat during post-mortem aging (Koohmaraie & Geesink, 2006). μ-Calpain and m-calpain are calcium-dependent enzymes and heterodimers composed of distinct 80 kDa catalytic subunits and a common 28 kDa regulatory subunit (Goll, Thompson, Li, Wei, & Cong, 2003). Although both μ-calpain and m-calpain have proteolytic activity, only μ-calpain is thought to be the primary enzyme involved in post-mortem proteolysis, as the concentration of Ca^{2+} in post-mortem muscle tissues cannot meet the needs to activate m-calpain (Geesink, Kuchay, Chishti, & Koohmaraie, 2006; Koohmaraie & Geesink, 2006). In addition to Ca^{2+}, many factors like pH and temperature influence the activity of μ-calpain (Bee, Anderson, Lonergan, & Huff-Lonergan, 2007; Mohrhauser, Lonergan, Huff-Lonergan, Underwood, & Weaver, 2014). As one of the well-characterized players in the calpain system, endogenous calpastatin is the only known protein inhibitor specific for μ-calpain and m-calpain (Crawford, 1990). Each calpastatin can inhibit four calpain molecules to limit post-mortem muscle proteolysis and meat tenderization (Goll et al., 2003; Kent, Spencer, & Koohmaraie, 2004).

Recently, as one of the most common post-translational modifications, protein phosphorylation has been studied with regard to meat quality, as it regulates protein functions in post-mortem muscles (Huang et al., 2011; Li et al., 2015; Li, Li, Gao, et al., 2017; Li, Li, Xin, et al., 2017). μ-Calpain can be phosphorylated by protein kinases like protein kinase A (PKA) and protein kinase C (PKC) in living tissues (Storr, Carragher, Frame, Parr, & Martin, 2011), but little is known about the regulatory mechanism of μ-calpain phosphorylation in relationship to its activity in post-mortem muscle. Previously, we observed that dephosphorylation of μ-calpain accelerated its degradation and activation, and phosphorylation altered the activity of μ-calpain (Du, Li, Li, Li, et al., 2017; Du, Li, Li, Shen, et al., 2017). Whether calpastatin has the same inhibitory effect on phosphorylated and dephosphorylated μ-calpain remains unknown. For this reason, here we evaluated the ability of calpastatin to inhibit phosphorylated and dephosphorylated μ-calpain *in vitro*, which will further our understanding of the influencing mechanism of μ-calpain activity and meat tenderization post-mortem.

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2. Materials and methods

2.1. Sample descriptions

The *longissimus lumborum* (LL) muscles were obtained 30 min post-mortem from both sides of the carcass of 3 Fat Tail Han Sheep (12 months) slaughtered at a local commercial meat processing company. The Animal Care and Ethics Committee, Institute of Food Science and Technology CAAS, approved the use of animals in the present study. The *longissimus lumborum* muscles were cut into small pieces, immediately frozen in liquid nitrogen and stored at −80 °C until protein extraction.

2.2. Calpastatin preparation

Muscle samples were homogenized in 1 vol of tissue ice-cold lysis buffer (100 mM Tris-HCl (pH 8.3), 10 mM EDTA) for 15 s three times with a 30-s cooling period between bursts. The homogenate was then heated at 95 °C for 15 min. The heat-stable proteins (supernatant) containing calpastatin were collected and concentrated by lyophilization. Bicinchoninic acid (BCA) assay (Thermo, Rockford, IL) was used to determine the protein concentration.

2.3. Incubation of μ-calpain with alkaline phosphatase or protein kinase A

Five hundred micrograms of purified μ-calpain (208712; Calbiochem, Merck KGaA, Darmstadt, Germany) were incubated in incubation buffer containing 50 mM Tris-HCl (pH 6.8), 10 mM $MgCl_2$, 10 mM DL-Dithiothreitol (DTT), 0.02 μM/μg ATP (designed as control) or buffer containing alkaline phosphatase (AP, 0.25 U/μg) or protein kinase A (PKA, 0.1 U/μg). To regulate the phosphorylation level of μ-calpain, all three treatments were incubated at 30 °C for 30 min. After incubation, 20 μL μ-calpain were combined with 460 μL Laemmli sample loading buffer, denatured at 100 °C for 5 min, and then stored at −80 °C until SDS-PAGE analysis.

2.4. Incubation of heat-stable proteins with phosphorylated/dephosphorylated μ-calpain

Seventy-two milligrams of freeze-dried proteins were dissolved in incubation buffer, divided equally into four groups, and added with 25, 50, 100, 150 units of AP/PKA treated μ-calpain, respectively. The final volume of each treatment was 460 μL adjusted with incubation buffer. Treated μ-calpain mixed just with incubation buffer was set as calpastatin-free control. Samples were incubated at 4 °C. At 1, 2, 12, 24 h incubation, samples were collected and denatured to stop the reaction. For casein zymography analysis, another 20 μL of incubated samples were collected at each time and mixed with a loading buffer of 150 mM Tris-HCl (pH 6.8), 10 mM EDTA, 0.75% 2-mercaptoethanol (MCE), 20% glycerol, 0.02% (*w/v*) bromophenol blue), immediately placed into liquid nitrogen and stored at −80 °C. Primary heat-stable proteins, untreated with μ-calpain, were prepared for calpastatin detection (SDS-PAGE and western blotting analysis) and defined as standard. Primary μ-calpain, untreated with AP/PKA, was also prepared for μ-calpain detection (SDS-PAGE and western blotting analysis) and defined as standard.

2.5. pH analysis

The measurement of pH value before or immediately after phosphorylation and dephosphorylation procedure was carried out by pH meter (FE20; Mettler Toledo, Urdorf, Switzerland).

2.6. SDS-PAGE and image analysis

The phosphorylation level analysis of proteins was performed as described in our previous studies (Du, Li, Li, Li, et al., 2017; Du, Li, Li, Shen, et al., 2017; Du et al., 2018). Five micrograms of proteins were loaded onto 12% polyacrylamide gels for SDS-PAGE of heat-stable proteins. For SDS-PAGE of μ-calpain in calpastatin-free control, 8% polyacrylamide gels were used. The phosphorylation level and the relative phosphorylation level of each sample were calculated as described in our previous studies (Du, Li, Li, Li, et al., 2017; Du, Li, Li, Shen, et al., 2017; Du et al., 2018).

Table 1
The pH values of μ-calpain solution measured before and after incubation at 30 °C for 30 min.

Treatment	0 min	30 min
AP	6.65 ± 0.02	6.57 ± 0.02
Control	6.68 ± 0.03	6.61 ± 0.03
PKA	6.65 ± 0.02	6.60 ± 0.02

AP: Alkaline phosphatase; PKA: Protein kinase A.

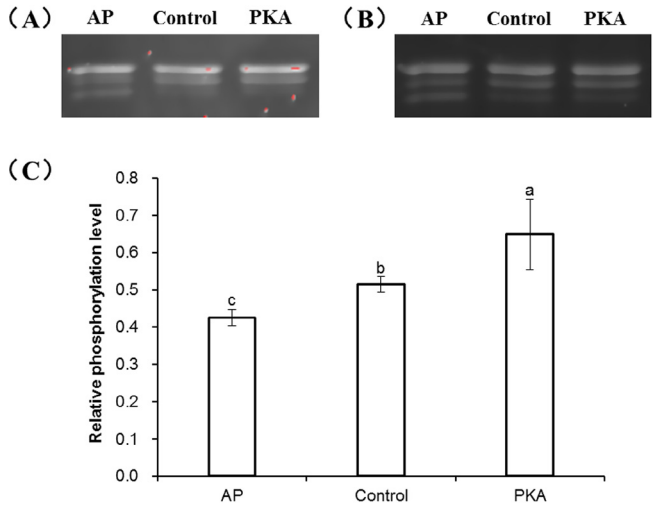

Fig. 1. Phosphorylation level of μ-calpain after incubation with AP and PKA at 30 °C for 30 min. (A) SDS-PAGE gels stained with Pro-Q Diamond. (B) SDS-PAGE gels stained with SYPRO Ruby. (C) Relative phosphorylation level of μ-calpain. Values with different letters are significantly different ($p < 0.05$). AP: alkaline phosphatase; PKA: protein kinase A.

2.7. Western blotting

Western blotting was performed as described in our previous studies (Du, Li, Li, Li, et al., 2017; Du, Li, Li, Shen, et al., 2017; Du et al., 2018). A monoclonal antibody against μ-calpain (1:1000 dilution; MA3-940, Thermo Scientific) or calpastatin (1:500 dilution; C270, Sigma-Aldrich) was used as a primary antibody. The sum of 80, 78, 76 kDa forms of μ-calpain was considered as total μ-calpain. The ratio of 76 kDa form of μ-calpain to the total μ-calpain was defined as the degradation rate of μ-calpain. The percentage of the degradation rate of standard μ-calpain was defined as the relative degradation rate of μ-calpain.

2.8. Casein zymography

Casein zymography was used to detect the activity of μ-calpain based on the method of Veiseth, Shackelford, Wheeler, and Koohmaraie (2001) with minor modifications. 12.5% casein gels (acrylamide: *N,N′*-bis-methylene acrylamide = 75:1) were pre-run at 100 V at 4 °C for 15 min with a running buffer (25 mM Tris-HCl, 192 mM glycine, 0.05% MCE, 1 mM EDTA, pH 8.3) before loading samples. The gels were then run at 100 V, 4 °C for 7 h. After electrophoresis, gels were incubated in buffer (50 mM Tris-HCl, 0.05% MCE, 4 mM $CaCl_2$, pH 7.5) with shaking

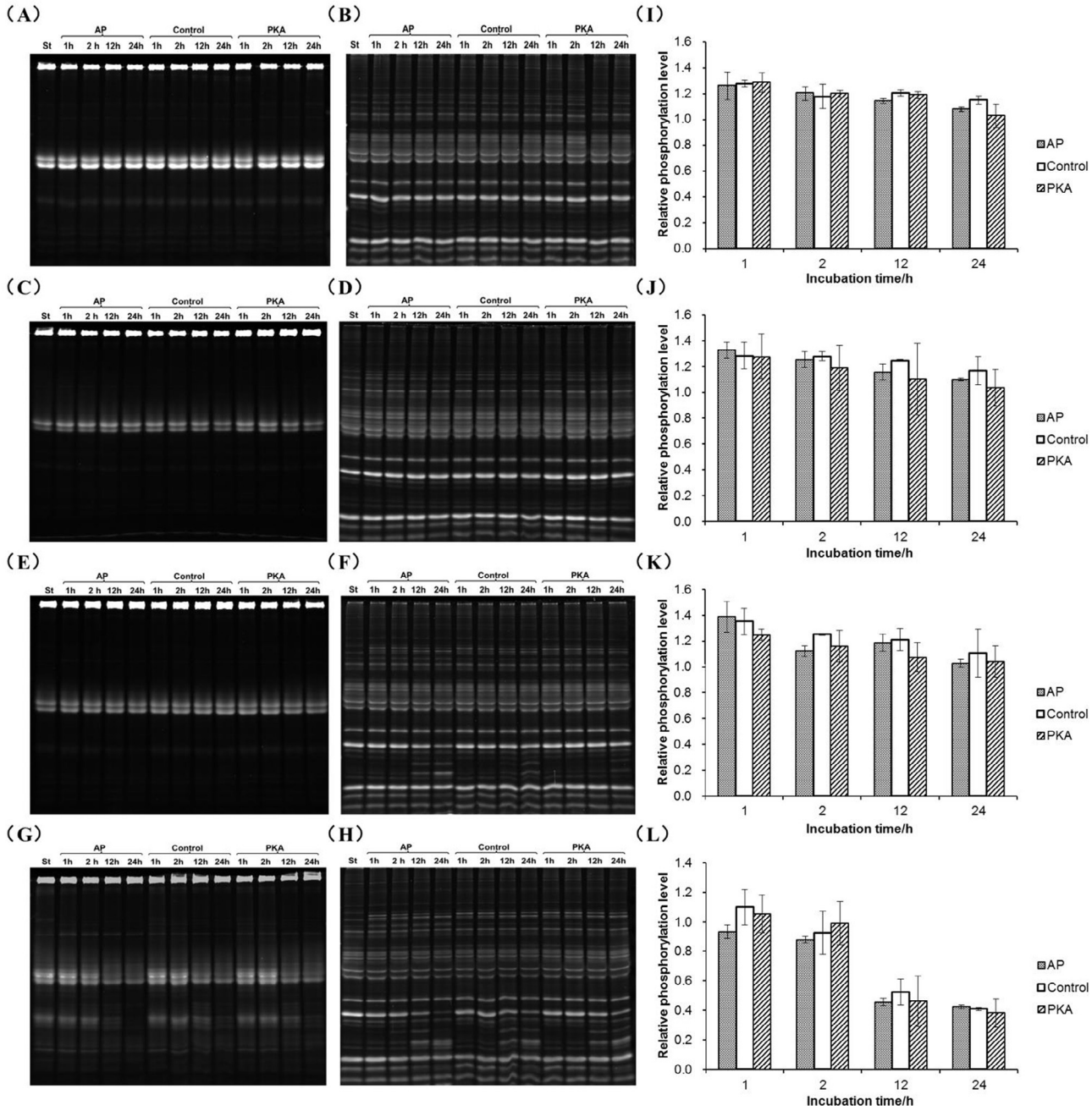

Fig. 2. Phosphorylation of heat-stable proteins during incubation with μ-calpain. (A), (B) Proteins incubated with 25 units μ-calpain. (C), (D) Proteins incubated with 50 units μ-calpain. (E), (F) Proteins incubated with 100 units μ-calpain. (G), (H) Proteins incubated with 150 units μ-calpain. (A), (C), (E), (G) SDS-PAGE gels stained with Pro-Q Diamond. (B), (D), (F), (H) SDS-PAGE gels stained with SYPRO Ruby. (I), (J), (K), (L) Relative phosphorylation level of proteins incubated with 25, 50, 100, 150 units μ-calpain, respectively. AP: alkaline phosphatase; PKA: protein kinase A.

at room temperature for 1 h (changing the buffer every 20 min). After that, gels were incubated under the same conditions for a further 16 h. Finally, the gels were then stained with Coomassie blue (R-250) overnight and destained with 7% acetic acid and 20% methanol.

2.9. Statistical analysis

Analysis of data was conducted using SPSS Statistic 21.0 (IBM Corporation, Armonk, NY). Differences among individual means were compared using Duncan's multiple range test ($p < 0.05$).

3. Results and discussion

3.1. pH value

Table 1 shows the pH values of μ-calpain solution before and after incubation with AP/PKA. No significant difference was detected between AP, control and PKA-treated μ-calpain at 0 and 30 min ($p > 0.05$). Biochemical changes in post-mortem muscles are pH dependent, especially the activity of enzymes. μ-Calpain is a neutral protease with optimum pH 7.5 (Pulford et al., 2009). Muscles with higher pH values had been shown to have greater degradation degree and activation rate of μ-calpain than muscles with lower pH values (Bee et al., 2007). Thus, the results indicated that the activity of μ-calpain

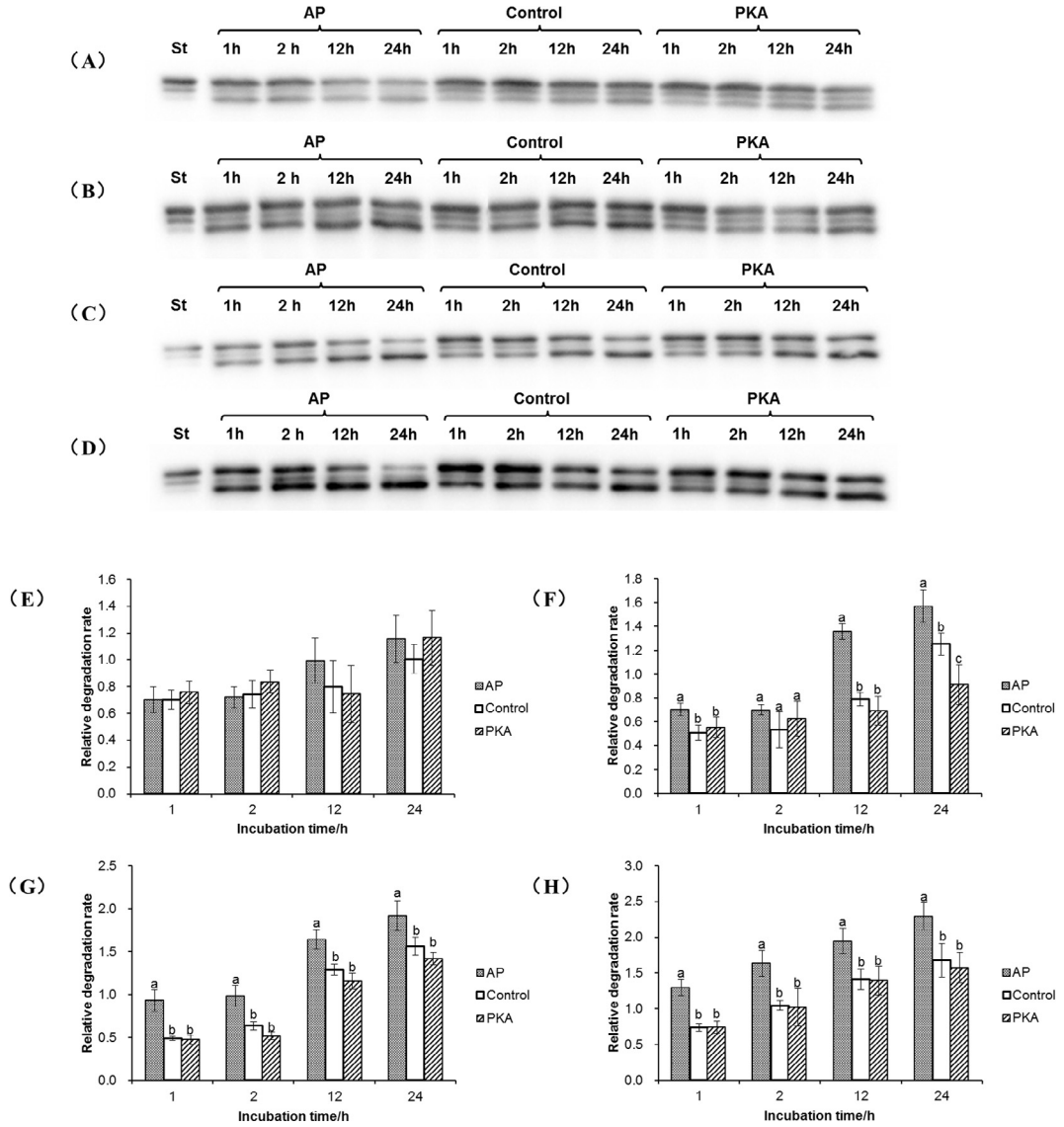

Fig. 3. Degradation of 80 kDa μ-calpain subunit during incubation. A–D, The degradation of 80 kDa μ-calpain subunit incubated with 25 (A), 50 (B), 100 (C), 150 units (D) μ-calpain. E–H, Relative degradation rate of 80 kDa μ-calpain subunit incubated with 25 (E), 50 (F), 100 (G), 150 units (H) μ-calpain. Values with different letters differ at the same incubation time ($p < 0.05$). AP: alkaline phosphatase; PI: phosphatase inhibitor.

was not affected by pH in three treatments.

3.2. Phosphorylation level of proteins

As shown in Fig. 1A, μ-calpain treated with alkaline phosphatase had lower band intensity than that of the control after staining with Pro-Q Diamond. PKA group has the most abundant phosphorylated μ-calpain. Non-significant difference in total proteins was determined among the three treatments (Fig. 1B). Correspondingly, the phosphorylation level of μ-calpain was highest in the PKA group and lowest in the AP group ($p < 0.05$) (Fig. 1C). These data showed that μ-calpain incubated with AP and PKA significantly modulated its phosphorylation level, as expected.

To determine whether AP/PKA added to alter the phosphorylation level of μ-calpain changed the phosphorylation status of heat stable proteins during incubation at 4 °C, the phosphorylation level of heat-stable proteins after incubation with μ-calpain for 1, 2, 12 and 24 h was monitored. Heat-stable proteins incubated with 25, 50, 100 and 150 units of μ-calpain presented non-significant differences in phosphorylation level between three treatments ($p > 0.05$) (Fig. 2I–L). The intensity of protein bands on both Pro-Q Diamond stained and SYPRO Ruby stained gels showed no significant difference between AP, control and PKA treatment (Fig. 2A–H). The amount of ATP added to regulate the phosphorylation level of μ-calpain can only be used to phosphorylate less than 200 μg proteins. When the AP/PKA treated μ-calpain was mixed with heat-stable proteins, the protein content of each mixture was more than 7000 μg. Under such circumstances, the added ATP was not adequate to influence the phosphorylation status of heat-stable proteins and the amount of AP/PKA was also limited in each treatment, resulting in a lack of significant differences of phosphorylation level of proteins between three treatments in the four incubation systems, which achieved the effect we desired.

3.3. μ-Calpain activity

The 80 kDa subunit of intact μ-calpain progressively degrades to 78 kDa and then 76 kDa forms post-mortem, which is considered as the activation process of μ-calpain (Goll et al., 2003). In the present study, the degradation degree of μ-calpain increased as the amount of μ-calpain added to the system increased (Fig. 3A–D). When heat-stable

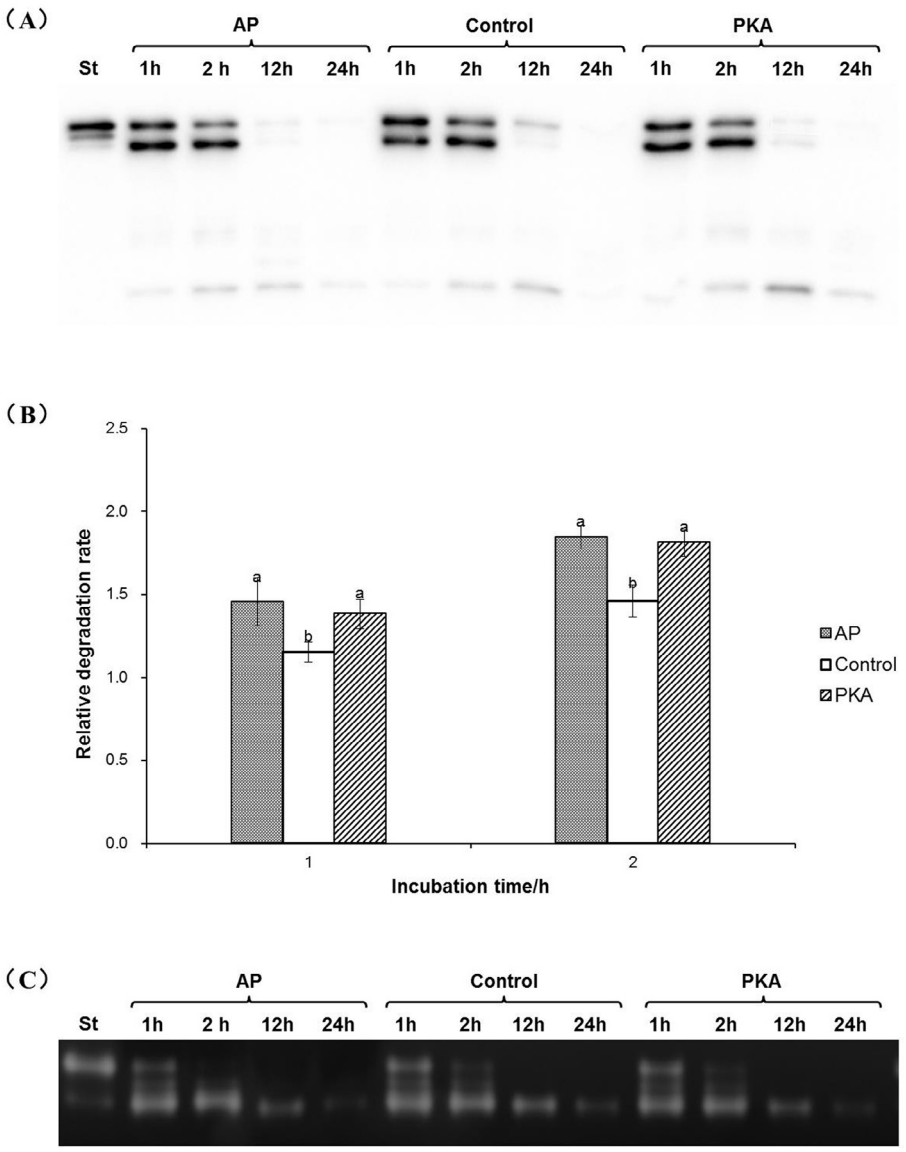

Fig. 4. Western blotting analysis and casein zymography analysis of μ-calpain incubated in the absence of calpastatin. (A) Detection of degradation of 80 kDa μ-calpain subunit by western blot. (B) Relative degradation rate of 80 kDa μ-calpain subunit. (C) Casein zymography analysis of μ-capain. Values with different letters show significant difference in the results in different groups at the same incubation time ($p < 0.05$). AP: alkaline phosphatase; PI: phosphatase inhibitor.

proteins were incubated with 25 units of μ-calpain, there was no significant degradation rate of μ-calpain between AP, control and PKA groups ($p > 0.05$) (Fig. 3E). Overexpression of calpastatin reduced the degradation extent and degradation rate of μ-calpain, indicating a decrease in post-mortem proteolysis (Kent et al., 2004). Therefore, the non-significant difference of degradation rate of μ-calpain between the three treatments was caused by a relative excess of calpastatin to 25 unit μ-calpain. When the amount of μ-calpain on incubation was more than 50 units, the degree of degradation of μ-calpain in AP group was the greatest and the corresponding degradation rate of μ-calpain was significant higher than the other two groups ($p < 0.05$) (Fig. 3B–D, F–H). μ-Calpain in AP and PKA groups had significant higher degradation rates than the control group in a calpastatin-free control system during incubation ($p < 0.05$) (Fig. 4A and B). Dephosphorylation and phosphorylation induced by PKA of μ-calpain accelerated its degradation and activation. Studies have shown that dephosphorylation improves the activity of μ-calpain (Du, Li, Li, Li, et al., 2017) but different protein kinases play different roles in regulating μ-calpain activity (Storr et al., 2011; Leloup et al., 2010). In the calpastatin-free control system in the present study, PKA phosphorylated μ-calpain also enhances the activity of μ-calpain, which is consistent with the results of our recent research (Du, Li, Li, Shen, et al., 2017; Du et al., 2018). However, μ-calpain in PKA treatment showed significantly lower degradation rate than control at 24 h in 50 unit μ-calpain incubation system ($p < 0.05$) (Fig. 3F). Besides, in 100 and 150 unit μ-calpain incubation system, there were no significant differences in the degradation rate of μ-calpain between PKA and control treatments during incubation ($p > 0.05$) (Fig. 3G and H). These results suggested that the degradation of PKA phosphorylated μ-calpain could probably be easily inhibited by calpastatin.

Casein zymography detects the native and autolyzed μ-calpain, which has the proteolytic activity to degrade casein in gels. Casein zymography analysis of μ-calpain incubated without calpastatin is shown in Fig. 4C. The top row of bands is native μ-calpain, the bottom row of bands is autolyzed μ-calpain. The results showed that μ-calpain in AP and PKA group autolyzed faster than that of the control. After 24 h incubation, μ-calpain was still detected in the control group but was not detected in AP and PKA groups, indicating that without calpastatin, dephosphorylation and PKA phosphorylation positively regulate μ-calpain degradation/autolysis. When 25 units of μ-calpain were

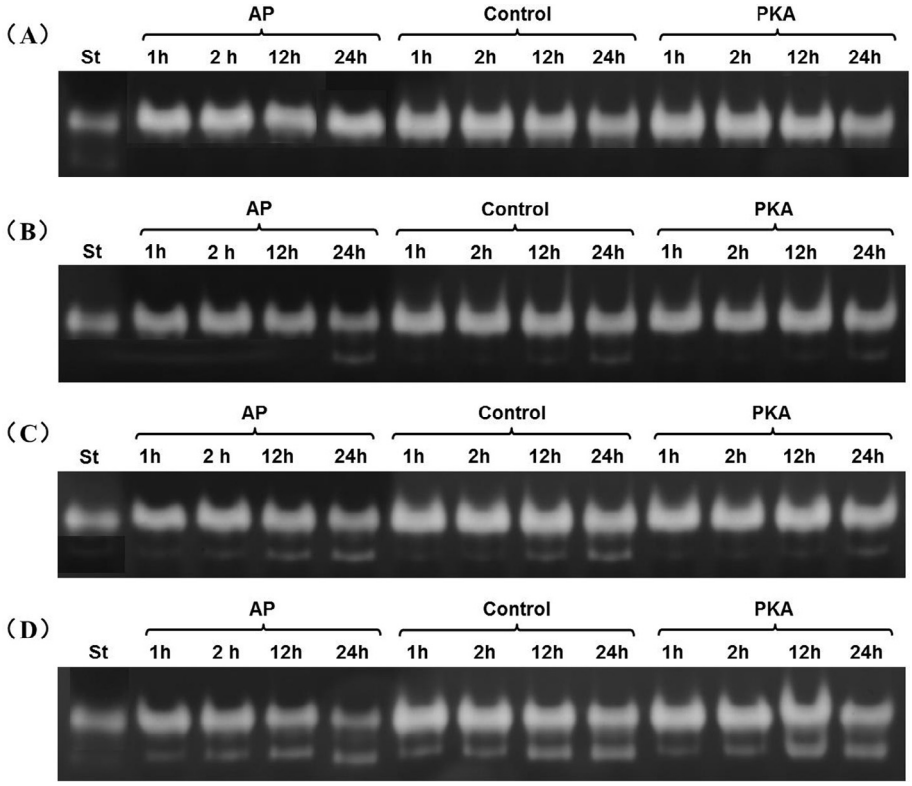

Fig. 5. Casein zymography analysis of μ-capain incubated with calpastatin. (A), (B), (C), (D) 25, 50, 100, 150 unit μ-calpain was incubated with heat stable proteins, respectively. AP: alkaline phosphatase; PKA: protein kinase A.

added to heat-stable proteins, the autolysis of μ-calpain was prevented and the intensity of intact μ-calpain was not different between the three treatments (Fig. 5A). As the content of μ-calpain added to heat-stable proteins increased, the autolyzed μ-calpain was detected in casein gels (Fig. 5B–D). In 50 and 100 unit μ-calpain incubation systems, the intensity of intact μ-calpain in AP group decreased faster than in the other two groups. In addition, the amounts of autolyzed μ-calpain were higher in the control group than that of the PKA group at 24 h incubation. But when the incubation system contained 150 units of μ-calpain, the autolysis status of μ-calpain in control and PKA group showed no difference. These results were consistent with the degradation rate of μ-calpain measured by western blotting. Increased degradation and activity of PKA phosphorylated μ-calpain was limited by calpastatin.

Previous studies have reported that dephosphorylation of μ-calpain enhanced its activity (Du, Li, Li, Li, et al., 2017; Du, Li, Li, Shen, et al., 2017). However, phosphorylation of μ-calpain induced by different kinases may have opposite effects. Protein kinase Cι (PKCι) induced μ-calpain and m-calpain phosphorylation enhances their activity and is associated with increased cell migration (Xu & Deng, 2006), While m-calpain phosphorylated by PKA reduces its activity by blocking the binding sites of m-calpain to its activator (Leloup et al., 2010; Shao et al., 2006), calpain phosphorylated by different protein kinases usually occurred at different amino acid residues, accounting for the different roles they played in regulating calpain activity (Storr et al., 2011). Our recent study shows that increased phosphorylation level of sarcoplasmic proteins induced by phosphatase inhibitor (PI) inhibits μ-calpain activity (Du, Li, Li, Li, et al., 2017). PKA was also reported to positively regulate μ-calpain activity (Du, Li, Li, Shen, et al., 2017). Both alkaline phosphatase and phosphatase inhibitor have broad-spectrum ability to decrease or increase the phosphorylation level of proteins. Phosphatase inhibitor improved the phosphorylation of proteins by inhibiting dephosphorylation process rather than working directly on protein kinases, indicating that the overall effects of phosphorylation induced by all protein kinases on μ-calpain activity might be negative. Besides, in the present study, calpastatin had higher inhibitory ability on the activity of PKA phosphorylated μ-calpain, which might be another reason why PI-treated sarcoplasmic proteins showed lower μ-calpain activity.

3.4. Calpastatin degradation

The undegraded calpastatin in lamb longissimus muscles is about 130 kDa in size and degrades gradually to small fragments during post-mortem storage (Doumit & Koohmaraie, 1999). The discrete degradation products of calpastatin include immunoreactive peptides of about 100, 80 and 50 kDa. The degradation of calpastatin during incubation with μ-calpain is shown in Supplementary Material, Fig. 6. In the 25 unit μ-calpain system, there were no distinctly changes of calpastatin in AP, control and PKA groups during incubation. As the amounts of μ-calpain in the incubation system increased, calpastatin degradation increased. The 130, 100 and 80 kDa calpastatins were no longer detectable in all three treatments when 150 units of μ-calpain were added (Supplementary Material, Fig. 6D). Apparently, calpastatin in the AP group degraded much faster than in the control and PKA groups in 100 and 150 unit μ-calpain incubation systems (Supplementary Material, Fig. 6C and D). In addition, the PKA group presented the lowest degradation rate of calpastatin when samples were incubated with 50 units of μ-calpain (Supplementary Material, Fig. 6B). Proteolytic enzymes like μ-calpain, m-calpain, cathepsin and proteasome degrade calpastatin, but μ-calpain was the major contributor to this process in post-mortem muscles (Doumit & Koohmaraie, 1999; Huang et al., 2014). Thus, the μ-calpain added to systems was not only inhibited by calpastatin but also degraded calpastatin in turn. Therefore, the relatively stable state of calpastatin during incubation with 25 units of μ-calpain could be illustrated by the greatly suppressed and indifferent μ-calpain activity in the three treatments. Meanwhile, the results of calpastatin degradation in 50, 100 and 150 unit μ-calpain incubation

systems agreed well with the corresponding activity of μ-calpain. Calpastatin in skeletal muscle contains four repeated calpain-inhibiting domains (Lee et al., 1992). The ability of μ-calpain to degrade calpastatin reduces activity of calpastatin, but cannot completely remove the inhibition of μ-calpain by calpastatin (DeMartino, Wachendorfer, McGuire & Croall, 1988; Doumit & Koohmaraie, 1999; Koohmaraie, 1990). PKA-treated μ-calpain degraded calpastatin greater than the control according to the results of μ-calpain activity in a calpastatin-free system. Considering μ-calpain activity in PKA treatment was extensively inhibited by calpastatin, the weakened proteolytic ability of μ-calpain contributed to the reduced degradation of calpastatin than in the other two treatments in 50 unit μ-calpain incubation system. Consequently, PKA-phosphorylated μ-calpain endured more inhibitory effects of calpastatin.

4. Conclusion

Calpastatin effectively inhibited μ-calpain activity *in vitro*. Both dephosphorylation and PKA phosphorylation positively regulate the activity of μ-calpain, but PKA phosphorylated μ-calpain was more sensitive to calpastatin. Further study is needed to figure out the interaction between calpastatin and phosphorylated μ-calpain to further understand the influencing mechanism of μ-calpain activity in postmortem muscle.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2018.09.073.

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