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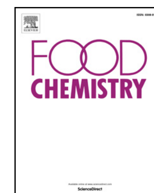

Calpastatin inhibits the activity of phosphorylated μ-calpain *in vitro*

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A B S T R A C T

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 postmortem 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.

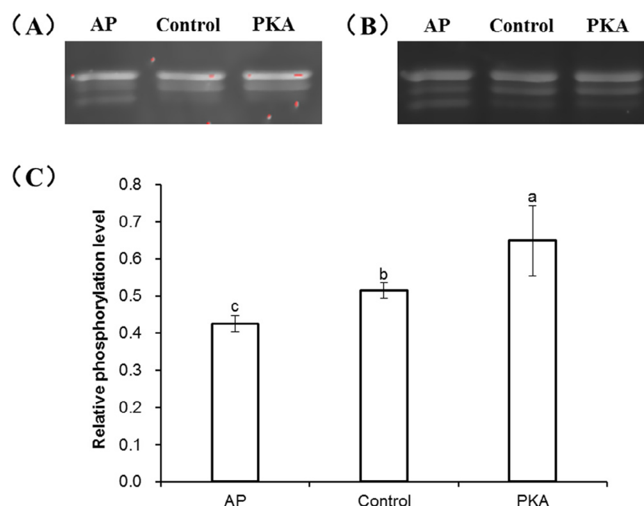

Fig. 1. Phosphorylation level of μ-calpain after incubation with AP and PKA at 30 °C for 30 min. (A) SDS-PAGE gels stained with Pro-Q Diamond. (B) SDS-PAGE gels stained with SYPRO Ruby. (C) Relative phosphorylation level of μ-calpain. Values with different letters are significantly different ($p < 0.05$).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

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