THE EFFECTS OF PHOSPHORYLATION ON THE INTERACTION BETWEEN CALPASTATIN AND μ-CALPAIN

Manting Du, Xin Li, Zheng Li, Chengli Hou and Dequan Zhang*

Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences/Key Laboratory of Agro-Product Processing, Ministry of Agriculture, Beijing 100193, P R China

*Corresponding author email: dequan_zhang0118@126.com

I. INTRODUCTION

Enzymes of the calpain system, especially μ-calpain, are considered to be primarily responsible for the postmortem proteolytic tenderization of meat [1]. As one of the well-characterized players in calpain system, endogenous calpastatin is the only known protein inhibitor specific for calpain [2]. Studies have shown that, μ-calpain and calpastatin is phosphorylated by protein kinases like protein kinase A (PKA) and PKC in living tissues [3], and phosphorylation alters its functions. Our early reports found that dephosphorylation and PKA phosphorylation of μ-calpain promoted its degradation and activation [4-5]. The objective of this study is to figure out the effects of phosphorylation on the interaction between μ-calpain and calpastatin in postmortem muscle.

II. MATERIALS AND METHODS

For treatment 1, purified μ-calpain was treated with alkaline phosphatase (AP) or PKA to dephosphorylate or phosphorylate μ-calpain, respectively. Heat stable proteins, contain calpastatin, were extracted from lamb muscles. 100 units AP/PKA treated μ-calpain was incubated with 0 mg or 6 mg heat stable proteins at 0.05 mM Ca^{2+}, 4℃ for 1, 2, 12, 24 h. For treatment 2, heat stable proteins were treated with AP or PKA first. 6 mg AP/PKA treated heat stable proteins were incubated with 100 units untreated μ-calpain at 0.05 mM Ca^{2+}, 4℃ for 1, 2, 12, 24 h. All the procedures were repeated in triplicate. The degradation of μ-calpain during incubation was determined by western blot and the phosphorylation sites of calpastatin in treatment 2 were identified by iTRAQ. Analysis of data was conducted using SPSS Statistic 21.0 (IBM Corporation, USA). Differences among individual means were compared using Duncan's multiple range test ($P < 0.05$).

III. RESULTS AND DISCUSSION

When AP/PKA treated μ-calpain was incubated without calpastatin, μ-calpain in AP and PKA group had significant higher degradation rate than control group ($P<0.05$) (Fig. 1A, 1D), which means that dephosphorylation and PKA phosphorylation of μ-calpain promoted its degradation and activation. When AP/PKA treated μ-calpain was incubated with calpastatin, there were no significant differences in the degradation rate of μ-calpain between PKA and control group ($P>0.05$) (Fig. 1B, 1E), indicating that PKA phosphorylated μ-calpain was more sensitive to calpastatin. When μ-calpain was incubated with AP/PKA treated calpastatin, the degradation rate of μ-calpain in AP group was significant higher than control group, than that of in PKA group ($P<0.05$) (Fig. 1C, 1F). It is reported that phosphorylation of calpastatin modulates its inhibitory activity. Calpastatin phosphorylated by PKA presents greater inhibition to m-calpain. In the present study, calpastatin with higher phosphorylation level presented much higher inhibitory ability, which demonstrated that PKA phosphorylation of calpastatin also improved the inhibitory ability of calpastatin.

Two phosphopeptides contain five phosphorylation sites of calpastatin were identified. Ser 649 was the only significantly differential displayed phosphorylation sites ($P<0.05$) (Table 1). Phosphorylation of calpastatin at Ser 649 was significant different between three groups. Ser 649 locates at region A in inhibitory domain IV, interacts with calpain before inhibition, positively regulates the inhibitory ability of calpastatin by promoting the combination of calpastatin and calpain.

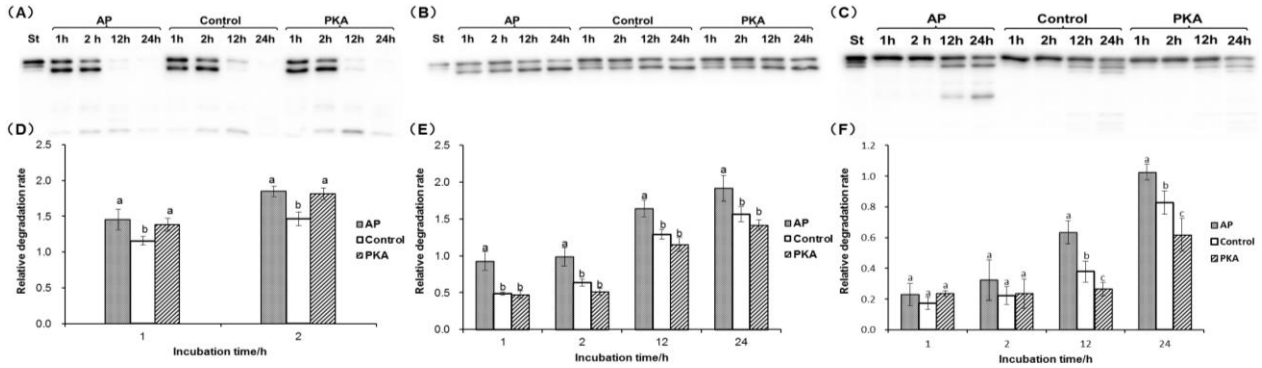

Figure 1. Degradation of μ-calpain

Western blotting analysis of μ-calpain during incubation. A-C, The degradation of μ-calpain 80 kDa subunit. D-F, Relative degradation rate of μ-calpain 80 kDa subunit. A, D, AP/PKA treated μ-calpain incubated without heat stable proteins. B, E, AP/PKA treated μ-calpain incubated with heat stable proteins. C, F, AP/PKA treated heat stable proteins incubated with μ-calpain. Values with different letters show significant difference in the results in different groups at the same incubation time ($P < 0.05$). AP: alkaline phosphatase; PKA: protein kinase A.

Table 1 Detection of phosphorylated peptides and phosphorylated sites of calpastatin

Phosphopeptides	Phosphorylation sites	AP VS C		PKA VS C	
		R	P	R	P
DNKELDDALDQLSDSLGQR	Ser 649	0.68	0.01	1.58	0.02
DFTVPSDTSSPQFEDAK	Ser 597, Ser 598	0.92	0.77	1.18	0.58
DFTVPSDTSSPQFEDAK	Thr 596, Ser 598	0.91	0.69	1.08	0.99
DNKELDDALDQLSDSLGQR	Ser 647	1.06	0.49	1.25	0.38
DFTVPSDTSSPQFEDAK	Ser 597, Ser 598	0.88	0.52	1.10	0.91

AP: alkaline phosphatase; PKA: protein kinase A; C: Control. R: ratio; P: significance.

IV. CONCLUSION

Calpastatin presents greater inhibition to PKA phosphorylated μ-calpain. Phosphorylation enhances the inhibitory ability of calpastatin. PKA phosphorylates calpastatin at Ser 649 plays positive role in regulating inhibitory ability of calpastatin by promoting the interaction between calpastatin and calpain.

ACKNOWLEDGEMENTS

The authors thank the financial support from “National Agricultural Science and Technology Innovation Program” and “National Natural Science Foundation (31471604)” in China.

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