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THE EFFECT OF OXIDATION ON THE INTERACTION OF CALPASTATIN WITH μ-CALPAIN

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Introduction

Postmortem proteolysis of muscle proteins is known to influence functional characteristics in meat including tenderness and water-holding capacity. The calpain proteinases, particularly μ-calpain, play a major role in the proteolysis of key proteins in postmortem muscle. Because of the cysteine residue found in the active site of calpains, the oxidative state of the environment can affect calpain activity. Previous research (Maddock et al., 2004) has shown oxidation by H_2O_2 significantly limits activity of μ-calpain *in vitro*; indicating that oxidation of calpain could be a source of variation in postmortem proteolysis. Rowe at al. (2004) determined that irradiation of beef steaks early postmortem inactivated μ-calpain and decreased postmortem proteolysis.

The calpain inhibitor, calpastatin binds to the calpain heterodimer in the presence of calcium at three sites, domain VI on the 28-kDa subunit and domain IV on the 80-kDa subunit (Nishimura and Goll, 1991) and also to or near the active site of calpain (Goll et al., 1992). The influence of oxidation on ability of calpastatin to inhibit calpain has been described (Maddock et al., 2004), but a specific mechanism is not yet defined. Previous research found that a) calpastatin decreased μ-calpain degradation of desmin and b) oxidation clearly decreased desmin degradation by μ-calpain (Figure 1; Maddock et al., 2004). However, increased degradation of desmin was observed in the presence of H_2O_2 as increased amounts of calpastatin were added to the digests. It is notable that oxidation of the μ-calpain/calpastatin complex resulted in greater desmin degradation (Maddock et al., 2004).

Objectives

These observations lead to the hypothesis that calpastatin, when bound to μ-calpain, can protect μ-calpain from being oxidized. Therefore, the objective of this study was to determine if, in the presence of calpastatin, oxidative conditions allowed for activation of μ-calpain.

Methodology

Purified porcine μ-calpain (0.6 units) was incubated in 165 mM NaCl, 50 mM HEPES, pH 6.5, on ice for 1 h. Treatment groups consisted of 1) 100 μM $CaCl_2$; 2) 4 mM N-ethylmaleimide (NEM) or 0.170 μM H_2O_2; 3) either 4 mM NEM or 0.170 μM H_2O_2

with 100 µM $CaCl_2$; 4) 1.2 units purified porcine calpastatin and 100 µM $CaCl_2$; and 5) 1.2 units calpastatin, 100 µM $CaCl_2$, then either 4 mM NEM or 0.170 µM H_2O_2 ; or 6) 4 mM NEM or 0.170 µM H_2O_2. A control of µ-calpain incubated in 50 mM HEPES, pH 6.5 on ice for 1 h was used. After incubation, reactions were stopped with 20 mM EDTA. Samples were aliquoted for casein zymography and SDS-PAGE analysis. Comparisons were made between treatment groups on µ-calpain activity and autolysis.

Casein zymography and native gels. Casein zymography was used to determine remaining µ-calpain activity after incubation. Native gels were used to confirm µ-calpain was present in the sample. Gel samples were made for casein zymography and nondenaturing polyacrylamide gels by diluting the sample 70:30 (sample: electrophoresis sample buffer [20% glycerol, 0.1% bromphenol blue, 0.75% 2-mercaptoethanol (MCE), 150 mM Tris-HCl, pH 6.8]. Samples were loaded onto nondenaturing acrylamide gels containing casein and identical nondenaturing gels that did not contain casein. Gels were run at a constant voltage of 75 V for approximately 18 hours. Casein gels were incubated in 5 mM $CaCl_2$, 0.1 % MCE, 50 mM Tris-HCl, pH 7.5 solution overnight at room temperature to active any potentially active µ-calpain. Both casein and native gels were stained in 0.1 % Coomassie brilliant-blue R-250, 40% methanol, and 7% acetic acid solution and then destained in a 40% methanol, 7% acetic acid solution. Clear zones on the casein zymograms indicated activation of µ-calpain. Stained protein on the native gels confirmed the presence of µ-calpain in the gel sample that was used in the casein zymograms when no active µ-calpain was detected.

*SDS-PAGE. G*el samples were prepared for SDS-PAGE by diluting samples 70:30 (sample: buffer tracking dye solution [3 mM EDTA, 3% SDS, 20% glycerol, 0.003% pyronin Y, and 30 mM Tris-HCl, pH 8.0]. Samples were run on a 10% polyacrylamide separating gel at a constant voltage of 120 V for approximately 3.5 hours. Gels were stained in 0.1% Coomassie brilliant blue R-250, 40% methanol, and 7% acetic acid solution, and destained in a 40% methanol, 7% acetic acid solution. Differences in autolysis of the 80 kDa subunit of µ-calpain to a 78-kD and a 76-kDa autolysis product were evaluated.

Results & Discussion

Previous research (Maddock et al., 2004), using purified porcine myofibrils as a substrate, determined that µ-calpain proteolytic activity was inhibited by calpastatin and oxidation (Figure 1) as determined by degradation of desmin. Calpastatin inhibited µ-calpain activity in the samples that had not been oxidized with H_2O_2, as shown by an increase in intact desmin. Oxidation with H_2O_2 clearly inhibited desmin degradation by µ-calpain, particularly when no calpastatin was used in the experiment. However, inclusion of the oxidant with µ-calpain and calpastatin proved to stimulate proteolysis of desmin, indicating increased µ-calpain activity. This lead to the hypothesis that calpastatin, when bound to µ-calpain, can prevent µ-calpain from being oxidized.

After incubation, casein zymograms, native gels, and SDS-PAGE were run on each sample. Clear zones on the casein zymograms indicate that active µ-calpain was present in the samples, which demonstrates that µ-calpain in the experiments was not inactivated, either by autolysis or irreversible oxidation. The stained native gels (Figure 2A) correspond to the casein gels and indicate the presence of µ-calpain in gel samples loaded

onto the casein zymograms. SDS-PAGE gels (Figure 2A) indicate activation of µ-calpain based on autolysis of the large 80 kDa subunit degrading to a 78 kDa and 76 kDa subunit. Autolysis is often used as an indicator of µ-calpain activation and can also directly cause inactivation of µ-calpain (as reviewed by Croall and DeMartino, 1991) and as shown in Figure 2A (Lane 10). Incubation of µ-calpain with NEM resulted in loss of µ-calpain proteolytic activity (Figure 2B). NEM binds irreversibly to reduced cysteine residues on proteins (Riordan and Vallee, 1972), essentially acting as an oxidizer. Therefore NEM may bind the active site cysteine residue of µ-calpain, preventing activation. When NEM was incubated with µ-calpain in the presence of calcium, autolysis of µ-calpain did not occur (Figure 2B), indicating that µ-calpain was likely not activated. Calpastatin inhibited µ-calpain autolysis. µ-Calpain activity was observed on casein zymograms (Figure 2A [Lane 1] and 2B) after incubation with calpastatin and calcium. When NEM was added after the formation of the µ-calpain/calpastatin complex (Figure 2A [Lane 3] and 2B), autolysis of µ-calpain did occur. Therefore, calpastatin alone inhibited µ-calpain autolysis, but the addition of NEM to the reaction after the formation of the µ-calpain/calpastatin complex allowed for autolysis and inactivation of µ-calpain. This is an interesting observation in that NEM caused complete loss of µ-calpain activity and prevented autolysis, calpastatin prevented autolysis, but together calpastatin and NEM permitted autolysis of µ-calpain to occur.

Incubation of µ-calpain with H_2O_2 resulted in no loss of proteolytic activity (Figure 2A [Lane 7] and 2B) as observed in the casein zymograms. When calcium was used in the incubation of µ-calpain with H_2O_2, autolysis did occur (Figure 2 [Lane 2] and 2B) and autolytic inactivation of µ-calpain activity was observed. As previously observed with NEM, when H_2O_2 was added to the reactions after the calpastatin/µ-calpain complex formed, autolysis of µ-calpain occurred (Figure 2 [Lanes 5 and 9] and 2B) and inactivation of µ-calpain also was observed as indicated by the casein zymograms. Conversely, when H_2O_2 was added to µ-calpain before the addition of calpastatin and calcium (Figure 2 [Lanes 6 and 8] and 2B), no autolysis of µ-calpain was observed and proteolytic activity was still apparent on the casein zymograms, indicating that µ-calpain had not been activated. Collectively, the results indicated that oxidation of the µ-calpain/calpastatin complex promotes autolysis, activation, and autolytic inactivation of µ-calpain to occur. This conclusion is consistent with the preliminary data (Figure 1) that indicated greater proteolytic activity of µ-calpain occurred in the presence of calpastatin and H_2O_2. Conversely, exposure of µ-calpain to an oxidant (NEM or H_2O_2) before exposure to calpastatin inhibits autolysis of calpain. Interestingly, the reagents used in the study appeared to affect the µ-calpain/calpastatin complex similarly, but when used alone, NEM causes complete and irreversible loss of proteolytic activity, whereas H_2O_2 inactivation of µ-calpain is reversible and proteolytic activity is recovered if reducing conditions are introduced.

Conclusions

Because oxidative conditions do occur in meat during the storage period, they can have an effect the activity of µ-calpain and its effects on protein proteolysis. Further research must be conducted to understand the interaction of µ-calpain with calpastatin, particularly in postmortem muscle. The data from this study make strides toward

understanding the mechanisms of activation of µ-calpain and how specific inherent factors can affect calpain activity.

References

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Figures

Figure 1. Relative intensity of intact desmin after incubation with µ-calpain for 60 min at pH 6.5 (n=4).

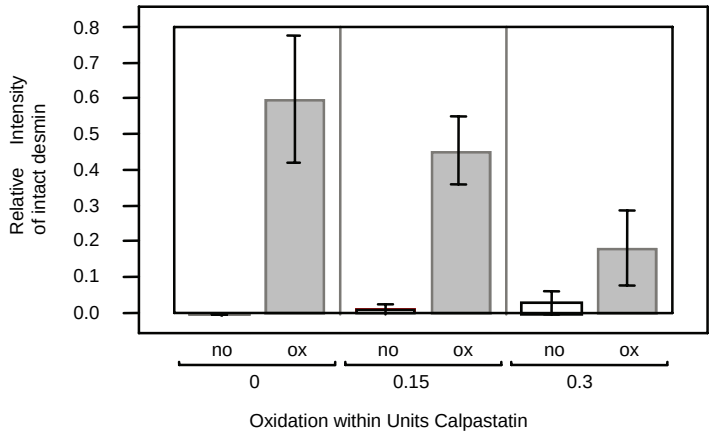
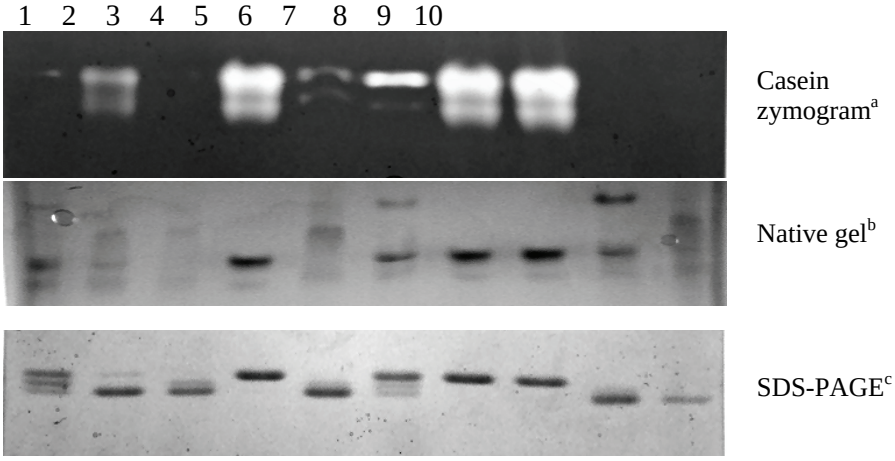


Figure 1. Relative intensity of intact desmin based on densitometry measured and standardized with a control sample from western blots of purified myofibrils digested with µ-calpain for 60 min. The x-axis depicts presence of H_2O_2 in digests within calpastatin treatments.

Figure 2. µ-calpain incubations at pH 6.5

A



B

Treatment (Lane on gels)	Proteolytic activity	Autolysis
C) µ-calpain (4)	+++	---
1) µ-calpain + $CaCl_2$ (10)	-	+++
2a) µ-calpain + NEM (NA[d])	---	---
2b) µ-calpain + H_2O_2 (7)	+++	---
3a) µ-calpain + NEM + $CaCl_2$ (NA[d])	---	---
3b) µ-calpain + H_2O_2 + $CaCl_2$ (2)	+	+++
4) µ-calpain + calpastatin + $CaCl_2$ (1)	+	+
5a) µ-calpain + calpastatin + $CaCl_2$ + NEM (3)	--	++
5b) µ-calpain + calpastatin + $CaCl_2$ + H_2O_2 (5, 9)	+-	+++
6) µ-calpain + H_2O_2 + calpastatin + $CaCl_2$ (6.8)	+++	-

[a,b,c] Proteolytic activity of µ-calpain is depicted by casein zymogram and autolysis of µ-calpain is depicted by SDS-PAGE gel. Native gel is used to show location of protein on casein zymograms.
[d] NA (Not shown on gels)

Figure 2. A) Casein zymograms gel depicting available µ-calpain activity as shown by a clear zone after incubation under conditions described in Figure 2B. Native gel depicting presence of µ-calpain in sample to give indication as to state of µ-calpain loaded in each sample on casein zymograms. SDS-PAGE depicting autolysis of the 80 kDa band to a 78 kDa and 76 kDa band indicate activation of µ-calpain during the incubations. B) Description of different treatments used with µ-calpain and description of observable proteolytic activity in casein zymograms and autolytic activity based on SDS-PAGE. All treatments are described in order of addition to incubation buffer. The number in parentheses behind treatment description corresponds to the lane on Figure 2A.