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Parallel session 2: Protein oxidation

PS2.01 Protein oxidation and regulation of µ-calpain: implications for meat quality. 141.00

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***Abstract*—Postmortem proteolysis of structural proteins results in myofibrillar fragmentation and improved meat tenderness. The activity of the endogenous proteinas, µ-calpain, is responsible for many of these postmortem changes in protein integrity and meat tenderness. The active site cysteine of calpain not only facilitates effective catalysis, it also makes the enzyme sensitive to oxidation characterized by decreased activity in the presence of oxidizing conditions. The effects of an oxidizing environment on calpain activity are important to understand because oxidative stress has been linked to variations in protein metabolism in muscle as well as variation in meat quality and tenderness. Our investigations have used LC-MS/MS analysis of the oxidized µ-calpain to reveal a disulfide bound between Cys(108) and Cys(115) in µ-calpain. The disulfide bond was confirmed by reduction of the peptide. Formation of the disulfide bound is limited in the control (without oxidation reagent) and further limited in a concentration dependent manner when a reducing agent is added, however the active site disulfide is still present at all conditions indicating that the formation disulfide bond could also occur in-vivo. It is likely that there is an equilibrium between reduced and oxidized µ-calpain in muscle and meat. Because oxidation reactions are more rapid and less easily reversed in postmortem muscle, the oxidation of proteins, in particular calpains, can make a significant contribution to variation in meat tenderness.**

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Improving and maintaining meat quality and value is at the heart of enhancing the viability of the meat industry in the rapidly growing and extremely competitive global market. Progress in this area depends on the acquisition and application of knowledge regarding events and mechanisms that control the variation of traits that are key to consumer acceptance. Meat tenderness is considered to be among the most important value-determining attributes[1]. Deviations in meat tenderness diminish the value of fresh meat and certainly drive the consumers' decisions whether or not to make repeat purchases. It is therefore critical to identify the events and conditions within the muscle and meat that cause reduction in quality.

Factors that have long been recognized as contributors to meat tenderness include 1) myofibrillar fragmentation [2, 3], 2) sarcomere length [4], 3) connective tissue amount and maturation [4], 4) pH [5], and 5) intramuscular lipid content (marbling) [6]. Meat tenderness is clearly a multi-factorial trait. The extent that each of these traits influences tenderness is co-dependent on other traits. For example, pork loin lipid content is known to influence pork tenderness at intermediate pH, but not high or low pH [6]; Proteolysis is a critical component to tenderness of pork only if sarcomeres are shorter than 2.0 µm[4]. A variable that influences development of tenderness that has not received a great deal of attention is protein oxidation. Protein oxidation can interfere with the development of tenderness in two ways: 1) promotion of cross-linking and polymerization of myosin[7], and 2) inactivation of endogenous proteinases (µ-calpain) that contribute to myofibrillar fragmentation [8, 9]. This paper will present evidence that oxidation of µ-calpain results in inactivation and that the consequences of µ-calpain oxidation include modifications of the active site cysteine.

A. Calpain proteinases

Calpains are a family of intracellular calcium-dependent cysteine proteases that require both calcium and a reduced environment for activity. The calpain

system is composed of several isoforms of the proteolytic enzyme calpain, and an endogenous inhibitor of the calpains, calpastatin [10].The most well characterized isoforms are μ - calpain and m-calpain. These isoforms require the presence of calcium to be active and are named in reference to the amount of calcium each requires for activity. In general, μ-calpain requires between 5 and 65 μM Ca^{2+} for half-maximal activity, while m-calpain requires between 300-1000 μM Ca^{2+} for half-maximal activity. These two enzymes cleave the same myofibrillar proteins that are degraded during postmortem aging [11, 12] without degrading actin and myosin [13]. High calpastatin activity in post rigor muscle results in very little proteolysis and tenderization of beef [14]. These lines of evidence support the conclusion that calpain, specifically μ-calpain, is responsible for postmortem protein degradation and tenderization.

Both μ- and m-calpain are heterodimers composed of an 80 kDa catalytic subunits and a common 28 kDa regulatory subunit [15]. The 28 kDa subunit is identical in both μ-calpain and m-calpain. The 80 kDa subunits of μ- and m-calpains are similar, but are products of different genes [15]. Calcium is necessary for calpain activity, however both μ- and m-calpain autolyze when incubated with calcium. Autolysis reduces the mass of the 80 kDa subunit of μ-calpain to 76 kDa (via a 78 kDa intermediate autolysis product) and the mass of the 80 kDa subunit of m-calpain to 78 kDa. The 28 kDa subunit of both enzymes is reduced to 18 kDa. Autolysis also reduces the Ca^{2+} concentration required for half-maximal activity of both enzymes. Extended autolysis leads to inactivation of the enzymes [16]. Autolysis occurs under situations that allow activity, both in living cells and in postmortem muscle, but the physiological significance of autolysis in living muscle is not clear [17-19]. Both autolyzed and unautolyzed forms of the enzymes have been shown to have activity. However, the autolyzed form of μ-calpain appears to be more hydrophobic and binds tightly to subcellular organelles, including myofibrils [20]. Presence of the autolyzed form of μ-calpain in post-mortem muscle indicates previous activation of μ-calpain.

B. Protein Oxidation in Meat

During postmortem storage, oxidation of meat proteins is increased by several sources of reactive oxygen species [8, 9, 21]. This results in the conversion of some amino acid residues, including histidine, to carbonyl derivatives [21, 22] and can cause the formation of intra and/or inter protein disulfide cross-links [21, 23]. In general, both of these changes can influence the functionality of proteins [24].

The obvious role of the calpains in postmortem tenderization and the absolute calcium requirement for activity lead to the conclusion that increasing calcium concentration in meat either through infusion or post rigor injection would increase proteolysis and tenderization [25] .This approach was quite effective as long as active calpain was still available to be activated. Unfortunately a consequence of this process is a calcium stimulated discoloration and associated tissue oxidation. In an attempt to reverse or avoid this, the antioxidant status of the beef was altered through dietary supplementation of alpha-tocopherol, resulting in an increase in muscle alpha-tocoperhol levels. Under these circumstances, calcium chloride injection of beef resulted in more rapid degradation of myofibrillar protein and in more rapid development of tender beef [26]. Additional experiments have shown that more rapid protein degradation is observed in steaks from cattle fed diets high in alpha-tocopherol [8, 9]. These experiments provided the basis for continued work to determine how oxidative modifications of calpain can influence meat tenderness.

Because μ-calpain and m-calpain enzymes contain both histidine and SH-containing cysteine residues at their active sites, they are particularly susceptible to inactivation by oxidation. In fact, evidence suggests oxidizing conditions inhibits proteolysis by μ-calpain [19, 27, 28]. Oxidation of proteins can thus decrease the normal progression of calpain catalyzed proteolysis and tenderization observed with postmortem storage. Irradiation was used to produce oxidizing environments in beef steaks early postmortem. This approach results in a significant decrease in the rate and extent of protein degradation. It is noteworthy that this decrease in protein degradation occurs under conditions that arrest the activation of μ-calpain[8, 9].

C. Determination of the nature of μ-calpain oxidation.

It is important to define the nature of the inactivation of the calpain. There are several key observations regarding μ-calpain oxidation that can provide cues to define the consequences of oxidation:

- μ-Calpain isolated from the irradiated beef can be activated if exposed to a reducing agent such as mercaptoethanol or dithiothreitol, specifically noting that the oxidation event is *reversible[8, 9]*.
- Purified μ-calpain is inactivated in the presence of low concentrations (< 100μM) hydrogen peroxide, and this inactivation is *reversible* with reducing agents [29].
- Oxidation of purified μ-calpain with hydrogen peroxide severely slows down progression of autolysis in the presence of calcium[30].
- Oxidation of purified μ-calpain with hydrogen peroxide results in a proteinase that is not susceptible to inactivation by alkylating agents such as N-ethylmaleimide or cysteine proteinase inhibitors such as E-64. This provides evidence that the target of this oxidation is a sulfhydryl group and likely the active site of calpain[30].

Taken together, the conclusion can be made that a primary calpain oxidation product is a reversible modification of a sulfhydryl group. A reversible oxidation product of the reaction between cysteine and H_2O_2 is sulfenic acid. A nucleophilic reagent (dimedone), [31] was used to irreversibly attack the sulfenate sulfur, displacing hydroxide and forming a thiol adduct and thus irreversibly blocking activation of the active site cysteine. Inability of calpain become active after oxidation and incubation with dimedone would be direct evidence that the modification is a sulfenic acid. Since calpain activity was detected after this treatment, formation of sulfenic acid is eliminated as a possible modification in response to oxidation, leaving only disulfide bonds as the only possible modifiction.

Direct evidence of a specific intramolecular disulfide bond was provided with LC MS/MS analysis [30]. A modification in the μ-calpain peptide 105-133 was revealed as a disulfide bond between Cys(108) and Cys(115) (active site cysteine). It is important to note that the disulfide bond was detected primarily when calpain was oxidized with hydrogen peroxide, but was also present to a lesser extent even when calpain was not exposed to the oxidizing agent.

D. Effects of oxidation on calpain/calpastatin interaction

In a series of controlled experiments [29] using highly purified μ-calpain and calpastatin and highly purified myofibrils as a substrate, demonstrated that calpastatin inhibited μ-calpain activity at pH 7.5, and 6.5 (Figure 1, Controls). The addition of H_2O_2 (to stimulate protein oxidation) caused a decrease in activity of μ-calpain (Figure 1, Oxidized samples) noted by an increase in intact desmin. What was not expected, however, was that oxidation of the calpain/calpastatin complex resulted in *increased degradation* of desmin in purified myofibrils (Figure 1) at pH 6.5. This is noted by less intact desmin in the oxidized samples that contain calpastatin. It appears that the addition of H_2O_2 in the presence of calpastatin-μ-calpain complex results in greater degradation of intact desmin. These effects were confirmed using a synthetic substrate. These results may indicate another regulatory mechanism for calpastatin.

E. Summary

Oxidative processes are balanced with the antioxidant systems in cells, but the conditions of this balance are drastically altered in postmortem muscle. Oxidation of proteins can alter function and solubility of many proteins, but prevention or regulation of oxidation of proteins like calpain can regulate protease activity. The extent of oxidation of muscle μ-calpain in perimortem and early postmortem period should be considered a source of variation in fresh meat tenderness.

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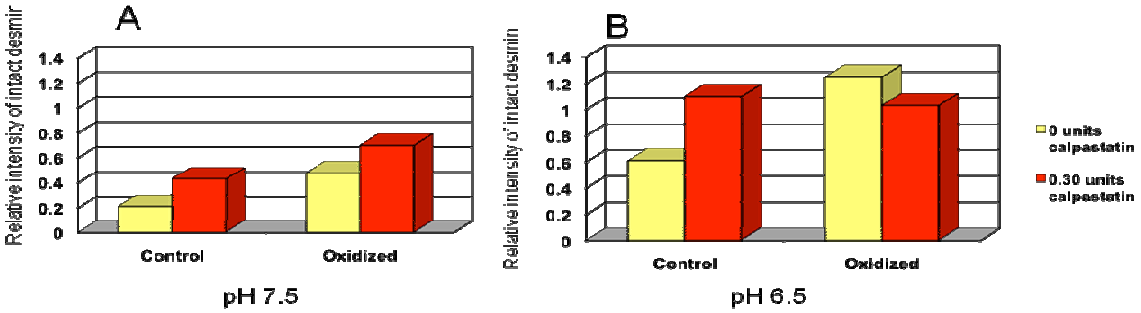

Figure 1. Effect of oxidation on inhibition of μ-calpain by calpastatin [29]. The relative intensity of intact desmin after incubation of highly purified myofibrils with calpain (0.45 units) in 50 mM HEPES at the pH noted and presence of 1 mM $CaCl_2$ for 15 minutes. Intensity of the subject band was reported as a ratio of a reference, undigested myofibril sample. Oxidation treatment consisted of the addition of 0 (Control) or 100 μM hydrogen peroxide (Oxidized). Calpastatin was included at 0 or 0.3 units of activity per incubation. A) Results after incubation at pH 7.5. B) Results after incubation at pH 6.5.