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Influence of oxidation on myofibrillar proteins degradation from bovine via μ-calpain

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ABSTRACT

The objective of this study was to investigate the effect of oxidation on proteolysis of myofibrillar proteins via μ-calpain. Myofibrillar proteins prepared from bovine muscle were exposed to *in vitro* oxidation to produce varying levels of protein modification by use of H_2O_2 and Fe^{2+}. The protein oxidation level was measured by the carbonyl content. Modified proteins were then incubated with active μ-calpain and the rates of protein degradation were analyzed via SDS–PAGE and western blotting. The results revealed that increased protein oxidation enhanced the degradation of myosin heavy chain (MHC) and α-actinin, reduced the degradation of 38 kDa troponin-T, but had little influence on the 30 kDa degradation fragment derived from troponin-T and the degradation of actin. These findings demonstrated that the oxidative modification of myofibrillar proteins changed their susceptibility to μ-calpain and provided a mechanistic link connecting oxidation with myofibrillar proteolysis.

1. Introduction

Oxidation is widespread in postmortem storage and processing, which is considered one of the major factors reducing quality and acceptability of meat (Morrissey, Sheehy, Galvin, Kerry, & Buckley, 1998). In live animal there is relatively high antioxidant capacity. However, during the conversion of muscle to meat, there is a high oxidant stress and relatively low antioxidant capacity to reverse the oxidative reactions. It is the difference between these two scenarios that makes oxidative stress important to meat quality (Warner, Dunshea, Ponnampalam, & Cottrell, 2005). Among meat eating quality parameters, tenderness is generally considered to be the most important characteristics by consumers. In practice, oxidation during the immediate postmortem period appears to inhibit meat tenderisation (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004). Moreover, oxidation of muscle tissue occurring as a result of the availability of oxygen during natural oxidation and modified atmosphere packaging may also have deleterious consequences for the tenderization during storage of meat prior to retail display (Sorheim, Wahlgren, Nilsen, & Lea, 2004).

Oxidation occurred in meat postmortem storage has mainly two pathways: lipid oxidation and protein oxidation. Lipid oxidation has been well investigated, which is demonstrated to be an important factor affecting meat color, flavor and so on (Morrissey et al., 1998). Actually, proteins from animal tissues are also one of the targets for oxygen radial attack *in vivo* and *in vitro* (Eymard, Baron, & Jacobsen, 2009; Mercier, Gatellier, & Renerre, 2004). In muscle cells, proteins are close to free radial initiators exposing them to oxidative reactions. They are the primary targets of oxygen radicals *in vivo*. Proteins in muscle can be modified by reactive oxygen species (ROS) that include free radical but also by non-radical species such as H_2O_2 and lipid hydroperoxides. Protein oxidation can also negatively impact the functional and edible properties of meat especially tenderness (Xiong, 2000). Increasing protein oxidation induced by irradiation has been shown to be associated with increased shear force values at later times postmortem (Rowe et al., 2004), while vitamin E supplementation has positive effects on texture of animal tissue (Dal Bosco, Castellini, Bianchi, & Mugnai, 2004).

It has been well established that improvement of meat tenderness during postmortem ageing mainly results from the weakening and alteration of the myofibrillar structure by endogenous proteolytic enzymes (Huang, Huang, Xue, Xu, & Zhou, 2011; Koohmaraie & Geesink, 2006). Several lines of evidence suggest a major role of calpains and especially of μ-calpain in postmortem tenderization of meat (Huff-Lonergan, Zhang, & Lonergan, 2010; Koohmaraie & Geesink, 2006). Protein oxidation can lead to radical formation, formation of amino acid derivatives, protein break down and polymerization. These damages of protein result from oxygen radicals increased the breakdown of cellular proteins. Studies revealed that increase of oxidation can enhance protein degradation by proteases (Grune, Klotz, Gieche, Rudeck, & Sies, 2001). Overall, the interaction between proteolysis and oxidation of proteins has for years been extensively studied in biomedical sciences. However, to our knowledge, the link between enzymatic proteolysis and oxidative processes is rather poorly documented in meat science. Therefore,

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this study was undertaken to further investigate the potential contribution of oxidation to postmortem tenderization of meat by examining the role of oxidation in the degradation of bovine myofibrillar proteins via μ-calpain, and aimed to offer the potential to increase our understanding of the fundamental cellular mechanisms underlying the proteolytic breakdown of muscle proteins during meat ageing.

2. Materials and methods

2.1. Animals and muscle sampling

Five 2.5 years old Chinese yellow cattle (Luxi bulls with live weight 450 ± 50 kg) were slaughtered humanely at a commercial meat processing company (Hansen Meat Co. Ltd., Anhui, China) according to the requirements of National Standards of PR China "Operating Procedures of Cattle Slaughter". After animal exsanguination, longissimus thoracis (LT) muscles were excised from the right side of carcasses within 30 min and were frozen rapidly in liquid nitrogen for subsequent analysis.

2.2. Myofibril preparation

Myofibril isolations were carried out as described previously (Goll, Young, & Stromer, 1974) with minor modifications. Minced muscle was homogenized in approximately 10 vol (v/w) isolation buffer (20 mM potassium phosphate, 0.1 M KCl, 2 mM $MgCl_2$, and 2 mM EGTA, pH 6.8), using a polytron at a speed of 13,000 rpm for 10 s. The muscle homogenate was centrifuged at 1000*g* for 10 min, and the supernatant was discarded. The pellet was washed two more times with 8 vol of the same isolation buffer using the same blending and centrifugation condition as indicated above. The myofibril pellet was then washed two more times with 8 vol of isolation buffer indicated above containing 1% Triton X-100 (20 mM potassium phosphate, 0.1 M KCl, 2 mM $MgCl_2$, and 2 mM EGTA, 1% Triton X-100, pH 6.8). Then, the pellet was washed twice with 8 vol of 100 mM KCl. In the last step, the myofibrils were suspended in incubation buffer without DTT (5 mM Hepes, 100 mM NaCl, 0.1% Chaps, and 5 mM NaN_3, pH 6.5). The protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Shanghai, China).

2.3. In vitro oxidation of myofibrillar proteins

The protein samples were randomly divided into four groups. Proteins in group 1 were not exposed to oxidizing conditions and designated as the control group. The remaining three samples were divided into separate groups and were exposed to varying levels of hydrogen peroxide (H_2O_2) and iron (Fe^{2+}) to generate three differing levels of hydroxyl radical (**.**OH) production and, therefore, three distinct levels of protein modification: 300 μM H_2O_2 and 600 μM Fe^{2+}; 400 μM H_2O_2 and 800 μM Fe^{2+}; and 500 μM H_2O_2 and 1000 μM Fe^{2+}. The different levels of protein oxidation generated by these concentrations were measured by estimation of total carbonyl content. A Fe^{2+}/H_2O_2 ratio of 2:1 was used to obtain the maximum effect of the oxidative system recommended by Yoon and Lee (2001). Each protein oxidation treatment was performed at 37 °C for 30 min. Following the oxidation process, the proteins were quickly removed from the oxidizing medium by centrifugation at 5000*g* for 5 min, and then placed on ice to inhibit further oxidation.

2.4. Protein carbonyls

Carbonyl measurement is the most common method for determining protein oxidation. Protein carbonyls were measured by estimation of total carbonyl groups according to the method of Oliver, Alin, Moerman, Goldstein, and Stadtman (1987) with minor modifications. From two fractions of 50 μl protein samples, one aliquot was treated with 2 ml of 2.0 M HCl (control) and the other was treated with 2.0 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.0 M HCl for 1 h at room temperature. After incubation, the two fractions were then precipitated with 2.0 ml of 20% trichloroacetic acid. The precipitate was washed twice with 4.0 ml of ethanol:ethylacetate (1:1, v/v) solution to remove unreacted DNPH and blow-dried. The pellet was then dissolved in 1.5 ml of 6.0 M guanidine hydrochloride with 20 mM potassium phosphate buffer (pH 2.3). The absorbance was measured at 370 nm. The protein concentration was analyzed by measuring the absorption at 280 nm using bovine serum albumin (BSA) as standard. The amount of protein carbonyl content was expressed as nmol of mg protein using an absorption coefficient of $2.1 \times 10^4\ M^{-1}\ cm^{-1}$ for protein hydrazones.

2.5. Incubations

To evaluate the impact of protein oxidation on the rates of myofibrillar protein breakdown via μ-calpain, we incubated both control and oxidized myofibrillar protein samples with known quantities of purified and active μ-calpain. Specifically, 600 μg of myofibrillar protein was placed in a reaction medium containing 5 μl of active μ-calpain (Merk, Germany). Two microliters of 500 μM Ca^{2+} was then added to the medium and the samples were incubated at 37 °C for 30 min. At the completion of this incubation period, proteolytic activity was immediately stopped by placing the samples on ice, followed by the addition of treatment buffer (125 mM Tris, 4% SDS, 20% glycerol). The samples were heated in a 50 °C water bath for 20 min, and then centrifuged for 30 min (16,000*g*). Protein concentration was determined with the BCA Protein Assay Kit (Pierce, USA), and diluted to 4 mg/ml using treatment buffer containing 10% MCE and 0.001% bromophenol blue. Samples were well mixed and heated at 50 °C for 20 min, and then stored at −80 °C for subsequent SDS–PAGE and western blotting.

2.6. SDS–PAGE and western blotting

Polyacrylamide gels are used to investigate the rate of proteolysis and the protein fragmentation patterns generated by digestion of myofibrillar proteins by μ-calpain. A 12.5% polyacrylamide separating gel was used for detecting the myofibril proteins Whanges and determination of myosin heavy chain (MHC). Ten percent polyacrylamide separating gels were used to detect the changes of actin, troponin-T, while 7.5% gel for α-actinin. A 4.5% polyacrylamide gel was used for stacking gel. The gels were run on the Bio-Rad Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA) at a constant voltage of 120 V.

After electrophoresis, 12.5% gel were stained using Coomassie brilliant blue R-250 and the separated proteins in 10% and 7.5% gels were blotted onto polyvinylidene difluoride (PVDF) membranes in transfer buffer containing 25 mM Tris, 192 mM glycine, and 10% (v/v) methanol. The electroblotted membranes were probed with antibodies specific for bovine skeletal muscle actin, troponin-T, and α-actinin. Actin was incubated with a 1:400 dilution of primary polyclonal antibody produced in rabbit (Sigma–Aldrich, St. Louis, USA). Troponin-T was incubated with a 1:500 dilution of monoclonal antibody detecting specific band of 38 kDa which produced in rabbit (Sigma–Aldrich, St. Louis, USA), and α-actinin was incubated with a 1:500 dilution of primary monoclonal antibody produced in mouse (Sigma–Aldrich, St. Louis, USA). After incubation, membranes were washed with PBS–Tween (TTBS: 0.05% Tween 20, 20 mM Tris, 137 mM NaCl, 5 mM KCl), and either sheep anti-rabbit (troponin-T and actin) or sheep