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Proteomic profiling of oxidized cysteine and methionine residues by hydroxyl radicals in myosin of pork

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ABSTRACT

In order to investigate the effects of hydroxyl radicals on the myosin of pork, with focus on reducible and non-reducible oxidation of specific cysteine and methionine residues, extracted myofibrillar protein from *longissimus dorsi* of pork was incubated with H_2O_2 for 24, 48 and 72 h, respectively. The thiol contents and crosslinking of myofibrillar protein were analyzed after oxidation of the protein. Moreover, cysteine (labeled with *N*-ethylmaleimide (NEM) and iodoacetamide (IAM) before and after reduction, respectively) and methionine oxidation were detected by LC/MS using label-free quantitation. The result revealed that cysteine at head of myosin tended to form sulfinic and sulfonic acid, while the cysteine at coiled tail of myosin easily generated disulfide under same condition. Furthermore, it was also revealed that the methionine at the coiled tail of myosin was more easily oxidized than that of the head.

1. Introduction

It is recognized that protein oxidation is an important parameter for quality loss during processing and storage of muscle foods (Soladoye, Juárez, Aalhus, Shand, & Estévez, 2015; Zhang, Xiao, & Ahn, 2013). Protein oxidation is usually caused by reactive oxygen species (ROS) which will directly or indirectly damage all biomolecules, including proteins, lipids, DNA, and carbohydrates (Shacter, 2000). ROS such as the superoxide anion, and the hydroxyl radical are generated under numerous conditions *in vivo*. They are formed in the course of normal metabolism through leakage of electrons from the electron transport chain and by the activities of oxidoreductase enzymes (Shacter, 2000). The oxidation of proteins in meat has previously been suggested to reduce the eating quality of meat by decreased tenderness and juiciness (Huff-Lonergan & Lonergan, 2005). The main oxidative modifications of protein take place at the side chains of amino acids, which include thiol oxidation, aromatic hydroxylation, and formation of carbonyl groups (Stadtman, 1992). Recently, intermolecular disulfide crosslinking of myosin was found to take place in pork packed in a high-oxygen atmosphere (70% O_2/30% CO_2), and cross-linking caused by protein oxidation was attributed to less tender meat (Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007). The disulfide bonds were formed by the oxidation of thiols from meat. Yuanh, Elisabeth, Josephg, and Stevenm (2010) reported that beef steaks of *longissimus lumborum* in high-oxygen atmosphere system showed lower subjective tenderness scores than samples from vacuum packaging, which was associated with the formation of cross-linkages between myosin and titin and was independent of the postmortem protein degradation and the activity of μ-calpain.

Cysteine and methionine are the most susceptible to oxidation by all forms of ROS due to their sulfur atoms (Garrison, 1987). Cysteine in proteins not only frequently participate in enzymatic reactions, but also are subjected to a variety of covalent post-translational modifications (PTMs), which function as important mediators of redox signaling and regulation (Bachi, Dalle-Donne, & Scaloni, 2013; Held & Gibson, 2012). One-electron oxidation of cysteine with radical oxidant can generate thiyl radicals. These species have two major pathways: reaction with other thiol/thiolate to form disulfide, or reaction with O_2 to generate thiyl peroxylradicals (Schöneich, 2008; Wardman & Von, 1995). The two-electron oxidation between cysteine and oxidants can result in the formation of sulfenic acid (CysSOH), sulfinic acid ($CysSO_2H$), and sulfonic acid ($CysSO_3H$) (Claiborne et al., 1999). These species are unstable and can yield oxyacids by hydrolysis reactions or disulfide bonds by reacting with another thiol group (Turell et al., 2008). The oxidation of cysteine to form disulfide would cause protein crosslinkage and thus influence the meat quality as described above.

Methionine is another reactive amino acid to oxidants and the oxidation of methionine residues in proteins has been shown to result in structural changes (Liu, Gaza-Bulseco, Xiang, & Chumsae, 2008; Liu,

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Ren, et al., 2008) and decreased stability (Liu, Gaza-Bulseco, et al., 2008; Liu, Ren, et al., 2008). Proteomics analysis revealed that myosin functional and structural changes are associated with oxidation of multiple methionine residues in the myosin catalytic domain and essential light chain (Prochniewicz et al., 2008). It is not clear which methionine or how many methionine residues are responsible for the observed functional and structural changes in skeletal myosin. So, it is necessary to identify the oxidized cysteine and methionine at the proteome level that exploits the speed and resolution of mass spectrometry (Rudolph & Freeman, 2009; Thamsen & Jakob, 2011).

Several methods have been used to identify cysteines that are sensitive to oxidation. Immunologic methods including one-dimensional or two-dimensional gel electrophoresis are often sensitive to measure changes in specific proteins but cannot study specific cysteine residues. As a result, tandem mass spectrometry (MS/MS) methods are being increasingly used to study specific cysteine residues in proteins (Aebersold & Mann, 2003; Wong & Liebler, 2008) with the trap of alkylating agents iodoacetamide (IAM) or *N*-ethylmaleimide (NEM). The method usually was as follows: first, the unmodified cysteines were blocked by alkylation; then the oxidized cysteines were reduced, and the reduced cysteines were labeled with reagents containing a biotin tag. Then the labeled proteins were enriched by purification for proteomic analysis. When coupled with LC-MS/MS, those proteins that had been modified, as well as the specific cysteine residues at which the modifications occurred, can be identified (Derakhshan, Wille, & Gross, 2007).

In order to investigate the specific cysteine and methionine oxidation thoroughly in pork, especially cysteine and methionine residue located in different isoforms of myosin, to establish a subtle relationship between the different parts of myosin (head and tail) and to better understand the oxidation of myosin, we isolated myofibrillar proteins from pork and oxidized the myofibrillar protein for 24, 48 and 72 h, respectively by hydroxyl radicals. After oxidation, NEM and IAM were used to label the cysteine before and after reduction, respectively. At last, we identified specific and oxidized cysteines and methionines in myosin using label-free quantitation by LC/MS-MS.

2. Materials and method

2.1. Materials

The *longissimus dorsi* muscle of pork was bought from the local supermarket and used within 72 h postmortem. The samples were taken from the last rib of the muscle and used for the extraction of myofibrillar protein.

2.2. Methods

2.2.1. Extraction of myofibrillar protein

The myofibrillar protein from the muscle was extracted by differential centrifugation according to the method described by Goll, Young, and Stromer (1974). Minced muscle was suspended in 10 volumes (v/w) standard salt solution (100 mM KCl, 20 mM potassium phosphate, pH6.8, 2 mM $MgCl_2$, 2 mM EGTA) by homogenizing for 10 s for three times (paused for 5 s each, 20,000 rpm) in centrifuge tube using a homogenizer (Ultra-Turrax T25, Janke and Kunkel IKA Labortechnik, Denmark). The mixture was centrifuged at 1000*g* for 10 min to obtain the pellets. This step was repeated three times. At the third time, the mixture was passed through the nylon net to remove the connective tissue, and then centrifuged at 1000*g* for 10 min to obtain the pellets. This step was repeated. And then the pellets were suspended in 6 volumes (v/w) of standard solution plus 1% Triton X-100 (v/w) by homogenizing for 10 s to remove the membranes that normally adhere to the surface of myofibrils. The mixture was centrifuged at 1500*g* for 10 min to obtain the pellets. This step was repeated. At last, the pellets were added in 8 volumes (v/w) of 100 mM KCl, homogenized for 3 s and centrifuged at 1500*g* for 10 min. This step was repeated 6 times. The sediment containing the myofibril protein was suspended in 2 volumes (v/w) of 100 mM KCl and used for further analysis.

2.2.2. Determination of protein concentration

100 μL of myofibrillar protein solution was diluted by 900 μL 5% SDS and heated at 85 °C for 30 min. The sample was diluted with MilliQ water and the protein content were determined by measuring the absorbance at 280 nm using a standard curve prepared from 0 to 3 mg/mL BSA using Nanodrop 1000 (Thermo Fisher Scentific, Hvidovre, Denmark) with 5% SDS as a blank.

2.2.3. Oxidation by hydroxyl radicals

The method of oxidation was according to Xiong, Blanchard, Ooizumi, and Ma (2010), with some modifications. Myofibrillar protein (4 mg/mL) was suspended in 15 mM piperazine-*N*,*N* bis (2-ethane sulfonic acid) (PIPES) buffer at pH 6.0 that contained 0.6 M NaCl, and the sample was incubated at 4 °C for 24, 48 and 72 h, respectively, with an iron-catalyzed oxidizing system that generates hydroxyl radicals (10 μM $FeCl_3$, 0.1 mM ascorbic acid, 10 mM H_2O_2 (fresh)). Oxidation was terminated by washing the mixture with 5 volumes of 15 mM PIPES buffer twice and with cold, deionized water once to remove hydroxyl radicals. The pellets contained oxidized myofibrillar protein, which was obtained by centrifugation at 10,000*g* for 15 min. The control was the myofibrillar protein that was treated only with the washing process.

2.2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of myofibrillar proteins

SDS-PAGE was used to determine the protein profile of myofibrillar proteins. The myofibrillar protein (0.5 mg/mL) were mixed with sample buffer with and without 100 mM TCEP and incubated at 80 °C for 4 min. An aliquot of 10 μL samples was loaded on a NuPAGE 4–12% Bis-Tris gel (Novex by Life Technologies, USA). The gel was running at 200 V for about 45 min with MES SDS Running buffer (Novex by Life Technologies, USA). Then the gel was sank into the Coomassie Brilliant Blue stain overnight until the gel turned blue. Then, the protein bands were clear. And then the gel was transferred to a new container with MilliQ water to destain the redundant color until only the protein bands were blue and the stain in other parts of gels was removed.

2.2.5. Determination of thiol contents

The thiol contents of the myofibrillar protein were determined by Ellman's reagent (Ellman, 1959) with some modifications as follows. 0.125 mL of the myofibrillar protein solution (around 5 mg/mL) was mixed with 0.75 mL SDS buffer (5% SDS in 0.1 M Tris buffer, pH8.0) in a 1 mL plastic cuvette. The absorbance was read at 412 nm and referred as $Abs_{(412)pre}$. Then, 0.125 mL 5, 5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was added to the cuvette and left to react at room temperature in the dark for 30 min. The absorbance was read at 412 nm and referred as $Abs_{(412)post}$ using a UV–VIS spectrophotometer (Helios Omega, Thermo Fisher Scientific, Hvidovre, Denmark).The concentration was determined from the linear regression of the standard curve of the cysteine ($y = 0.0016x + 0.0367$, $R^2 = 0.9969$; y means the absorbance, x means the concentration of cysteine (μM) followed the calculations below:

$$\text{thiol contents} = (Abs(412)_{post} - Abs(412)_{pre} - 0.0367) \div 0.0016 \times DF$$

DF is the dilution factor (DF = 8).

2.2.6. Preparation of protein alkylated with NEM and with IAM

The free cysteines were labeled with NEM. NEM can permanently block sulfhydryl to prevent disulfide bond formation in the pH range 6.5–7.5 and the reaction is not reversible. The samples were then reduced by Tris(2-carboxyethyl)phosphine (TCEP) and digested by trypsin followed by alkylation using IAM, which is an alkylating agent