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Comparative profiling of sarcoplasmic phosphoproteins in ovine muscle with different color stability

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ABSTRACT

The phosphorylation of sarcoplasmic proteins in postmortem muscles was investigated in relationship to color stability in the present study. Although no difference was observed in the global phosphorylation level of sarcoplasmic proteins, difference was determined in the phosphorylation levels of individual protein bands from muscles with different color stability. Correlation analysis and liquid chromatography – tandem mass spectrometry (LC-MS/MS) identification of phosphoproteins showed that most of the color stability-related proteins were glycolytic enzymes. Interestingly, the phosphorylation level of myoglobin was inversely related to meat color stability. As the phosphorylation of myoglobin increased, color stability based on a* value decreased and metMb content increased. In summary, the study revealed that protein phosphorylation might play a role in the regulation of meat color stability probably by regulating glycolysis and the redox stability of myoglobin, which might be affected by the phosphorylation of myoglobin.

1. Introduction

Meat color is the most intuitive basis for consumers to evaluate meat quality, which influences the purchase decision to a large extent (Mancini & Hunt, 2005). Discoloration is a common and serious problem for both meat producers and retailers, which shortens the shelf life of meat, causes price discount and even waste of meat. Many factors affect meat color stability, including extrinsic factors such as gender, age, ante-mortem stress and postmortem handling of carcass, and intrinsic factors such as muscle pH and muscle fiber types (AMSA., 2012).

Reversible phosphorylation is the most common post-translational modification of proteins, which regulates most aspects of cellular biological processes (Graves & Krebs, 1999). With the employment of proteomic tools, several studies have recently reported that protein phosphorylation is involved in postmortem meat quality formation. Li et al. (2015) reported that phosphorylation of myofibrillar proteins and glycolytic enzymes, as well as pork quality, was affected by season, indicating that phosphoproteome might mediate the effects of environment temperature on postmortem glycolysis and meat quality development. A study by Huang, Larsen, and Lametsch (2012) revealed the phosphoproteome changes in muscle early postmortem and suggested that phosphorylation of myofibrillar proteins influenced the development of rigor mortis and meat quality. Another study has reported that postmortem glycolysis and muscle pH decline are related to protein phosphorylation, which might regulate the activity or stability of glycolytic enzymes (Huang et al., 2011). Indeed, profiling of phosphoproteome in porcine *longissimus dorsi* (LD) muscle shows that proteins involved in glucose metabolism and muscle contraction are the two largest groups of proteins with significantly changed phosphorylation within 24 h postmortem (Huang, Larsen, Palmisano, Dai, & Lametsch, 2014). Using the same technology, Chen et al. (2016) reported that lamb tenderness was closely associated with the phosphorylation status of myofibrillar proteins. In addition, Li et al. (2012) reported that electrical stimulation changed the phosphorylation of several proteins involved in energy metabolism in postmortem muscle and accelerated activation of μ-calpain. In summary, all these studies show that proteins involved in glycolysis and muscle contraction are reversibly phosphorylated in postmortem muscle, which then regulate muscle pH decline, rigor mortis and meat tenderness.

In contrast to muscle pH and meat tenderness of which the phosphoproteome has been intensively studied, little is known about the influence of protein phosphorylation on meat color stability. A previous

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study (Li et al., 2017) shows that meat color stability is related to the global phosphorylation status of sarcoplasmic proteins. To better understand the biochemistry of meat color stability, protein phosphorylation was comparatively profiled in muscles with different color stability. It is hoped that information from this study may advance our understanding of the regulation of meat color stability.

2. Materials and methods

2.1. Animals and sample collections

Sixty Bayan nur sheep (male, about 8-month old, average carcass weight 24.74 ± 2.05 kg, un-castrated) were slaughtered in a commercial slaughter plant in Linhe, Inner Mongolia, China. The *longissimus thoracis et lumborum* (LTL) muscles were removed right after exsanguination. LTL muscles from each side of the carcasses were fabricated into six steaks, individually wrapped in an oxygen-permeable polyvinylchloride (PVC) film, and stored at 4 °C for 8 days to simulate retail display. The first steak from the left side was used for pH measurement at 45 min and 24 h postmortem. Muscle pH values were measured using a portable pH meter (Testo205 pH meter, Lenzkirch, Germany) by directly inserting the spear-tipped glass electrode pH probe into muscles in a direction perpendicular to muscle fibers. For each muscle, measurement was performed at three random locations. The second steak was used for color determination. The rest of the steaks were used for muscle sample collection at 45 min, 6 h, 24 h, 2 d, 3 d, 4 d, 5 d, 6 d, 7 d and 8 d postmortem. Muscle samples were immediately snap frozen in liquid nitrogen and stored at −80 °C until use. Samples used in the present study were collected at 45 min, 6 h, 24 h, 3 d, 5 d and 7 d postmortem.

2.2. Instrumental color measurement

Meat color (CIE-L*a*b*) was measured at four random locations on each LTL muscles through the packaging film using a CM-600D Minolta colorimeter (Konica Minolta Sensing Americas Inc., Ramsey, NJ, USA). The standard white plate was covered with the same PVC film for calibration. A CIE D 65 10° standard observer angle and a 2.54 cm diameter aperture were used throughout (AMSA, 2012). Reflectance values were recorded from 360 to 740 nm at 10 nm intervals. R630/580 was calculated by the ratio of reflectance at 630 to 580 nm as an indicator of meat color stability (AMSA, 2012). The reflectance values at 474, 525 and 572 nm were calculated by linear interpolation and the Kubelka–Munk K/S values were calculated using the equation $K/S = (1 - R)^2/(2R)$, where R = % reflectance. The percentage of deoxymyoglobin (DeoxyMb), oxymyoglobin (OxyMb), and metmyogobin (MetMb) was calculated using equations DeoxyMb = [1.5 − (K/S474)/(K/S525)], OxyMb = [1 − (K/S610)/(K/S525)] and MetMb = [2 − (K/S572)/(K/S525)] (AMSA, 2012; Li, Lindahl, Zamaratskaia, & Lundström, 2012; Mancini, Hunt, & Kropf, 2003; Stewart, Zipser, & Watts, 1965). The L*, a*, and b* values were obtained from the instrument directly.

2.3. Grouping

No extreme pH values were detected in carcasses. R630/580 is an indirect indicator of meat discoloration, which was used to follow color changes during display. A larger ratio indicates greater redness contributed by either OxyMb and/or DeoxyMb and thus means greater color stability (AMSA, 2012; Joseph, Suman, Rentfrow, Li, & Beach, 2012). LTL muscles from the 60 carcasses were ranked according to the R630/580 value on day 7 and numbered as NO. 1 to 60 from high to low R630/580 values. Five muscles of highest a* values were selected from NO. 1 to 10 and grouped as high color stability (high, n = 5). Five muscles of middle a* values were selected from NO. 25 to 34 and grouped as moderate color stability (moderate, n = 5). Five muscles of lowest a* values were selected from NO. 51 to 60 and grouped as low color stability (low, n = 5). R630/580 values, a* values, and b* values are statistically significantly different among three groups.

2.4. Protein extraction

The sarcoplasmic proteins were extracted as previously described with minor modification (Chen et al., 2016; Huang et al., 2014; Lametsch et al., 2006; Sayd et al., 2006). 1 g (0.2 g from each carcass, n = 5) frozen muscle was homogenized using an Ultra TurraxT10 basic S25 (IKA Labortechnik, Staufen, Germany) in 6 ml ice-cold extraction buffer (100 mM Tris, 10 mM DTT, pH 8.3) containing complete protease inhibitors (Roche, Mannheim, Germany, one slice per 50 ml solution) and phosphatase inhibitor PhosStop (Roche, Mannheim, Germany, one slice per 10 ml solution). The homogenate was centrifuged for 30 min at 10,000 × *g*, 4 °C. The supernatant was collected and stored at -80 °C until further analysis. Protein concentration was determined by BCA assay (Pierce Chemical Company, Rockford, IL, USA).

2.5. Gel electrophoresis and image analyses

Gel based separation of proteins and determination of protein phosphorylation level were performed as previously described with some modification (Chen et al., 2016; Huang et al., 2011; Huang et al., 2012; Li, Zhou et al., 2015; Li, Fang et al., 2015). The sarcoplasmic proteins were diluted to 2 μg/μl with ultrapure water, and added with same volume of isovolumetric 2× loading buffer (100 mM Tris-HCl, pH 6.8, 40 g/l SDS, 1 g/l bromophenol blue, 250 g/l glycerin). The mixture was boiled for 5 min. 5 μl of samples was loaded in triplicates onto polyacrylamide gels for SDS-PAGE. A sample collected at 45 min postmortem from high color stability group was loaded onto each gel and used as a reference for normalization among different gels in densitometric analysis. The gels were run at 70 V and then at 110 V when the dye front came into the separating gel.

After electrophoresis, gels were stained with Pro-Q Diamond dye (Invitrogen, Eugene, OR, USA) to detect phosphoproteins and then stained with SYPRO Ruby dye (Invitrogen, Eugene, OR, USA) to detect total proteins. The gel images were collected by using the ChemiDoc™MP Imaging System (Bio-Rad, Hercules, CA, USA). Quantity One 4.6.2 software (Bio-Rad, Hercules, CA, USA) was used to quantify the intensity of protein bands on both Pro-Q Diamond and SYPRO Ruby images. The global phosphorylation level (P/T ratio) of each sample was calculated as the total protein band intensity on the Pro-Q Diamond image (P) divided by the total protein band intensity on the SYPRO Ruby image (T). The phosphorylation level of the reference sample on each gel was used for normalization among gels. Thus, the global phosphorylation level of each sample was finally expressed as the ratio of the P/T ratio of one sample to the P/T ratio of the reference sample on the same gel. The phosphorylation level (P/T ratio) of each protein band was calculated as the ratio of the intensity of phosphoproteins (P) on the Pro-Q Diamond image to the intensity of total proteins (T) on the SYPRO Ruby image and finally normalized by the reference sample.

2.6. Protein identification by LC-MS/MS

Protein identification by LC-MS/MS was performed as previously described (Canto et al., 2015; Chen et al., 2016). Briefly, a total of 11 protein bands visualized by Coomassie-Blue staining on gels were excised and subjected to in-gel trypsin digestion. After proteins were reduced with 10 mmol/l DTT, gel pieces were washed with 25 mmol/l NH_4HCO_3 and dehydrated with 100% acetonitrile. Dry gel pieces were added with trypsin solution (sequencing grade; Roche, Mannheim, Germany, 0.1 μg/μl of trypsin dissolved in 25 mmol/l of NH_4HCO_3) and incubated on ice for 1 h. The proteins were digested overnight at 37 °C and then stopped by 0.1% trifluoroacetic acid. Tryptic peptides were resuspended in mobile phase A (2% acetonitrile, 0.1% formic acid) and