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Myoglobin Post-Translational Modifications Influence Color Stability of Beef *Longissimus Lumborum*

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Abstract: Post-translational modifications (PTM) of proteins play critical roles in biological processes. PTM of muscle proteins influence meat quality. Nonetheless, myoglobin (Mb) PTM and their impact on fresh beef color stability have not been characterized yet. Therefore, our objectives were to identify Mb PTM in beef *longissimus lumborum* muscle during postmortem aging and to characterize their influence on color stability. The *longissimus lumborum* muscles from 9 ($n = 9$) beef carcasses (24 h postmortem) were subjected to wet aging for 0, 7, 14, and 21 d. At the end of each wet-aging period, steaks were fabricated. One steak for analyses of PTM was immediately frozen at −80°C, whereas other steaks were assigned to refrigerated storage in the darkness under aerobic packaging. Instrumental color and biochemical attributes were evaluated on day 0, 3, or 6 of storage. Mb PTM were analyzed using two-dimensional electrophoresis and tandem mass spectrometry. Surface redness (a^* value), color stability, and Mb concentration decreased ($P < 0.05$) upon aging. Gel image analyses identified 6 Mb spots with similar molecular weight (17 kDa) but different isoelectric pH. Tandem mass spectrometry identified multiple PTM (phosphorylation, methylation, carboxymethylation, acetylation, and 4-hydroxynonenal alkylation) in these 6 isoforms. The amino acids susceptible to phosphorylation were serine (S), threonine (T), and tyrosine, whereas other PTM were detected in lysine (K), arginine (R), and histidine residues. Additionally, distal histidine (position 64), critical to heme stability, was found to be alkylated. Overall, Mb PTM increased with aging. The aging-induced PTM, especially those occurring close to hydrophobic heme pocket, could disrupt Mb tertiary structure, influence heme affinity, and compromise oxygen binding capacity, leading to decreased color stability of fresh beef. Furthermore, PTM at K45, K47, and K87 were unique to Mb from non-aged beef, whereas PTM at R31, T51, K96, K98, S121, R139, and K147 were unique to Mb from aged counterparts, indicating that these Mb PTM could be used as novel biomarkers for fresh beef color stability.

Key words: aging, beef color stability, *longissimus lumborum*, myoglobin, post-translational modifications
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Introduction

The color of fresh beef is critical to meat industry because it is a major quality attribute influencing consumers' purchasing decisions at the point of sale (Suman et al., 2014). Consumers often use the cherry-red color as an indicator of the wholesomeness of fresh meats (Faustman and Cassens, 1990; Mancini and Hunt, 2005; Suman et al., 2014; Neethling et al., 2017). Meat discoloration results in consumer rejection, leading to huge economic loss. The U.S. beef industry incurs an annual revenue loss of $1 billion as a result of discoloration (Smith et al., 2000). Myoglobin (Mb) is the sarcoplasmic heme protein responsible for meat color. The concentration and redox forms of Mb determine the fresh meat color. Furthermore, the primary structure of Mb dictates its tertiary structure and, in turn, influences its functional properties as an oxygen carrier and its interactions with biomolecules, ultimately affecting meat color (Faustman et al., 2010; Suman and Joseph, 2013; Ramanathan et al., 2020a, 2020b).

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Post-translational modifications (PTM) are covalent changes in proteins by the addition or removal of modifying group(s) at one or more amino acids in the primary structure (Lodish, 1981; Han and Martinage, 1992; Mann and Jensen, 2003). PTM can modulate proteins' functionality, localization, turnover, and interactions with other proteins (Seo and Lee, 2004; Rakhit et al., 2014; Müller, 2017). Previous investigations have documented that PTM in calpain (Liu et al., 2016; Du et al., 2019), myofibrillar proteins (Huang et al., 2012; Li et al., 2017), and metabolic enzymes (Anderson et al., 2014; Cruzen et al., 2015; Carlson et al., 2017; Huang et al., 2018; Liu et al., 2018) in skeletal muscles of livestock (cattle, pig, and sheep) influenced meat tenderness and muscle to meat conversion. The aforementioned studies suggested the potential of protein PTM as novel biomarkers for meat quality (Li et al., 2021).

PTM such as oxidation (Bostelaar et al., 2016; Lindsay et al., 2016), methylation (Santucci et al., 1993), carboxymethylation (Ray and Gurd, 1967; Harris and Hill, 1969), phosphorylation (Stewart et al., 2004; Hojlund et al., 2009; Hohenester et al., 2013), and acetylation (Livingston et al., 1985) influenced the functionality of mammalian Mb. Additionally, alkylation (nucleophilic adduction by reactive aldehydes) compromises stability of beef Mb (Alderton et al., 2003; Suman et al., 2006, 2007; Yin et al., 2011; Elroy et al., 2015; Viana et al., 2020).

Mb interacts reciprocally with small biomolecules, proteins, and cellular components in the muscle food matrix during postmortem aging (Richards, 2013; Ramanathan et al., 2020a, 2020b). The biomolecular interactions in postmortem skeletal muscles govern Mb chemistry and color of fresh meats (Fox, 1966; Giddings, 1977; Livingston and Brown, 1981; Seideman et al., 1984; Renerre, 1990; Faustman et al., 2010; Suman and Nair, 2017). Logically, *in situ* PTM in Mb can impact fresh meat color stability through modulating the heme protein's structural and functional properties as well as interactions with other biomolecules (Suman and Joseph, 2013).

Recent investigations (Li et al., 2018a, 2018b) identified phosphorylation in glycolytic enzymes and Mb in sheep *longissimus* muscle and suggested that phosphorylation might be involved in meat color stability. Nonetheless, *in situ* PTM in beef Mb and their impact on fresh beef color stability have not been characterized yet. Therefore, the objective of the current study was to characterize the Mb PTM in beef *longissimus lumborum* (LL) muscle during postmortem aging and their influence on fresh beef color stability.

Materials and Methods

Beef fabrication

Beef carcasses ($n = 9$; USDA Choice; A maturity; black-hided crossbred heifers) were obtained from the USDA-inspected meat laboratory at the University of Kentucky (Lexington, KY). LL muscles from the right side of carcasses were removed and divided into 4 equal-length sections after 24 h postmortem. The muscle sections were vacuum packaged (99% vacuum; Sipromac Model 600A; Drummondville, Quebec, Canada) in Prime Source vacuum pouches (3 mil, Bunzl Koch Supplies Inc., Kansas City, MO) and randomly assigned to wet aging at 2°C for either 0, 7, 14, or 21 d. At the end of each wet-aging period, the muscle sections were removed from the vacuum package and fabricated into four 1.92-cm-thick steaks. One steak from each muscle section allotted for proteome analyses was immediately vacuum packaged and frozen at −80°C until used. The remaining 3 steaks were allotted to refrigerated storage for evaluation of the color traits. The 3 steaks utilized for color evaluation were individually placed on Styrofoam trays and aerobically overwrapped with oxygen-permeable film (15,500–16,275 $cm^3/m^2/24$ h oxygen transmission rate at 23° C). Packages were randomly assigned for refrigerated storage (2°C) for either 0, 3, or 6 d in the darkness (Mancini et al., 2009; Nair et al., 2018a, 2018b). At each storage time point, Mb concentration, meat pH, instrumental color, metmyoglobin reducing activity (MRA), and lipid oxidation were evaluated.

Instrumental color

The surface color of steaks was measured instrumentally at each time point using a HunterLab LabScan XE colorimeter (Hunter Associations Laboratory, Reston, VA) with 2.54-cm-diameter aperture, illuminant A, and 10° standard observer. The colorimeter was calibrated with standard black and white plates. On day 0 of storage, the oxygen-permeable film was removed from the packages, and the steaks were bloomed for 2 h at 2°C before the instrumental color attributes were evaluated. CIE (1976) L^* (lightness), a^* (redness), and b^* (yellowness) values were measured at 6 random locations on the oxygen-exposed surface of each steak (American Meat Science Association, 2012). Additionally, the reflectance was measured from 700 to 400 nm, and the ratio of reflectance at 630 nm and 580 nm (R630/580) was obtained as an indirect estimate of surface color stability (American Meat Science Association, 2012).

Meat pH

The pH value of raw steak samples was determined according to the method of Strange et al. (1977). Triplicate 5-g muscle samples were homogenized with 25 mL of distilled deionized water (at 25°C), and the pH was measured utilizing an Accumet AR25 pH meter (Fisher Scientific, Pittsburg, PA).

Lipid oxidation

Lipid oxidation was measured using the thiobarbituric acid assay (Yin et al., 1993). Triplicate 5-g of sample—from the surface and interiors of the steaks—was homogenized with 22.5 mL of 11% trichloroacetic acid solution and filtered through Whatman No. 1 paper (GE Healthcare, Little Chalfont, UK). One milliliter of aqueous filtrate was mixed with 1 mL of aqueous thiobarbituric acid and incubated at 25°C for 20 h. The absorbance values at 532 nm were measured utilizing a UV-2401PC spectrophotometer (Shimadzu Inc., Columbia, MD) and were presented as thiobarbituric acid reactive substances (TBARS).

Metmyoglobin reducing activity

MRA was measured at 3 time points (day 0, 3, and 6) of each aging period. MRA was evaluated according to Sammel et al. (2002). Triplicate 2.45-cm samples removed from the oxygen-exposed steak surface were submerged in 0.3% sodium nitrate (Sigma-Aldrich Co., St. Louis, MO) solution for 20 min at room temperature to facilitate metmyoglobin formation. After 20 min, the samples were removed from the solution, blotted dry, and vacuum packaged. The reflectance spectra were measured from 700 to 400 nm on the light-exposed surface using a HunterLab LabScan XE colorimeter immediately after vacuum packaging in order to calculate pre-incubation surface metmyoglobin values (American Meat Science Association, 2012). The samples were then incubated at 30°C for 2 h allowing for metmyoglobin reduction, and then surface reflectance was rescanned to calculate post-incubation metmyoglobin values (American Meat Science Association, 2012). MRA was calculated using the following equation:

$$\text{MRA} = 100 \times [(\% \text{ pre-incubation surface metmyoglobin} - \% \text{ post-incubation surface metmyoglobin}) / \% \text{ pre-incubation surface metmyoglobin}].$$

Myoglobin concentration

The Mb concentration was measured at 3 time points (day 0, 3, and 6) of each aging period. Triplicate 5-g samples were homogenized with 45 mL ice-cold 40 mM sodium phosphate buffer at pH 6.8 (Faustman and Phillips, 2001). The homogenate was filtered through Whatman No. 1 paper, and the absorbance of the filtrate was measured at 525 nm (A_{525}) utilizing a UV-2401PC spectrophotometer (Shimadzu Inc., Columbia, MD) with 40 mM sodium phosphate buffer as a blank. The Mb concentration was calculated using the following equation:

$$\text{Mb (mg/g)} = [A_{525} / (7.6 \text{ mM}^{-1} \text{ cm}^{-1} \times 1 \text{ cm})] \times (17{,}000/1{,}000) \times 10$$

where 7.6 $\text{mM}^{-1} \text{cm}^{-1}$ = mM absorptivity coefficient of Mb at 525 nm; 1 cm = light path length of cuvette; 17,000 Da = average molecular weight of Mb; and 10 = dilution factor.

Isolation of sarcoplasmic proteome

The sarcoplasmic proteomes from samples ($n = 9$) frozen (−80°C) on each of the aging days (0, 7, 14, and 21) were extracted according to the method of Joseph et al. (2012). Frozen samples were thawed overnight at 2°C. Five grams of muscle tissue devoid of any visible fat and connective tissue was homogenized in 25 mL of ice-cold extraction buffer (40 mM Tris, 5 mM ethylenediaminetetraacetic acid [pH 8]) using a Waring blender (Model No. 51BL32; Waring Commercial, Torrington, CT). The homogenate was then centrifuged at 10,000 × g for 15 min at 4°C. The supernatant consisting of sarcoplasmic proteome extract was filtered through Whatman No. 1 paper (GE Healthcare) and used for subsequent analyses (Joseph et al., 2012; Nair et al., 2018a, 2018b).

Two-dimensional electrophoresis

The protein concentration of the sarcoplasmic proteome extract was determined in duplicate employing the Bradford assay (Bradford, 1976) utilizing the Bio-Rad Protein Assay kit (Bio-Rad Laboratories Inc., Hercules, CA). Nine hundred micrograms of sarcoplasmic proteome was mixed with rehydration buffer (Bio-Rad Laboratories Inc.) optimized to 7 M urea, 2 M thiourea, 20 mM dithiothreitol, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.5% Bio-Lyte 5/8 ampholyte and 0.001% Bromophenol blue. The mixture of sarcoplasmic protein and rehydration buffer was loaded into immobilized pH gradient strips (pH 5–8; 17 cm; Bio-Rad Laboratories Inc.) and subjected to passive rehydration for 16 h (Joseph et al., 2012). First-dimension isoelectric focusing,

which enables the separation of proteins based on their isoelectric point (pI), was performed using a Protean isoelectric focusing cell system (Bio-Rad Laboratories Inc.). A low voltage (50 V) was applied during the initial active rehydration for 4 h, followed by a linear increase in voltage, and a final rapid voltage ramping to attain a total of 60 kVh. Following this, the immobilized pH gradient strips were equilibrated with equilibration buffer I (6 M urea, 0.375 M Tris-HCl [pH 8.8], 2% sodium dodecyl sulfate [SDS], 20% glycerol, and 2% [w/v] dithiothreitol; Bio-Rad Laboratories Inc.) followed by equilibration buffer II (6 M urea, 0.375 M Tris-HCl [pH 8.8], 2% SDS, 20% glycerol, and 2.5% [w/v] iodoacetamide), each for 15 min. Second-dimension separation of protein was achieved by 13.5% SDS-polyacrylamide gel electrophoresis (38.5:1 ratio of acrylamide to bis-acrylamide) in a Protean II Multicell system (Bio-Rad Laboratories Inc.). The equilibrated strips were loaded onto 18.5 cm × 20 cm lab cast SDS-polyacrylamide gel electrophoresis gels with an agarose overlay, and the electrophoresis was completed using running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at room temperature. A constant voltage of 100 V was applied for approximately 16 h to allow the separation of proteins in the second dimension. Beef LL muscle during the aging days (0, 7, 14, and 21) from all the carcasses ($n = 9$) was analyzed in duplicate, resulting in a total of 72 gels.

Gel staining and image analyses

Gels were stained for phosphorylated protein using Pro-Q Diamond (Invitrogen, Carlsbad, CA) according to manufacturer recommendations. Gels were immersed in fixing solution (50% methanol and 10% acetic acid) and incubated twice at room temperature with gentle agitation for 30 min. Fixed gels were then immersed in ultrapure water in order to remove all the methanol and acetic acid. Pro-Q Diamond phosphoprotein gel stain was used to stain the gels for 2 h in the dark, followed by destaining in destaining solution (20% acetonitrile, 50 mM sodium acetate [pH 4]) for 30 min 3 times. Gels were washed with ultrapure water 2 times before they were imaged (532 nm laser; excitation: 555 nm; emission: 580 nm) using a TyphoonTM FLA 9500 biomolecular imager (GE Healthcare). After gel imaging, gels were stained with Sypro Ruby Protein gel stain (Invitrogen, Carlsbad, CA) overnight in the dark and were transferred to a clean container, where they were destained twice with destaining solution (10% methanol, 7% acetic acid) for 30 min and rinsed with ultrapure water. Gels were then imaged (473 nm laser; excitation: 450 nm; emission: 610 nm) utilizing a TyphoonTM FLA 9500 biomolecular imager (GE Healthcare). Gel images stained with Pro-Q Diamond and Sypro Ruby were analyzed using PDQUEST software (Bio-Rad Laboratories Inc.).

Liquid chromatography-electrospray ionization-tandem mass spectrometry

The protein gel spots with similar molecular weight of 17 kDa were excised and subjected to dithiothreitol reduction, iodoacetamide alkylation, and in-gel trypsin digestion using a standard protocol (Shevchenko et al., 2006). The resulting tryptic peptides were extracted, concentrated, and subjected to shot-gun proteomics analysis as previously described in Kamelgarn et al. (2018). Nano-liquid chromatography (LC)–tandem mass spectrometry (MS/MS) analysis was performed using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with an Eksigent Nanoflex cHiPLC system (Eksigent, Dublin, CA) through a nano-electrospray ionization source. The peptide samples were separated with a reversed-phase cHiPLC column (75 μm × 150 mm) at a flow rate of 300 nL/min. Mobile phase A was water with 0.1% (v/v) formic acid, whereas B was acetonitrile with 0.1% (v/v) formic acid. A 50-min gradient condition was applied: initial 3% mobile phase B was increased linearly to 40% in 24 min and further to 85% and 95% for 5 min each before it was decreased to 3% and re-equilibrated. The mass analysis method consisted of 1 segment with 10 scan events. The first scan event was an Orbitrap MS scan (300–1,800 m/z) with 60,000 resolution for parent ions followed by data-dependent MS/MS for fragmentation of the 10 most intense multiply charged ions with the collision-induced dissociation method.

Identification of PTM in myoglobin

The LC-MS/MS data were submitted to a local mascot server for MS/MS protein identification via Proteome Discoverer (version 1.3, Thermo Fisher Scientific, Waltham, MA) against a custom database containing only beef Mb protein (MYG_BOVIN) downloaded from UniProt (https://www.uniprot.org/uniprot/P02192). Typical parameters used in the MASCOT MS/MS ion search were as follows: trypsin digestion with a maximum of 2 miscleavages; 10 ppm precursor ion and 0.8-Da fragment ion mass tolerances; methionine oxidation; lysine acetylation; lysine and arginine methylation; serine, threonine, and tyrosine

phosphorylation; 4-hydroxynonenal (HNE) alkylation at histidine, and lysine.

Statistical analysis

The LL muscles from 9 ($n = 9$) beef carcasses were utilized for the current study. A split-plot design with a completely randomized block design in the whole plot was utilized to evaluate the effects of postmortem aging time and display day on steak color and biochemical properties. In the whole plot, each LL muscle ($n = 9$) was cut into 4 sections. Each section within a muscle served as a whole plot unit and was randomly assigned to one of 4 aging periods (0, 7, 14, and 21 d). The subplot experimental units consisted of steaks fabricated from each aged section assigned for 0, 3, or 6 d of refrigerated storage. Random effects included muscle (block) and muscle × aging period (error A) for the whole plot and unspecified residual error (error B) for the subplot. The analysis of variance was determined utilizing the Mixed Procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC), and the differences among means were detected using the least significant difference at a 5% level.

Results and Discussion

Instrumental color and biochemical attributes

***L** value (lightness).** There was a significant aging × storage interaction ($P < 0.05$; Table 1) for lightness (L^* value). While non-aged steaks exhibited lower ($P < 0.05$) L^* value than aged counterparts on day 0 of storage, all the steaks demonstrated similar lightness ($P > 0.05$) at the end of storage (6 d). Non-aged steaks demonstrated an increase ($P < 0.05$) in L^* value during the storage, whereas L^* value remained stable ($P > 0.05$) in the aged ones. Overall, aging resulted in an increase in lightness, which was consistent with the observations of Marino et al. (2014), Obuz et al. (2014), English et al. (2016), and Nair et al. (2018a) in beef longissimus muscle.

Table 1. Surface lightness (L^* value), redness (a^* value), yellowness (b^* value), meat pH, and Mb concentration of aged[1] beef *longissimus lumborum* steaks ($n = 9$) during refrigerated storage (2°C) for 6 d under aerobic packaging[2]

Parameter	Aging days	Storage days		
		0	3	6
L^* value	0	38.22 ± 1.80^{c}	41.60 ± 0.97^{b}	42.11 ± 1.46^{ab}
	7	42.28 ± 1.48^{ab}	42.77 ± 1.31^{ab}	43.87 ± 1.16^{a}
	14	42.88 ± 1.33^{ab}	43.02 ± 0.95^{ab}	42.68 ± 1.05^{ab}
	21	42.99 ± 1.51^{ab}	43.08 ± 1.66^{ab}	42.20 ± 0.90^{ab}
a^* value	0	24.79 ± 0.43^{a}	25.05 ± 0.77^{a}	23.48 ± 0.98^{ab}
	7	25.77 ± 0.62^{a}	24.60 ± 0.42^{a}	21.81 ± 0.93^{b}
	14	24.56 ± 0.57^{a}	23.53 ± 0.86^{ab}	17.26 ± 2.19^{c}
	21	24.85 ± 0.34^{a}	23.19 ± 0.77^{ab}	16.35 ± 2.08^{c}
b^* value	0	19.55 ± 0.63^{ab}	20.33 ± 0.63^{a}	19.86 ± 0.54^{ab}
	7	20.31 ± 0.46^{a}	19.25 ± 0.30^{ab}	17.66 ± 0.39^{b}
	14	18.99 ± 0.53^{ab}	18.22 ± 0.62^{b}	15.91 ± 0.85^{c}
	21	19.51 ± 0.40^{ab}	18.47 ± 0.40^{b}	15.99 ± 0.81^{c}
Meat pH	0	5.53 ± 0.02^{c}	5.54 ± 0.02^{c}	5.56 ± 0.02^{bc}
	7	5.62 ± 0.02^{b}	5.63 ± 0.02^{b}	5.61 ± 0.01^{bc}
	14	5.56 ± 0.02^{bc}	5.62 ± 0.02^{bc}	5.77 ± 0.06^{a}
	21	5.60 ± 0.02^{bc}	5.63 ± 0.01^{b}	5.80 ± 0.06^{a}
Mb concentration (mg/g)	0	6.00 ± 0.33^{a}	5.34 ± 0.20^{b}	5.08 ± 0.21^{c}
	7	5.14 ± 0.21^{bc}	4.98 ± 0.23^{cd}	5.21 ± 0.24^{bc}
	14	4.74 ± 0.24^{d}	4.68 ± 0.31^{d}	4.58 ± 0.25^{d}
	21	4.63 ± 0.25^{d}	4.79 ± 0.34^{cd}	4.51 ± 0.28^{d}

[1]Aged in vacuum packaging at 2°C.

[2]Results expressed as mean ± SEM.

[a–d]Means without common superscript within an attribute are different ($P < 0.05$).

Mb = myoglobin; SEM = standard error of the mean.

***a** Value (redness).** An aging × storage interaction ($P < 0.05$; Table 1) was observed for surface redness (a* value). All steaks demonstrated similar redness ($P > 0.05$) on days 0 and 3 of storage. A decrease in redness upon aging ($P < 0.05$) was observed on day 6 of storage, with steaks aged for 14 and 21 d exhibiting the lowest redness. In general, redness of non-aged steaks remained stable during the storage ($P > 0.05$), whereas redness of aged ones decreased ($P < 0.05$). In agreement, Liu et al. (1996) observed that prolonged aging of beef LL accelerated the loss of redness. Postmortem aging can influence the cellular mechanisms that determine the Mb redox chemistry and therefore impact the meat color stability (Ledward, 1985; Tang et al., 2005; King et al., 2012). In addition, previous investigations reported that the decrease of redness in LL muscle during aging was possibly due to the negative effects of storage time on mitochondria-mediated metmyoglobin reduction (Mancini and Ramanathan, 2014) and mitochondrial degeneration (Mitacek et al., 2019). The increase in pH (Table 1) and lipid oxidation (Table 2) during aging and storage observed in the present study could also be responsible for the surface discoloration. The increase in pH observed in LL muscle could enhance mitochondria activity (Ramanathan and Mancini, 2018), resulting in the decrease of oxymyoglobin content as well as redness. Lipid oxidation, on the other hand, accelerates metmyoglobin formation, thus promoting discoloration in fresh meat (Faustman et al., 2010).

***b** Value (yellowness).** An interaction between aging and storage ($P < 0.05$; Table 1) was observed for yellowness (b* value). While all steaks demonstrated similar yellowness at the beginning of the storage (day 0), those aged for 14 and 21 d had lower ($P < 0.05$) yellowness on storage days 3 and 6 than their counterparts aged for 0 and 7 d. While the yellowness of non-aged steaks remained stable during storage, aged counterparts exhibited a decrease ($P < 0.05$) in yellowness. Overall, an increase in aging time resulted in rapid loss of yellowness during the storage in beef LL steaks. Our observation was consistent with previous investigations that documented that aging (Obuz et al., 2014) and storage (Joseph et al., 2012; Canto et al., 2015) resulted in the decrease of yellowness in beef LL steaks. On the contrary, Marino et al. (2014) documented that yellowness of *longissimus dorsi* muscle was not influenced by aging.

R630/580 (color stability). The R630/580 indicates surface color stability; a greater ratio reflects lower metmyoglobin content and thus greater color stability. There was no aging × storage interaction ($P > 0.05$; Table 2) for R630/580. Nevertheless, both aging and storage led to the decrease ($P < 0.05$) of surface color stability of LL muscle. Muscles aged for 14 and 21 d demonstrated lower ($P < 0.05$) color stability

Table 2. Surface color stability (R630/580), MRA, and lipid oxidation of aged[1] beef *longissimus lumborum* steaks ($n = 9$) during refrigerated storage (2°C) for 6 d under aerobic packaging[2]

Parameter	Aging days	Storage days		
		0	3	6
R630/580	0	6.06 ± 0.34^{ax}	5.12 ± 0.33^{ay}	4.52 ± 0.34^{az}
	7	5.50 ± 0.26^{ax}	4.80 ± 0.22^{ay}	3.97 ± 0.27^{az}
	14	5.09 ± 0.28^{bx}	4.46 ± 0.30^{by}	3.14 ± 0.51^{bz}
	21	5.16 ± 0.23^{bx}	4.39 ± 0.30^{by}	2.94 ± 0.46^{bz}
MRA (%)	0	48.88 ± 3.99^{x}	27.75 ± 2.22^{y}	22.78 ± 3.47^{y}
	7	45.05 ± 2.02^{x}	30.39 ± 3.45^{y}	19.16 ± 4.51^{y}
	14	48.35 ± 3.94^{x}	29.18 ± 5.04^{y}	31.35 ± 8.08^{y}
	21	41.76 ± 2.39^{x}	21.41 ± 5.05^{y}	34.16 ± 10.74^{xy}
Lipid oxidation[3]	0	0.016 ± 0.002^{bz}	0.028 ± 0.004^{by}	0.035 ± 0.004^{bx}
	7	0.020 ± 0.002^{az}	0.047 ± 0.009^{ay}	0.067 ± 0.018^{ax}
	14	0.029 ± 0.004^{az}	0.046 ± 0.012^{ay}	0.062 ± 0.013^{ax}
	21	0.027 ± 0.008^{az}	0.048 ± 0.009^{ay}	0.067 ± 0.015^{ax}

[1]Aged in vacuum packaging at 2°C.

[2]Results expressed as mean ± SEM.

[3]Result expressed as absorbance at 532 nm.

[a,b]Means within a column without common superscript within an attribute are different ($P < 0.05$).

[x–z]Means within a row without common superscript are different ($P < 0.05$).

MRA = metmyoglobin reducing activity; R630/580 = ratio of reflectance at 630 nm and 580 nm; SEM = standard error of the mean.

than those aged for 7 d or less. In agreement, previous studies (Lindahl, 2011; English et al., 2016) recorded that beef discoloration increased with aging. Longer aging could decrease mitochondria function and metabolites required to generate NADH and therefore compromises color stability of beef (Ramanathan and Mancini, 2018). In addition, LL steaks exhibited a decrease ($P < 0.05$) in surface color stability during storage, and this observation was consistent with the results from previous investigations (Joseph et al., 2012; Canto et al., 2016; Nair et al., 2018a).

pH. There was an aging × storage interaction ($P < 0.05$; Table 1) for pH. The pH of steaks aged for 0 and 7 d remained stable over the storage, whereas steaks aged for 14 and 21 d exhibited an increase ($P < 0.05$) on day 6 of storage. In general, aging beyond 7 d resulted in higher pH of beef LL steaks at the end of 6 d of storage. The increase in the pH of aged meat during storage could be due to the proteolytic degradation of muscle fibers and the generation of basic metabolites (Lawrie, 1998). Likewise, Jayasooriya et al. (2007), Obuz et al. (2014), and Colle et al. (2015) documented that the pH of beef LL muscle increased with aging.

Myoglobin concentration. There was an aging × storage interaction ($P < 0.05$; Table 1) for Mb concentration. While all steaks experienced a decrease in Mb concentration during storage, the decline in non-aged steaks (0 d)—which demonstrated the highest ($P < 0.05$) Mb concentration—was more pronounced than their aged counterparts. Previous research (Jeong et al., 2009; King et al., 2011; McKenna et al., 2005) also indicated that the high content of Mb in muscles was associated with rapid discoloration. Muscle with a high level of Mb also contains a high concentration of iron, which is a prooxidant indirectly favoring the formation of metmyoglobin, leading to the decline in color stability (Farouk et al., 2007; Purchas et al., 2010).

Metmyoglobin reducing activity. The MRA indicates the ability of meat to reduce ferric metmyoglobin to ferrous redox forms (oxymyoglobin or deoxymyoglobin). The higher MRA suggests the greater inherent ability of muscle to reduce metmyoglobin, thus improving meat color stability. There was neither an aging × storage interaction ($P > 0.05$) nor an effect of aging ($P > 0.05$) for MRA (Table 2). However, storage influenced ($P < 0.001$) MRA, with the greatest ($P < 0.05$) values observed at the beginning of the storage (day 0). MRA decreased during storage in muscles aged for 0 and 7 d, whereas MRA in 21-d-aged steaks had a tendency ($P > 0.05$) to increase from day 3 to day 6 of storage. Nair et al. (2018a) documented a similar pattern in beef *psoas major* muscle. Likewise, Bekhit et al. (2001) observed that sheep *longissimus* muscle at 6 wk postmortem had 20% higher MRA than that at 48 h postmortem. Nair et al. (2018a) suggested that the tendency of increased MRA with storage could be possibly due to the increased degradation of mitochondria—releasing more mitochondrial enzymes—and subsequently increase of MRA without improving surface redness.

Lipid oxidation. There was no aging × storage interaction ($P > 0.05$) for TBARS (Table 2). However, there was an effect of storage ($P < 0.001$) and aging ($P = 0.0062$) on lipid oxidation. In agreement, several previous investigations documented that lipid oxidation in beef LL muscle increased with longer aging periods (Mancini and Ramanathan, 2014; Colle et al., 2015; English et al., 2016) and storage times (McKenna et al., 2005; Joseph et al., 2012; Mancini and Ramanathan, 2014; Colle et al., 2015; Canto et al., 2016). The observed increase in TBARS upon aging and storage could be attributed to the decrease in the redox capacity of meat and the generation of free radicals, which trigger the chain reaction and enhance lipid oxidation in meat (Min and Ahn, 2005). Previous investigations documented a decline in antioxidant capacity (Imazaki et al., 2018; Mitacek et al., 2019) and a decrease in the abundance of antioxidant enzymes (Nair et al., 2018b) in postmortem beef muscles upon aging. Endogenous antioxidants in postmortem skeletal muscles minimize lipid oxidation and metmyoglobin formation (Decker et al., 2000). A close positive correlation between lipid oxidation and Mb oxidation has been reported previously (Faustman and Cassens, 1990; Suman and Joseph, 2013). Lipid oxidation–induced Mb oxidation promotes the accumulation of metmyoglobin and leads to surface discoloration (Faustman et al., 2010).

Differential PTM between myoglobin isoforms

PTM can cause a shift in the pI of proteins by adding, removing, or changing titratable groups; this change in pI enables two-dimensional gel electrophoresis (2-DE) to resolve PTM-induced isoforms of proteins (Halligan et al., 2004). The representative 2-DE images (Figure 1) of beef sarcoplasmic proteome stained with Pro-Q Diamond (Invitrogen) for phosphorylated protein (Figure 1A) and Sypro Ruby (Invitrogen) for total protein (Figure 1B) are presented. Six Mb isoforms appeared in the gel images with similar molecular weight (17 kDa) but different pI, presenting the "beads on a string" appearance. These 6 Mb spots

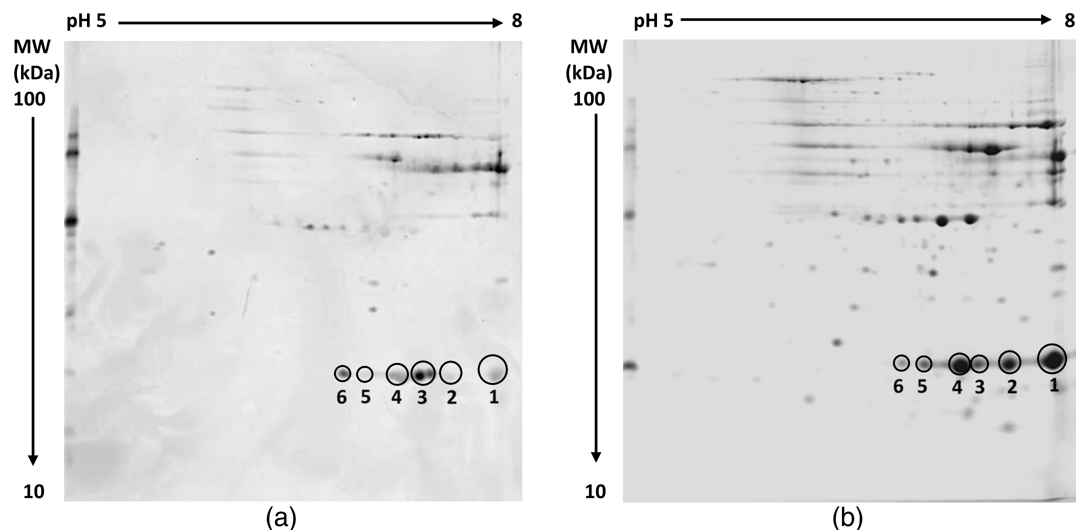

Figure 1. Representative two-dimensional gel electrophoresis map of sarcoplasmic proteome extracted from beef *longissimus lumborum*. The gel was stained with Pro-Q Diamond for (a) phosphorylated protein and with Sypro Ruby for (b) total protein. Myoglobin spots with the same number are located at the same position (isoelectric point and molecular weight [MW]) across the gels.

migrated different distances to the acidic side of the gel and exhibited different pI, indicating that they were post-translationally modified to different degrees. Similarly, Canto et al. (2015) identified 4 spots with similar molecular weight and different pI on 2-DE as beef Mb, yet the PTM in those spots were not identified.

Data in Table 3 indicated that the greatest number of phosphorylation sites were detected in isoform 3 (spot 3), whereas the sites of acetylation and carboxymethylation were most abundant in isoform 2 (spot 2). The largest number of methylation sites were observed in isoform 4 (spot 4). Phosphorylation, acetylation, methylation, and carboxymethylation could contribute to the pI shift by the addition of phosphates (McLachlin and Chait, 2001; Halligan et al., 2004; Jensen, 2004), acetyl group (Kumar et al., 2004; Xie et al., 2007), methyl group (Zhu et al., 2005; Xie et al., 2007), and carboxylic acid (Kung, 1976), respectively. The addition of negatively charged groups to the protein would cause its migration toward the acidic side of the gel (Anderson et al., 2014). Nonetheless, the magnitude of the pI shift will be dependent on the number and chemistry of the titratable groups added to the protein. Kumar et al. (2004) observed a direct relationship between the number of residues phosphorylated in a protein and the pI shift. Furthermore, Halligan et al. (2004) indicated that phosphates add 1.5 negative charge to the protein at a pH near 6.5. On the other hand, the pI shift due to acetylation is generally small (<0.2 pH; Zhu et al., 2005). Kumar et al. (2004) indicated that phosphorylation causes a greater shift in pI than acetylation and methylation. Therefore, the combination of different PTM could have contributed to the pI shifts of the isoforms 2, 3, and 4.

Spot 1 (the most alkaline isoform with the greatest pI) representing the native form of Mb was observed to have HNE alkylation at the greatest extent (Table 3). HNE could adduct to the imidazole group of histidine residues and the amine group of lysine, forming Michael adducts (Esterbauer et al., 1991). The addition of HNE has a minimal effect on the pI compared with other PTM, which introduce negatively charged groups (phosphates, acetyl group, and carboxylic acid) to the protein. This could possibly be the reason why spot 1 exhibited no change in the pI.

The Mb isoforms in spots 5 and 6 migrated further to the acidic side of the gel, suggesting that they were post-translationally modified at a greater degree than the other isoforms. However, the number of sites of PTM were lower in these 2 isoforms than in the other ones (Table 3). Phosphorylation was the major PTM in the isoforms 5 and 6 (Table 3), and this could have contributed to greater shift in pI of these 2 isoforms compared with the other isoforms in which a variety of PTM were identified.

PTM patterns in beef myoglobin

The position and identity of amino acids in beef Mb that underwent various PTM are summarized in Figure 2. The amino acids susceptible to phosphorylation were serine (S), threonine (T), and tyrosine (Y), whereas other PTM are detected in lysine (K), arginine

Table 3. Post-translational modifications and their locations in myoglobin isoforms isolated from beef *longissimus lumborum* muscle from all aging days

	Myoglobin spots[a]					
Post-translational modifications	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Spot 6
Phosphorylation	51 Threonine 58 Serine 67 Threonine	67 Threonine 103 Tyrosine	34 Threonine 67 Threonine 70 Threonine 103 Tyrosine 108 Serine 121 Serine 132 Serine	34 Threonine 51 Threonine 58 Serine	34 Threonine 67 Threonine 70 Threonine 103 Tyrosine 132 Serine	70 Threonine 103 Tyrosine 108 Serine 121 Serine
Acetylation	50 Lysine 63 Lysine 77 Lysine 78 Lysine 79 Lysine 118 Lysine	56 Lysine 63 Lysine 77 Lysine 78 Lysine 79 Lysine 87 Lysine 118 Lysine 133 Lysine	50 Lysine 56 Lysine 63 Lysine 77 Lysine 78 Lysine 79 Lysine 118 Lysine	63 Lysine 77 Lysine 78 Lysine 79 Lysine	63 Lysine 77 Lysine 78 Lysine 147 Lysine	–
Methylation	31 Arginine 42 Lysine 56 Lysine 62 Lysine 63 Lysine 118 Lysine 133 Lysine 139 Arginine	31 Arginine 42 Lysine 62 Lysine 63 Lysine 98 Lysine 102 Lysine 118 Lysine	31 Arginine 42 Lysine 77 Lysine 78 Lysine 79 Lysine 118 Lysine 139 Arginine	31 Arginine 42 Lysine 56 Lysine 62 Lysine 63 Lysine 96 Lysine 98 Lysine 118 Lysine 133 Lysine	77 Lysine 78 Lysine 139 Arginine	56 Lysine
Carboxymethylation	62 Lysine 63 Lysine 77 Lysine 78 Lysine 102 Lysine 118 Lysine 133 Lysine 147 Lysine	56 Lysine 62 Lysine 63 Lysine 77 Lysine 78 Lysine 79 Lysine 87 Lysine 96 Lysine 98 Lysine 102 Lysine 133 Lysine 147 Lysine	56 Lysine 62 Lysine 63 Lysine 77 Lysine 78 Lysine 79 Lysine 102 Lysine 118 Lysine 133 Lysine 147 Lysine	62 Lysine 63 Lysine 77 Lysine 78 Lysine 118 Lysine 133 Lysine	–	62 Lysine 77 Lysine 78 Lysine
HNE alkylation	45 Lysine 47 Lysine 56 Lysine 62 Lysine 63 Lysine 64 Histidine 77 Lysine 78 Lysine 79 Lysine	77 Lysine 78 Lysine 79 Lysine	77 Lysine 78 Lysine 79 Lysine	56 Lysine 62 Lysine 63 Lysine 64 Histidine	–	77 Lysine

[a]Spot number refers to the numbered spots in gel images (Figure 1A–1B).

HNE = 4-hydroxynonenal.

Sequence No.	10	20	30	40	50
Phosphorylation	GLSDGEWQLV	LNAWGKVEAD	VAGHGQEVLI	RLFTGHPETL	EKFDKFKHLK
Acetylation	GLSDGEWQLV	LNAWGKVEAD	VAGHGQEVLI	RLFTGHPETL	EKFDKFKHLK
Methylation	GLSDGEWQLV	LNAWGKVEAD	VAGHGQEVLI	RLFTGHPETL	EKFDKFKHLK
Carboxymethylation	GLSDGEWQLV	LNAWGKVEAD	VAGHGQEVLI	RLFTGHPETL	EKFDKFKHLK
HNE Alkylation	GLSDGEWQLV	LNAWGKVEAD	VAGHGQEVLI	RLFTGHPETL	EKFDKFKHLK

Sequence No.	60	70	80	90	100
Phosphorylation	TEAEMKASED	LKKHGNTVLT	ALGGILKKKG	HHEAEVKHLA	ESHANKHKIP
Acetylation	TEAEMKASED	LKKHGNTVLT	ALGGILKKKG	HHEAEVKHLA	ESHANKHKIP
Methylation	TEAEMKASED	LKKHGNTVLT	ALGGILKKKG	HHEAEVKHLA	ESHANKHKIP
Carboxymethylation	TEAEMKASED	LKKHGNTVLT	ALGGILKKKG	HHEAEVKHLA	ESHANKHKIP
HNE Alkylation	TEAEMKASED	LKKHGNTVLT	ALGGILKKKG	HHEAEVKHLA	ESHANKHKIP

Sequence No.	110	120	130	140	150	153
Phosphorylation	VKYLEFISDA	IIHVLHAKHP	SDFGADAQAA	MSKALELFRN	DMAAQYKVLG	FHG
Acetylation	VKYLEFISDA	IIHVLHAKHP	SDFGADAQAA	MSKALELFRN	DMAAQYKVLG	FHG
Methylation	VKYLEFISDA	IIHVLHAKHP	SDFGADAQAA	MSKALELFRN	DMAAQYKVLG	FHG
Carboxymethylation	VKYLEFISDA	IIHVLHAKHP	SDFGADAQAA	MSKALELFRN	DMAAQYKVLG	FHG
HNE Alkylation	VKYLEFISDA	IIHVLHAKHP	SDFGADAQAA	MSKALELFRN	DMAAQYKVLG	FHG

Figure 2. Post-translational modifications (phosphorylation, acetylation, methylation, carboxymethylation, 4-hydroxynonenal [HNE] alkylation) and their locations in the amino acid sequence of beef myoglobin. Post-translationally modified residues are underlined.

(R), and histidine (H) residues. Moreover, lysine residues at positions 56, 63, 77, 78, 79, 118, and 132 were susceptible to several PTM, namely acetylation, methylation, and carboxymethylation. A variety of factors, such as the number of PTM in a molecule of protein as well as their chemistry and location(s), influence protein functionality (Kumar et al., 2004).

Phosphorylation sites in myoglobin. Phosphorylation is the most common PTM and is a key regulator of biological/cellular processes (Graves and Krebs, 1999; Hunter, 2000). Phosphorylation has been reported in Mb from beluga whale (Stewart et al., 2004), human (Hojlund et al., 2009), pork (Huang et al., 2011; Lametsch et al., 2011), and sheep (Li et al., 2017, 2018a, 2018b). Moreover, Li et al. (2018a, 2018b) documented that the degree of phosphorylation in sheep Mb was inversely related to lamb color stability. Nonetheless, the impact of phosphorylation on the functionality of beef Mb is yet to be understood.

Phosphorylation was identified in the serine (positions 58, 108, 121, and 132), threonine (positions 34, 51, 67, and 70), and tyrosine (position 103) residues of beef Mb (Figure 2). In agreement, previous studies reported that serine, threonine, and tyrosine are the 3 amino acids that are most susceptible to phosphorylation (Hunter, 2012; Ardito et al., 2017; Lin, 2018). Similar to our results, Li et al. (2018b) documented phosphorylation at T34, T51, S58, T67, T70, Y103, S121, and S132 in sheep Mb. Furthermore, Hojlund et al. (2009) identified phosphorylation at T67 and T70 in human Mb.

In the three-dimensional model of beef Mb (Figure 3), T34, T51, T70, and S121 residues are located on the surface and thus are readily accessible to protein kinase, which is the enzyme responsible for catalyzing phosphorylation. In support, Stewart et al. (2004) suggested that S117 in beluga whale Mb was a site for phosphorylation and is located on the Mb surface making it easily accessible to protein kinase. A phosphate group added to Mb could form intra- and inter-molecular hydrogen bonds or salt bridges and thus alter the interaction of the heme protein with other small biomolecules (Hunter, 2012; Ardito et al., 2017).

Threonine at position 67 (T67) is located in the vicinity of distal histidine (position 64) in beef Mb

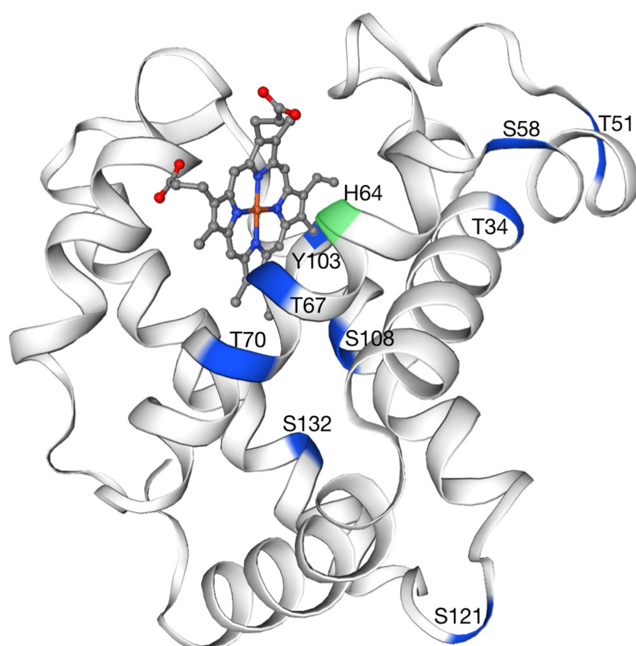

Figure 3. Threonine (T34, T51, T67, and T70), serine (S58, S108, S121, and S132), and tyrosine (Y103) residues in beef myoglobin are indicated in blue, whereas distal histidine (H64) is in green. The residues in blue were phosphorylated. The three-dimensional homology model of beef myoglobin was downloaded from SWISS-MODEL (Waterhouse et al., 2018).

(Figure 3), which is critical to the oxygen binding capability and redox stability (Suman and Joseph, 2013). The addition of a negative phosphate group on T67 influences the distal histidine's spatial interaction with hydrophobic heme pocket, increases the heme pocket's polarity, and decreases oxygen binding (Cameron et al., 1993). In partial agreement, Livingston et al. (1986) suggested that T67 in Mb from yellowfin tuna, turtle, and sperm whale could be involved with ligand binding. Likewise, Stewart et al. (2004) reported that the substitution of valine at position 67 with threonine in beluga whale Mb influenced the distal histidine's role in oxygen binding ability of heme. Additionally, Li et al. (2018b) suggested that the phosphorylation of sheep Mb at S132 might compromise the heme protein's oxygen binding ability and thus plays a negative role in color stability. Sheep and beef cattle Mb share 98.7% similarity in amino acid sequence (Suman and Joseph, 2013); therefore, it is possible that the phosphorylation at S132 in beef Mb could be detrimental to its oxygen binding capability and redox stability.

Acetylation sites in myoglobin. Protein acetylation is involved in essential biological processes (Zhao et al., 2010; Kwan et al., 2016; Ali et al., 2018). Previous investigations have documented acetylation of lysine in Mb from yellowfin tuna (Rice et al., 1979), cattle (Livingston et al., 1985), bullet tuna (Ueki et al., 2005), and pig (Jiang et al., 2019). In the present study, 10 lysine residues (positions 50, 56, 63, 77, 78, 79, 87, 118, 133, and 147) were found to be acetylated in beef Mb. These 10 positively charged lysine residues are located on the surface in beef Mb. Charged residues play critical roles in protein stability through the formation of ionic networks (Strickler et al., 2006; Pace et al., 2009; Raghunathan et al., 2013). Therefore, the addition of acetyl groups may neutralize the positively charged lysine residues, disrupting the charge distribution and altering the ionic network of Mb. Attachment of acetyl groups to surface lysine residues in Mb could decrease the protein's hydrophilicity and induce unfolding, and this in turn could expose the heme pocket to oxidizing agents, thus promoting heme iron release and jeopardizing oxygen binding ability. In partial agreement, Azami-Movahed et al. (2018) observed that horse apomyoglobin underwent acetylation-induced conformational changes with less ordered tertiary structure and absence of stable hydrophobic patches due to heme pocket disruption. Likewise, Nguyen et al. (2000) suggested that the N-terminus acetylation altered orientation of heme and proximal histidine imidazole plane in Mb from *Aplysia limacine* (sea hare). Moreover, Jiang et al. (2019) observed acetylation of K43 and K78 in pig Mb and suggested that lysine acetylation may be related to meat quality.

Methylation sites in myoglobin. Methylation is a distinct PTM that contributes to minimal change in size and electrostatic status to lysine and arginine residues (Luo, 2018). This PTM is also involved in a number of biological processes (Ong et al., 2004; Uhlmann et al., 2012). Methylation has been identified in histone (Greer and Shi, 2012; Lanouette et al., 2014; Clarke, 2018; Luo, 2018), ribosomal protein (Pang et al., 2010), tumor suppressor p53 (Huang and Berger, 2008), heat shock proteins (Abu-Farha et al., 2011), myosin (Li et al., 2015), and hemoglobin (Chen et al., 2017).

Methylation sites were detected in arginine (positions 31 and 139) and lysine (positions 42, 56, 62, 63, 77, 78, 79, 96, 98, 102, 118, and 133) residues in beef Mb. The aforementioned methylation sites are adjacent to hydrophobic residues such as leucine (L), isoleucine (I), alanine (A), and phenylalanine (F) in the amino acid sequence (Figure 2). This observation was consistent with previous report (Bremang et al., 2013), which reported that the amino acid residues near the methylated sites in proteins were predominantly hydrophobic in nature. Moreover, the methylation site K56 in Mb (Figure 2) conforms with the methionine-lysine

methylation motif reported by Pang et al. (2010), indicating that K56 might be methylated by a specific methyl transferase. The addition of methyl groups could increase hydrophobicity and steric hindrance and, in turn, alter the stability of proteins (Bremang et al., 2013). The methylation at K62 and K63 could impact distal histidine's (H64) interactions with heme pocket (Figure 4) and compromise Mb redox stability. Likewise, the addition of methyl groups at K96 and K98 might affect the proximal histidine (H93) in the vicinity (Figure 4), which connects heme to the globin chain, and consequently influence the oxygen binding ability/oxygen affinity. Additionally, the 11 sites (K56, K62, K63, K77, K78, K79, K96, K98, K102, K118, and K133) of methylation were also susceptible to other PTM (acetylation, carboxymethylation, and HNE alkylation), indicating the existence of PTM crosstalk (Aggarwal et al., 2020). PTM crosstalk is defined as the interactions between co-occurring multiple PTM, which can positively or negatively influence each other's occurrence (van der Laarse et al., 2018). The crosstalk among PTM could alter protein functions (Zhang et al., 2015). Furthermore, methylation could decrease protein stability by acting in combination with other PTM (Pang et al., 2010; Moore and Gozani, 2014; Zhang et al., 2015; Wu et al., 2017). Therefore, it is highly possible that the interplay between the PTM at aforementioned 11 lysine residues (methylation sites) could decrease Mb redox stability.

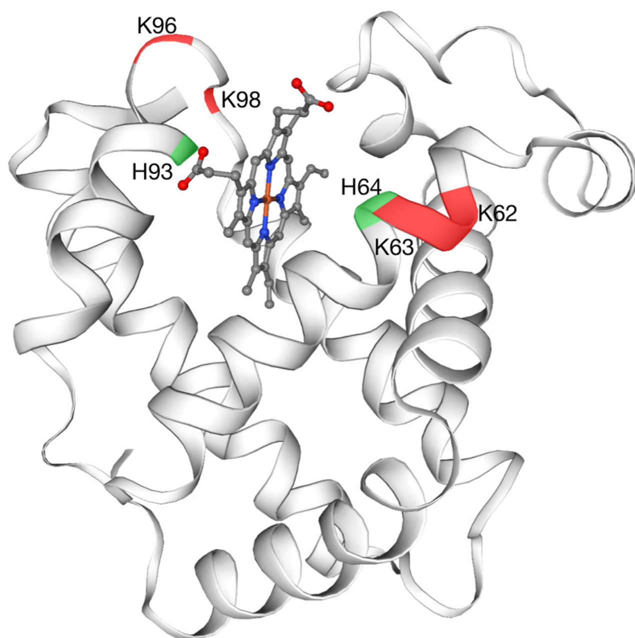

Figure 4. Distal (H64) and proximal (H93) histidines in beef myoglobin are indicated in green, whereas lysine residues (K62, K63, K96, and K98) are in red. The three-dimensional homology model of beef myoglobin was downloaded from SWISS-MODEL (Waterhouse et al., 2018).

Carboxymethylation sites in myoglobin. Carboxymethylation is a nonenzymatic PTM and is a potential metabolic modulator in chemotaxis, neurosecretory regulation, and diabetes (Diliberto et al., 1976; Curtiss and Witztum, 1985; Hackett and Campochiaro, 1988; Fang et al., 2010). Carboxymethylation could be achieved *in vitro* chemically (using bromoacetate) in the Mb from human (Harris and Hill, 1969), sperm whale (Banaszak et al., 1963; Ray and Gurd, 1967; Schlecht, 1969; Hugli and Gurd, 1970; Wu et al., 1972) and harbor seal (Nigen and Gurd, 1973). However, Mb carboxymethylation has not been investigated in postmortem skeletal muscle tissue.

Carboxymethylation was identified in 13 lysine residues (positions 56, 62, 63, 77, 78, 79, 87, 96, 98, 102, 118, 133, and 147) in beef Mb. Carboxymethylation introduces negatively charged carboxylic acid to the positively charged lysine residues located on the Mb surface, which could alter the ionic network and lead to conformational changes (Fang et al., 2010). Furthermore, carboxymethylation at K62 and K63, which are adjacent to the distal histidine (H64; Figure 4), might induce a spatial rearrangement of heme pocket, influencing the oxygen affinity of beef Mb and the color stability of steaks. Additionally, the loss of positive charge of K96 and K98 in the vicinity of proximal histidine (H93) could disrupt the heme iron-proximal histidine bond, compromising the heme affinity and damaging Mb helical structure (Smerdon et al., 1993; Hargrove et al., 1996). In agreement, Wu et al. (1972) observed that carboxymethylated sperm whale Mb (chemically modified) underwent autoxidation faster than its unmodified counterpart, indicating that carboxymethylation could compromise Mb redox stability.

HNE alkylation sites in myoglobin. HNE is an α,β-unsaturated aldehyde formed as a result of oxidation of ω-6 polyunsaturated fatty acids (Esterbauer et al., 1991), which are present abundantly in the membrane phospholipids of skeletal muscles (Wood et al., 2008). The electrophilic nature of carbon 3 in HNE enables it to covalently bind to nucleophilic sidechains of lysine and histidine residues (Esterbauer et al., 1991; Uchida and Stadtman, 1992). Alkylation of proteins by HNE has been reported to cause cytotoxicity (Codreanu et al., 2014; Yang et al., 2015). In *in vitro* model systems, HNE alkylation through Michael addition has been identified at histidine residues of Mb from beef (Alderton et al., 2003; Suman et al., 2006, 2007), pork (Suman et al., 2006, 2007; Elroy et al., 2015),

sheep (Yin et al., 2011), horse (Faustman et al., 1999), sperm whale (Tatiyaborworntham et al., 2012), yellowfin tuna (Lee et al., 2003), ostrich (Nair et al., 2014), emu (Nair et al., 2014), turkey (Naveena et al., 2010), and chicken (Naveena et al., 2010). However, *in situ* HNE alkylation of Mb in muscle foods has yet to be reported.

In the present study, histidine (position 64) and lysine (positions 45, 47, 56, 62, 63, 77, 78, and 79) residues were modified by HNE alkylation in beef Mb. Histidine and lysine residues form cyclic hemiacetal derivatives (Uchida and Stadtman, 1992, 1993; Uchida, 2003) via Michael addition with HNE. Alkylation at distal histidine (position 64), which is critical for heme stability, could destabilize heme iron's ability to bind with oxygen and therefore compromise Mb redox stability and beef color stability (Suman and Joseph, 2013). In agreement, previous investigations (Alderton et al., 2003; Suman et al., 2007; Viana et al., 2020) observed that HNE alkylation at H64 enhances oxidation of beef Mb. Additionally, the Michael adducts formed at lysine residues (positions 45, 47, 56, 62, 63, 77, 78, and 79)—especially at K62 and K63, which are located adjacent to the distal histidine (Figure 4)—could compromise hydrophobic interactions stabilizing the tertiary structure of Mb, leading to globin unfolding (Ueki and Ochiai, 2006).

Aging-induced PTM influence beef color stability

The sites of PTM in beef Mb during 21 d of postmortem aging are presented in Table 4. Total PTM sites increased with aging from day 0 to day 14, whereas it decreased thereafter. The decrease in number of detected PTM from day 14 to day 21 of aging is possibly due to the observed decrease in the Mb concentration (Table 1) as a result of protein degradation and drip loss.

Increased numbers of phosphorylation, methylation, and carboxymethylation sites were observed in Mb from aged beef compared with Mb from non-aged counterparts (Table 4). The increased number of phosphate groups, methyl groups, and carboxylic acid adducted to Mb could disrupt the ionic network, which is critical to the heme protein's stability. Moreover, phosphorylation at T51 and S121; methylation at R31, K77, K78, K79, K96, K98, K102, K133, and R139; and carboxymethylation at K56, K96, K98, K118, and K147 were unique to Mb from aged beef, whereas carboxymethylation at K79 and K87 were unique to Mb from non-aged counterparts (Table 5). The PTM at K96 and K98 observed in Mb from aged beef could influence the interactions between proximal histidine (H93) and heme iron (Figure 4), compromising the protein's heme affinity and redox state (Hargrove et al., 1996; Grunwald and Richards, 2006). Previous studies have indicated that minor variations in amino acid sequence of Mb, especially those close to the heme pocket, could alter autoxidation rate (Kitahara et al., 1990; Tada et al., 1998), heme affinity (Grunwald and Richards, 2006), and structural stability (Ueki and Ochiai, 2004, 2006). Therefore, the decreased color stability in aged beef could be attributed to the increased number of PTM (phosphorylation, methylation, carboxymethylation).

Mb acetylation sites decreased during postmortem aging (Table 4). In agreement, a decrease in acetylation was observed in proteins during postmortem aging of pork *longissimus* muscle (Jiang et al., 2019). In addition, the dynamic acetylation-deacetylation of lysine residues could influence the conversion of muscle to meat and meat quality (Jiang et al., 2019). Acetylation can regulate stability of Mb and its interactions with other proteins by converting positively charged NH_4^+ cation on lysine and arginine residues into a neutral moiety (Kumar et al., 2004; Zhu et al., 2005; Krueger and Srivastava, 2006; Xie et al., 2007). Moreover, acetylation at K87 and K118 were unique to Mb from non-aged beef, whereas acetylation at K56 and K147 were unique to Mb from aged beef (Table 5). Therefore, aging might cause the deacetylation at K87 and K118—and acetylation at K56 and K147—and, in turn, regulate Mb redox stability and color stability of beef.

The number of HNE alkylation sites in Mb did not change during 14 d of aging (Table 4). Nevertheless, HNE alkylation at K45 and K47 were only detected in Mb from non-aged beef, whereas K78 and K79 were adducted by HNE only in Mb from aged counterparts (Table 5). Covalent binding of HNE to lysine residues compromises the tertiary structure of proteins and increases its susceptibility to oxidation (Isom et al., 2004; Szapacs et al., 2006); therefore, aging-induced HNE alkylation of lysine residues might play a critical role in Mb redox stability (Suman and Joseph, 2013). Additionally, distal histidine (H64) was alkylated in Mb from both non-aged and aged beef (Table 5). Our results were different from *in vitro* studies of Suman et al. (2006) and Viana et al. (2020), in which beef Mb was incubated with HNE at meat conditions. While Suman et al. (2006) identified 4 HNE adducted histidine residues (position 36, 81, 88, and 152) of beef Mb, the present study found only 1 histidine (H64)

Table 4. Post-translational modifications and their locations in myoglobin isolated from beef *longissimus lumborum* muscle during postmortem aging

Post-translational modifications	Aging days			
	0 d	7 d	14 d	21 d
Phosphorylation	34 Threonine	34 Threonine	34 Threonine	34 Threonine
	58 Serine	51 Threonine	58 Serine	67 Threonine
	67 Threonine	58 Serine	67 Threonine	70 Threonine
	70 Threonine	67 Threonine	70 Threonine	103 Tyrosine
	103 Tyrosine	70 Threonine	103 Tyrosine	108 Serine
	108 Serine	103 Tyrosine	108 Serine	132 Serine
	132 Serine	108 Serine	121 Serine	
		121 Serine	132 Serine	
		132 Serine		
Acetylation	50 Lysine	56 Lysine	50 Lysine	63 Lysine
	63 Lysine	63 Lysine	56 Lysine	77 Lysine
	77 Lysine	77 Lysine	63 Lysine	78 Lysine
	78 Lysine	78 Lysine	77 Lysine	147 Lysine
	79 Lysine	79 Lysine	78 Lysine	
	87 Lysine	133 Lysine	79 Lysine	
	118 Lysine			
	133 Lysine			
Methylation	42 Lysine	31 Arginine	31 Arginine	31 Arginine
	56 Lysine	42 Lysine	42 Lysine	42 Lysine
	62 Lysine	56 Lysine	56 Lysine	63 Lysine
	63 Lysine	62 Lysine	62 Lysine	77 Lysine
	118 Lysine	63 Lysine	63 Lysine	78 Lysine
		77 Lysine	77 Lysine	98 Lysine
		78 Lysine	78 Lysine	102 Lysine
		79 Lysine	98 Lysine	118 Lysine
		96 Lysine	102 Lysine	133 Lysine
		98 Lysine	118 Lysine	
		118 Lysine	133 Lysine	
		139 Arginine		
Carboxymethylation	62 Lysine	56 Lysine	56 Lysine	62 Lysine
	63 Lysine	62 Lysine	62 Lysine	63 Lysine
	77 Lysine	63 Lysine	63 Lysine	77 Lysine
	78 Lysine	77 Lysine	77 Lysine	78 Lysine
	79 Lysine	78 Lysine	78 Lysine	133 Lysine
	87 Lysine	96 Lysine	102 Lysine	
	102 Lysine	98 Lysine	118 Lysine	
	133 Lysine	102 Lysine	147 Lysine	
		118 Lysine		
		133 Lysine		
		147 Lysine		
HNE alkylation	45 Lysine	56 Lysine	56 Lysine	77 Lysine
	47 Lysine	62 Lysine	62 Lysine	
	56 Lysine	63 Lysine	63 Lysine	
	62 Lysine	64 Histidine	64 Histidine	
	63 Lysine	77 Lysine	77 Lysine	
	64 Histidine	78 Lysine	78 Lysine	
	77 Lysine	79 Lysine	79 Lysine	
Total number of post-translational modification sites	35	45	40	25

HNE = 4-hydroxynonenal.

Table 5. Differential PTM[‡] identified in myoglobin isolated from non-aged[1] and aged[2] beef *longissimus lumborum* muscle

Non-aged beef			Aged beef		
Position[†]	Residue	Modification	Position[†]	Residue	Modification
			31	**Arginine**	**M**[*]
34	Threonine	P	34	Threonine	P
42	Lysine	M	42	Lysine	M
45	**Lysine**	**H**[Ψ]			
47	**Lysine**	**H**[Ψ]			
50	Lysine	A	50	Lysine	A
			51	**Threonine**	**P**[*]
56	Lysine	M H	**56**	**Lysine**	**A**[*] M **C**[*] H
58	Serine	P	58	Serine	P
62	Lysine	M C H	62	Lysine	M C H
63	Lysine	M A C H	63	Lysine	M A C H
64	Histidine	H	64	Histidine	H
67	Threonine	P	67	Threonine	P
70	Threonine	P	70	Threonine	P
77	Lysine	A C H	**77**	**Lysine**	A **M**[*] C H
78	Lysine	A C	**78**	**Lysine**	A **M**[*] C **H**[*]
79	**Lysine**	A **C**[Ψ]	**79**	**Lysine**	A **M**[*] **H**[*]
87	**Lysine**	**A**[Ψ] **C**[Ψ]			
			96	**Lysine**	**M**[*] **C**[*]
			98	**Lysine**	**M**[*] **C**[*]
102	Lysine	C	**102**	**Lysine**	**M**[*] C
103	Tyrosine	P	103	Tyrosine	P
108	Serine	P	108	Serine	P
118	**Lysine**	**A**[Ψ] M	**118**	**Lysine**	M **C**[*]
			121	**Serine**	**P**[*]
132	Serine	P	132	Serine	P
133	Lysine	A C	**133**	**Lysine**	A **M**[*] C
			139	**Arginine**	**M**[*]
			147	**Lysine**	**A**[*] **C**[*]
Total	22	35	Total	26	49

[‡]Differential PTM and their locations are listed in boldface.

[1]Non-aged = aged for 0 d.

[2]Aged = aged in vacuum packaging at 2°C for 7, 14, or 21 d.

[†]Amino acid positions in the sequence of myoglobin from both non-aged and aged beef are placed in the same row for direct comparison.

[*]PTM unique to myoglobin isolated from beef *longissimus lumborum* muscle aged in vacuum packaging at 2°C for 7, 14, or 21 d.

[Ψ]PTM unique to myoglobin isolated from non-aged beef *longissimus lumborum* muscle.

A = Acetylation; C = Carboxymethylation; H = 4-hydroxynonenal alkylation; M = Methylation; P = Phosphorylation; PTM = post-translational modification.

modified by HNE. Viana et al. (2020) observed the number of HNE adduction sites increased with storage, with 6 histidines (positions 24, 36, 64, 93, 113, and 152) adducted after 21 d of incubation at pH 5.6 and 4°C; however, in the current study, only 1 HNE alkylation site (K77) was detected after 21 d of aging. The lower number of HNE alkylation sites observed *in situ* in beef Mb in the present study than *in vitro* (Suman et al., 2006; Viana et al., 2020) could be possibly due to (1) HNE adducting to several proteins other than Mb in muscle food matrix or (2) the formation of other *in situ* PTM in Mb adversely influencing HNE alkylation.

Overall, greater numbers of PTM were identified in Mb from aged beef than in Mb from non-aged counterparts (Table 5). These aging-induced PTM, especially those occurring close to hydrophobic heme pocket, could disrupt Mb tertiary structure, heme affinity, and oxygen binding capacity, leading to the decreased color stability in aged beef observed in the present study. Furthermore, PTM at K45, K47, and K87 were unique to Mb from non-aged beef, whereas PTM at R31, T51, K96, K98, S121, R139, and K147 were unique to Mb from aged counterparts (Table 5), indicating that these Mb PTM sites could be used as biomarkers for fresh beef color stability.

Conclusions

Mb in fresh beef LL muscle underwent PTM (phosphorylation, methylation, carboxymethylation, acetylation, and HNE alkylation) during postmortem aging. Increased numbers of phosphorylation, methylation and carboxymethylation sites were detected in Mb from aged beef compared with Mb from non-aged counterparts, whereas acetylation sites decreased during aging. While the number of HNE alkylation sites remained the same during 14 d of aging, HNE adduction at K78 and K79 were unique to aged beef, indicating that HNE alkylation of lysine residues might play a critical role in Mb redox stability. The aging-induced PTM could compromise Mb redox stability by adding modifying groups to amino acids—especially those close to the hydrophobic heme pocket—and thus accelerate Mb oxidation and beef discoloration. These *in situ* Mb PTM could be utilized as novel biomarkers for fresh beef color stability.

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