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Yifei Wang
University of Kentucky, ywa323@uky.edu
Author ORCID Identifier:

https://orcid.org/0000-0002-2285-2357

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Yifei Wang, Student

Dr. Surendranath P. Suman, Major Professor

Dr. David L. Harmon, Director of Graduate Studies

MYOGLOBIN POST-TRANSLATIONAL MODIFICATIONS
AND FRESH BEEF COLOR STABILITY

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture, Food and Environment
at the University of Kentucky

By

Yifei Wang
Lexington, Kentucky

Director: Dr. Surendranath P. Suman, Professor of Animal and Food Sciences
Lexington, Kentucky
2020

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https://orcid.org/0000-0002-2285-2357

ABSTRACT OF THESIS

MYOGLOBIN POST-TRANSLATIONAL MODIFICATIONS
AND FRESH BEEF COLOR STABILITY

Surface color of fresh beef is the major trait influencing consumers' purchase decisions. Fresh beef color is determined by the myoglobin (Mb) redox stability. Post-translational modifications (PTMs) play a critical role in regulating Mb structure and functionality. This thesis focuses on the PTMs in Mb and their impact on fresh beef color stability.

In the first experiment, Mb PTMs in beef longissimus lumborum (LL) muscle during postmortem aging and their influence on fresh beef color stability were examined. Beef LL muscle from nine (n = 9) beef carcasses (24 h postmortem) were subjected to wet-aging for 0, 7, 14 and 21 d. On each aging day, steaks were fabricated. Instrumental color and biochemical attributes of aerobically packaged steaks were evaluated on d 0, 3, and 6 of storage. Mb PTMs were analyzed on 0, 7, 14 and 21 d of wet-aging using two-dimensional electrophoresis and tandem mass spectrometry. Aging decreased ($P < 0.05$) surface redness, color stability, and Mb concentration. Gel image analyses identified six Mb spots with similar molecular weight (17 kDa) but different isoelectric pH. Tandem mass spectrometry identified multiple PTMs (phosphorylation, methylation, carboxymethylation, acetylation, and HNE alkylation) in these isoforms. The amino acids susceptible to phosphorylation were serine, threonine, and tyrosine, whereas other PTMs are detected in lysine, arginine, and histidine residues. Overall, Mb PTMs increased with aging. The aging-induced PTMs, especially those occurring close to hydrophobic heme pocket, could disrupt Mb tertiary structure, influence heme affinity, and compromise oxygen binding capacity, leading to surface discoloration.

The second experiment was carried out to characterize the influence of vitamin E supplementation to beef cattle on the Mb PTMs in post-mortem LL muscle. Beef LL muscle samples (24 hours postmortem) were obtained from the carcasses of nine (n = 9) vitamin E-fed (VITE; 1000 IU vitamin E for 89 days) and nine (n = 9) control (CONT; diet without supplemental vitamin E) heifers. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate Mb from other sarcoplasmic proteins of beef LL muscle. Tandem mass spectrometry identified multiple PTMs (phosphorylation,

acetylation, alkylation, methylation, dimethylation, trimethylation, and carboxymethylation) in protein bands (17 kDa) representing Mb. Differential occurrence of acetylation, methylation, dimethylation and trimethylation were identified in Mb from CONT and VITE samples. Additionally, PTMs at lysine residues (K87, K96, K98 and K102) were unique to CONT, whereas PTMs at K118 were unique to VITE. Overall, supplementation of vitamin E decreased the numbers of post-translationally modified residues in myoglobin. These findings suggested that dietary supplementation of vitamin E in beef cattle might protect residues in Mb, especially those located spatially close to proximal histidine, from undergoing PTMs, and thereby improving Mb redox stability.

KEYWORDS: Post-translational modifications, Myoglobin, Beef color stability, Aging, Vitamin E

Yifei Wang
Student's signature

12/7/2020
Date

MYOGLOBIN POST-TRANSLATIONAL MODIFICATIONS
AND FRESH BEEF COLOR STABILITY

By

Yifei Wang

Dr. Surendranath P. Suman
Director of Thesis

Dr. David L. Harmon
Director of Graduate Studies

12/7/2020
Date

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CHAPTER 1

Review of Literature

1.1. Meat color

Beef industry is a critical contributor to the global agriculture economy (FAO, 2019). The U.S. as the world's largest beef producer, and the retail equivalent value of U.S. beef industry was $107 billion in 2018 (USDA ERS, 2019). The color of fresh beef is of prime importance to the beef industry, because it significantly influences the consumers' purchase decisions (Seideman et al., 1984; Mancini and Hunt, 2005; Suman et al., 2014). Discoloration of packaged fresh beef is caused a number of factors (Seideman et al., 1984). Consumers consider any deviation from cherry-red color as an indicator of spoilage. Although the microbial safety of the discolored fresh beef is not always compromised, they had to be sold at discounted prices or manufactured as lower-value products (Suman and Joseph, 2013; Suman et al., 2014). The economic loss caused by these practices is estimated to be more than $1 billion annually in U.S. beef industry in 2000 (Smith et al., 2000), and this figure is anticipated to have increased in the past 20 years. Therefore, exploring the mechanisms of meat color and approaches to improve beef color stability would critically aid U.S. agriculture economy.

Myoglobin (Mb) is the sarcoplasmic heme protein responsible for meat color (Livingston and Brown, 1981). Livestock Mb is composed of 153 amino acids, and has the molecular weight of approximately 17,000 Da. It consists of a heme prosthetic group and a globin moiety (Suman and Joseph, 2003). The globin peptide chain folds into eight helical segments wrapping the heme, enabling the latter's water solubility and protecting the heme iron from oxidation (Suman and Joseph, 2013). In live animals, Mb is responsible to bind oxygen and deliver it to mitochondria, allowing the physiological functions of

muscle tissue (Wittenberg and Wittenberg, 2003). In fresh meat, the conjugated double bonds in heme group within Mb enable it to absorb visible light and thus serves as a pigment (Suman and Joseph, 2013).

The fresh meat color perceived by consumers is primarily governed by the concentration and chemical form of Mb (Seideman et al., 1984). Variation in meat color stability may arise from numbers of factors. The endogenous factors including pH, muscle source, lipid oxidation, and mitochondrial activity influence meat color (Mancini and Hunt, 2005). Additionally, Mb concentration and meat color intensity are also related to factors such as animal species, sex, breed, and age (Lawrie, 1998).

The ultimate pH of the muscle, which is largely determined by immediate antemortem or postmortem conditions, is a critical endogenous factor contributing to the meat color stability. Short-term excitement of animals immediately before slaughter can result in a low ultimate pH, causing muscle fibrils to be more “open” and scatter light, and expose Mb to be more readily oxidized to metmyoglobin (MetMb; Walters, 1975). On the contrary, long-term stress and depletion of nutrients can give rise to a high ultimate pH, which prevents the oxygen to combine with Mb forming cherry-red oxymyoglobin (OxyMb; Urbain, 1952). The high pH also causes the muscle fibers to be swollen and tightly packed together, forming a barrier to the diffusion of oxygen and the absorption of light, and thus leads to a dark appearance (Walters, 1975).

Meat color stability is muscle-specific, and this is due to the differences in anatomical locations, physical functions, and metabolism of skeletal muscles (Hunt and Hedrick, 1977). The muscles used in locomotion require more oxygen for energy

production and contain a higher concentration of Mb (Seideman et al., 1984). The beef muscles demonstrating greater rate of oxygen consumption rate (O'Keeffe and Hood, 1982) and lower MetMb reduction rate (Ledward, 1985) are color-labile, whereas muscles exhibited greater reducing activities are color-stable (Reddy and Carpenter, 1991). Joseph et al. (2012) reported that antioxidant and chaperone proteins, which protect Mb from oxidation, are over-abundant in color-stable longissimus lumborum (LL) than color-labile psoas major (PM). Additionally, Nair et al. (2018a) documented more abundant glycolytic enzymes in LL than PM, indicating muscles with greater color stability have greater capacity to regenerate NADH for subsequent MetMb reduction and therefore stabilizing beef color.

Lipid oxidation is not only responsible for rancid odor, but also Mb oxidation and meat discoloration. Lipid oxidation is the result of the interactions between unsaturated fatty acid and oxygen, generating primary and secondary lipid oxidation products, such as aldehyde and ketones. The reactive secondary products of lipid oxidation can covalently adduct with OxyMb and accelerate its oxidation to MetMb (Faustman et al., 2010). Of specific, 4-hydroxynoenal (HNE) is a well-documented secondary product of n-6 polyunsaturated fatty acid oxidation in meat (Sakai et al., 1995, 1998). HNE is a highly reactive electrophilic molecule reacting with the histidine and lysine residues in proteins by 1,4-adduction to form Michael adducts (Sakai et al., 1995). Previous studies (Alderton et al., 2003; Faustman et al., 1999; Suman et al., 2006, 2007; Naveena et al., 2010) documented that HNE adduction at histidine residues of Mb via Michael addition compromised Mb redox stability by altering its tertiary structure and thus accelerating

Mb oxidation. Therefore, the strategies to delay the lipid oxidation can decrease Mb oxidation and enhance meat color stability (Faustman et al., 2010).

Mitochondria can impact meat color stability by influencing oxygen consumption and Mb redox state. In postmortem muscle, mitochondria continue to metabolize oxygen (Tang et al., 2005), and the competition between Mb and mitochondria for diffused oxygen is critical for the development of bright cherry-red surface color of meat. An increase in mitochondria activity can decrease oxygen partial pressure via respiration, which can limit available oxygen to bind with Mb, resulting in the conversion of OxyMb to Deoxymyoglobin (DeoxyMb; Tang et al., 2005) and darker meat color. On the other hand, limiting mitochondrial respiration by rotenone (Cornforth and Egbert, 1985) or low temperature (Bendall and Taylor, 1972) favors the formation of bright-red color. In addition, mitochondrial activity can affect MetMb reducing activity (MRA) through four different pathways: electron-transport mediated MRA (Tang et al., 2005; Ramanathan and Mancini, 2010; Ramanathan et al., 2010), under anaerobic conditions (Watts et al., 1996; Lanier et al., 1978), NADH-dependent reductase activity (Giddings, 1977; Arihara et al., 1995), and non-enzymatic MRA (Elroy et al., 2015). To conclude, mitochondria influence both meat color development (via oxygen consumption) and color stability (via MRA).

Several exogenous factors, for example the presence of ligands and antioxidants, also play important roles in fresh meat color. Strategies to preserve meat color were developed by employing antioxidants or applying various packaging methods to manipulate the ligands available for binding with Mb. Ligands, such as oxygen and CO,

can bind the sixth position of heme iron of Mb, forming OxyMb or COMb, respectively, and provide the desirable cherry-red color. In aerobically packaged meat, oxygen reacts with Mb in the fresh-cut meat, forming OxyMb and a cherry-red color, which is known as blooming in the industry. However, in less than a week, conventionally bloomed meat will turn to brown color due to the oxidation of OxyMb to MetMb (McMillin, 2008). Therefore, novel meat packaging strategies are desirable in order to increase the color shelf-life of fresh meat. Modified atmosphere packaging (MAP) systems containing high oxygen (80% oxygen and 20% carbon dioxide) and low CO (0.4% CO) have been developed to maintain color shelf life of meat up to 14 days (Church, 1994; Eilert, 2005; Fu et al., 2017). Previous studies (Jakobsen and Bertelsen 2000, Jayasingh et al. 2002) reported that the elevated level of oxygen in high-oxygen MAP could saturate Mb on meat surface, and thereby slow the MetMb formation. Nevertheless, high-oxygen MAP was reported to accelerate lipid oxidation, off-flavor development (Jakobsen and Bertelsen, 2000, Jayasingh et al. 2002), premature browning during cooking (Torngren, 2003; Seyfert et al., 2004a,b; John et al., 2004, 2005; Suman et al., 2005,2009,2010b,2011; Mancini et al., 2010,2011), and bone darkening of bone-in cuts (Mancini et al., 2005). To extend meat color stability and avoid drawbacks of aerobic packaging, an anaerobic MAP technology with 0.4% CO (CO-MAP) was approved in the US (FDA, 2004). The use of CO in MAP enables the formation of COMb and provides cherry-red color, which is indistinguishable from OxyMb by human eyes (Cornforth and Hunt 2008). CO-MAP packaged ground beef and steaks have the shelf life up to 28 days and 35 days, respectively (Hunt et al., 2004). Additionally, no bone darkening or premature browning were observed in CO-MAP meat (Torngren, 2003;

Seyfert et al., 2004a,b; John et al., 2004, 2005; Mancini, et al., 2005,2009,2010; Suman et al.,2009,2010b,2011). However, since CO-MAP packaging can dramatically improve color stability, there is a possibility that the product could be spoiled even though it still appears fresh (Jayasingh et al., 2001; Cornforth and Hunt, 2008).

Antioxidants can minimize oxidation-induced discoloration of meat (Decker et al., 2000), and could be applied both pre-harvest and post-harvest (Faustman et al., 2010). Feeding meat-producing animal with vitamin E can delay the oxidation of lipids as well as Mb and can thus increase meat color stability (Faustman et al., 1989a,b; Kerry et al., 2000; Arnold et al., 1993). Kim (2018) observed over-abundant antioxidant proteins and glycolytic enzymes in non- vitamin E-supplemented beef compared with vitamin E-supplemented beef, and documented that the strong antioxidant protection offered by vitamin E might lead to the less expression of antioxidant proteins and glycolytic enzymes that generate antioxidant metabolites in vitamin E-supplemented beef. Additionally, dietary supplementation of plant extracts rich in antioxidant compounds also contribute to increase the color shelf life of meat. Employing tea catechins, rosemary extract (O'Grady et al., 2006), and plant extract containing polyphenols (Gobert et al., 2010) in beef cattle diet can improve the color stability of beef. Moreover, the discoloration in lamb could be slowed down by dietary supplementation with turmeric (Karami et al., 2011), and rosemary diterpenes (Ortuno et al., 2015). In addition, synthetic and natural antioxidant can be also applied in meat system by injecting to whole-muscle cuts or incorporating into comminuted meats. Previous studies suggested that potassium lactate (Mancini et al., 2005; Knock et al., 2006; Mancini et al., 2009,2010; Suman et al.,

2009,2010c; Ramanathan et al., 2011), calcium lactate (Lawrence et al., 2004), and sodium acetate (Livingston et al., 2004), pyruvate (Ramanathan et al., 2011) and succinate (Mancini et al., 2011; Ramanathan et al., 2011) can benefit beef color stability. A variety of natural antioxidants, including phosvitin (Jung et al., 2012), pomegranate extract (Qin et al., 2013), fenugreek (Hettiarachchy et al., 1996), grape-seed extract (Kulkarni et al., 2011), chitosan (Georgantelis et al., 2007; Suman et al., 2010a,2011), rosemary (Sanchez-Escalante et al., 2001), and olive-leaf extract (Hayes et al., 2010) can be incorporated in meat system to improve meat color. Furthermore, the effect of antioxidants can be packaging-dependent (Suman et al., 2009,2010a,b,c,2011; Mancini et al., 2010,2011). Injection enhancement of beef with succinate, pyruvate, and lactate is more evident in high-oxygen packaging than aerobic packaging and vacuum packaging (Ramanathan et al., 2011). Chitosan (Suman et al., 2010a) and lactate (Mancini et al., 2009; Suman et al., 2010c) enhanced beef demonstrated greater redness in CO-MAP and aerobic packaging than high-oxygen packaging and vacuum packaging.

1.2. Myoglobin chemistry

Mammalian Mb has an iron-based heme moiety surrounded by a globin peptide chain of 153 amino acids. The primary structure of Mb determines its tertiary structure, and in turn governs the volume of the heme cavity, net charge, oxidation-reduction properties, the interactions with other biomolecules, and ultimately influences meat color (Suman and Joseph, 2013). Although Mb amino acid sequence varies with species (Brown and Mebine, 1969; Enoki et al., 2008; Suman and Joseph, 2013), the distal

(position 64) and proximal (position 93) histidines are conserved in livestock (mammalian) as well as poultry (avian) Mbs. The iron atom can accept six electrons in its outer orbit, forming six coordinate bonds. Four of these bonds are with pyrrole nitrogen atoms, and the fifth is with proximal histidine which connects heme to the globin chain. The distal histidine (position 64) is in the vicinity of the heme, but does not bond with heme. The sixth coordination site is available to bind with different ligands, which influences the redox state of the Mb (Han et al., 1970; Han et al., 1972; Mancini and Hunt, 2005; Suman and Joseph, 2013; Faustman and Suman, 2017). Distal histidine received considerable research attention due to its spatial interaction with hydrophobic heme pocket, which limits the size of the ligands and protect heme by preventing its interaction with large molecules (Cornforth and Jayasingh, 2004). Furthermore, the number of histidines were observed to be directly proportional to the susceptibility of mammalian Mb to HNE alkylation (Yin et al., 2011).

In fresh meat, Mb exists mainly in three redox forms, namely DeoxyMb, OxyMb, and MetMb. The equilibrium and relative proportions of these three forms of Mb determine the color of fresh meat during the postmortem storage and retail display (Watts et al., 1996; Suman and Joseph, 2013). DeoxyMb exists when no ligands are bound with the sixth coordination site of heme, and the iron is in ferrous state (Fe^{2+}). Purplish-red DeoxyMb is associated with color of fresh-cut and vacuum-packaged meat. When meat is exposed to air, an oxygen molecule binds to the sixth coordination site forming OxyMb, which provides an attractive cherry-red color. The partial pressure of oxygen in situ is critical to the formation of OxyMb. Brown MetMb results from the oxidation of the

ferrous (Fe^{2+}) iron of heme in DeoxyMb or OxyMb to ferric state (Fe^{3+}), and is associated with meat discoloration. MetMb has a water molecule bound at the sixth coordinate of the ferric heme and it is not able to carry oxygen.

During the storage of meat, accumulation of MetMb at the meat surface due to the oxidation is the major factor causing discoloration (Ledward et al., 1971). The redox reactions of MetMb formation can be reversible depending on the MetMb reducing activity (MRA), cofactors, and oxygen availability. MetMb reducing enzymes or reducing equivalents can reduce MetMb to DeoxyMb, which can then be oxygenated to bright red OxyMb. Therefore, MRA is a critical component in meat color stability. NADH is a reducing equivalent for both enzymatic and non-enzymatic MetMb reduction (Renerre and Labas, 1987; Echevarne et al., 1990). The process of producing NADH continually depleted postmortem, however, NADH can be regenerated by dehydrogenase enzymes from cytoplasmic or mitochondria in postmortem muscle (Stewart et al., 1965; Watts et al., 1966; Giddings, 1977). Previous studies indicated that enhancing beef with succinate (Ramanathan et al., 2011), lactate (Kim et al., 2006) or glycolytic and tricarboxylic acid (TCA) cycle substrates (Saleh and Watts, 1968) increased MRA due to the regeneration of reducing equivalents such as NADH. On the other hand, Jerez et al. (2003) suggested that limiting NADH content inhibited postmortem glycolysis, leading to decreased meat color stability.

Mb and mitochondria are dynamic components in live skeletal muscles, which facilitate oxygen delivery and energy production, respectively. In postmortem muscles, oxygen is used for Mb oxygenation, mitochondria oxygen consumption, lipid and protein

oxidation, and microbial growth. Noticeably, greater amount of oxygen utilized for mitochondria consumption, the less will be available for binding to Mb. Therefore, mitochondrial oxygen consumption and oxygen-consuming enzymes have a significant impact in Mb redox state (Tang et al., 2005; Ramanathan et al., 2018; Ramanathan et al., 2019).

1.3. Post-translational modification (PTM) of protein

Post-translational modification (PTM) refers to the covalent processing events protein may undergo after translation by addition or removal of modifying groups to amino acids (Lodish, 1981; Han and Martinage, 1992; Mann and Jensen, 2003). Instead of merely being "decorations" of protein, PTM determines tertiary and quaternary structures of proteins and modulates the functional properties of proteins (Mann and Jensen, 2003; Seo and Lee, 2004; Nicolis et al., 2008). PTM plays a fundamental role in regulating biological processes as it determines protein's functionality, localization, turnover and interaction with other proteins (Seo and Lee, 2004; Muller, 2017). PTMs influence a number of critical signaling events, and thus identification of the diverse realm of protein PTMs is important for generating deeper insight in cell regulation and protein biological function (Witz et al., 2007; Seo and Lee, 2004; Krueger and Srivastava, 2006). In addition, post-translationally modified proteins are associated with numerous diseases (Jensen, 2004; Conibear et al., 2019). In this perspective, investigation of PTMs is significant for biomarkers development and disease pathogenesis in medical sciences.

PTMs can cause the isoelectric point shift of the protein by modifying the titratable groups, which enable the 2-dimensional electrophoresis to resolve many PTM-induced isoforms (Halligan et al., 2004). Advances in mass spectrometry, a fundamental tool for detecting covalent modifications, make it possible to identify PTMs more rapidly and specifically (Jensen, 2004; Schwammle and Vaudel, 2017; Thygesen et al., 2018). Phosphorylation, acetylation, methylation, carboxymethylation and HNE alkylation are the common PTMs, and they are briefly introduced below.

1.3.1. Phosphorylation

Protein phosphorylation is the most common PTM that is involved in modulating molecular interactions in cellular pathways (Graves and Krebs, 1999; Pawson, 2002). Phosphorylation takes place on serine (Ser), threonine (Thr) and tyrosine (Tyr) residues in eukaryotic cells (Ham, 2011). Phosphorylation causes an acidic shift in the protein's isoelectric pH and a mass increment of 80 Da by replacing the neutral hydroxyl groups on Ser, Thr and Tyr with negatively charged phosphoryl group (McLachlin and Chait, 2001; Halligan et al., 2004; Jensen, 2004). The phosphate group covalently attached to the protein can form either intra- or inter-molecular hydrogen bonds or salt bridges, and thus modify the protein from hydrophobic nonpolar to hydrophilic polar, promoting protein conformational changes and protein-protein interactions (Hunter, 2012; Ardito et al., 2017).

Activation of protein kinases leads to the addition of phosphate groups to the amino acids. Phosphatases, which works on the contrary of kinase, are responsible for the

removal of the phosphate groups from the target proteins by hydrolyzing phosphoric acid monoesters (Ardito et al., 2017). Protein kinases and phosphatases work together to adjust phosphorylation states and control cellular processes (Ham, 2011). Protein phosphorylation plays a critical role in glycolysis metabolism (Graves and Krebs, 1999), which is an enzymatic conversion of glucose to pyruvate. Most enzymes involved in glycolysis are identified as phosphoproteins, including rate limiting enzymes glycogen phosphorylase (GP), enolase, phosphofructokinase (PFK) and pyruvate kinase (PK; Huang et al., 2011).

Previous studies documented that meat tenderness is influenced by phosphorylation through its regulatory role in myofibrillar proteins (Chen et al., 2016; Li et al., 2017b; Liu et al., 2018), calpain system (Du et al., 2017, 2018) as well as glycolytic enzymes (Huang et al., 2011, 2012; Anderson et al., 2014). Moreover, phosphorylation was identified to impact meat color through influencing redox stability of myoglobin (Li et al., 2017; 2018). Additionally, Underwood et al. (2008) reported that protein phosphorylation is closely associated with intramuscular fat content through regulation of adenosine monophosphate-activated protein kinase (AMPK).

1.3.2. Acetylation

The acetylation usually happens on N-terminal when the growing polypeptide chains are still attached to the ribosomes (Kwan et al., 2016). It plays a role similar to phosphorylation. By transferring an acetyl group, acetylation increases the size of target amino acid’s side chain and neutralizes its positive charge, leading to a 42-Da mass

increase and isoelectric point shift (Parker et al., 2010; Kumar et al., 2004). Acetylation can regulate proteins' stability and their interaction with other proteins by converting positively charged ammonia 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). Furthermore, acetylation is critical in modulating the interactions between lysine residues of histones with negatively charged DNA, and it has been found to be involved in the regulation of intracellular compartmentalization, cell signaling, chromatin structure, cytokine signaling and apoptosis. (Sterner and Berger, 2000; Annunziato and Hansen, 2000; Zhao et al., 2010; Seto and Yoshida, 2014; Kwan et al., 2016; Ali et al., 2018; Christensen et al., 2019). Additionally, most enzymes involved in intermediate metabolism, including glycolysis, glycogen metabolism, gluconeogenesis, the TCA cycle, fatty acid oxidation, and the urea cycle have been identified to be acetylated (Zhao et al., 2010; Guan and Xiong, 2011; Menzies and Auwerx, 2013).

1.3.3. Methylation

Methylation refers to the addition of variable number of methyl groups to a protein, usually at lysine and arginine residues (Ong et al., 2004; Bermang et al., 2013). Methylation reactions do not contribute to any negative charge on the protein, but neutralize the positive charge of lysine and arginine, and thus reduce their hydrophilicity and alter the protein conformation (Kumar et al., 2004; Krueger and Srivastava, 2006; Uhlmann et al., 2012). The increased steric hindrance and decreased hydrogen bonding of methylated amino acids could impact the interactions between proteins and other

cellular substrates (Ong et al., 2004). Methylation has been associated with cell proliferation, which is a process commonly involved in cell growth and division, resulting in an increase in the number of cells (Kwan et al., 2016). Methylation has been widely studied in histone, where it acts as an epigenetic regulator of chromatin structure and gene expression (Greer and Shi, 2012; Lanouette et al., 2014; Clarke, 2018; Luo, 2018). In addition, methylation also plays a critical role in non-histone proteins, and thus is heavily involved in DNA repair, RNA processing and cellular signaling (Ong et al., 2004; Uhlmann et al., 2012).

1.3.4. Carboxymethylation

Carboxymethylation usually occurs at lysine, glutamate, aspartic acid, and cysteine residues. Carboxymethylation can cause a mass increment of 58 Da to the target amino acids by introducing a negatively charged carboxylic acid (Kung, 1976; Fang et al., 2010; Conibear et al., 2019). Carboxymethylation on lysine is a non-enzymatic PTM and results in a glycation end product (Reddy et al., 1995; Conibear et al., 2019). The addition of carboxylic acid leads to a net charge change and alter the interactions of the lysine side chains. Previous studies reported that carboxymethylation is a potential modulator in chemotaxis, neurosecretory regulation and diabetes metabolism (Diliberto et al., 1976; Curtiss and Witztum, 1985; Hackett and Campochiaro, 1988).

1.3.5. HNE alkylation

Reactive lipid oxidation products adduct at nucleophilic residues in proteins, and this process is called protein alkylation. The 4-hydroxyl-2-nonenal (HNE), one of the most abundant lipid-derived compounds, is generated through the β-cleavage of hydroperoxides from n-6 polyunsaturated fatty acids (Esterbauer et al., 1991), and has been studied as a model aldehyde to react with peptides and proteins. The conjugated double bond within the HNE molecule makes it a highly reactive electrophile, allowing it to covalently bind to the specific nucleophilic site of the protein to yield a hemiacetal structure (Esterbauer et al., 1991; Carini et al., 2004). HNE is found to adduct histidine, lysine, and cysteine residues via Michael addition, resulting in mass shift of 156 Da (Uchida et al., 1992; Faustman et al., 1999; Alderton et al., 2003; Fenaille and Guy, 2003; Suman et al., 2006, 2007). This modification can happen during food processing and storage, deteriorating the color, flavor, functional/physical properties and nutritional values of foods (Zamora and Hidalgo, 2001). Furthermore, in biological systems, protein alkylation by HNE contributes to oxidative stress and cellular toxicity (Codreanu et al., 2014; Yang et al., 2015).

1.4. Protein PTMs and meat quality

After slaughter, the metabolism in skeletal muscle cells changes in order to maintain homeostasis (Morgan et al., 1993). Biochemical changes in response to stoppage of respiration and blood circulation have a dramatic impact on the meat quality, such as tenderness, water-holding capacity, and color. Muscle proteins are the biomolecules

contributing to the meat quality development and could undergo a multitude of post-translational modifications (PTMs), such as phosphorylation (Hojlund et al., 2009; Li et al., 2020), acetylation (Jiang et al., 2019; Li et al., 2020), oxidation (Feng et al., 2008), nitration (Kanski et al., 2005), and glycosylation (Martin-Rendon and Blake, 2003). These PTMs play critical roles in regulation of postmortem muscle metabolism and meat quality development through modulating protein structure and functions (Li et al., 2020).

1.4.1. Muscle to meat conversion

A series of important physical-biochemical changes take place at the beginning of postmortem when muscle is converted to meat. These changes include pH decline, programmed cell death, rigor mortis, and proteolysis. PTMs are involved in the process of meat quality development, and could be affected by conditions prior and after animal slaughter. Physiological changes in response to pre-slaughter stress, such as handling and transport, might alter protein modification patterns and thus influence meat quality traits, especially the extent and rate of pH decline (Ferguson and Warner, 2008). After slaughter, muscle remains functional and metabolically active for several days (Paredi et al., 2012). A shift in the energy metabolism occur shortly after slaughter (Jia et al., 2006), and this dramatic metabolic change can result in the activation of PTMs to regulate the activity of proteins in order to cope with the shortage of ATP and the development of rigor mortis (Huang and Lametsch, 2013).

Conversion of muscle to meat has been schematized into seven steps by D’Alessandro and Zolla (2013), including blood supply loss, glycolysis ensues, apoptosis,

onset of rigor, calcium- dependent and independent proteases, oxidative stress, and proteolysis. Among these steps, glycolysis and apoptosis are critically regulated by various PTMs, including phosphorylation (Shen et al., 2006; Huang et al., 2011; Paredi et al., 2012), acetylation (Li et al., 2016; Jiang et al., 2019) and S-nitrosylation (Liu et al., 2018; Zhang et al., 2019).

1.4.1.1. PTM and glycolysis in postmortem muscle

Glycolysis is the core metabolic pathway converting glycogen into lactate and H+, producing ATP under anaerobic conditions. After exsanguination, muscle cells switch from aerobic metabolism to glycolytic one to generate ATP in order to maintain homeostasis and cell function. While glycolysis progresses in postmortem muscles, concomitant accumulation of lactate and pH decline critically influence meat tenderness (Silva et al., 1999) and water-holding capacity (Huff-Lonergan and Lonergan, 2005).

Glycogen phosphorylase (GP) catalyzes the breakdown of glycogen to glucose-1 phosphate. Previous investigations (Johnson, 1992; Schwagele et al., 1996; Sprang et al., 1988) suggested that phosphorylation at serine at position 14 in GP resulted in structural changes of the protein, which represents the first step in the transformation of the enzyme to its active form. The amino- and carboxyl-terminal domains of GP rotated apart due to phosphorylation, enabling the increased access of substrates to the catalytic site (Sprang et al., 1991). Furthermore, a positive correlation was observed between GP activity and its phosphorylation level in postmortem sheep muscle, and the increased GP activity due to phosphorylation could accelerate the glycogenolysis, leading to a low

glycogen content and decline in pH (Chen et al., 2019a). However, S-nitrosylation of GP in pork could inhibit its activity, resulting in a reduced rate of glycolysis (Zhang et al., 2019). S-nitrosylation might modify the reactive cysteine residues in GP with NO moiety, and thus modulating GP's activity (Hess et al., 2005).

Pyruvate kinase (PK) is a critical rate limiting enzyme in glycolysis, catalyzing the irreversible conversion of phosphoenolpyruvate to pyruvate. Huang et al. (2011) observed that the the phosphorylation of PK contributed to the fast glycolysis and rapid pH decline in pork. On the contrary, Liu et al. (2018) and Chen et al. (2018, 2019b) documented a higher phosphorylation level of PK in slow pH decline group of postmortem ovine muscles compared to rapid pH decline group. According to Silva et al. (2019), the remarkable increase in PK phosphorylation during aging of beef longissimus muscle could be associated with activating glycolytic pathway to produce ATP. Additionally, pre-slaughter stress was found to increase glycolysis through the acetylation of PK in postmortem mice longissimus muscle (Li et al., 2016). Nonetheless, deacetylation of lysine residues in PK was detected in stressed pork longissimus muscle compared to the control ones, suggesting that the differentially acetylated residues are possibly the consequence of pre-slaughter stress regulated postmortem glycolysis (Zhou et al., 2019).

Phosphofructokinase (PFK) catalyzes the first step in the glycolytic pathway, the conversion of fructose-6-phosphate and ATP to fructose-1,6-bisphosphate and ADP. PFK with higher level of phosphorylation demonstrated higher activity in ovine muscle (Chen et al., 2019a). Additionally, previous studies (Marsin et al., 2000; Hardie et al., 2004; Sambandam and Lopschuk, 2003) showed that PFK is activated after phosphorylation by

AMP-activated protein kinase (AMPK). Phosphorylation of AMPK was found to regulate glycolysis in pork longissimus muscle through its activation of PFK (Shen et al., 2006).

Phosphoglucomutase-1 (PGM1) is involved in both glycolysis and glycogenesis, reversibly catalyzing the conversion of glucose 1-phosphate to glucose 6-phosphate. Rodrigues et al. (2017) suggested that the higher level of PGM-1 phosphorylation contributed to faster glycolysis in beef muscles. The phosphorylated PGM-1 was found to prevent sarcomere shortening during conversion of muscle to meat (Silva et al., 2019). Additionally, a higher level of phosphorylated PGM1 was identified in tender beef longissimus muscles compared to their tough counterparts (Anderson et al., 2014). Nevertheless, Zhou et al. (2019) indicated that pre-slaughter stress may up-regulate glycolysis in porcine muscle through the downregulation of lysine acetylation of PGM1.

Triosephosphate isomerase (TPI) is responsible for catalyzing the reversible conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate. The phosphorylation of TPI could result in the loss of catalytic activity in HeLa cells (Lee et al., 2010). In addition, pork muscles with slow pH decline demonstrated higher levels of TPI phosphorylation compared with the fast pH decline muscles (Huang et al., 2011).

1.4.1.2. PTM and apoptosis in postmortem muscle

Mitochondrial energy production drops in the postmortem muscle due to the lack of oxygen, leading to the accumulation of the reactive oxygen species (ROS; Murphy, 2009; Kim et al., 2007). The proteins, DNA, mitochondria, and cells that are prone to the oxidative stress would in turn engage in a programmed cellular procedure, also named

apoptosis (Kerr et al., 1972). Apoptosis is the preferential way the cells use to suicide themselves, avoiding the risk to damage surrounding cells (Longo et al., 2015).

Heat shock protein (HSP) is responsible for cell protection and is involved in stress resistance and apoptotic signaling pathways (Ouali et al., 2006; Takayama et al., 2003). PTMs could induce structural changes in HSPs, including the reversible changes in oligomeric substructure or reversible cycles of dissociation and association, and in turn modulate HSPs' interactions with target proteins and their chaperone-like activity (Mymrikov et al., 2011). Phosphorylation of HSP27 was identified in human (Loktionova and Kabakov, 1998; Liu et al., 2018), beef (Mato et al., 2019) and pork (Huang et al., 2011) muscles. Loktionova and Kabakov (1998) reported that phosphorylation of myocardial HSP27 is a key regulator in the protection of actin from fragmentation during injury. Charette et al. (2000) and Huot et al. (1996) observed that phosphorylated HSP27 can protect cells from exposing to stress factors and can inhibit stress-induced apoptosis. These observations could be due to the fact that phosphorylation at serine residues in HSP72 presumably lead to its dissociation, which enables HSP27 to become a lower molecular weight oligomer, which in turn critically promotes its activity in protecting cells from stress induced-apoptosis (Theriault et al., 2004; Yuan and Rozengurt, 2008; Mymrikov et al., 2011). Moreover, acetylation of HSP70 was identified in pork (Jiang et al., 2019; Zhou et al., 2019), indicating HSP acetylation might be involved in the regulation of postmortem cell stress response and apoptosis, and thus may affect meat quality. Furthermore, Guillemin et al. (2011) documented a positive correlation between HSPs and meat tenderness. In agreement, higher level of phosphorylated HSPB6 was observed

in tender beef compared to the tough beef (Mato et al., 2019). This could be attributed to fact that the phosphorylated HSPB6 interacts with universal adapter protein 14-3-3, which is a phospho-binding protein regulating essentially every major cellular function (Pennington et al., 2018). Phosphorylated HSPB6 displaces phosphorylated cofilin complex with protein 14-3-3, inducing the fragmentation of actin filaments (Mymrikov et al., 2011) leading to tenderization.

1.4.2. Meat tenderness

Meat tenderness is one of the most important quality attributes critical to consumers' acceptability and their re-purchase decisions (Huffman et al., 1996; Miller et al., 2001; Hughes et al., 2014). The architecture and the integrity of the skeletal muscle cell as well as the events that modify muscle proteins determine the meat tenderness (Huff Lonergan et al., 2010). During postmortem aging, proteolysis of myofibrillar and other structural proteins have been considered as one of the key mechanisms for tenderization (Huff Lonergan et al., 2010; Wu et al., 2014). PTMs can alter the protein properties by changing their structural conformation and regulating muscle protein functions (Hu et al., 2006; Li et al., 2020). PTMs, including phosphorylation, acetylation, and S-nitrosylation were found to be involved in the tenderization through regulating the activities of structural proteins and endogenous enzymes.

1.4.2.1. PTMs and myofibrillar proteins

Degradation of myofibrillar proteins is the fundamental phenomenon during meat tenderization (Te Pas et al., 2009; Choi and Kim, 2009; Habek et al., 2010). The extent of weakening of myofibrillar structure directly affects meat tenderness (Hollung et al., 2014). Previous investigations suggested that the PTMs in myofibrillar proteins, such as myosin, titin, troponin, nebulin and desmin, influence meat tenderness of beef, pork and lamb (Li et al., 2020).

Differentially phosphorylated myofibrillar proteins were identified in tough and tender beef. Muroya et al. (2007) discovered that myosin regulatory light chain (MyLC2) of beef longissimus muscle was double phosphorylated on the N-terminal region during the rigor formation, indicating the involvement of MyLC2 phosphorylation in rigor mortis. Furthermore, Franco et al. (2015) studied the proteome changes in beef longissimus thoracis muscle in response to pre-slaughter stress, and found that MyLC2 was highly phosphorylated in Dark Firm Dry (DFD) meat. Similarly, Mato et al. (2019) documented that DFD beef longissimus thoracis muscle demonstrated higher phosphorylation in myofibrillar proteins, including MyLC2, actin, troponin-T compared with normal beef, indicating that these phosphorylated myofibrillar proteins could be candidate biomarkers of DFD meat. Additionally, higher phosphorylation levels in titin and troponin T-fast skeletal muscle type were detected in tough beef longissimus muscle (from Maremmana breed) compared with tender counterparts, suggesting that phosphorylation of structural proteins is inversely related to meat tenderness (D'Alessandro et al., 2012a). D'Alessandro et al. (2012b) further studied the phosphorylation level of structural

proteins in beef longissimus muscle from Chianina breed, and found that tropomyosin beta was highly phosphorylated in tender beef, while alpha-actin was highly phosphorylated in tough beef.

Bos indicus beef is generally tougher than Bos taurus beef. Rodrigues et al. (2017) compared the phosphoproteome profile of beef from Angus (Bos taurus) and Nellore (Bos indicus) cattle, and reported a higher phosphorylation level of MyLC2 and alpha-actin in Nellore, whereas troponin-T was highly phosphorylated in Angus. Silva et al. (2019) investigated the differential phosphoproteome between Nellore bulls and steers after 14 days postmortem aging. Phosphorylated fast skeletal muscle troponin T was overabundant in beef from steers, which demonstrated higher tenderness compared with bulls, while phosphorylated myosin light chain was more abundant in 14-d aged beef compared with non-aged counterparts (Silva et al., 2019). Additionally, the abundance of phosphorylated alpha-actin and MyLC2 was positively correlated with sarcomere shortening (Silva et al., 2019).

Lamb tenderness was also found to be regulated by phosphorylation of myofibrillar proteins. Chen et al. (2016) analyzed the phosphorylation patterns of myofibrillar proteins in tender and tough lamb longissimus muscles, and found higher phosphorylation level of beta-actin and MyLC2 in tough group than in tender group. The phosphorylated myofibrillar proteins could influence rigor mortis through contractile machinery and ultimately affect lamb tenderness (Chen et al., 2016). Similarly, Li et al. (2017a) examined the phosphoproteome of sheep longissimus thoracis et lumborum muscles with different tenderness and identified higher level of phosphorylated

tropomyosin alpha-1 chain in the tough group, whereas alpha-actinin-3 and myosin binding protein H were highly phosphorylated in the tender group. Moreover, Li et al. (2017b) incubated the myofibrillar proteins from sheep longissimus muscle, including MyHCs, actin, desmin and troponin-T, with protein kinase and phosphatase to control phosphorylation level in-vitro, followed by μ-calpain hydrolysis, and observed that phosphorylation prevents the myofibrillar proteins to be degraded by μ-calpain. In support, Li et al. (2018) modulated phosphorylation level of myofibrillar protein by adding either protein kinase inhibitor or phosphatase inhibitor to sheep longissimus thoracis lumborum muscles, and observed that the dephosphorylation enhanced the degradation of myofibrillar proteins.

Phosphorylation and acetylation have been reported to be involved in pork rigor mortis and tenderness development. Huang et al. (2012) investigated the phosphorylation of the myofibrillar proteins in the three groups of pigs with different pH, and found that the highest phosphorylation level in the pork from fast pH decline rate group. Additionally, myosin-binding protein C, tropomyosin, and MyLC2 were found to be highly phosphorylated, and their phosphorylation levels were affected by the pH decline rate and postmortem time, suggesting that the phosphorylation of myofibrillar protein might be related to the rigor mortis as well as meat quality development (Huang et al., 2012). Moreover, Zhou et al. (2019) studied the acetylation of myofibrillar proteins from pork longissimus muscle in response to ante-mortem stress. The results indicated that pork loins from control pigs were more tender than those from animals subjected to transport stress. Furthermore, multiple myofibrillar proteins (nebulin, myosin binding

protein C, myosin light chain 3, tropomyosin 2, myosin heavy chain 7B) were differentially acetylated in pork from control animals and those exposed to ante-mortem stress, indicating the potential role of acetylation in stress response and tenderness (Zhou et al., 2019).

1.4.2.2. PTMs and calpain system

During postmortem aging, proteolysis of muscle proteins by endogenous enzymes governs meat tenderization (Dransfield, 1993; Koohmaraie and Geesink, 2006; Taylor et al., 1995). There are several proteolytic systems present in the muscle, and the calpain system is considered to be the major protease contributing to tenderization during aging (Huff-Lonergan et al., 1996; Koohmaraie, 1990; Taylor et al., 1995; Goll et al., 1992). The calpain system consists of several isoforms of calpain, and an endogenous calpain inhibitor, named calpastatin (Sentandreu et al., 2002). The µ-calpain and m-calpain are the most characterized isoforms among all members of calpain.

Previous investigations indicated that phosphorylation and S-nitrosylation influenced meat tenderness by regulating calpain and calpastatin function (Li et al., 2020). The µ-calpain is phosphorylated by protein kinase A (PKA) and protein kinase C (Storr et al., 2011). The phosphorylation of µ-calpain could regulate its proteolysis activity as well as its location within cells (Vazuez et al., 2008; Xu and Deng, 2006a, b). Du et al. (2017) investigated the effects of in-vitro phosphorylation on the activity of µ-calpain and its sensitivity to temperature and Ca^{2+} concentration. The alkaline phosphatase (AP) and phosphatase inhibitor (PI) were utilized to modulate µ-calpain's phosphorylation level,

and the results showed that a higher degradation rate of μ-calpain in the AP group than PI group, indicating phosphorylation plays a negative role in μ-calpain degradation and activation (Du et al., 2017). Du et al. (2018) further observed both phosphorylation by PKA and dephosphorylation by AP could increase the activity of μ-calpain. Two serine residues (S255, S256) located on domains II and III of μ-calpain were phosphorylated by PKA. Additionally, phosphorylation by PKA resulted in increased α-helix content of domains II and III. These observations suggested the phosphorylation at S255 and S256 could regulate the μ-calpain activity through changing its structure. Recently, Du et al. (2019) examined the ability of calpastatin to inhibit phosphorylated and dephosphorylated μ-calpain in-vitro, and found calpastatin presented greater inhibition to the PKA-phosphorylated μ-calpain compared with AP-dephosphorylated and control ones. These results indicated that while both dephosphorylation and PKA phosphorylation could positively regulate the activity of μ-calpain and that PKA phosphorylated μ-calpain was more sensitive to calpastatin.

Protein S-nitrosylation is a ubiquitous PTM, that has been shown to regulate protein conformation and activity in biological systems (Hess et al., 2005; Stamler and Meissner, 2001). S-nitrosylation is achieved by coupling NO moiety with a reactive cysteine thiol of protein (Hess et al., 2005). The active site cysteine of calpain makes the enzyme sensitive to S-nitrosylation. Previous studies (Ascenzi et al., 2001; Lametsch et al., 2008) indicated modifications of cysteine residues in calpain's active site through oxidation and S-nitrosylation can inhibit its autolytic and proteolytic activities. Liu et al. (2016) evaluated the effect of S-nitrosylation on the autolysis and catalytic ability of μ-

calpain in-vitro. The results suggested that S-nitrosylated μ-calpain, by nitric oxide donor S-nitrosoglutathione (GSNO), decreased its ability to degrade pork myofibrillar proteins, including titin, nebulin, troponin-T, and desmin. Moreover, the five S-nitrosylated cysteine residues (positions 49, 142, 351, 384, and 592) could be responsible for the decreased μ-calpain activity (Liu et al., 2016). Similarly, Zhang et al. (2018) determined the effect of nitrosylation on the μ-calpain proteolysis activity in beef semimembranosus muscle and found that the degradation of desmin and troponin-T was decreased by GSNO treatments and was increased by nitric oxide synthase (NOS) inhibitor. These results indicated that protein nitrosylation plays a negative role in meat tenderness through regulating calpain autolysis and myofibrillar protein degradation during postmortem aging.

1.4.3. Meat color

Meat color is a critical determinant of consumer acceptance of fresh meat (Mancini and Hunt, 2005; Suman et al., 2014; Neethling et al., 2017). The fresh meat that fails to meet the consumer-preferred cherry-red color are often be reduced in price or be processed into lower-value products before the microbial quality is compromised (Suman et al., 2014). Discoloration of meat not only leads to an annual economic loss of $1 billion to the U.S. meat industry, but also is a wastage of highly nutritious food (Smith et al., 2000). The fresh meat color is mainly determined by the concentration and the redox forms of Mb (Seideman et al., 1984). Although meat discoloration is inevitable, understanding the biochemical pathways involved in Mb redox stability could contribute

to the development of novel strategies to improve color stability (Suman and Joseph, 2013; Ramanathan et al., 2020a, b). Phosphorylation is the most common protein PTM and is a key regulator of biological process (Graves and Krebs, 1999). Previous investigations identified phosphorylation in Mb and glycolytic enzymes and indicated a potential role of phosphorylation in meat color stability (Li et al., 2020).

1.4.3.1. Phosphorylation and myoglobin redox stability

Li et al. (2017) evaluated the effect of phosphorylation on the color stability of ground lamb meat by utilizing phosphatase inhibitor (increase phosphorylation) and protein kinase inhibitor (decrease phosphorylation) to modulate phosphorylation of sarcoplasmic protein. The results indicated that the redness of the highly phosphorylated lamb declined faster than the low phosphorylated counterparts, indicating meat color stability was inversely related to the phosphorylation of sarcoplasmic proteins. Li et al. (2018a) further determined the phosphorylation levels of sarcoplasmic proteins from lamb longissimus thoracis et lumborum whole muscle cuts with different color stability and identified 9 phosphorylated glycolytic enzymes as color stability-related proteins. Moreover, the degree of phosphorylation of Mb was found to be inversely related to the lamb color stability. Higher content of OxyMb was observed in low phosphorylated meat than the highly phosphorylated counterpart, while the highest content of MetMb was determined in the highly phosphorylated group indicating that Mb redox stability was negatively influenced by phosphorylation. Li et al. (2018b) attempted to further understand the regulatory role of protein phosphorylation in meat color stability, and

investigated the color-related proteins and their phosphorylation sites. By performing quantitative phosphoproteomic analysis among lamb longissimus thoracis et lumborum muscles with different color stability, Li et al. (2018b) reported 27 key color-related phosphoproteins, including Mb and glycolytic enzymes. Additionally, phosphorylation at Mb S133 was significantly higher in color-labile lamb compared with the color-stable counterparts, suggesting a negative role of phosphorylation at S133 in meat color stability.

1.4.3.2. Phosphorylation of glycolytic enzymes

Phosphorylation of glycolytic enzymes, including glycogen phosphorylase, pyruvate kinase, phosphofructokinase, phosphoglucomutase-1, triosephosphate isomerase, as well as its impact on postmortem glycolysis and meat pH were reviewed in section 1.4.1. Interestingly, those glycolytic enzymes were also reported to be positively correlated with redness and MetMb reducing activity in beef (Gagaoua et al., 2020). Previous investigations indicated that the abundance of glycogen phosphorylase and triosephosphate isomerase were related to the color stability of semitendinosus (Wu et al., 2015), longissimus lumborum (Wu et al., 2016), and psoas major (Wu et al., 2016) muscles from Chinese Luxi yellow cattle. Phosphoglucomutase-1 was found positively correlated with surface redness of beef semitendinosus (Yu et al., 2017), and longissimus lumborum (Canto et al., 2015; Nair et al., 2018a), but negatively correlated with redness in beef psoas major (Wu et al., 2016). In general, the greater glycolytic metabolism in the postmortem muscle indicates a possible low oxygen consumption, which could minimize

Mb autoxidation, leading to lower MetMb accumulation and improved color stability (O'Keeffe and Hood, 1982; Renerre and Labas, 1987). In addition, the glycolytic enzymes contribute to the meat color stability through NADH regeneration in postmortem muscles (Ramanathan et al., 2010; Ramanathan and Mancini, 2010; Ramanathan et al., 2020a, b). Different isoforms of color-related enzymes, including creatine kinase (Joseph et al., 2012; Nair et al., 2016,2018a,b), glycogen phosphorylase (Wu et al., 2015), beta-enolase (Nair et al., 2016,2018a,b), triosephosphate isomerase (Wu et al., 2016; Nair et al., 2016,2018a,b), phosphoglucomutase-1 (Canto et al., 2015; Nair et al., 2018a) and glyceraldehyde-3-phosphate dehydrogenase (Wu et al., 2016; Canto et al., 2015; Nair et al., 2018a) exhibiting different isoelectric pH have been identified in the two-dimensional gel electrophoresis, indicating that these enzymes might undergo PTMs. Nonetheless, the PTMs in abovementioned enzymes were not identified.

Li et al. (2018a) identified 9 phosphorylated glycolytic enzymes that were correlated with lamb color stability. Further studies (Li et al., 2018b) analyzed the influence of specific phosphorylation sites on the functionality of these glycolytic enzymes and lamb color stability. The results indicated that phosphorylation of pyruvate kinase at S113, triosephosphate isomerase at S160, fructose-bisphosphate aldolase C at S124 or S127, and phosphoglucomutase-1 isoform X2 at Y550, S553 or Y554 could negatively impact meat color stability. In addition, phosphorylation of phosphoacetylglucosamine mutase at S64, beta-enolase isoform X1 at S272, fructose-bisphosphate aldolase A isoform X1 at S39, and 6-muscle type isoform X2 at S448 were positively related to meat color stability (Li et al., 2018b). Interestingly, the phosphorylation of glucose-6-phosphate

isomerase was up-regulated at S533, while down-regulated at S532 in the low color stability group (Li et al., 2018b). This observation suggested that differences in the phosphorylation sites within a protein could play different roles in regulating the protein's functionality and meat color stability.

In summary, protein phosphorylation influences meat color stability through regulating Mb redox stability and post-mortem glycolysis. Nonetheless, Mb phosphorylation patterns during the post-mortem aging, the involvement of other Mb PTMs in color stability, and the PTMs of Mb from different beef muscles are yet to be characterized.

1.5. PTMs in myoglobin

Diverse PTMs, such as phosphorylation (Stewart et al., 2004; Hojlund et al., 2009; Huang et al., 2011; Lametsch et al., 2011; Li et al., 2017a,b, 2018a,b), acetylation (Livingston et al., 1985; Noble et al., 1967; Jiang et al., 2019), methylation (Santucci et al., 1993), carboxymethylation (Ray and Gurd, 1967; Hugli and Gurd, 1970; Schlecht, 1969; Banaszk et al., 1963; Wu et al., 1972; Harris and Hill, 1969) and HNE alkylation (Faustman et al., 1999; Alderton et al., 2003; Suman et al., 2006, 2007; Elroy et al., 2015), could influence the structure and function of mammalian Mbs.

1.5.1. Phosphorylation of myoglobin

Reversible phosphorylation, which can activate (or deactivate) numerous enzymes to regulate their functions, has been considered as the most common PTM (Cohen, 2002;

Grave and Krebs, 1999; Tripodi et al., 2015; Berndt et al., 2017). Previous investigations documented phosphorylation of Mbs 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., 2017a,b, 2018a,b).

While phosphorylation in Mb has been detected by gel-based phosphoproteomic analysis of sarcoplasmic proteins in postmortem pork muscles (Huang et al., 2011; Lametsch et al., 2011), the sites of phosphorylation were not identified in these studies. Potassium phosphoramidate was utilized to chemically phosphorylate histidine residues in horse Mb, and the result indicated that the degree of phosphorylation may vary with site accessibility (Hohenster et al., 2013). In agreement, Stewart et al. (2004) discovered two potential phosphorylation sites (S117 and Y147) in beluga whale (*Delphinapterus leucas*) Mb. These two amino acid residues are located on the surface, and thus are readily accessible for protein kinase, which is the enzyme responsible for transferring phosphate groups from ATP to proteins. Hojlund et al. (2009) analyzed the phosphoproteome of human skeletal muscle, and identified phosphorylation at T68, T71 and S145 in human Mb. Moreover, Li et al. (2017a, 2018a, b) studied the influence of Mb phosphorylation on the color of sheep longissimus thoracis et lumborum muscles by analyzing sarcoplasmic phosphoproteins and reported that the phosphorylation level of Mb was inversely related to the lamb color stability. Furthermore, phosphorylation at S4, T35, T52, S59, T68, T71, Y104, S122 and S133 was detected in sheep Mb (Li et al., 2018b). The phosphorylation level at S133 was significantly greater in color-labile lamb compared with color-stable counterparts, indicating the negative role of S133 phosphorylation in meat color stability

(Li et al., 2018b). Interestingly, the phosphorylation level of Mb was greater in tough sheep longissimus thoracis et lumborum muscle compared with tender counterparts (Li et al., 2017b). These observations suggested that phosphorylation of Mb could be utilized as a biomarker for meat color as well as tenderness.

1.5.2. Acetylation of myoglobin

Acetylation was identified in Mbs from yellowfin tuna (Rice et al., 1979), bullet tuna (Ueki et al., 2005), sea hare (Nguyen et al., 2000), horse (Noble et al., 1967), cattle (Livingston et al., 1985), and pig (Jiang et al., 2019). N-terminus acetylation was detected in Mbs from yellowfin tuna (Rice et al., 1979) and bullet tuna (Ueki et al., 2005). Nguyen et al. (2000) studied the influence of N-terminus acetylation on the structure of *Aplysia limacina* (sea hare) Mb and observed that the absence of N-acetyl group in the recombinant wild-type Mb altered the orientations of heme and proximal histidine imidazole plane. Furthermore, lysine residues in beef Mb were chemically acetylated using acetic anhydride (Livingston et al., 1985), and the results indicated that lysine acetylation did not affect beef Mb's oxygen affinity, whereas it severely decreased the enzymatic reduction rate of Mb. Additionally, Azami-Movahed et al. (2018) documented that lysine acetylation in horse apomyoglobin resulted in a less-ordered tertiary structure and absence of stable hydrophobic patches due to heme pocket disruption. These observations could be attributed to the disruption of charge distribution induced by lysine acetylation, which alter the ionic network on the protein surface and led to destabilization of protein structure (Azami-Movahed et al., 2018). Moreover, Jiang et al. (2019) identified

the acetylation at K43 and K78 in Mb from pork longissimus muscle and indicated that lysine acetylation was involved in the conversion of muscle to meat. In summary, acetylation could compromise Mb's structural stability by modulating the charge distribution, and thereby regulating it functionality.

1.5.3. Methylation of myoglobin

Methylation is a common PTM catalyzed by methyltransferase. Methyl groups bind with nitrogen or oxygen on amino acid sidechains, leading to N- or O-methylation, respectively (Kwan et al., 2016). While methylation has not been reported in globin portion of Mb, several previous studies investigated methylation of heme groups in Mbs from horse (Santucci et al., 1993) and sperm whale (La Mar et al., 1986). These studies observed that methylation of heme did not influence heme re-orientation in horse and sperm whale Mbs.

1.5.4. Carboxymethylation of myoglobin

Carboxymethylation is a non-enzymatic PTM. In-vitro carboxymethylation by bromoacetate was documented in Mbs from human (Harris and Hill, 1969), sperm whale (Ray and Gurd, 1967; Hugli and Gurd, 1970; Schlecht, 1969; Banaszk et al., 1963; Wu et al., 1972) and harbor seal (Nigen and Gurd, 1973). Harris and Hill (1969) documented carboxymethylation at histidine, lysine, and methionine residues in human Mb. Similarly, histidine residues in sperm whale Mb were also susceptible to carboxymethylation. Hugli and Gurd (1970) identified the carboxymethylation at histidine residues (positions 12, 36,

81, 113, 116 and 119) in sperm whale Mb. Moreover, Nigen and Gurd (1973) observed similar carboxymethylation patterns in harbor seal and sperm whale Mbs and reported reactive histidine residues (positions 8, 81, 113, and 116) and unreactive histidines (positions 24, 64, 82,93, and 97) in both species. Banaszk et al. (1963) documented that the addition of carboxymethyl groups might introduce a minor rearrangement of the protein structure, which enables a second carboxymethylation step to be facilitated at a particular histidine residue. In addition, Schlecht (1969) observed that the isoelectric point of sperm whale Mb was altered upon carboxymethylation, indicating that the addition of carboxymethyl group altered charge distribution in Mb. Furthermore, Wu et al. (1972) reported that carboxymethylated sperm whale OxyMb underwent autoxidation faster than its unmodified counterparts. These studies suggested that carboxymethylation could compromise Mb redox stability through altering the heme protein's net charge and structural properties.

1.5.5. HNE alkylation of myoglobin

HNE is an α,β-unsaturated aldehyde formed as a result of oxidation of n-6 polyunsaturated fatty acids (Esterbauer et al., 1991) and has been detected in fresh beef, pork and fish (Sakai et al., 1995,1998,2004). HNE can inactivate enzymes and alter protein structure by covalently binding with lysine, cysteine, arginine, and histidine residues (Esterbauer et al., 1991; Uchida and Stadtman, 1992; Szweda et al., 1993). HNE has been used as a model aldehyde to investigate lipid oxidation-induced oxidation of Mb from beef (Alderton et al., 2003; Suman et al., 2006, 2007; Maheswarappa et al., 2016), 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).

In in-vitro model systems, HNE alkylation occurred exclusively at histidine residues in Mb (Alderton et al., 2003; Suman et al., 2006, 2007; Yin et al., 2013; Nair et al., 2014). Faustman et al. (1999) observed that the HNE alkylation accelerated horse OxyMb oxidation and resulted in mass increment of 156 Da, indicating that the HNE adducts were formed through Michael addition. Alderton et al. (2003) further identified six nucleophilic histidines (positions 24, 64, 93, 116, and 152) in beef Mb that were readily adducted by HNE, including the proximal (H93) and distal (H64) histidines associated with the heme group. Likewise, Naveena et al. (2010) documented covalent HNE adduction at histidine residues (positions 64 and 93) in chicken Mb. Suman et al. (2006, 2007) compared HNE-induced redox instability in beef and pork Mbs at different storage conditions and reported that beef Mb was more susceptible to HNE alkylation than pork Mb. Both mono- and di-adducts were detected in beef Mb, while only mono-adducts were present in pork Mb (Suman et al., 2006). Additionally, the preferential HNE adduction at proximal histidine (H93) was exclusively detected in beef Mb, but absent in pork Mb, and this could render beef Mb a more favorable candidate for HNE adduction compared with pork Mb (Suman et al., 2007). The observed species-specificity in lipid oxidation-induced Mb redox instability suggested the important role of Mb primary structure in acceleration of heme oxidation (Suman et al., 2007). Furthermore, Yin et al. (2011) compared HNE-induced

OxyMb oxidation from various meat-producing species, including beef, pork, horse, sheep, deer, chicken, and turkey. The results indicated greater oxidation rate in Mbs containing greater number of histidine residues, suggesting a correlation between the number of histidine residues and HNE-induced Mb redox instability. Nair et al. (2014a) further investigated lipid oxidation induced-oxidation in emu and ostrich Mbs. Tandem mass spectrometry results revealed that HNE adducted histidine 36 in ostrich Mb, whereas histidine 34 and 36 were adducted in emu Mb, indicating that the variation in primary amino acid sequence of Mb could influence their redox stability in the presence of prooxidants. In addition, buffalo and goat Mbs share 95.4% sequence similarity (Suman and Joseph, 2013), and five histidine residues (positions 24, 36, 81, 88, and 119) in both Indian water buffalo and goat Mbs were found covalently modified by HNE in-vitro (Maheswarappa et al., 2016). This observation suggested that Mbs with similar primary structure might undergo HNE alkylation in a similar fashion.

In summary, HNE alkylation is influenced by species-specific variations in amino acid sequence and the number as well as the locations of histidine residues in the Mb (Suman et al., 2007; Yin et al., 2011). HNE adduction, especially at the hydrophobic heme pocket, could induce conformational changes in Mb, which exposes the heme pocket to oxidizing environment, and thus lead to increased Mb oxidation (Alderton et al., 2003; Lee et al., 2003; Suman et al., 2007).

CHAPTER 2

Myoglobin post-translational modifications influence color stability of beef longissimus lumborum

Abstract:

Post-translational modifications (PTMs) of proteins play critical roles in biological processes. PTMs of muscle proteins influence meat quality. Nonetheless, myoglobin (Mb) PTMs and their impact on fresh beef color stability have not been characterized yet. Therefore, our objectives were to identify Mb PTMs in beef longissimus lumborum (LL) muscle during postmortem aging and to characterize their influence on color stability. The LL muscles from nine (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 PTMs 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 d 0, 3, or 6 of storage. Mb PTMs were analyzed using two-dimensional electrophoresis and tandem mass spectrometry. Surface redness (a^* value), R630/580 (color stability), and Mb concentration decreased ($P < 0.05$) upon aging. Gel image analyses identified six Mb spots with similar molecular weight (17 kDa) but different isoelectric pH. Tandem mass spectrometry identified multiple PTMs (phosphorylation, methylation, carboxymethylation, acetylation, and HNE alkylation) in these six isoforms. The amino acids susceptible to phosphorylation were serine (S), threonine (T), and tyrosine, whereas other PTMs are 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 PTMs increased with aging. The aging-induced PTMs, 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, PTMs at K45, K47, and K87 were unique to Mb from non-aged beef, whereas PTMs at R31, T51, K96, K98, S121, R139, and K147 were unique to Mb from aged counterparts, indicating these Mb PTMs could be used as novel biomarkers for fresh beef color stability.

Keywords: aging, beef color stability, longissimus lumborum, myoglobin, post-translational modifications

2.1. Introduction

The color of fresh beef is critical to meat industry as it is a major quality attribute influencing the consumers' purchase decisions at the point of sale (Suman et al., 2014). Consumers often use the cherry-red color as an indicator of 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 the 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, and ultimately affect meat color (Faustman et al., 2010; Suman and Joseph, 2013; Ramanathan et al., 2020a, b).

Post-translational modifications (PTMs) 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). PTMs can modulate proteins' functionality, localization, turnover, and interactions with other proteins (Seo and Lee, 2004; Rakhit et al., 2014; Muller, 2017; Li et al., 2020). Previous investigations have documented that PTMs in calpain (Liu et al., 2016; Du et al., 2019), myofibrillar proteins (Huang et al., 2012; Li et al., 2017; Li et al., 2020), as well as metabolic enzymes (Anderson et al., 2014; Cruzen et al., 2015; Carlson et al., 2017; Liu et al., 2018; Huang 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 PTMs as novel biomarkers for meat quality.

PTMs such as oxidation (Lindsay et al., 2016; Bostelaar 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; Li et al., 2020), and acetylation (Livingston et al., 1985) influenced the functionality of mammalian Mbs. 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 muscle food matrix during postmortem aging (Richards, 2013; Ramanathan et al., 2020a, b). 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 PTMs 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, b) 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 PTMs in beef Mb and their impact on fresh beef color stability have not been characterized yet. Therefore, the objective of

current study was to characterize the Mb PTMs in beef longissimus lumborum muscle during postmortem aging and their influence on fresh beef color stability.

2.2. Materials and methods

2.2.1. 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). Longissimus lumborum (LL) muscle 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 days. 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 three steaks were allotted to refrigerated storage for evaluation of the color traits. The three 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 days in the darkness (Mancini et al., 2009; Nair et al., 2018). At each storage time point, Mb

concentration, meat pH, instrumental color, metmyoglobin reducing activity (MRA) and lipid oxidation were evaluated.

2.2.2. 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 steaks were bloomed for 2 h at 2 °C before evaluating the instrumental color attributes. CIE (1976) L^* (lightness), a^* (redness), and b^* (yellowness) value 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).

2.2.3. Meat pH

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

2.2.4. Lipid oxidation

Lipid oxidation was measured using the thiobarbituric acid assay (Yin et al., 1993). Triplicate five grams of sample were 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, USA), and were presented as thiobarbituric acid reactive substances (TBARS).

2.2.5. Metmyoglobin reducing activity (MRA)

The MRA was measured at three time points (d 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 was 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). The MRA was calculated using the equation:

MRA = 100 × [(% pre-incubation surface metmyoglobin – % post-incubation surface metmyoglobin) / % pre-incubation surface metmyoglobin].

2.2.6. Myoglobin concentration

The Mb concentration was measured at three time points (d 0, 3, and 6) of each aging period. Triplicate five-gram 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, USA) with 40 mM sodium phosphate buffer as a blank. The Mb concentration was calculated using the following equation:

$$\text{Myoglobin (mg/g)} = [A_{525} / (7.6\ \text{mM}^{-1}\text{cm}^{-1} \times 1\ \text{cm})] \times (17{,}000/ 1000) \times 10$$

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.

2.2.7. 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-gram of muscle tissue devoid of any visible fat and connective tissue was homogenized in 25 mL 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., 2018).

2.2.8. Two-dimensional electrophoresis (2-DE)

The protein concentration of the sarcoplasmic proteome extract was determined in duplicate employing the Bradford assay (Bradford, 1976) utilizing 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 DTT, 4% CHAPS, 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 (IPG) 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 (IEF) which enables the separation of proteins based on their isoelectric point (pI) was performed using a Protean IEF 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. Further, the IPG 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, 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, 2.5% [w/v] iodoacetamide), each for 15

min. Second dimension separation of protein was achieved by 13.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE; 38.5:1 ratio of acrylamide to bis-acrylamide) in a Protean II Multicell system (Bio-Rad Laboratories Inc.). The equilibrated strips were loaded on to 18.5 cm × 20 cm lab cast SDS-PAGE 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.

2.2.9. Gel staining and image analyses

Gels were stained for phosphorylated protein using Pro-Q Diamond (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. Gels were immersed into fix solution (50% methanol and 10% acetic acid) and incubated twice at room temperature with gentle agitation for 30 min. Fixed gels were then immersed into 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 for three times. Gels were washed with ultrapure water for two times before they were imaged (532 nm laser; excitation: 555 nm; emission: 580 nm) using 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 were rinsed with ultrapure water. Gels were then imaged (473 nm laser; excitation: 450 nm; emission: 610 nm) utilizing 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.).

2.2.10. 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. 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, while 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 one segment with 10 scan events. The first scan event was an

Orbitrap MS scan (300–1800 m/z) with 60,000 resolution for parent ions followed by data dependent MS/MS for fragmentation of the 10 most intense multiple charged ions with collision induced dissociation method.

2.2.11. Identification of PTMs 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 two 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) modification on histidine, and lysine.

2.2.12. Statistical analysis

The LL muscle from 9 beef carcasses (n = 9) were utilized for current study. The experiment design was a split-split plot with randomized block design in the whole plot with nine replicates, wherein LL muscle from each carcass served as blocks, and aging time (0, 7, 14, and 21 d) as subplot. For the data of instrumental color and biochemical attributes, storage time (0, 3, and 6 d) was set as a sub-sub plot. The data were analyzed using PROC MIXED procedure of SAS version (SAS Institute Inc., Cary, NC), and the

differences among means were detected using the least significant difference at a 5% level.

2.3. Results and discussion

2.3.1. Instrumental color and biochemical attributes

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 0 d 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. (2018) in beef longissimus muscle.

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 which determine the Mb redox chemistry and therefore impact the meat color stability (Ledward, 1985; Tang et al., 2005; King et al., 2012). In addition, Mancini and Ramanathan

(2014) 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. 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, and thus promotes discoloration in fresh meat (Faustman et al., 2010).

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 which 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.

The ratio of reflectance at 630 nm and 580 nm (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 that those aged for 7 days 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., 2018).

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 days resulted in higher pH of beef LL steaks at the end of 6 days 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.

There was an aging × storage interaction ($P < 0.05$; Table 1) for Mb concentration. While all steaks experienced a decrease in Mb concentration during the 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 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).

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, and thus improves the meat color stability. There was neither 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 (d 0). MRA decreased during the 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 d 3 to d 6 of storage. Nair et al. (2018) documented a similar pattern in beef psoas major muscle. Likewise, Bekhit et al. (2001) observed that sheep longissimus muscle at 6 weeks postmortem had 20% higher MRA than those at 48 h postmortem. Nair et al. (2018) 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 increased MRA without improving the surface redness.

There was no storage × aging 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). 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).

2.3.2. Differential PTMs between myoglobin isoforms

PTMs can cause a shift in the isoelectric point (pI) of proteins by adding, removing, or changing titratable groups; this change in pI enables 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 for phosphorylated protein (Figure 1A) and Sypro Ruby 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 six Mb spots migrated different distances to the acidic side of the gel and exhibited different pI, indicating 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 PTMs 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, 1979), respectively. The addition of negatively charged groups to the protein would cause its migration towards the acidic side of the gel (Anderson et al., 2014). Nonetheless, the magnitude of 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 greater shift in pI than acetylation and methylation. Therefore, the combination of different PTMs 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 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 PTMs, 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 PTMs were lower in these 2 isoforms than in the other ones (Table 3). Phosphorylation was the major PTMs in the isoforms 5 and 6 (Table 3), and this could have contributed to greater shift in pI of these 2 isoforms compared to the other isoforms in which a variety of PTMs were identified.

2.3.3. PTMs patterns in beef myoglobin

The position and identity of amino acids in beef Mb that underwent various PTMs are summarized in Figure 2. The amino acids susceptible to phosphorylation were serine (S), threonine (T), and tyrosine (Y), whereas other PTMs are detected in lysine (K), arginine (R), and histidine (H) residues. Moreover, lysine residues at positions 56, 63, 77, 78, 79, 118, and 132 were susceptible to several PTMs, namely acetylation, methylation and carboxymethylation. A variety of factors such as the number of PTMs in a molecule of protein as well as their chemistry and location(s) influence protein functionality (Kumar et al., 2004).

2.3.3.1. 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; Li et al., 2018a,b). Moreover, Li et al. (2018a, b) 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 three amino acids that 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), 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.

2.3.3.2. Acetylation sites in myoglobin

Protein acetylation is involved in essential biological processes (Kwan et al., 2016; Ali et al., 2018; Zhao et al., 2010). Previous investigations 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, ten lysine residues (positions 50, 56, 63, 77, 78, 79, 87, 118, 133, and 147) were found to be acetylated in beef Mb. These ten 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 expose the heme pocket to oxidizing agents, 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.

2.3.3.3. 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 MK 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 influencing 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 PTMs (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 PTMs, which can positively or negatively influence each other's occurrence (van der Laarse et al., 2018).

The crosstalk among PTMs could alter protein functions (Zhang et al., 2015). Furthermore, methylation could decrease protein stability by acting in combination with other PTMs (Pang et al., 2010; Wu et al., 2017; Zhang et al., 2015; Moore and Gozani, 2014). Therefore, it is highly possible that the interplay between the PTMs at aforementioned 11 lysine residues (methylation sites) could decrease Mb redox stability.

2.3.3.4. Carboxymethylation sites in myoglobin

Carboxymethylation is a non-enzymatic PTM and is a potential metabolic modulator in chemotaxis, neurosecretory regulation, and diabetes (Fang et al., 2010; Diliberto et al., 1976; Curtiss and Witztum, 1985; Hackett and Campochiaro, 1988). Carboxymethylation could be achieved in-vitro chemically (using bromoacetate) in the Mb from human (Harris and Hill, 1969), sperm whale (Ray and Gurd, 1967; Hugli and Gurd, 1970; Schlecht, 1969; Banaszk et al., 1963; 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.

2.3.3.5. 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 Mbs 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 destabilized 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).

2.3.4. Aging-induced PTMs influence beef color stability

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

Increased number 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 PTMs 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 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 PTMs (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 days 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 compromise the tertiary structure of proteins and increase 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 et al., 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 four HNE adducted histidine residues (position 36, 81, 88 and 152) of beef Mb, the present study found only one histidine (H64) 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 days incubation at pH 5.6 and 4°C; however, in the current study only one HNE alkylation site (K77) was detected after 21 days 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; (2) the formation of other in-situ PTMs in Mb adversely influencing HNE alkylation.

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

2.4. Conclusions

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

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Table 2.1: Surface lightness (*L** value), redness (*a** value), yellowness (*b** value), meat pH, and myoglobin (Mb) concentration of aged[1] beef longissimus lumborum steaks (n = 9) during refrigerated storage (2°C) for 6 days 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	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 ± standard error of the mean (SEM).
[a-d] Means without common superscript within an attribute are different ($P < 0.05$).

Table 2.2: Surface color stability (R630/580), metmyoglobin reducing activity (MRA) and lipid oxidation of aged[1] beef longissimus lumborum steaks (n = 9) during refrigerated storage (2°C) for 6 days 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 ± standard error of the 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).

Table 2.3: Post-translational modifications and their locations in myoglobin isoforms isolated from beef longissimus lumborum muscle from all aging days.

Post-translational modifications	Myoglobin spots [a]					
	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

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

Table 2.3 (continued): Post-translational modifications and their locations in myoglobin isoforms isolated from beef longissimus lumborum muscle from all aging days.

Post-translational modifications	Myoglobin spots [a]					
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Spot 6
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 (Figures 1 A and B).

Table 2.4: 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 58 Serine 67 Threonine 70 Threonine 103 Tyrosine 108 Serine 132 Serine	34 Threonine 51 Threonine 58 Serine 67 Threonine 70 Threonine 103 Tyrosine 108 Serine 121 Serine 132 Serine	34 Threonine 58 Serine 67 Threonine 70 Threonine 103 Tyrosine 108 Serine 121 Serine 132 Serine	34 Threonine 67 Threonine 70 Threonine 103 Tyrosine 108 Serine 132 Serine
Acetylation	50 Lysine 63 Lysine 77 Lysine 78 Lysine 79 Lysine 87 Lysine 118 Lysine 133 Lysine	56 Lysine 63 Lysine 77 Lysine 78 Lysine 79 Lysine 133 Lysine	50 Lysine 56 Lysine 63 Lysine 77 Lysine 78 Lysine 79 Lysine	63 Lysine 77 Lysine 78 Lysine 147 Lysine
Methylation	42 Lysine 56 Lysine 62 Lysine 63 Lysine 118 Lysine	31 Arginine 42 Lysine 56 Lysine 62 Lysine 63 Lysine 77 Lysine 78 Lysine 79 Lysine 96 Lysine 98 Lysine 118 Lysine 139 Arginine	31 Arginine 42 Lysine 56 Lysine 62 Lysine 63 Lysine 77 Lysine 78 Lysine 98 Lysine 102 Lysine 118 Lysine 133 Lysine	31 Arginine 42 Lysine 63 Lysine 77 Lysine 78 Lysine 98 Lysine 102 Lysine 118 Lysine 133 Lysine

Table 2.4 (continued): 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
Carboxymethylation	62 Lysine 63 Lysine 77 Lysine 78 Lysine 79 Lysine 87 Lysine 102 Lysine 133 Lysine	56 Lysine 62 Lysine 63 Lysine 77 Lysine 78 Lysine 96 Lysine 98 Lysine 102 Lysine 118 Lysine 133 Lysine 147 Lysine	56 Lysine 62 Lysine 63 Lysine 77 Lysine 78 Lysine 102 Lysine 118 Lysine 147 Lysine	62 Lysine 63 Lysine 77 Lysine 78 Lysine 133 Lysine
HNE alkylation	45 Lysine 47 Lysine 56 Lysine 62 Lysine 63 Lysine 64 Histidine 77 Lysine	56 Lysine 62 Lysine 63 Lysine 64 Histidine 77 Lysine 78 Lysine 79 Lysine	56 Lysine 62 Lysine 63 Lysine 64 Histidine 77 Lysine 78 Lysine 79 Lysine	77 Lysine
Total number of post-translational modification sites	35	45	40	25

Table 2.5: Differential post-translational modifications identified in myoglobin isolated from non-aged[1] and aged[2] beef longissimus lumborum muscle.

Non-aged beef			Aged beef		
Position	Residue	Modification [a]	Position	Residue	Modification [a]
34	Threonine	P	**31**	**Arginine**	**M***
42	Lysine	M	34	Threonine	P
45	**Lysine**	**H$^{\Psi}$**	42	Lysine	M
47	**Lysine**	**H$^{\Psi}$**	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$^{\Psi}$**	**79**	**Lysine**	A **M*** **H***
87	**Lysine**	**A$^{\Psi}$ C$^{\Psi}$**	**96**	**Lysine**	**M* C***
102	Lysine	C	**98**	**Lysine**	**M* C***
103	Tyrosine	P	**102**	**Lysine**	**M*** C
108	Serine	P	103	Tyrosine	P
118	**Lysine**	**A$^{\Psi}$** M	108	Serine	P
132	Serine	P	**118**	**Lysine**	M **C***
133	Lysine	A C	**121**	**Serine**	**P***
			132	Serine	P
			133	**Lysine**	A **M*** C
			139	**Arginine**	**M***
			147	**Lysine**	**A* C***
Total	22	35	Total	26	49

[a] P = Phosphorylation; A = Acetylation; M = Methylation; C = Carboxymethylation; H = HNE alkylation
[1] Non-aged = aged for 0 d
[2] Aged = aged in vacuum packaging at 2°C for 7, 14, or 21 d
Differential PTMs and their locations are listed in boldface
$^{\Psi}$ PTMs unique to Mb isolated from non-aged beef longissimus lumborum muscle
* PTMs unique to Mb isolated from beef longissimus lumborum muscle aged in vacuum packaging at 2°C for 7, 14, or 21 d

Figure 2.1: Representative two-dimensional gel electrophoresis map of sarcoplasmic proteome extracted from beef longissimus lumborum separated using an immobilized pH gradient (IPG) 5 to 8 strip in the first dimension and 13.5% SDS gel in the second dimension. 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 (pI and MW) across the gels.

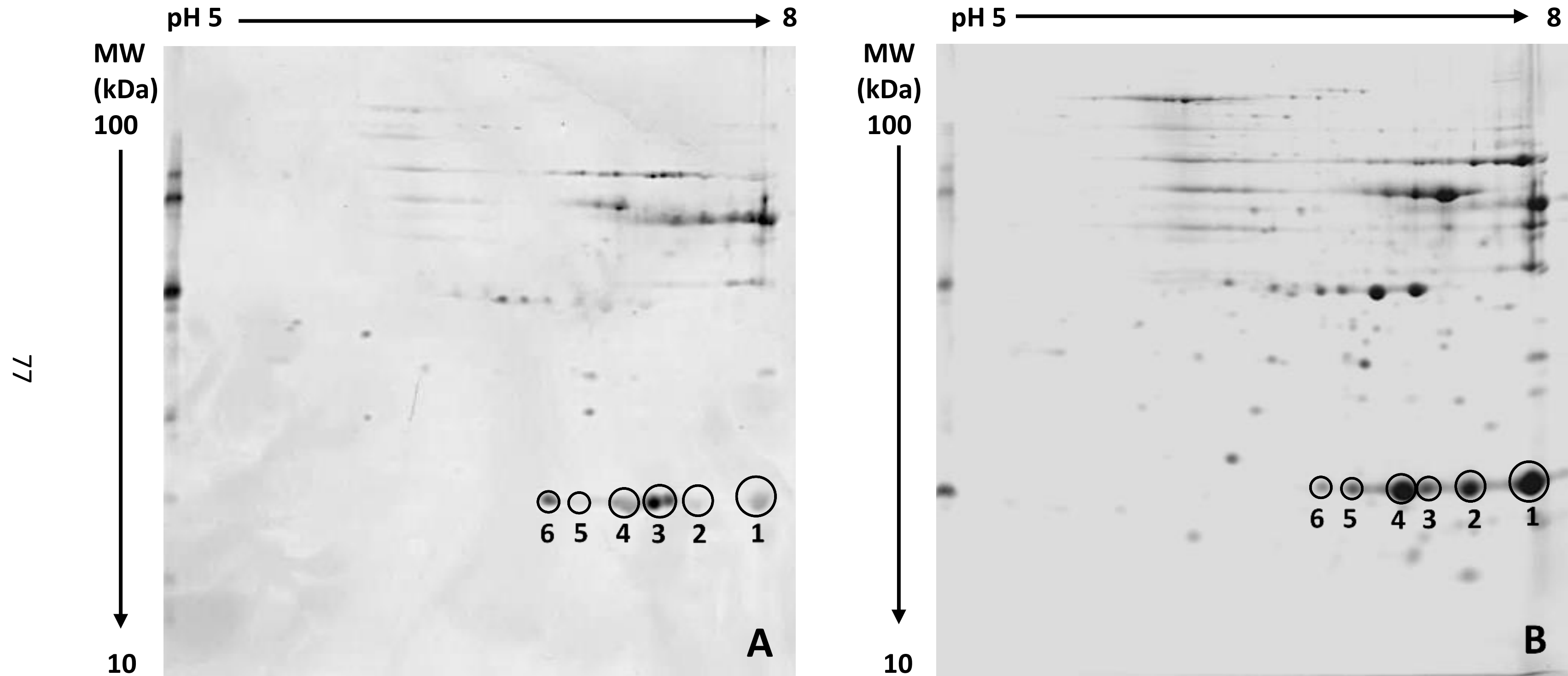


Figure 2.2: Post-translational modifications (phosphorylation, acetylation, methylation, carboxymethylation, HNE alkylation) and their locations in the amino acid sequence of beef myoglobin. Post-translationally modified residues are underlined.

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 2.3: 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 3-dimensional homology model of beef myoglobin was downloaded from SWISS-MODEL.

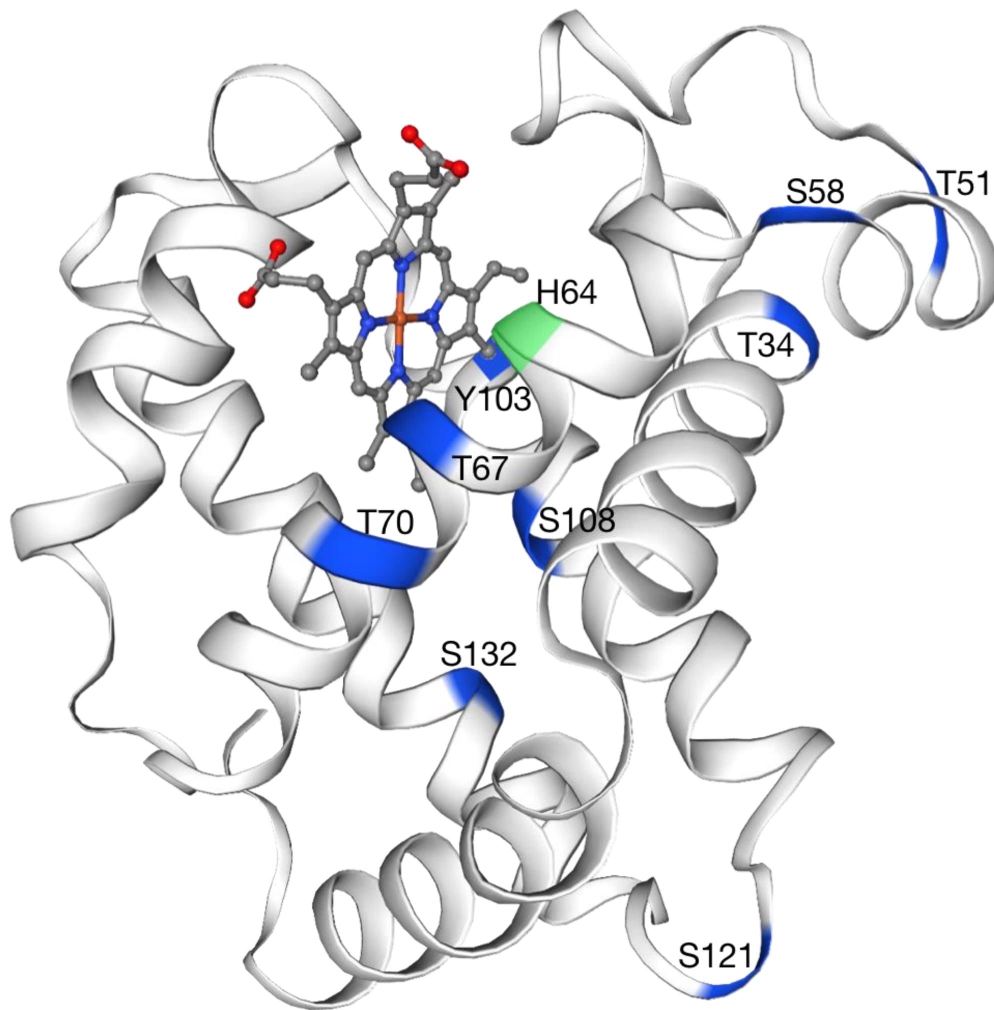
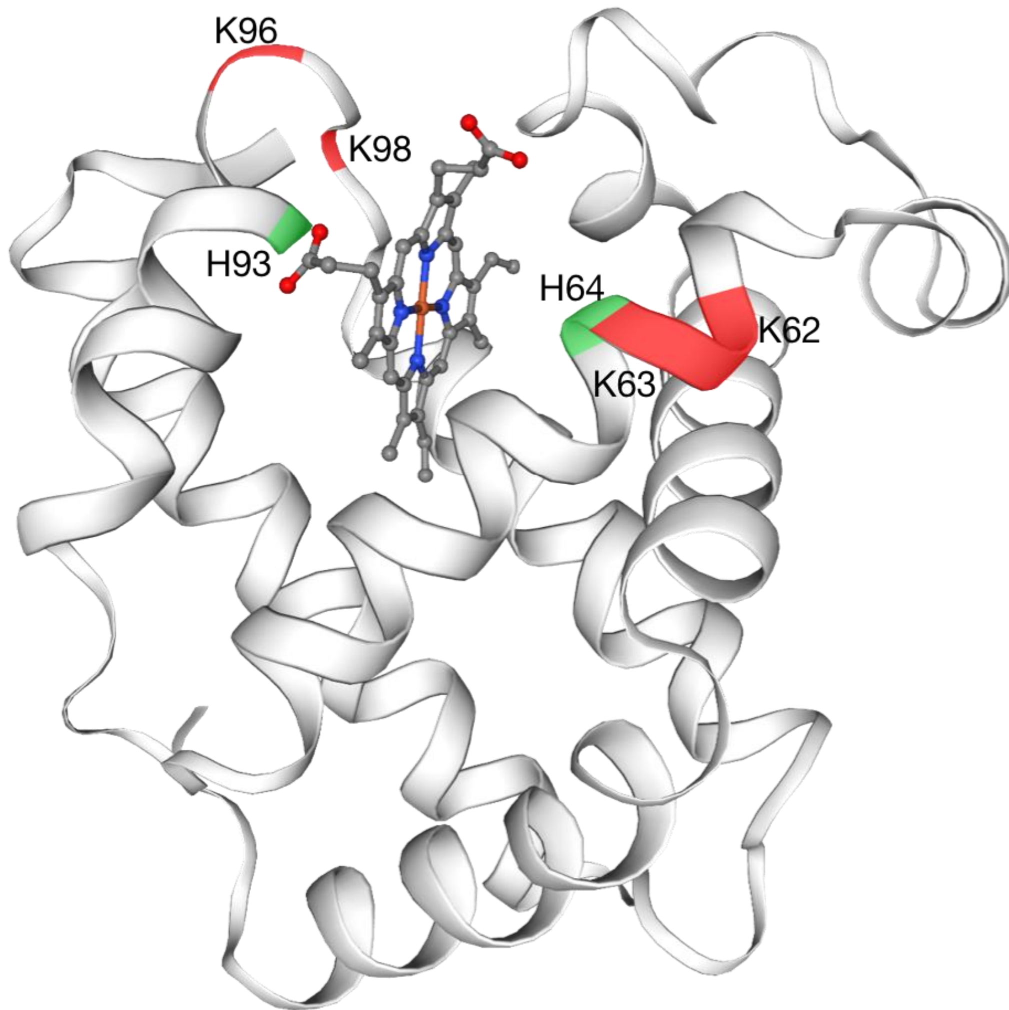


Figure 2.4: Distal (H64) and proximal (H93) histidines in beef myoglobin are indicated in green, whereas lysine residues (K62, K63, K96 and K98) are in red. The 3-dimensional homology model of beef myoglobin was downloaded from SWISS-MODEL.



CHAPTER 3

Supranutritional supplementation of vitamin E influences myoglobin post-translational modifications in postmortem beef longissimus lumborum muscle

Abstract:

Post-translational modifications (PTMs) in myoglobin (Mb) can influence fresh meat color stability. Dietary supplementation of vitamin E improves beef color stability by delaying lipid oxidation-induced Mb oxidation and influences proteome profile of postmortem beef skeletal muscles. Nonetheless, the influence of vitamin E on Mb PTMs in post-mortem beef skeletal muscles has yet to be investigated. Therefore, the objective of current study was to examine the effect of dietary vitamin E on Mb PTMs in postmortem beef longissimus lumborum (LL) muscle. Beef LL muscle samples (24 hours post-mortem) were obtained from the carcasses of nine (n = 9) vitamin E-fed (VITE) (1000 IU vitamin E for 89 days) and nine (n = 9) control (CONT) (diet without supplemental vitamin E) heifers. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate Mb from other sarcoplasmic proteins of beef LL muscle. Tandem mass spectrometry identified multiple PTMs (phosphorylation, acetylation, HNE alkylation, methylation, dimethylation, trimethylation, and carboxymethylation) in the protein bands (17 kDa) representing Mb. The amino acids susceptible to phosphorylation were threonine (T) and tyrosine (Y), whereas lysine (K) residues were prone to other PTMs. Same sites of phosphorylation (T34, T67, Y103), carboxymethylation (K77, K78) and HNE alkylation (K77, K78, K79) identified in Mbs from CONT and VITE samples, indicating these PTMs were not influenced by the vitamin E supplementation in cattle. Nonetheless, differential occurrence of acetylation, methylation, dimethylation and trimethylation were identified in Mb from CONT and VITE samples. Overall, a greater number of amino acids were modified in CONT than VITE, suggesting that the supplementation of vitamin

E decreased the numbers of post-translationally modified residues in Mb. Additionally, PTMs at K87, K96, K98 and K102 were unique to CONT, whereas PTMs at K118 were unique to VITE. These findings suggested that dietary supplementation of vitamin E in beef cattle might protect amino acid residues in Mb, especially those located spatially close to proximal histidine, from undergoing PTMs, and thereby improving Mb redox stability.

Keywords: vitamin E, myoglobin, post-translational modifications, beef color stability

3.1. Introduction

Meat discoloration is due to the conversion of cherry-red oxymyoglobin (OxyMb) to brownish metmyoglobin (MetMb) and adversely affects consumer perception of quality (Suman et al., 2014; Neethling et al., 2017). The secondary products of lipid oxidation can accelerate OxyMb oxidation through alkylation of myoglobin (Mb) leading to meat discoloration (Faustman et al., 1999). Pre-harvest applications of dietary antioxidants in animal production can minimize lipid oxidation-induced OxyMb oxidation (Faustman et al., 2010).

Vitamin E (α-tocopherol) is a lipid-soluble antioxidant that protects highly oxidizable polyunsaturated fatty acids from oxidation by reactive oxygen species and free radicals (Buttriss and Diplock, 1988). Dietary supplementation of vitamin E demonstrated a dual protective effect for both lipid and Mb oxidation in beef (Faustman et al., 1989, Arnold et al., 1992; Sanders et al., 1997; Zerby et al., 1999; Lynch et al., 1999) and lamb (Wulf et al., 1995; Guidera et al. 1997; Strohecker et al., 1997; Gonzalez-Calvo et al., 2015). Vitamin E also retards lipid and OxyMb oxidation in microsomes (Yin et al., 2013) and liposome (Yin et al., 1993) models in-vitro. In addition, dietary delivery of vitamin E improved pigment and lipid stability more efficiently than addition of this ingredient to postmortem muscles (Mitsumoto et al., 1993). The effect of vitamin E on meat color stability is believed through a direct protective effect for lipid, and an indirect effect for minimizing OxyMb oxidation (Faustman et al., 2010; Ramanathan et al., 2020a, b).

Post-translational modification (PTM) refers to the covalent changes that proteins undergo after translation (Lodish, 1981; Han and Martinage, 1992; Mann and Jensen,

2003). PTMs determine protein structure and modulates the protein properties by addition or removal of modifying groups to amino acids (Mann and Jensen, 2003; Seo and Lee, 2004). PTM plays a fundamental role in regulating biological processes as it determines protein's functionality, localization, turnover, and interactions with other biomolecules (Seo and Lee, 2004; Muller, 2017). Diverse PTMs such as phosphorylation (Stewart et al., 2004; Huang et al., 2011; Hohenester et al., 2013; Li et al., 2020), acetylation (Livingston et al., 1985; Jiang et al., 2019; Li et al., 2020), carboxymethylation (Ray and Gurd, 1967; Hugli and Gurd, 1970), and oxidation (Lindsay et al., 2016; Bostelaar et al., 2016) have been reported to regulate the structure and functionality of mammalian Mb. Furthermore, HNE alkylation accelerated in-vitro OxyMb oxidation by covalently binding to histidine residues (Faustman et al., 1999; Alderton et al., 2003; Suman et al., 2006, 2007; Yin et al., 2011; Nair et al., 2014; Elroy et al., 2015).

Li et al. (2018a, b) documented that the phosphorylation level of sheep Mb in inversely related to the color stability of longissimus muscle. Moreover, the results from Chapter 2 indicated that PTMs (including phosphorylation, acetylation, methylation, carboxymethylation, and alkylation) compromised beef Mb redox stability and color stability. These observations suggested that PTMs play a critical role in Mb functionality and fresh meat color stability.

The effect of vitamin E on fresh beef color have been extensively studied from the standpoint of lipid oxidation-induced Mb oxidation. Recent study indicated dietary supplementation of vitamin E influenced the mitochondrial (Zhai et al., 2018) and sarcoplasmic (Kim, 2018) proteome profile of postmortem beef longissimus lumborum

(LL) muscle. Nonetheless, investigations were not undertaken on the potential effect of vitamin E on Mb PTMs in beef skeletal muscle. Therefore, the objective of current study was to examine the influence of dietary vitamin E supplementation on the Mb PTMs in postmortem beef LL muscle.

3.2. Materials and methods

3.2.1. Animal production and muscle sample collection

The muscle samples were obtained from a feeding study (Harsh et al., 2018) completed at the University of Illinois. All protocols were approved by the University of Illinois Institutional Animal Care and Use Committee (Protocol #15008). Eighteen Angus × Simmental heifers were used in a randomized complete block design with treatment factors including daily dietary inclusion of no supplemental (CONT) or 1,000 IU vitamin E/animal per day (VITE). Heifers were managed as a group on a trace mineral maintenance diet prior to trial initiation and were administered an implant of 140 mg trenbolone acetate and 14 mg estradiol (Component TE-H; Elanco Animal Health, Greenfield, IN). After being weighed on day 1 and 0, heifers were stratified by bodyweight (n = 9 heifers per treatment). Diets were the same for the two dietary treatments with the exception of vitamin E inclusion as feed supplement. Diets were formulated to meet or exceed NRC (2000) recommendations and contained 20% corn silage, 35% modified wet distillers grains with solubles, 35% dry rolled corn, and 10% supplement (dry matter basis). Dietary vitamin E (dl-alpha-tocopheryl acetate) was provided to VITE heifers, and individual feed

intakes of all heifers were collected with a GrowSafe feeding system (GrowSafe Systems Ltd., Airdrie, AB, Canada).

Heifers were weighed at 28-day intervals and fed for ad libitum intake daily for a total of 89 days on feed. Heifers were housed in 4.88 m × 4.88 m pens in a confinement barn with slatted, concrete floors covered with interlocking rubber matting. On day 90, heifers were slaughtered humanely under USDA inspection at a commercial slaughter facility. At 24 h postmortem, a 2.54-cm section of LL was excised from between the 12th and 13th rib section of the carcasses, immediately vacuum-packaged, frozen at –80 °C, and shipped in dry ice to the University of Kentucky. The results of growth performance, carcass quality, color attributes, and lipid oxidation are discussed in Harsh et al. (2018).

3.2.2. Isolation of sarcoplasmic proteome

The sarcoplasmic proteomes from beef LL muscle were extracted according to the method of Joseph et al. (2012). Frozen samples were thawed overnight at 2 °C. Five-gram of muscle tissue devoid of any visible fat and connective tissue was homogenized in 25 mL 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, Little Chalfont, UK), and used for subsequent analysis.

3.2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The sarcoplasmic proteomes from beef LL muscle were separated based on molecular weight using SDS-PAGE gels as described by Laemmli (1970). A stacking gel with 3% of acrylamide and a resolving gel with 13% acrylamide were used. The diluted protein samples (3 mg/mL) dissolved in the SDS-PAGE sample buffer (10% [w/v] SDS, glycerol, 0.1% [w/v] bromophenol blue, 0.5 M Tris-HCl, pH 6.8) were boiled with 10% β-mercaptoethanol in the water bath for 5 min. Aliquots of 20 μg of protein per well were loaded to SDS-PAGE gels in a mini PROTEAN Tetra cell system (Bio-Rad Laboratories Inc.). The molecular weight standard (Bio-Rad Laboratories Inc.) used consisted of myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), lysozyme (14 kDa), and aprotinin (6.5 kDa). The gels were stained with Colloidal Coomassie Blue for 48 h, and destained until sufficient background was cleared.

3.2.4. Liquid chromatography-electrospray ionization-tandem mass spectrometry

The protein gel bands (17 kDa) representing Mb in the SDS-PAGE gels were excised and subjected to dithiothreitol reduction, iodoacetamide alkylation, and in-gel trypsin digestion using a standard protocol. 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 while 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 one segment with 10 scan events. The first scan event was an Orbitrap MS scan (300–1800 m/z) with 60,000 resolution for parent ions followed by data dependent MS/MS for fragmentation of the 10 most intense multiple charged ions with collision induced dissociation (CID) method.

3.2.5. Identification of PTMs 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 two miscleavages; 10 ppm precursor ion and 0.8-Da fragment ion mass tolerances; methionine oxidation; lysine acetylation; lysine mono-, di-, and tri-methylation; arginine mono- and di-methylation; serine, threonine and tyrosine phosphorylation; 4-hydroxynonenal (HNE) modification on histidine, and lysine.

3.3. Results and discussion

3.3.1. Influence of vitamin E supplementation on beef myoglobin PTMs

Tandem mass spectrometry identified multiple PTMs (such as phosphorylation, methylation, dimethylation, trimethylation, carboxymethylation, acetylation, and HNE alkylation) in protein bands (17 kDa) representing Mb. PTMs in Mb from CONT and VITE beef are presented in Table 1. The amino acids susceptible to phosphorylation were threonine (T) and tyrosine (Y), whereas lysine (K) residues were prone to other PTMs. While Mb from CONT and VITE exhibited same number of PTMs, a greater number of amino acids were modified in CONT than VITE (16 vs 13). Mb from CONT and VITE demonstrated similar pattern in phosphorylation (T34, T67, Y103), carboxymethylation (K77, K78), and HNE alkylation (K77, K78, K79) sites, indicating these PTMs were not influenced by the vitamin E supplementation in beef animals. Nonetheless, differential occurrence of acetylation, methylation, dimethylation and trimethylation were identified in Mb from CONT and VITE samples (Table 1; Figure 1), and are discussed below.

Seven lysine residues were acetylated in Mb from both CONT and VITE animals. While six of acetylation sites (K50, K63, K77, K78, K79, and K147) were observed in both in CONT and VITE, acetylation at K87 and K118 were unique to CONT and VITE, respectively (Table 1). The addition of acetyl group neutralizes the positive charge of lysine, and thereby disrupting the ionic network and Mb tertiary structure. Moreover, the acetylation-induced conformational changes in Mb could result in an increased tendency for unfolding, compromising the heme stability and oxygen affinity (Azami-Movahed et al., 2018; Nguyen et al., 2000). Compared with K118, K87 is in closer proximity to the

proximal histidine (H93), which is bound to heme moiety (Figure 1). Thus, the acetylation of distantly located K118 could have much lesser impact on the hydrophobic heme pocket than the acetylation in K87. In partial agreement, Suman et al. (2006) documented that the HNE alkylation at H88, which is adjacent to K87, appeared to compromise Mb redox state due to potential interference with proximal histidine. In general, the lysine acetylation in VITE occurred at residues far from proximal histidine may result in minimum damage of Mb redox stability, which could also explain the better color stability in vitamin E-supplemented beef (Faustman et al., 1989).

The ε-amine moiety of lysine can be methylated up to three times from unmodified lysine to mono-, di-, and tri-methylated forms, altering the biophysical properties (i.e., pKa value and size) of this residue (Luo, 2018). Differential methylation, di-methylation and tri-methylation sites were detected in Mb from CONT and VITE samples (Table 1). While K31 and K42 were methylated in both CONT and VITE Mbs, methylation at K98 and K118 were unique to CONT and VITE, respectively. The addition of methyl groups increases the overall size and hydrophobicity of the lysine sidechains (Bremang et al., 2013; Luo, 2018), which in turn could induce conformational changes and compromise Mb redox stability. The K98 lies in the closer vicinity of proximal histidine (H93) compared with the K118 (Figure 1). Consequently, the methylation at K98 in CONT might result in a greater damage on the heme iron-proximal histidine bond and heme affinity than at K118 in VITE. The removal of heme could lead to the decrease of helical content, which is detrimental to the Mb tertiary structure (Ochiai, 2011), and thereby resulting in meat discoloration (Suman and Joseph, 2013). Additionally, lysine di-

methylation at K118 and K133 were only detected in VITE beef. While K96, K102 and K133 were tri-methylated in CONT, only K118 were tri-methylated in VITE. As lysine methylation progressed, the adduction of increased number of methyl groups could lead to a decrease in lysine's hydrophilicity and the residue's capability to form hydrogen bonds (Luo, 2018); this in turn could compromise protein hydrophilicity and stability (Hamamoto et al., 2015). Therefore, a tri-methylated lysine could contribute to a greater level of hydrophobicity to proteins than a di-methylated lysine. Accordingly, the three tri-methylated lysine residues (K96, K102, and K133) observed in CONT could be more deleterious to Mb tertiary structure than the two di-methylated (K118 and K133) and one tri-methylated (K118) lysine residues in VITE samples; thus, Mb from VITE samples might have better redox stability than its counterpart from CONT samples. Furthermore, K96 and K102 are closer to proximal histidine (H93) spatially compared to K118 and K133 within Mb tertiary structure (Figure 1), so that the observed tri-methylation at K96 and K102 in CONT could have a greater impact on the hydrophobic heme pocket and Mb redox stability compared to the di-methylation at K118 and K133 in VITE. Overall, the location and number of mono-, di-, and tri-methylated lysine residues observed in CONT might be more detrimental to Mb redox stability than those in VITE, which could lead to the lower color stability of beef from non-vitamin E fed animals than the beef from vitamin E-supplemented cattle (Faustman et al., 1989).

Previous investigations documented that dietary supplementation of cattle with vitamin E (i.e., α-tocopherol) could improve OxyMb stability (Chan et al., 1996; Faustman et al., 1989) and beef color stability (Faustman et al., 1989; Arnold et al., 1993; Lanari et

al., 1993; Sherbeck et al., 1995). The observed color-stabilizing effect of α-tocopherol was believed to be achieved by direct inhibition of lipid oxidation, and thereby indirectly delaying OxyMb oxidation (Faustman et al., 2010; Ramanathan et al., 2020a). Secondary products of lipid oxidation have been shown to accelerate OxyMb oxidation through alkylation of Mb (Witz, 1989; Faustman et al., 1999). 4-Hydroxy-2-nonenal (HNE), a well-documented secondary product of linoleic acid oxidation, was observed to covalently adduct with histidine residues in beef Mb via Michael addition, exposing heme and subsequently accelerating Mb oxidation (Faustman et al., 1999; Alderton et al., 2003; Suman et al., 2006; Suman et al., 2007). Therefore, α-tocopherol, the lipid-soluble and a chain-breaking antioxidant, was expected to decrease HNE generation from lipid oxidation and in turn improve Mb stability. Nonetheless, current study observed HNE alkylation at three lysine residues (K77, K78, and K79) in Mbs from both CONT and VITE groups (Table 1), suggesting that this PTM was not influenced by the dietary supplementation of vitamin E.

Interestingly, our results indicated the supplementation of vitamin E seems to exert its protective effect on Mb by influencing several other PTMs such as acetylation, methylation, di-methylation and tri-methylation. Additionally, the supplementation of vitamin E decreased the numbers of post-translationally modified residues in Mb. PTMs at K87, K96, K98 and K102 were unique to CONT, whereas PTMs at K118 were unique to VITE. These observations indicated that dietary supplementation of vitamin E in beef cattle might protect residues in Mb, especially those located spatially close to proximal histidine, from undergoing PTMs, and thereby improving Mb redox stability.

3.4. Conclusions

Dietary supplementation of vitamin E decreased the number of post-translationally modified residues in Mb from beef LL. While phosphorylation, carboxymethylation, and alkylation of Mb were not influenced by vitamin E supplementation, differential acetylation, methylation, dimethylation and trimethylation sites were identified in Mb from CONT and VITE beef cattle. The unique PTMs in CONT Mb (K87, K96, K98 and K102) were spatially closer to proximal histidine compared to the unique PTM (K118) in Mb from VITE samples, and thus could be more detrimental to Mb redox stability due to the potential interference with proximal histidine. The strong antioxidant protection offered by vitamin E might have minimized the occurrence of PTMs at residues located spatially close to proximal histidine in Mb and could have contributed to the improved beef color stability.

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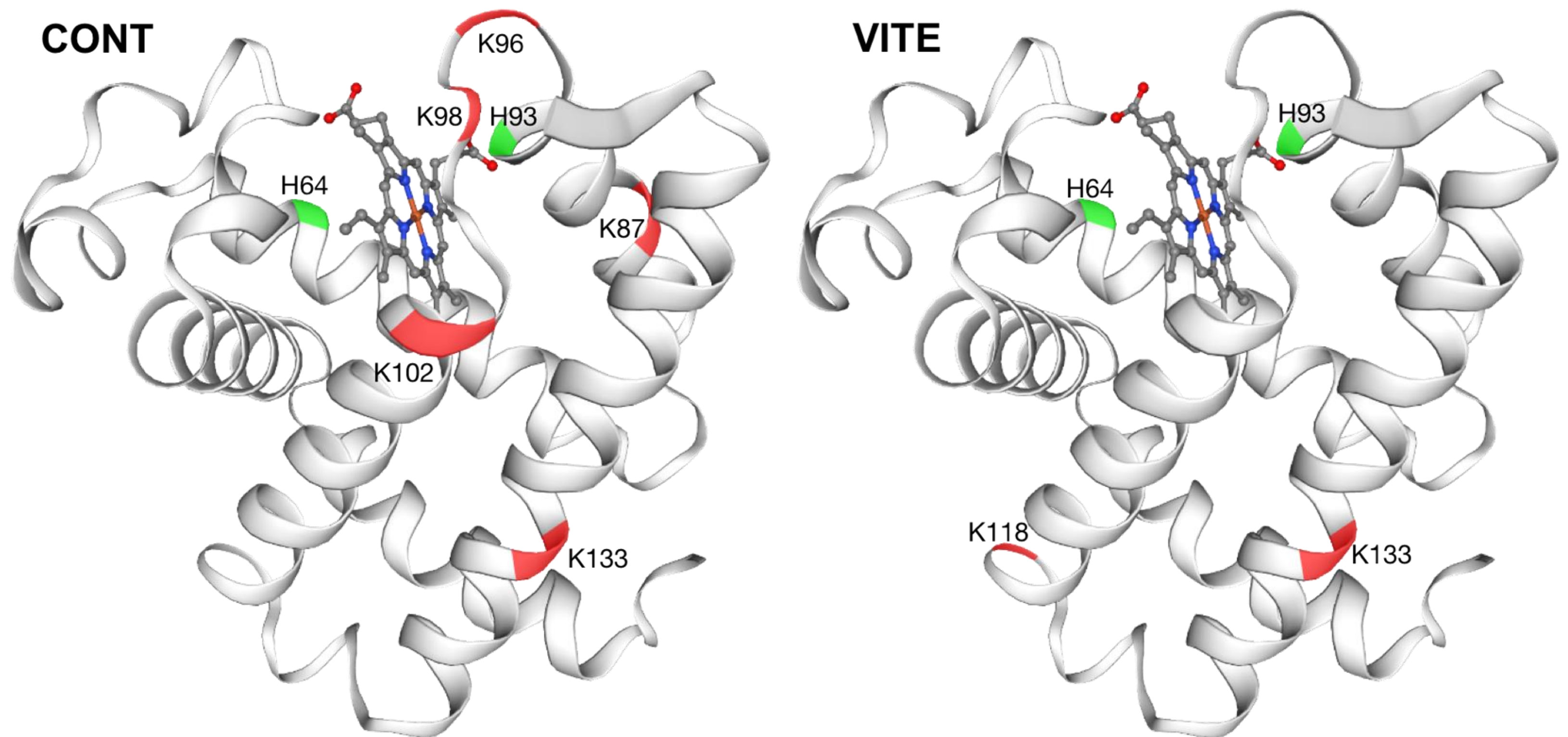
Table 3.1: Impact of supranutritional supplementation of vitamin E on myoglobin post-translational modifications in postmortem beef longissimus lumborum muscle

Post-translational modifications [a]	CONT [b]	VITE [b]
Phosphorylation	34 Threonine 67 Threonine 103 Tyrosine	34 Threonine 67 Threonine 103 Tyrosine
Carboxymethylation	77 Lysine 78 Lysine	77 Lysine 78 Lysine
HNE Alkylation	77 Lysine 78 Lysine 79 Lysine	77 Lysine 78 Lysine 79 Lysine
Acetylation	50 Lysine 63 Lysine 77 Lysine 78 Lysine 79 Lysine **87 Lysine$^{\Psi}$** 147 Lysine	50 Lysine 63 Lysine 77 Lysine 78 Lysine 79 Lysine **118 Lysine*** 147 Lysine
Methylation	31 Arginine 42 Lysine **98 Lysine$^{\Psi}$**	31 Arginine 42 Lysine **118 Lysine***
Dimethylation	–	**118 Lysine*** **133 Lysine***
Trimethylation	**96 Lysine$^{\Psi}$** **102 Lysine$^{\Psi}$** **133 Lysine$^{\Psi}$**	**118 Lysine***
Total number of post-translationally modified residues	16	13

[a] Position and residue are listed for the post-translational modifications
[b] CONT: non-vitamin E diet; VITE: 1000 IU vitamin E diet for 89 days
Differential PTMs are listed in boldface
$^{\Psi}$ PTMs unique to myoglobin from controlled beef
* PTMs unique to myoglobin from vitamin E supplemented beef

Figure 3.1: Differential post-translational modifications at lysine (K) residues in myoglobins from longissimus lumborum muscle of CONT (non-vitamin E diet) and VITE (1000 IU vitamin E diet for 89 days) fed heifers. Differential post-translational modifications are indicated in red, whereas distal (H64) and proximal (H93) histidines in beef myoglobin are in green. The 3-dimensional homology model of beef myoglobin was downloaded from SWISS-MODEL.



SUMMARY

Color stability is critical to fresh beef retailing and has significant economic impact. The redox state of myoglobin (Mb) determines fresh beef color and is influenced by the heme protein's interactions with cellular organelles and biomolecules in the postmortem skeletal muscle. Post-translational modifications (PTMs) can modulate proteins' functionality and interactions with other proteins by the addition or removal of modifying group(s) to amino acids in primary structure. Diverse PTMs, including oxidation, methylation, phosphorylation, acetylation and alkylation, have been reported to influence the functionality of mammalian Mbs. Nonetheless, the in-situ PTMs in beef Mb and their impact on fresh beef color stability have not been characterized. Therefore, the objectives of this thesis research were to characterize the PTMs in beef Mb and their influence on fresh beef color stability.

The first experiment examined the influence of Mb PTMs on the color stability of fresh beef longissimus lumborum (LL) muscle during postmortem aging. The results indicated that postmortem wet-aging decreased ($P < 0.05$) surface redness, color stability, and Mb concentration. Mb in fresh beef LL muscle underwent PTMs (phosphorylation, methylation, carboxymethylation, acetylation, and HNE alkylation) during postmortem aging. Aging resulted in an increase in the number of phosphorylation, methylation and carboxymethylation sites and a decrease in the number of acetylation sites in beef Mb. While the number of alkylation sites remained the same during 14 days of aging, HNE adduction at lysine (positions 78 and 79) residues were unique to aged beef, indicating

HNE alkylation of lysine residues might play a critical role in Mb redox stability. Furthermore, PTMs at lysine (positions 45, 47, and 87) residues were unique to Mb from non-aged beef, whereas PTMs at arginine (positions 31, and 139), threonine (position 51), serine (position 121), and lysine (positions 96, 98, and 147) residues were unique to Mb from aged counterparts. These aging-induced PTMs could compromise Mb redox stability by adding modifying groups to amino acids, especially to those residues close to hydrophobic heme pocket, and thus accelerating Mb oxidation and beef discoloration.

The second experiment examined the influence of vitamin E supplementation to beef cattle on Mb PTMs in beef LL muscle. Beef LL samples (24 h postmortem) were obtained from the carcasses of vitamin E-fed (VITE; 1,000 IU vitamin E for 89 days) and control (CONT; diet without supplemental vitamin E) heifers. Dietary supplementation of vitamin E decreased the number of post-translationally modified residues in Mb from beef LL. Differential occurrence of acetylation, methylation, dimethylation and trimethylation sites were detected in lysine (K) residues due to vitamin E supplementation. The unique PTMs in CONT Mb (K87, K96, K98 and K102) were spatially closer to proximal histidine compared to the unique PTM (K118) in Mb from VITE samples, and thus could be more detrimental to Mb redox stability due to the potential interference with proximal histidine. These findings indicated that vitamin E might protect Mb from PTMs, especially those spatially close to proximal histidine, and therefore improve Mb redox stability and beef color stability.

In summary, in-situ PTMs compromised Mb redox stability and beef color stability by adding modifying groups to amino acids, especially to those residues close to

hydrophobic heme pocket. Dietary supplementation of vitamin E might offer the antioxidant protection by minimizing the occurrence of PTMs at residues located spatially close to proximal histidine in Mb and thus contribute to improved beef color stability.

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VITA

Yifei Wang
University of Kentucky
Department of Animal and Food Science

EDUCATION

B.S. (2017) Food Science and Engineering, Sichuan University, China

PROFESSIONAL POSITIONS

Graduate Research Assistant (2017 – Present), Department of Animal and Food Sciences, University of Kentucky, Lexington, KY
Graduate Teaching Assistant (Spring 2019), Sensory Evaluation of Foods (FSC 430), Department of Animal and Food Sciences, University of Kentucky, Lexington, KY

AWARDS AND HONORS

2019 First Place, Proposal Division, 9th Annual Poster Symposium, Animal and Food Sciences Graduate Association, University of Kentucky

PUBLICATIONS

Peer-reviewed journal articles

1. Viana, F.M.; **Wang, Y.**; Li, S.; Conte-Junior, C.A.; Chen, J.; Zhu, H.; Suman, S.P. 2020. Thermal instability induced by 4-hydroxy-2-nonenal in beef myoglobin. *Meat and Muscle Biology*, 4(1): 18, 1–7. doi:10.22175/mmb.9479
2. Salim, A.P.A.; **Wang, Y.**; Li, S.; Conte-Junior, C.A.; Chen, J.; Zhu, H.; Rentfrow, G.; Suman, S.P. 2020. Sarcoplasmic proteome profile and internal color of beef longissimus lumborum steaks cooked to different endpoint temperatures. *Meat and Muscle Biology*, 4(1): 15, 1–15. doi:10.22175/mmb.9470
3. Xue, F.; **Wang, Y.**; Zuo, Y.; He, Q.; Sun, Q.; Zeng, W. 2017. The effects of different sterilization methods on bamboo shoots' microbial indicators and sensory qualities. *The Food Industry*, 02:54–58. (Published in Chinese)

Scientific abstracts, full-paper conference proceedings, and technical articles

1. Whalin, J.; Benjamin, D.; Choudhury, F.A.; Wu, Y.; **Wang, Y**.; Suman, S.P.; Shohet, J.L.; Richards, M.P. Hydroxyl radical footprinting of bovine myoglobin using plasma induced modification of biomolecules (PLIMB). 66th International Congress of Meat Science and Technology, August 2 – 7, 2020, Virtual meeting, USA, Abstract # 208.
2. Suman, S.P.; Rentfrow, G.; **Wang, Y.**; Nair, M.N. 2019. Muscle-specific color stability in fresh meats from ruminants. Meatingplace Online. http://www.meatingplace.com/Industry/TechnicalArticles/Details/87676
3. Nair, M.N.; Rentfrow, G.; Canto, A.C.V.C.S.; **Wang, Y.**; Li, S.; Joseph, P.; Ramanathan, R.; Hoffman, L.C.; Sun, Q.; Salim, A.P.A.; Conte-Junior, C.A.; Mancini, R.; Suman, S.P.

A global perspective on muscle-specific color stability in fresh meats. In proceedings of American Meat Science Association Annual Reciprocal Meat Conference, June 2019, Fort Collins, CO.

4. Salim, A.P.A.; Suman, S.P.; Li, S.; **Wang, Y.**; Chen, J.; Zhu, H.; Conte-Junior, C.A. Endpoint temperature influences sarcoplasmic proteome profile of cooked beef longissimus lumborum. American Meat Science Association Annual Reciprocal Meat Conference, June 23 – 26, 2019, Fort Collins, Colorado. Abstract # 141.
5. Viana, F.M.; Conte-Junior, C.A.; **Wang, Y.**; Li, S.; Chen, J.; Zhu, H.; Suman, S.P. Thermal stability of beef myoglobin is compromised by reactive lipid oxidation products. American Meat Science Association Annual Reciprocal Meat Conference, June 23 – 26, 2019, Fort Collins, Colorado. Abstract # 140.

Yifei Wang