Comparison of myoglobin, hemoglobin, and cytochrome *c* oxidation properties

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1. Introduction

Many factors influence the acceptability of meat products; however, meat color plays a major impact in purchasing decisions. With meat color being the most prominent sign of wholesomeness for the consumer, there is continuous pressure to understand the fundamental basis behind meat discoloration. Although myoglobin is the major protein contributes to meat color, hemoglobin and cytochrome *c* also influence meat color. Each of these proteins exhibits varying characteristics and attributes pertaining to meat discoloration.

Myoglobin is responsible for delivering oxygen to mitochondria to carry out various metabolic processes in living tissues (Suman & Poulson, 2013). However in meat, the protein has a major responsibility to provide color characteristics (Mancini & Ramanathan, 2008). Myoglobin consists of a centrally located iron atom surrounded amino acids. The central heme can coordinate with six bonds. Four of the bonds are connected to pyrrole groups, one to histidine and the remaining bond is available to bind to oxygen and other small ligands (Suman & Poulson, 2013). Myoglobin can be present in various redox forms. The important ones to distinguish between in this study are oxymyoglobin (OxyMb), deoxymyoglobin (DeoxyMb) and metmyoglobin (MetMb).

The hemoglobin is responsible for delivering oxygen to tissue cells from the lungs. This protein is also what gives oxygenated blood its bright red color and additionally plays a role in influencing meat color. The hemoglobin structure is similar to that of myoglobin except it contains four heme groups with each having the ability to bind oxygen (Shanmugraj et al., 2014). Conformational changes in the protein caused by attaching ligands are known to promote oxidation (Suman & Poulson, 2013). Hemoglobin can also be present in the same reduced and

oxidized forms of myoglobin, resulting in oxyhemoglobin (OxyHb), deoxyhemoglobin (DeoxyHb), and methemoglobin (MetHb).

Cytochrome *c* is an electron carrying mitochondrial protein that is extremely efficient in transporting electrons. The tertiary structure of cytochrome *c* consists of a single polypeptide chain with a covalently bonded heme ring (Salemme, 1977). Unlike myoglobin and hemoglobin proteins, cytochrome *c* does not have the 6^{th} position of the heme ring available to bind with ligands (Faustman et al., 1996). Mitochondria normally functions in aerobic conditions and are converted to anaerobic conditions when the animal is harvested (Ke et al., 2017). The restriction of oxygen causes a shift in homeostasis, affecting the ability of the mitochondria and cytochrome *c* to withstand oxidation. With this knowledge, the objective of this study was to examine the oxidative properties of myoglobin, hemoglobin, and cytochrome *c* to better understand the changes in meat color throughout a period of time.

2. Materials and methods

2.1 Preparation of reduced myoglobin, hemoglobin, and cytochrome *c*

Equine heart myoglobin, bovine hemoglobin, and bovine cytochrome *c* were used to study the differences in oxidation properties. Reduced forms of myoglobin, hemoglobin, and cytochrome *c* were prepared separately using a sodium phosphate buffer (0.15 mM). The three proteins were reduced by sodium hydrosulfite-mediated reduction. The remaining hydrosulfite was removed with the use of a PD- 10 column. Myoglobin concentration was confirmed using the absorbance 525 nm. The experiment was replicated three times under identical conditions.

2.2 Incubation

Oxymyoglobin, oxyhemoglobin, and reduced cytochrome *c* were incubated at 4 and 25 °C with the oxidation properties recorded at set time intervals for 5 days. The oxidation properties of the proteins incubated at 4°C were recorded on day 0, 1, 2, 3, 4, and 5, while the samples incubated at 25 °C were taken at 12-hour intervals for 96 hours. Samples were scanned spectrophotometrically at designated time intervals from 400 to 700 nm using a Shimadzu spectrophotometer.

2.3 Calculation of oxidation properties

All three proteins have different oxidation-reduction peaks. Calculations according to Tang et al. (2004) were used to determine the amount of reduced myoglobin present in the samples at each time and temperature designation to obtain absorbance readings. The equation used to determine the amount of reduced hemoglobin present in the samples was used according to Meng and Alayash (2017) at absorbance readings at 541, 560, 576, and 630 nm. The equation from Liu et al. (2017) was used to determine reduced cytochrome *c* from absorbance readings at 550 nm. Myoglobin and hemoglobin equations were used to compare the amount of proteins oxidized. All three proteins were compared using a ratio of the reduced form of protein to oxidized forms were calculated.

2.4 Analysis

The objective of the study was to determine the oxidative properties of myoglobin, hemoglobin and cytochrome *c*. It is well known that exposure to oxygen over a period of time promotes autoxidation of proteins. The mean values of total oxidation and reduction of the

proteins were used to compare oxidation properties. The mean percentages of remaining reduced forms of the proteins were subtracted from the starting amount of the reduced form of the proteins to calculate the total amount oxidized.

Samples were kept at 25 °C and the protein readings used for analysis were from T0 to T36 to determine oxidative values. For samples kept at 4 °C, the protein absorbance readings were taken from D0 to D4 to determine oxidation values. These intervals were chosen because possible contamination effecting results surpassed these times.

3. Results and discussion

Absorbance readings recorded for the proteins were used to calculate the reduced form present at the various time intervals and temperatures. Ratios of reduced to oxidized proteins were used to compare results. Reduced forms of myoglobin showed peaks at 582 nm and 542 nm, coinciding with other findings from Suman and Poulson (2013). OxyHb showed peaks at 541 nm and 576 nm that support Meng and Alayash (2017). Cytochrome *c* in the reduced form showed peak readings at 550 nm and 520 nm, which can be compared to results from Liu et al (2017). Results of ratios are presented in tables 1 and 2.

Myoglobin, hemoglobin and cytochrome *c* exhibited differences in oxidation properties following incubation at 4 and 25 °C with readings at every 12 and 24 hours. Myoglobin oxidized the quickest, followed by hemoglobin, and lastly cytochrome *c*. Samples kept at 25°C were used for absorbance readings every 12 hours for 96 hours. Myoglobin showed to oxidize 100% and showed to greatest difference between T0 and T36 ratios of OxyMb to MetMb at 2.3. Hemoglobin followed behind by oxidizing 63% and showing a difference of 1.5 within the 36

hour time interval. Cytochrome *c* oxidized the slowest at 25°C with the difference between T0 and T36 of the reduced to oxidized form ratio was 0.11.

Samples stored at 4°C had absorbance readings taken every day for 4 days. These samples showed less variance between readings throughout the three trials than those kept at 25°C. The amount of reduced myoglobin present decreased by 26% with the ratio of reduced protein to oxidized at 0.77. Hemoglobin followed behind with the amount of oxidation being 22% and a ratio of 0.44. Lastly, the reduced form of cytochrome *c* presented a ratio of 0.09.

Each protein oxidized as expected, due to protein structure and the availability to lose electrons. The primary structure of myoglobin directly affects its tertiary structure, which is responsible for the way it reacts to biomolecules and directly influences the functional abilities to transport oxygen and serve as the main pigmentation in meat (Suman & Poulson, 2013). While myoglobin is a monomeric protein, hemoglobin is a tetrameric protein meaning it consists of four polypeptide chains. The four polypeptide chains result in four heme binding sites available for oxygen to attach as opposed to the single binding site in myoglobin (Cai et al., 2016). Although myoglobin and hemoglobin are structurally related, their primary structures are very different from one another and contribute to the fact that myoglobin has a higher affinity for oxygen than hemoglobin and oxidizes more rapidly (Cai et al., 2016). It has additionally been found that bovine myoglobin autoxidizes 11- fold faster than bovine hemoglobin according to Yin et al. (2017).

Cytochrome *c* proved to be the slowest of the three proteins to undergo oxidation with the greatest amount of its reduced form remaining at the end of the studies at 25 and 4 °C. Studies have been conducted relating mitochondrial damage to changes in oxygen consumption and overall appearance of meat color. Further examination has suggested the damage of

mitochondria causes cytochrome *c* to shift from their position within the inner membrane to the sarcoplasm, causing lower color stability of fresh steak cuts (Ke et al., 2017).

Each of the proteins exhibits different oxidative properties as shown by the different rates in which electrons were lost from their reduced forms. Autoxidation of myoglobin and hemoglobin promote oxidative reactions causing other proteins to be converted to their ferric (Fe 2++) forms (Vandewalle & Peterson, 1987). Reduced myoglobin is more apt to undergo autoxidation than hemoglobin due to clear allosteric properties of hemoglobin as well as the slow dissociation of protoporphyrin in reduced forms of hemoglobin (Cai et al., 2016). Myoglobin and hemoglobin become oxidized when the ferrous form of iron loses an electron and causes a molecule of water to become the ligand attached to the heme as opposed to oxygen. Limited studies have been conducted demonstrating the oxidation rate of cytochrome *c* in relation to myoglobin and hemoglobin oxidation rates. Conclusions made by Rodkey and Ball (1950) infer that groups attached to cytochrome iron are more strongly linked than hemoglobin, making the release of an electron more difficult. Amino acid groups also have an effect on the oxidation of proteins. Reduced cytochrome *c* contains amino acid attachments that create a more hydrophobic heme environment. Histidine and methionine are responsible for this and make it more difficult for the heme to obtain a positive charge, meaning it has a higher affinity for reducing electrons (Salemme, 1977). Examining amino acid ligands and structures can elaborate on the structural importance of these proteins in relation to oxidation and reduction.

Possible pitfalls involving this research lie within the collection of myoglobin samples kept at 4°C in round 1 of 3. These samples showed signs of contamination at T24 and were left out of the calculated averages of reduced myoglobin remaining. This was done to keep data

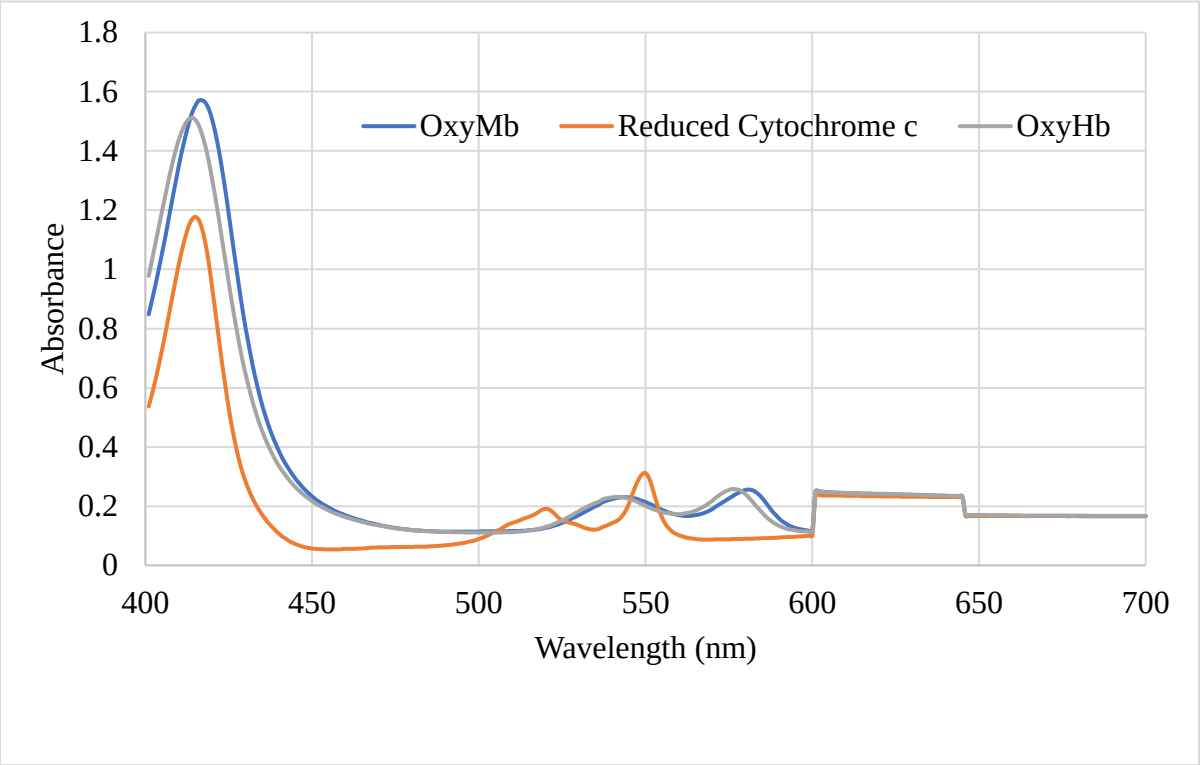
points effected by outside contamination from influencing the oxidation rates examined in the study.

Further studies should be conducted using beef extractions to analyze oxidation properties as they relate to a mixture of the three proteins. Little research has been done examining the effects of oxidation on myoglobin, hemoglobin and cytochrome *c* on each other. Future research opportunities should be used to examine the way proteins influence oxidation rates of one another and how they impact stability and meat color in the retail case.

4. Conclusions

Myoglobin, hemoglobin and cytochrome *c* are the primary proteins most associated with meat color. Oxidation of these proteins can have negative effects on color acceptability and overall appearance of fresh meat products. Understanding the ways in which protein oxidizes allows for further insight into how to limit the formation of metmyoglobin, methemoglobin and oxidized cytochrome *c*. The protein most abundant and most susceptible to oxidation is myoglobin, followed by the second most prevalent and prone to oxidation, hemoglobin, and lastly the protein present in the smallest amount and the slowest to oxidize was cytochrome *c*. Structural characteristics of the proteins play a role in their ability to resist oxidation. Further, understanding the factors affecting oxidation in meat products and enhance color stability while preventing product loss in the meat industry.

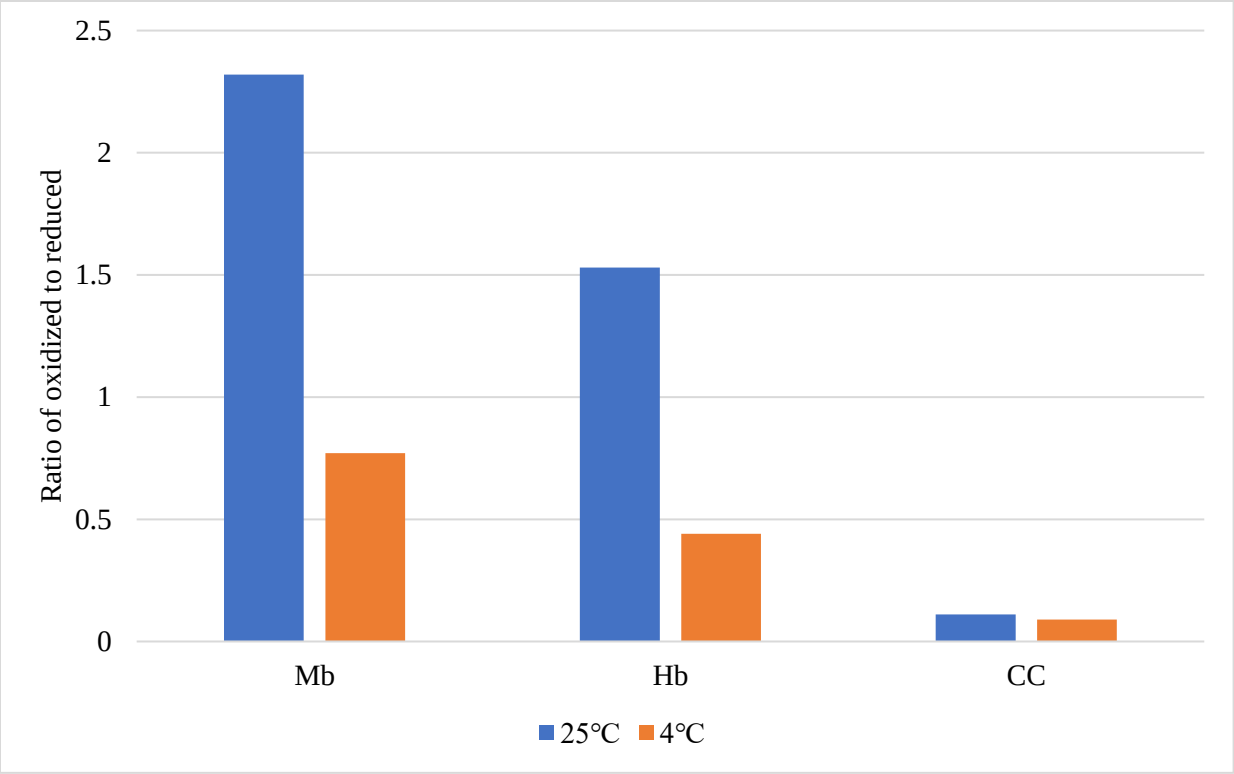
Figure 1



The absorbance spectra of reduced forms of myoglobin, hemoglobin and cytochrome *c*.

The wavelength is represented on the x-axis and absorbance on the y-axis.

Figure 2



The ratio of reduced form of protein to oxidized form at 25°C and 4°C. Values are the difference of ratios between T0 and T36 for 25°C and from D0 to D4 for 4°C.

Table 1

Ratio of oxidized form and reduced form of three different proteins incubated at 25 °C.

	25°C	% oxidized
Myoglobin	2.3	100%
Hemoglobin	1.5	63%
Cytochrome *c*	0.11	N/A

Table 2

Ratio of oxidized form and reduced form of three different proteins incubated at 4 °C.

	4°C	% oxidized
Myoglobin	0.77	26%
Hemoglobin	0.44	22%
Cytochrome *c*	0.09	N/A

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