Effect of Freezing and Microbial Growth on Myoglobin Derivatives of Beef

Mheni Ben Abdallah, John A. Marchello,* and Hamdi A. Ahmad

Department of Animal Sciences, University of Arizona, Tucson, Arizona 85721

The effect of freezing and bacterial growth on the discoloration of beef was assessed by measuring myoglobin derivatives myoglobin (MB), oxymyoglobin (MBO_2), and metmyoglobin (METMB) on the surfaces of fresh and frozen−thawed packaged beef cuts stored at 2 °C and analyzed after 0, 3, 6, 9, and 12 days of storage. MB, MBO_2, and METMB concentrations were measured spectrophotometrically. Frozen−thawed beef samples experienced less "blooming" (conversion of MB to MBO_2) and more rapid discoloration than fresh cuts during storage. By day 3, >20% METMB was formed in the frozen−thawed samples, whereas the fresh samples reached this value after day 6 of storage. The rates of MB oxidation were similar ($P > 0.05$) for sterile and frozen−thawed inoculated (*Pseudomonas fluorescens* at a rate of 1.5 colony forming units/cm^2·cm^2 area) samples from day 0 through day 6 of storage. For storage periods of less than a week, bacterial growth is not a major cause of meat discoloration. After day 6, the high bacterial growth rate resulted in a rapid increase in METMB formation. Possible mechanisms for MB oxidation in frozen−thawed beef are suggested.

Keywords: *Myoglobin; beef color; packaging; microbial*

INTRODUCTION

Increased consumer demand for safe, nutritious, and shelf-stable meat and meat products has driven the search for improved techniques to reduce quality loss during meat distribution and storage. With the strong trend toward centralized packaging and distribution of consumer-sized packages to the retail outlets (Tomioka, 1990), the color of meat becomes a very important factor influencing consumer acceptability and retail shelf life (Seideman et al., 1984). The consumer relates the color of meat to freshness and uses color as a quality standard (Pirko and Ayres, 1957; Seideman et al., 1984; Kropf, 1986; Renerre, 1990). Thus, for a retail meat distribution system such as central packaging to function properly, it is necessary to maintain the color at a desirable level (Seideman et al., 1984; Kropf, 1986). The overall redness of meat is largely governed by myoglobin and to a lesser extent by hemoglobin as well as the forms in which they exist. In a well-bled animal, the skeletal muscle has ≥90% of the total iron accounted for as myoglobin (Pegg and Shahidi, 1997).

Meat color is determined by the relative amounts of three derivatives of myoglobin: reduced myoglobin or myoglobin (MB) is purple, which is the predominant muscle color in the absence of O_2; oxymyoglobin (MBO_2), the oxygenated form of MB, is bright red; metmyoglobin (METMB), the oxidized form of MB, is brown (Hunt and Kropf, 1987; Cornforth, 1994). The brown color of METMB tends to turn the consumer away (Renerre, 1990).

A number of factors have been shown to cause the oxidation of myoglobin in prepackaged beef (Renerre, 1990; Madhavi and Carpenter, 1993). These include the type of muscle (Ledward, 1971; Hood, 1980; Renerre, 1984), storage temperature (Walters, 1975), oxygen pressure and modified atmosphere packaging (Bartkowski, et al., 1982; Okayoma, 1987; Young et al., 1988; Lamine, 1991; Luno et al., 1998) bacterial contamination (Robach and Costilow, 1961; Bala et al., 1977a,b; Ben Abdallah, 1992), packaging film permeability to gases (Pirko and Ayres, 1957; Ben Abdallah, 1992), and lipid oxidation (Greene, 1969; LaBrake and Fung, 1992; Gatellier et al., 1995; Chan et al., 1997).

Certain processes performed prior to packaging affect the color of the packaged meat cuts. Bevilacqua and Zaritzky (1986) found that vacuum aging before retail packaging significantly affects the formation of METMB in aerobically packaged meat. Moreover, Cheah and Ledward (1997) found that the degree of freshness of beef determines the success of inhibition of metmyoglobin formation.

Although freezing of meat offers a product with nutritional quality closest to that of fresh meat compared to other processes, it may significantly affect the organoleptic properties of meat such as color (Lanarie et al., 1993).

The objectives of the present study were (1) to determine the effect of freezing and thawing on the rate of METMB formation of packaged consumer retail frozen−thawed beef cuts compared to fresh during 12 days of storage and (2) to compare the influence of bacterial growth versus freezing and thawing on the time of storage and the magnitude of changes in the MB derivatives of packaged consumer retail beef cuts during 12 days storage.

MATERIALS AND METHODS

Sample Preparation. Cuts from frozen−thawed semimembranosus muscles (top round) were compared to fresh, unfrozen cuts from the same muscle. Steers weighing 450−500 kg were selected to provide the muscle cuts for this

* Address correspondence to this author at The University of Arizona Meat Laboratory, 4181 N. Campbell Ave., Tucson, AZ 85719 [telephone (520) 621-3951; fax (502) 621-9888; e-mail jam@ag.arizona.edu].

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experiment. After slaughter, the carcass was chilled to 5 °C, and after 4−6 days post-mortem, the semimembranosus muscle was removed. The fat was trimmed from each top round, and each cut was vacuum packaged. Those muscles that were selected for freezing and thawing were quick frozen at −30° C and stored at −18 °C for 3 days and thawed for 48 h at 5 °C in the dark. The fresh top rounds were stored at 5 °C in the dark.

Round cores with a surface area of 25 cm^2 and a thickness of 5 cm were removed from each muscle using a stainless steel corer, which was sterilized by dipping in 95% ethanol and flaming after each core was removed.

Packaging and Storage. The vials used for packaging the meat cores were straight-sided polyethylene cylindrical jars (Nalgene Co., Rochester, NY) with a 6 cm base diameter and a height of 6 cm. A specially designed fastening lid with a uniform hole 6 cm in diameter held the packaging film in place. The film used was a gas and moisture barrier (ethylene vinyl chloride copolymer, EVCC; W. R. Grace Co.). This film has an O_2 transmission rate of 0.0035 $cm^3/cm^2/24$ h and a CO_2 transmission rate of 0.0250 $cm^3/cm^2/24$ h.

Sample inoculation and packaging were done in a laminar flow hood (Forma Scientific) sterilized by both ultraviolet light and 70% ethanol. The cutting board was sterilized by 70% ethanol and exposed to the ultraviolet light overnight prior to the start of the experiment. Aseptic techniques were used throughout the sample preparation and packaging. The knives were cleaned and sanitized before each experiment. They were rinsed with distilled water and dipped in 95% ethanol and flamed before and after each cut. All of the vials and their lids were autoclaved on fast exhaust for 20 min at 121 °C and 1.3 kg/cm^2 pressure. The packaging films were sterilized by dipping them into 70% ethanol and exposing them to ultraviolet light overnight.

Prior to packaging, the outsides of the meat cores were sterilized by dipping them individually into boiling water for 5 s. This was to avoid any uncontrolled microbial contamination that could have occurred. Each sterile meat core was sliced longitudinally into halves, exposing two freshly cut muscle surfaces. The meat steaks obtained had an area of 25 cm^2 and a thickness of 2.5 cm. Samples that were judged visually similar at the exposed surfaces and as far as possible free from fat were used for the experiments. Meat cores for fresh treatment (20 samples) and for the frozen−thawed (20 samples) treatment were randomly placed in the sterile vials.

After the meat samples had been loaded into the vials, sterile film was placed across the vial mouths and the lids were screwed tight to hold the membrane in place and make the vial airtight. The closed vials were stored at 2 °C in the dark until the specific day of sampling.

Inoculation and Microbiological Analysis. Pure culture of *Pseudomonas fluorescens* was obtained from the Department of Microbiology (University of Arizona). Fresh suspensions were prepared in a sterile nutrient broth (Difco Laboratories, Detroit, MI). After 24 h of incubation at 28 °C, the bacterial cells were centrifuged at 5000 rpm for 10 min. The harvested cells were resuspended in sterile deionized water for meat inoculation. The inoculation on the meat surface was performed using a sterile needle (25 G $1^1/_2$) and 1 cm^3 syringe. A well-shaken 1 mL dilution of the culture was uniformly distributed over the surface of the meat cuts (25 cm^2 area). The purity was ensured by studying the morphology of the bacterial dilution used for inoculation under light microscopy. Gram stain was applied to a dry and heat-fixed smear of that bacterial dilution to determine the uniformity of our inoculum (Murray, 1981).

Sterile meat cores assigned to bacterial growth treatments (20 samples) were inoculated on the exposed surface with *P. fluorescens* at a rate of $\sim 1.5 \times 10^4/cm^2$ microbes. Vials were loaded with inoculated samples, covered with sterile film, and capped. Count colony method was used in this study to estimate the microbial population at the meat surface. The result was reported an the numbers of colony forming units (CFU) per square centimeter (Speck, 1984).

Sampling for microbiological analysis was carried out by the swab contact method (Speck, 1984). A sterile, moistened, 15.2 cm calcium alginate swab on a wood shaft was rubbed thoroughly over the designated surface area of 3.8 cm^2 defined by a sterile aluminum template. This process was repeated once, after which the swab tip was broken off in the dilution tube. The sample tube was then shaken vigorously, and appropriate serial dilutions were made with sterile 0.1% peptone. The dilutions prepared were then plated to evaluate the number CFUs in the samples using the pour plate technique. The Petri dishes were incubated at 28 °C for 48 h. The colonies were counted using a Quebec colony counter. Plates containing a range of 25−250 colonies were selected for enumerating the bacteria in the samples (Speck, 1984).

Myoglobin Derivative Fraction Measurement. A Beckman Model DK-2A ratio recording spectrophotometer attached to a single-board BCC-52 computer and an interface circuit was used as the system for reflectance measurement and recording of data. The spectrophotometer was calibrated against 10 certified standards having reflectances ranging from 2 to 80% (Labsphere, Inc., North Sutton, NH). To establish a calibration curve, the output of each standard was recorded at four different wavelengths, 450, 500, 550, and 600 nm. Data were electronically transferred to a digital computer for regression analysis (Jordan et al., 1991) The standards were the independent variables, and the instrument outputs were the dependent variables.

The percentages of MB, MBO_2, and METMB were determined on the meat disks using the Dean and Ball (1960) method. Two samples were taken from each core, placed on a special sample holder, and directly loaded into the spectrophotometer. Reflectance was determined against a white of barium sulfate at wavelengths of 473, 507, 573, and 597 nm. From each value of percent reflectance, the corresponding K/S was obtained using tables given by Judd (1952). Two ratios were calculated:

$$\text{ratio 1} = \frac{\frac{K}{S} \text{ at 507 nm}}{\frac{K}{S} \text{ at 573 nm}}$$

$$\text{ratio 2} = \frac{\frac{K}{S} \text{ at 473 nm}}{\frac{K}{S} \text{ at 597 nm}}$$

The percentages of METMB and MB were determined by ratios 1 and 2, respectively, using the plots given by Broumand et al. (1958). The percentage of MBO_2 was calculated using the following equation:

$$\% \text{ MBO}_2 = 100 - (\% \text{ METMB} + \% \text{ MB})$$

Measurement of pH. A sample of 2 g from the top 5 mm of the disks was used for measurement. The meat samples were blended for 1 min with 10 mL of distilled water at low speed. The pH was determined using an Orion Model 720 pH meter.

Statistical Analysis. Each treatment was replicated twice using meat from the same animal. Following an analysis of variance, significant differences between treatments were identified (for each sampling day) by the least significant differences (LSD) method at a 5% level of significance (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Frozen−Thawed Sterile Meat versus Fresh Sterile Meat. Sterile beef cores that were not inoculated had no microbial growth through the 12 days of storage at 2 °C. The frozen−thawed meat samples experienced less blooming (conversion of MB to MBO_2 during the

Table 1. Mean Percentages of MB, MBO_2, and METMB of Sterile Fresh versus Sterile Frozen−Thawed Beef by Day of Storage at 2 °C

day of sampling	treatment	% MB	% MBO_2	% METMB
0	sterile fresh	13.86	86.15	0.00[a]
	sterile thawed	20.77	74.39	4.85[b]
3	sterile fresh	2.09	97.92[a]	0.00[a]
	sterile thawed	14.36	60.88[b]	24.77[b]
6	sterile fresh	9.72	71.18[a]	19.11[a]
	sterile thawed	23.66	40.89[b]	35.46[b]
9	sterile fresh	7.22	56.82[a]	35.97[a]
	sterile thawed	22.65	24.76[b]	52.60[b]
12	sterile fresh	17.34	11.55	71.12
	sterile thawed	19.98	14.66	65.37

[a,b] Means within a sampling day for each myoglobin derivative with unlike superscripts were significantly different ($P < 0.05$).

initial period of storage) than did the fresh samples (Table 1). The reduced "bloom" in the initial period of storage for the frozen−thawed meat samples may be due to the denaturation of the globin moiety of the MB molecule during freezing, since Calvelo (1981) reported that during frozen storage protein denaturation, recrystallization, oxidation and moisture sublimation take place.

The sterile frozen−thawed meat samples reached 24% METMB level at day 3 and 36% at day 6, whereas the fresh sterile was still <20% METMB (19%) at day 6 and 35% at day 9 of storage (Table 1). When bright red beef and discolored beef are sold together, shopper discrimination against the discolored meat increases with the increase in METMB content (Hood and Riordan, 1973). Arnold et al. (1992) reported that on beef steaks, <20% MEMB was visually detectable discoloration. The ratio of sales of discolored beef to bright red beef is approximately 1:2 when 20% METMB is present in the discolored lot (Hood and Riordan, 1973). Furthermore, consumers will not purchase beef containing over 40% METMB on its surface (Kropf, 1986). Consequently, the frozen−thawed beef had a <3 day shelf life for color stability versus the unfrozen beef.

From a comparison of the results of sterile frozen−thawed and sterile fresh meat samples (Table 1), it can be seen that the proportion of METMB in the sterile frozen−thawed samples was significantly higher initially ($P < 0.05$) than that of the sterile fresh meat samples. After day 0, for the frozen−thawed samples, the percentage of METMB was higher ($P < 0.05$) and the percentage of oxymyoglobin was lower ($P < 0.05$) than those of the fresh, sterile samples (Table 1) through day 9. These results agree with the observation of Marriott et al. (1980), who reported that the color of thawed beef and pork deteriorated after 24 h of storage at 4 °C. At day 12, the concentration of METMB exceeded 60% for both treatments. The sterile fresh samples had 71.12% METMB, and the sterile thawed samples had 65.37% METMB. However, the difference was not significant ($P > 0.05$) (Table 1). This high METMB formation at day 12 for the sterile fresh meat may be explained by the longer storage time (beyond 9 days) during which MB undergoes autoxidation and METMB accumulated rapidly. Moreover, by 12 days of storage, it is possible that the fresh sterile beef samples had lost MRA and/or NAD.

The effects of freezing or freezing and thawing on beef discoloration were investigated (MacDougall, 1982; Lanari et al., 1989; Lanari and Zaritzky, 1992). MacDougall (1982) reported that the color of beef remained attractive for 2 months in the dark but for only 3 days in the light. Lanari et al. (1989) found that frozen−thawed samples had higher METMB concentrations compared to unfrozen beef samples refrigerated at 4 °C for 24 h. They investigated the kinetics of pigment autoxidations at different freezing temperatures, but they did not continue the comparison for additional storage days for unfrozen versus frozen−thawed. Repeated freeze−thaw cycles caused discoloration of semimenbranous muscle, and the effect was pronounced for control as compared with vitamin E-supplemented animals (Lanari et al., 1993).

Autoxidation involves a nonenzymatic spontaneous oxidation by free O_2, which determines the rate of discoloration (Giddings, 1974). Chow (1987) found that freezing at −20 °C and thawing in air at 4 °C caused autoxidation and insolubilization of MB reflected by accumualtion of 90−100% METMB. Autoxidation caused by the freezing and thawing is another possible explanation of fast discoloration in the frozen−thawed samples. Chu et al. (1987) proposed that the rapid increase in METMB of frozen beef stored at freezing−thawing transition phase (−2 °C) was probably due to ice crystal formation and cell distruption; such modifications allow catalysts of oxidative reactions to come into contact with MB and lipids. Fennema (1975) suggested that acceleration of reactions during freezing could be explained on the basis of concentration of reactants in the unfrozen phases during freezing. Lanari et al. (1989) stated that when meat is stored between −5 °C and −15 °C, the Fennema (1975) theory is a reasonable explanation for the METMB increase.

An enzymatic reduction system of METMB may exist in muscle according to Livingston and Brown (1981) and Arihara et al. (1995). After MB has been oxidized to METMB, an enzyme system in muscle with reducing ability, named the metmyoglobin reducing activity (MRA), can reduce METMB back to MB (purplish red form) (Kropf, 1986). In fact, Hagler et al. (1979) purified and characterized the first bovine muscle METMB reductase from heart muscle. The enzyme is classified as NADH-cytochrome b_5 reductase (Livingston et al., 1985). In addition, Arihara et al. (1997) found that muscle contains ~59 ± 20.9 μg/g of tissue of NADH cytochrome b_5 reductase. Cytochrome b_5 is an electron mediator, and its fate during freezing and thawing is not known. Therefore, it is possible that the freeze−thaw treatment could result in losses of MRA and/or the oxidation of NADH in the muscle. When meat is aerobically stored under refrigeration, most of the surface MB is in the MBO_2 form, which is continually deoxygenated and oxidized to METMB. If the meat is fresh, representing high MRA, the METMB thus formed is often reduced back to deoxymyoglobin (MB) and immediately oxygenated by the available oxygen in the headspace of the package. However, if the meat is not fresh and has lost MRA, METMB is constantly formed and will not be reduced back to deoxymyoglobin; thus, the percentage of METMB will increase very quickly with storage time. This could explain the rapid accumulation of METMB in the frozen−thawed beef cuts. Bevilaqua and Zaritsky (1986) proposed that meat color deterioration of aged meat is due to the loss of MRA in the tissue and the denaturation of the globin fraction of MB with time; however, there are no data supporting

this hypothesis. Giddings (1974) suggested that the loss of MRA in post-rigor muscle is due to many factors. Some of these factors are depletion and/or degradation of substances and cofactors and, ultimately, complete loss of mitochondrial structure integrity and functionality. Kariya et al. (1997) indicated that UDP-3 or UDP-4 ketoglucosamine reduces metmyoglobin in bovine cardiac muscle. Furthermore, these authors emphasize that this reducing activity depends on (NAD)(P)H.

Scope and Newbold (1968) reported that freeze−thawing accelerates the loss of NAD from muscle. The fast accumulation of METMB in thawed−frozen meat can be due to the fact that the METMB formed will no longer be reduced back to myoglobin because the reducing intermediates, particularly NADH, are no longer formed (O'Keefe and Hood, 1982). The freezing and thawing of meat result in the release of the enzyme β-hydroxyacyl CoA-dehydrogenase (HADH) from the mitochondrion into the sarcoplasm (Chen et al., 1989). The activity of this enzyme will deplete NADH to form hydroxybutyryl-COA as follows:

$$\text{acetoacetyl-COA} + \text{NADH}+\text{H}^+ \xrightarrow{\text{HADH}} \text{hydroxybutyryl-COA} + \text{NAD}^+$$

Therefore, the cofactors NADH + H^+ will be no longer be available for the METMB reducing enzymes. Consequently, the MRA will diminish considerably, allowing the accumulation of METMB in the surface of beef cuts. Ledward (1985) indicated that the activity of the METMB reducing system was the most important factor in the color stability of meat. Reddy and Carpenter (1991) found that very stable muscles, tensor faciae latae and longissimus dorsi, possess the highest MRA In contrast, O'Keefe and Hood (1982) and Renerre and Labas (1987) found no correlations between the MRA and color stability of various muscles. Echevarne et al. (1990) reported that the most unstable muscle from the color point of view (psoas major) presented the highest MRA and concluded that MRA lacks effectiveness in color stability regulation. On the other hand, Hadhavi and Carpenter (1993) found that psoas major steaks had greater metmyoglobin accumulation and lower MRA. Nonenzymatic reduction of METMB in beef could also occur (Renerre, 1990). Ledward (1972) reported that the aerobic reducing ability (ARA), which measures total METMB reduction (enzymatic and nonenzymatic), was better correlated with color stability as compared to MRA alone. The Ledward (1972) technique avoids the use of chemical oxidants and would appear to yield more reliable results for reduction measurement. Nonenzymatic reduction of METMB can be accomplished by NADH (NADPH), (Livingston and Brown, 1981) and vitamins C and E (Faustman and Cassens, 1990). Lipid and pigment oxidations in beef are interrelated (Faustmann, 1998). METMB can be activated with hydrogen peroxide (H_2O_2) to generate an active site for lipid oxidation (Kanner et al., 1987). Arnold et al. (1992) concluded that dietary suplementation of Holstein steers with vitamin E produced considerable improvement in lipid and color stablity of fresh retail cuts. Lanari et al. (1993) conducted similar experiments by using frozen beef after several thaw cycles; they reported that repeated freeze−thaw cycles caused fading of semimembranosus muscle color and the effect was more pronounced in meat from non-vitamin E-supplemented cattle. Thus, retarding the lipid oxidation resulted in a similar delay in metmyoglobin formation both in fresh and in frozen beef. In the present study, the sterile fresh and the sterile frozen−thawed beef originated from the semimenbranous muscle of the same animal, which was not suplemented with vitamin E, nor were the meat samples treated with antioxidant. However, it is possible that biochemical changes on endogenous antioxidants and/or cofactors caused by the freeze−thaw process could diminish nonenzymatic reduction of METMB, provoking MB to be oxidized at a faster rate for the frozen−thawed samples relative to the fresh samples.

Table 2. Mean Percentages of MB, MBO_2, and METMB of Sterile Frozen−Thawed versus Inoculated Frozen−Thawed Beef by Day of Storage at 2 °C

day of sampling	treatment	% MB	% MBO_2	% METMB
0	sterile	20.77	74.39	4.85
	inoculated	12.48	84.78	2.74
3	sterile	14.36	60.88	24.77
	inoculated	16.49	62.53	20.99
6	sterile	23.66	40.89	35.46
	inoculated	19.09	50.74	29.45
9	sterile	22.65	24.76[a]	52.60[a]
	inoculated	23.60	11.18[b]	65.23[b]
12	sterile	19.98	14.66[a]	65.37[a]
	inoculated	20.89	0.35[b]	78.77[b]

[a,b] Means within a sampling day for each myoglobin derivative with unlike superscripts were significantly different ($P < 0.05$).

Inoculated Frozen−Thawed Meat versus Sterile Frozen−Thawed Meat. Analysis of variance demonstrated that the rates of MB oxidation are similar ($P > 0.05$) for sterile verses inoculated frozen−thawed samples from day 0 up to day 6 of storage (Table 2). Samples of both treatments are characterized by fast discoloration and accumulation of 20% METMB by day 3 (Table 2). Samples of both inoculated and sterile frozen−thawed meat reached ~30% METMB by day 6 of storage. By day 9 of storage the inoculated frozen−thawed samples had a higher METMB concentration ($P < 0.05$) and a lower MBO_2 concentration than the frozen−thawed sterile control. This was true through day 12 ($P < 0.05$). At day 12, METMB reached 80% and the concentration of MBO_2 almost disappeared. However, for the frozen−thawed sterile samples, the concentration of METMB reached only 65% and ~15% percent of MBO_2 remained on the beef sample surface. After day 6 of storage, the higher rate of METMB accumulation in the inoculated samples can be attributed to microbial growth (Table 2). During the initial 6 days of storage, *P. fluorescens* was still in the lag phase and there was no increase in the bacterial numbers during this period (Figure 1). Within the second period (consisting of days 6−12), exponential growth occurred and the cell numbers reached 10^8 CFU/cm^2 by day 12 (Figure 1). During this period, the *P. fluorescens* cell numbers increase rapidly (Figure 1), thereby lowering O_2 availability on the surface of meat and thus enhancing METMB formation. According to George and Stratmann (1952) and Ledward (1970), low O_2 partial pressure favors oxidation of MBO_2 to METMB. For fresh inoculated meat the data show that when the bacteria did not enter an exponential growth phase, contribution of discoloration by bacterial growth was not significant. The results of this experiment showed that the fast discoloration (within the second to third days) of both inoculated and sterile frozen−thawed meat samples was not due to the reduc-

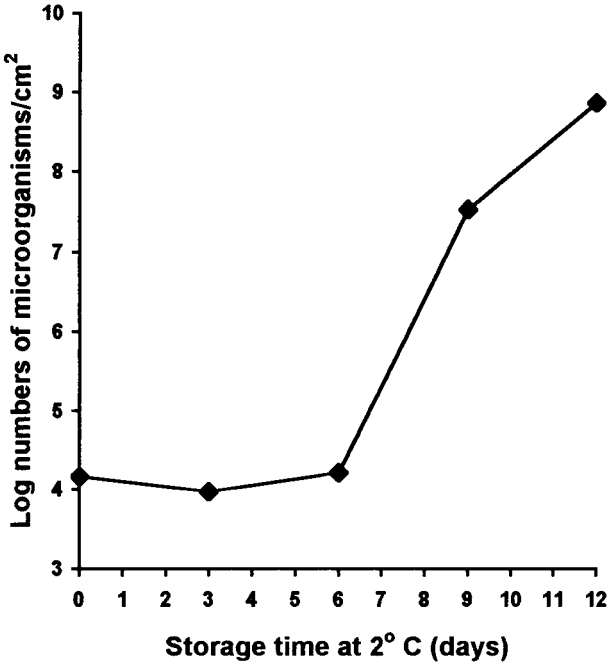

Figure 1. Means of log numbers of microorganisms in inoculated sterile frozen−thawed beef cuts.

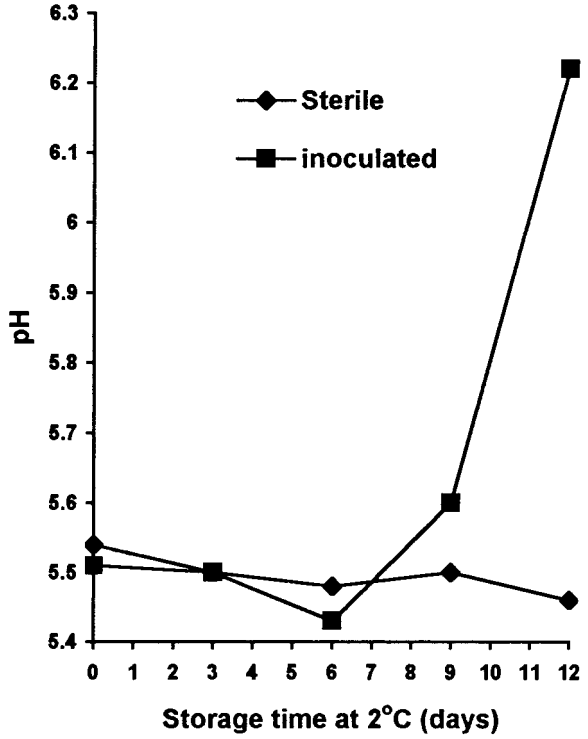

Figure 2. Mean values of pH of sterile frozen−thawed versus inoculated sterile frozen−thawed beef cuts.

tion of the oxygen tension on the meat surface but rather to myoglobin oxidation caused by the freeze−thaw treatment.

Results from the present study demonstrated that for ~1 week of storage, the effect of bacterial growth is not important in the discoloration of beef cuts. Frozen−thawed meat discolored rapidly because of the rapid oxidation of the myoglobin molecule due to freezing and thawing. The packaging procedure used cannot improve the color quality of the meat cuts. However, it can delay color deterioration for a time, which depends on the product packaged. Therefore, if the meat cuts already have some meat color change or loss of the MRA, and/or cofactors, the packaging procedure will not be effective in retaining the desired meat color.

Change in pH during Storage. The pH of the sterile samples remained constant throughout the 12 days of storage (Figure 2). Similar results were reported by Bala et al. (1977a,b) and Ockerman et al. (1969). The pH of the inoculated frozen−thawed beef samples increased from 5.52 to 6.22 during the 12 days of storage (Figure 2). When *Pseudomonas* species grow on the meat surface, they preferentially utilize glucose as substrate for growth (Jacoby, 1964). However, when glucose is exhausted, they attack amino acids (Gill, 1986). Proteolytic enzymes are produced only in the late exponential phase of growth (Gill and Penny, 1977). This usually takes place when the bacterial cell density reaches 10^8 cells/cm^2 (Gill, 1986). The increase of pH of the inoculated samples indicates that the *P. fluorescens* bacteria start degrading protein because glucose on the surface of the meat is no longer available for bacterial growth. These results agree with Bala et al. (1977a), who found that the pH increase in inoculated beef samples was due to the *P. fragi* breakdown of meat proteins. The ultimate pH of normal uninoculated meat is ~5.4−5.8 (Faustman and Cassens, 1990). The meat used in this study had a normal pH (Figure 2). Meat of high ultimate pH (>5.8) is more color stable than meat with normal pH (Renerre, 1990). The ultimate pH of meat is fairly constant for normal slaughtering practice. This normal pH is not critical for meat color consideration (Faustman and Cassens, 1990). Because microbial growth increased the pH of the inoculated sterile frozen−thawed meat, the higher percentage of METMB in the inoculated relative to the uninoclated samples was likely attributed to the bacterial growth rather than the increase of pH.

ABBREVIATIONS USED

MB, myoglobin; MBO_2, oxymyoglobin; METMB, metmyoglobin; MW, molecular weight; RPM, revolutions per minute; 25G, 25 gauge; CFU, colony forming units; *K*/*S*, *K* is the absorption coefficient and *S* is the scattering coefficient; LSD, least significant difference; MRA, myoglobin reducing activity; UDP-3 or UDP-4, uridine 5-diphosphate keto-D-glucose; NAD(P)H, nicotinamide adenine dinucleotide phosphate reduced form; HADH, β-hydroxyacyl CoA-dehydrogenase; NAD^+, nicotinamide adenine nucleotide oxidized form; NADH, nicotinamide adenine dinucleotide reduced form; ARA, aerobic reduction activity.

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LITERATURE CITED

Arihara, K.; Cassens, R. G.; Greaser, M. L.; Luchansky, J. B.; Mozdiak, P. E. Localization of metmyoglobin-reducing enzyme (NADH-cytochrome b5 reductase) system components in bovine skeletal muscle. *Meat Sci.* **1995**, *39* (2), 205−213.

Arihara, K.; Itoh, M.; Kondo, Y. Quantification of NADH-cytochrome b5 reductase (metmyoglobin-reducing enzyme) in bovine skeletal muscle by an immunoblotting assay. *Anim. Sci. Technol.* **1997**, *68*, 29−33.

Arnold, R. N.; Scheller, K. K.; Arp, S. C.; Williams, S. N.; Schaefer, D. M. Visual and spectrophotometric evaluations of beef color stabilty. *J. Food Sci.* **1992**, *57*, 518−520.

Bala, K.; Marshall, R. T.; Stringer, W. C.; Naumann, H. D. Effect of *Pseudomonas fragi* on the color of beef. *J. Food Sci.* **1977a**, *42*, 1176−1179.

Bala, K.; Marshall, R. T.; Stringer, W. C.; Naumann, H. D. Changes of color of aqueous beef extract caused by *Pseudomonas fragi*. *J. Food Prot.* **1977b**, *40*, 824−827.

Bartkowoski, L.; Dryden, F. D.; Marchello, J. A. Quality change of beef steaks stored in controlled gas atmosphere containing high or low levels of oxygen. *J. Food Prot.* **1982**, *45*, 41−45.

Ben Abdallah, M. Some Factors Influencing Myoglobin Derivatives on Refrigerated Packaged Beef. M.S. Thesis. University of Arizona, Tucson, AZ, 1992.

Bevialqua, A. E.; Zaritzky, N. E. Rate of pigment modifications in packaged refrigerated beef using reflectance spectrophotometery. *J. Food Process. Preserv.* **1986**, *10*, 1−18.

Calvelo, A. Recent studies on meat freezing. In *Developments in Meat Science*; Lawrie, R., Ed.; Applied Science Publishers: Englewood, NJ, 1981.

Chan, W. K. M.; Faustman, C.; Renerre, M. Model systems for studying pigment and lipid oxidation relevant to muscle-based foods. In *Natural Antioxidants. Chemistry, Health Effects, and Applications*; AOCS Press: Champaign, IL, 1997; pp 319−330.

Cheah, P. B.; Ledward D. A. Inhibition of metmyoglobin formation in fresh beef by pressure treatment. *Meat Sci.* **1997**, *45*, 411−718.

Chen, M. T.; Yang, W. D.; Go, S. L. Differentiation between fresh beef and thawed frozen beef. *Meat Sci.* **1989**, *24*, 223−226.

Chow, C. J.; Ochiai, Y.; Watabe, S.; Hashimoto, K. Autoxidation of bluefin tuna myoglobin associated with freezing and thawing. *J. Food Sci.* **1987**, *52*, 138−140.

Chu, Y. H.; Huffman, G. R.; Trout, G. R.; Egbert, W. R. Color and color stability of frozen restructured beef steaks: Effect of sodium chloride, tripolyphosphate, nitrogen atmosphere and processing procedure. *J. Food Sci.* **1987**, *52*, 869−875

Cole, A. B., Jr. Retail packaging systems for fresh meat cuts. *Reciprocal Meat Conference*; National Live Stock and Meat Board: Chicago, IL, 1986; Vol. 39, pp 106−111.

Cornforth, D. Color: its basis and importance. *Adv. Meat Res.* **1994**, *9*, 34−78.

Dean, R. W.; Ball, C. O. Analysis of the myoglobin fractions on the surfaces of beef cuts. *Food Technol.* **1960**, *14*, 271−286.

Echevarne, C.; Renerre, M.; Labas, R. Metmyoglobin reductase activity in bovine muscles. *Meat Sci.* **1990**, *27*, 161−172.

Eskin, N. A. M. Biochemical changes in raw foods. Meat and Fish. In *Biochemistry of Foods*; Eskin, N. A. M., Ed.; Academic Press: San Diego, CA, 1990.

Faustman, C. Beef Color Update: The Role for Vitamin E. *J. Anim. Sci.* **1998**, *76*, 1019−1026.

Faustman, C.; Cassens, R. G. The biochemical basis for discoloration in fresh meat. A review. *J. Musc. Food* **1990**, *1*, 217−243.

Fennema, O. R. Reaction kinetic in partially frozen aqueous systems. In *Water Relations in Foods*; Duckworth, R. B., Ed.; Academic Press: London, U.K., 1975; pp 440−455.

Gatellier, P.; Anton, M.; Renerre, M. Lipid peroxidation induced by H_2O_2-activated metmyoglobin and detection of a myoglobin-derived radical. *J. Agric. Food Chem.* **1995**, *43*, 651−656.

George, P.; Stratmann, C. J. The oxidation of myoglobin to metmyoglobin by oxygen. 2. The relation between the first-order rate constant and the partial pressure of oxygen. *Biochem. J.* **1952**, *51*, 418.

Giddings, G. G. Reduction of ferrmyoglobin in meat. *CRC Crit. Rev. Food Technol.* **1974**, *5*, 143−173.

Gill, C. O. The control of microbial spoilage in fresh meats. In *Advances in Meat Research. Vol. 2. Meat and Poultry Microbiology*; Pearson, A. M., Dutson, T. R., Eds.; Avi Publishing: Westport, CT, 1986.

Gill, C. O.; Penney, N. Penetration of bacteria into meat. *Appl. Environ. Microbiol.* **1977**, *33*, 1284−1286.

Greene, B. E. Lipid oxidation and pigment changes in raw beef. *J. Food Sci.* **1969**, *34*, 110−113.

Hagler, L.; Coppes, R. I., Jr.; Herman, R. H. Metmyoglobin reductase. Identification and purification of a reduced nicotinamide adenine dinucleotide-dependent enzyme from bovine heart with reduces metmyoglobin. *J. Biol. Chem.* **1979**, *254*, 6505−6511.

Hood, D. E. Factors affecting the rate of metmyoglobin accumulation in pre-packaged beef. *Meat Sci.* **1980**, *4*, 247−265.

Hood, D. E.; Riordan, E. B. Discoloration in pre-packaged beef: measurement by reflectance spectrophotometry and shopper discrimination. *J. Food Technol.* **1973**, *8*, 333−343.

Hunt, M. C.; Kropf, D. H. Color and appearance. In *Advances in Meat Research. Vol. 2. Meat and Poultry Microbiology*; Pearson, A. M., Dutson, T. R., Eds.; Avi Publishing: Westport, CT, 1987.

Jacoby, G. A. The induction and repression of amino acid oxidation in *Pseudomonas fluorescens*. *Biochem. J.* **1964**, *92*, 1−8.

Jordan, K. A.; Kuehl, R. O.; Swell, J. I. Planning the experiment. In *Instrumentation and Measurement for Environmental Sciences*; Henry, Z. A., Zoerbo, G. C., Birth, G. S., Eds.; American Society of Agricultural Engineers: St. Joseph, MI, 1991.

Judd, D. B. *Color in Business, Science, and Industry*; Wiley: New York, 1952.

Kanner, J.; German J. B.; Kinsella, J. E. Initiation of lipid peroxidation in biological systems. *CRC Crit. Rev. Food Sci. Nutr.* **1987**, *25*, 317.

Kariya M.; Sado, K.; Namiki, H. A novel UDP-sugar, UDP-3-ketoglucosamine from bovine heart muscle reduces metyoglobin with NAD(P)H. *J. Exp. Biol.* **1997**, *200*, 2977−2986.

Kropf, D. H.; Hunt, M. C.; Piske D. Color formation and retention in fresh meat. *Proceedings fo the Meat Industrial Research Conference*; American Meat Institute Foundation: Chicago, IL, 1986.

LaBrake, C. C.; Fung, L. W. M. Phospholipid vesicles promote human hemoglobin oxidation. *J. Biol. Chem.* **1992**, *267*, 16703.

Lamine, S. A predictive model of shelf life enhancement for meat packed under modified atmospheres. M.S. Thesis, University of Arizona, Tucson, AZ, 1991.

Lanari, M. C.; Bevilacqua, A. E.; Zaritzky, N. E. Pigment modifications during freezing and frozen storage of packaged beef. *J. Food Process Eng.* **1989**, *12*, 49−66.

Lanari, M. C.; Zaritzky, N. E. Effect of packaging and frozen storage temperature on beef pigments. *Int. J. Food Sci.* **1991**, *26*, 629−632.

Lanari, M. C.; Cassens, R. G.; Schaefer, D. M.; Scheller, K. K. Dietary vitamin E Enhances color and display life of frozen beef from holstein steers. *J. Food Sci.* **1993**, *58*, 701−704.

Ledward, D. A. Metamyoglobin formation in beef stored in carbon dioxide enriched and oxygen depleted atmospheres. *J. Food Sci.* **1970**, *35*, 3−37.

Ledward, D. A. Metmyoglobin formation in beef muscles as influenced by water content and anatomical location. *J. Food Sci.* **1971**, *36*, 138−140.

Ledward, D. A. Metmyoglobin reduction and formation in beef during aerobic storage at 1 °C. *J. Food Sci.* **1972**, *37*, 634−635.

Ledward, D. A. Post-slaughter influences on the formation of metmyoglobin in beef muscles. *Meat Sci.* **1985**, *15*, 149−171.

Livingston, D. J.; Brown, W. D. The chemistry of myoglobin and its reactions. *Food Technol.* **1981**, *35* (5), 244−252.

Livingston, D. J.; McLachlan, S. J.; LaMar, G. N.; Brown, W. D. Myoglobin: cytochrome b_5 interactions and the kinetic mechanism of metmyoglobin reductase. *J. Biol. Chem.* **1985**, *260*, 15699−15707.

Luno, M.; Beltran, J. A.; Roncales, P. Shelf-life extension and colour stabilization of beef packaged in a low O_2 atmosphere

containing CO: Loin steaks and ground meat. *Meat Sci.* **1998**, *48*, 75−84.
Macdougall, D. B. Changes in color and opacity of meat. *Food Chem*. **1982**, *9*, 75−88.
Madhavi, D. L.; Carpenter, C. E. Aging and processing affect color, metmyoglobin reductase and oxygen consumption of beef muscles. *J. Food Sci.* **1993**, *58*, 939−942.
Marriott, N. G.; Garcia, R. A.; Kurland, M. E.; Lee, D. R. Appearance and microbial quality of thawed retail cuts of beef, pork, and lamb. *J. Food Sci.* **1980**, *43*, 185−189.
Murray, R. G. E. Morphology. In *Manual of Methods for General Bacteriology*; Gerhardt, P., Murray, R. G. E., Costilow, R. N., Nester, E. W., Wood, W. A., Krieg, N. R., Phillips, G. B., Eds.; American Society for Microbiology: Washington, DC, 1981.
Ockerman, H. W.; Cahill, V. R.; Weiser, H. H.; Davis, C. E.; Siefker, J. R. Comparison of sterile and inoculated beef tissue. *J. Food Sci.* **1969**, *34*, 93−99.
Okayama, T. Effect of modified gas atmosphere packaging after dip treatment on myoglobin and lipid oxidation of beef steaks. *Meat Sci*. **1987**, *19*, 179−185.
O'Keefe, M.; Hood, D. E. Biochemical factors influencing metmyoglobin formation in beef from muscles of different color stability. *Meat Sci*. **1982**, *7*, 209−228.
Pegg, R.; Shahidi, F. Unraveling the Chemical Identity of Meat Pigments. *CRC Crit. Rev. Food Sci. Nutr*. **1997**, *37* (6), 561−589.
Pirko, P. C.; Ayres, J. C. Pigment changes in packaged beef during storage. *Food Technol*. **1957**, *11*, 461−468.
Reddy, I. M.; Carpenter, C. E. Determination of metmyoglobin reductase activity in bovine skeletal muscles. *J. Food Sci.* **1991**, *56*, 1161−1164.
Renerre, M. Variability of the color of beef between animals and between muscles. *Sci. Aliment.* **1984**, *4*, 567−584.
Renerre, M. Review: Factors involved in the discoloration of beef. *Int. J. Food Sci. Technol.* **1990**, *25*, 613−630.
Renerre, M.; Labas, R. Biochemical factors influencing metmyoglobin formation in beef muscles. *Meat Sci*. **1987**, *19*, 151−165.
Robach, D. L.; Costilow, R. M. Role of bacteria in the oxidation of myoglobin. *Appl. Microbiol.* **1961**, *9*, 529−533.
Scopes, R. K.; Newbold, R. P. Postmortem glycolysis in ox skeletal muscle. Effect of pre-rigor freezing and thawing on the intermediate metabolism. *Biochem. J*. **1968**, *30*, 197−200
Seideman, S. C.; Cross, H. R.; Smith, G. C.; Durland, P. R. Factors associated with fresh meat color: A review. *J. Food Qual.* **1984**, *6*, 211−237.
Speck, M. L. *Compendium of Methods for the Microbiological Examination of Foods*; American Public Health Association: Washington, DC, 1984.
Steel, R. G. D.; Torrie, J. H. Analyses of Variances II. Multiway classifications. In *Principles and Procedures of Statistics*; McGraw-Hill Book: New York, 1960.
Tomioka, Y. Fresh meat. In *Food Packaging*; Kadoya, T., Ed.; Academic Press: San Diego, CA, 1990.
Walters, C. L. Meat color: the importance of hem chemistry. In *Meat*; Cole, D. J. A., Lawrie, R. A., Eds.; AVI Publishing: Westport, CT, 1975.
Young, L. L.; Reviere, R. D.; Cole, A. B. Fresh red meats: A place to apply modified atmospheres. *Food Technol*. **1988**, *42* (9), 65−69.

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