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Thermal Instability Induced by 4-Hydroxy-2-Nonenal in Beef Myoglobin

Fernanda M. Viana[1,2,3], Yifei Wang[1], Shuting Li[1], Carlos A. Conte-Junior[2,3,4], Jing Chen[5], Haining Zhu[5], and Surendranath P. Suman[1]*

[1]Department of Animal and Food Sciences, University of Kentucky, Lexington, KY 40546, USA
[2]Instituto de Quimica, Centro de Tecnologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-909, Rio de Janeiro, Brazil
[3]Departamento de Tecnologia de Alimentos, Faculdade de Veterinaria, Universidade Federal Fluminense, Niteroi 24230-340, Rio de Janeiro, Brazil
[4]Instituto Nacional de Controle de Qualidade em Saúde, Fundação Oswaldo Cruz, Rio de Janeiro 21040-900, Rio de Janeiro, Brazil
[5]Proteomics Core Facility, University of Kentucky, Lexington, KY 40506, USA
*Corresponding author. Email: spsuma2@uky.edu (Surendranath P. Suman)

Abstract: The secondary products of lipid oxidation, such as 4-hydroxy-2-nonenal (HNE), compromise myoglobin (Mb) redox stability and can thus impact thermal stability. Previous studies examined HNE-induced redox instability in beef Mb, whereas investigations are yet to be undertaken to evaluate the relationship between lipid oxidation and thermal stability of beef Mb. Therefore, the objective of the present study was to investigate the direct influence of HNE on thermal stability of beef Mb at meat conditions. Beef oxymyoglobin (0.15 mM) was incubated with HNE (1.0 mM) at pH 5.6 and 4°C for 21 d in the dark. Metmyoglobin formation, percentage Mb denaturation (PMD), and HNE adduction sites in Mb were examined on days 0, 7, 14, and 21. The experiment was replicated 3 times ($n = 3$). The data were evaluated using the MIXED procedure of SAS, and the differences among means were detected at the 5% level using the least significant difference test. The HNE-treated samples exhibited greater ($P < 0.05$) metmyoglobin formation and PMD than the controls. Additionally, the PMD difference between HNE-treated and control samples increased ($P < 0.05$) over time. Mass spectrometric analyses indicated that the number of HNE adduction sites increased with storage, and 6 histidines (positions 24, 36, 64, 93, 113, and 152) were adducted on day 21. HNE adduction at the distal histidine (position 64), which is critical to heme stability, was observed only on days 14 and 21. An increase in PMD on days 14 and 21 in HNE-treated samples could be partially due to the adduction at distal histidine. These findings indicated that HNE compromises thermal stability of beef Mb, possibly through altering the conformation of the heme protein by nucleophilic adduction.

Key words: beef myoglobin, lipid oxidation, mass spectrometry, redox stability, thermal stability
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Introduction

Thermal processing of meat is necessary to destroy pathogens, thereby preventing foodborne illnesses. Although food thermometers are recommended to ensure that muscle foods are cooked to specified internal temperatures (USDA, 1997), a recent study indicated that only 13.5% of consumers use thermometers while cooking meats (Ramanathan et al., 2019). Despite not being a reliable parameter, color of cooked meat is often utilized to evaluate doneness and safety (Suman et al., 2016). Cooked color is primarily dictated by the heat-induced denaturation of myoglobin (Mb), which is the sarcoplasmic heme protein responsible for fresh meat color.

Mb denaturation temperature is governed by its redox state in raw meat; ferric metmyoglobin (MMb) undergoes denaturation at a lower temperature than ferrous oxymyoglobin (OMb) and deoxymyoglobin (DMb) forms (Machlik, 1965; Hunt et al., 1999; Sepe et al., 2005). Furthermore, Warren et al. (1996) demonstrated that vacuum-packaged ground beef patties containing mostly MMb developed brown color, while the ones in which DMb and OMb were predominant maintained the reddish-pink color when cooked to the same temperature. Therefore, factors governing the Mb redox stability in fresh meat can impact cooked meat color.

Mb redox stability is influenced by several extrinsic and intrinsic factors, including lipid oxidation (Suman and Joseph, 2013). Reactive secondary products of lipid oxidation form covalent adducts with OMb and accelerate its oxidation to MMb (Faustman et al., 2010). In this perspective, lipid oxidation–induced redox instability in beef Mb has been extensively investigated in model systems (Alderton et al., 2003; Suman et al., 2006, 2007; Yin et al., 2011; Nair et al., 2014a) using 4-hydroxy-2-nonenal (HNE), an α,β-unsaturated aldehyde generated by oxidation of n-6 polyunsaturated fatty acids. HNE adduction at histidine (HIS) residues compromises Mb redox stability by altering its tertiary structure and thereby exposing the heme iron to oxidation (Suman et al., 2007).

The interactions between Mb and other small biomolecules—such as lactate—governing the heme protein's thermal stability have been previously examined (Kim et al., 2010; Nair et al., 2014b). However, no investigations have been conducted on the direct influence of reactive lipid oxidation products on the thermal stability of beef Mb in model systems. Therefore, the objective of the present study was to examine the direct influence of HNE on thermal stability of beef Mb at a cooking temperature and pH typical of meat.

Materials and Methods

Chemicals and supplies

Sephacryl 200HR, ammonium sulfate, Tris-HCl, ethylenediaminetetraacetic acid (EDTA), sodium hydrosulfite, sodium citrate, and ethanol were obtained from Sigma Chemical Co. (St. Louis, MO). PD-10 columns were obtained from GE Healthcare (Piscataway, NJ), and HNE was obtained from Cayman Chemical Co. (Ann Arbor, MI). All chemicals were of reagent grade or greater purity.

Beef Mb purification

Beef Mb was isolated from cardiac muscles. Fresh hearts were obtained locally within 1 h of exsanguination, placed on ice, and transported to the laboratory. Mb was purified via ammonium sulfate precipitation and gel-filtration chromatography (Faustman and Phillips, 2001). Briefly, cardiac muscle sample was homogenized with 10 mM Tris-HCl, 1 mM EDTA buffer at pH 8.0, at a ratio of 1:3, and centrifuged (Thermo Fisher Scientific, Waltham, MA) at 5,000*g* for 10 min at 4°C. The supernatant was brought to 70% ammonium sulfate saturation and then centrifuged at 18,000*g* for 20 min at 4°C. The pellet was discarded, and the resulting supernatant was saturated with 100% ammonium sulfate, followed by centrifugation at 20,000*g* for 1 h at 4°C. The precipitate was resuspended and dialyzed against 10 mM Tris-HCl, 1 mM EDTA buffer at pH 8.0 and 4°C for 24 h with 3 buffer changes. Sephacryl 200HR gel-filtration column (2.5 × 100 cm) was used to separate Mb from hemoglobin with a 5 mM Tris-HCl, 1 mM EDTA elution buffer at pH 8.0 and 4°C, and the flow rate was 60 mL/h. Beef Mb isolated was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and appeared as only one band in the gels indicating high purity.

OMb preparation

Beef OMb was prepared by sodium hydrosulfite–mediated reduction of purified Mb (Brown and Mebine, 1969). Residual hydrosulfite was removed by passing through PD-10 desalting columns, and the pH of the OMb solution was adjusted to pH 5.6 with 50 mM sodium citrate buffer. The purity of OMb was ensured spectrophotometrically (UV-2401 PC, Shimadzu Inc., Columbia, MD), according to Tang et al. (2004).

Incubation of OMb with HNE

Beef OMb was incubated with HNE (0.15 mM Mb + 1.0 mM HNE) at pH 5.6 and 4°C (typical meat storage conditions) in 50 mM sodium citrate buffer in the dark for 21 d. The concentration of Mb reflected the Mb concentration reported for postmortem beef skeletal muscles (Rickansrud and Henrickson, 1967). Positive controls consisted of OMb plus a volume of ethanol equivalent to that used to deliver HNE to treatments. The samples were scanned spectrophotometrically (UV-2401PC, Shimadzu Inc., Columbia, MD) from 650 to 500 nm at specific time points of incubation (days 0, 7, 14, and 21), and the percentage of

MMb formation was calculated (Tang et al., 2004). Additionally, Mb samples (0.5 mL) were removed from the reaction assays, passed through a PD-10 desalting column (equilibrated with 50 mM sodium citrate buffer) to remove unreacted HNE, and frozen at −80°C for subsequent mass spectrometric analysis. The experiment was replicated 3 times ($n = 3$).

Thermal stability evaluation

The thermal stability of the controls and HNE-treated beef Mb samples was assessed by determining the percentage myoglobin denaturation (PMD) at 71°C, which is the recommended internal cooking temperature for meat (USDA, 1997). At days 0, 7, 14, and 21 of storage, the Mb samples were incubated at 71°C in a water bath for 10 min. Then, the samples were cooled immediately by immersing in slushed ice to prevent post-incubation temperature rise. After centrifugation at 16,000*g* for 2 min, Mb concentration was determined by measuring the absorbance at 525 nm (Tang et al., 2004) using a UV-2401PC spectrophotometer (Shimadzu Inc., Columbia, MD). PMD was calculated as follows:

$$\text{PMD} = 100 \times [(\text{pre-incubation Mb concentration} - \text{post-incubation Mb concentration}) \div \text{pre-incubation Mb concentration}]$$

Additionally, differences in PMD between control and HNE-treated samples were expressed as PMD difference.

Mass spectrometric analyses of Mb-HNE adducts

Control and HNE-treated Mb samples were subjected to dithiothreitol reduction, iodoacetamide alkylation, and in-solution trypsin digestion using a standard protocol (Washburn et al., 2001). The resulting tryptic peptides were extracted, concentrated, and subjected to shot-gun proteomics analysis as previously described in Kamelgarn et al. (2018). Liquid chromatography-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 phase 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 8 scan events. The first scan event was an Orbitrap mass spectrometry scan (300–1,800 m/z) with 60,000 resolution for parent ions followed by data-dependent MS/MS for fragmentation of the 10 most intense multiple-charged ions with the collision induced dissociation method.

Statistical analysis

The experimental designs for redox and thermal stability experiments were completely randomized designs, and each experiment was replicated 3 times ($n = 3$). Type-3 tests of fixed effects for HNE, incubation time, and their interactions were evaluated using the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC). The repeated option in MIXED was used to assess covariance structure (i.e., variance components) resulting from repeated measurements on the same sample during incubation. Least-square means were generated for significant F-tests ($P < 0.05$), and differences among means were detected at the 5% level using the least significant difference test.

Results

Mb oxidation in the presence of HNE

MMb formation increased ($P < 0.05$) during storage in controls and their HNE-treated counterparts. Nonetheless, HNE-treated samples demonstrated greater ($P < 0.05$) MMb levels than control ones from day 7 of storage (Figure 1).

Mb thermal stability in the presence of HNE

Control and HNE-treated samples demonstrated an increase ($P < 0.05$) in PMD values during storage (Figure 2). Furthermore, the HNE-treated samples exhibited greater ($P < 0.05$) PMD values than control samples on days 7, 14, and 21 of storage (Figure 2). The PMD difference (between HNE-treated and control samples) increased ($P < 0.05$) over time (Figure 3).

HNE adduction sites in beef Mb

MS/MS identified 6 HIS residues (positions 24, 36, 64, 93, 113, and 152) adducted by HNE in beef Mb (Table 1). While HNE adducts were observed in HIS

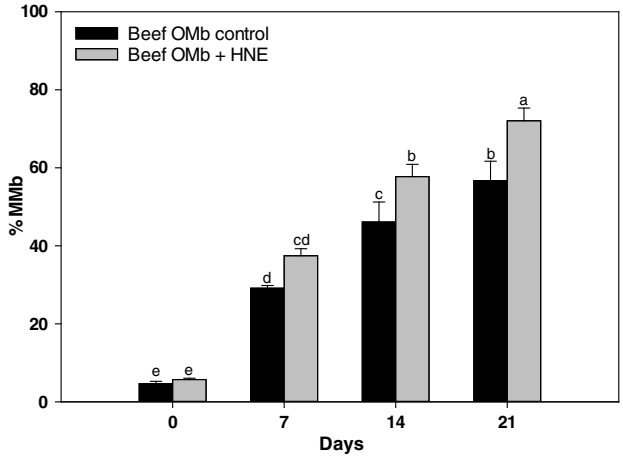

Figure 1. Percentage of metmyoglobin formation (% MMb) in beef oxymyoglobin (OMb; 0.15 mM) incubated with 4-hydroxy-2-nonenal (HNE; 1.0 mM) at pH 5.6 and 4°C in 50 mM sodium citrate buffer for 21 days. [a,b,c,d,e]Means without common superscripts are different ($P < 0.05$).

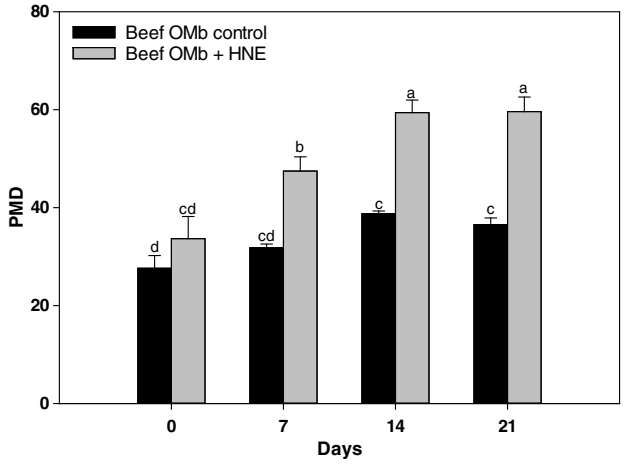

Figure 2. Percentage myoglobin denaturation (PMD) in beef oxymyoglobin (OMb; 0.15 mM) at 71°C, after incubation with 4-hydroxy-2-nonenal (HNE; 1.0 mM) at pH 5.6 and 4°C in 50 mM sodium citrate buffer for 21 days. [a,b,c,d]Means without common superscripts are different ($P < 0.05$).

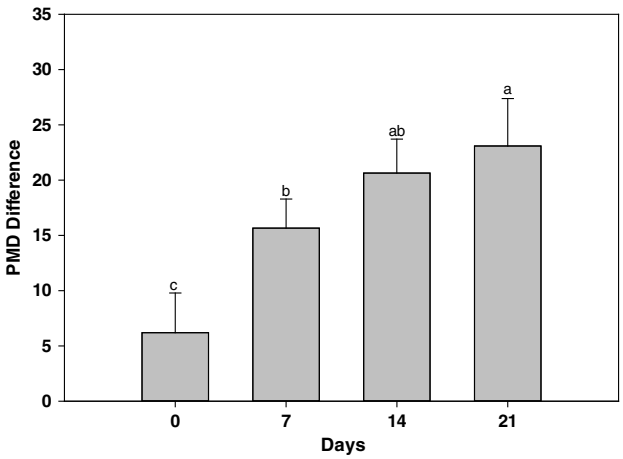

Figure 3. Percentage myoglobin denaturation (PMD) difference between 4-hydroxy-2-nonenal (HNE)-treated (1.0 mM) and control beef oxymyoglobin (OMb; 0.15 mM) samples at 71°C, after incubation at pH 5.6 and 4°C in 50 mM sodium citrate buffer for 21 days. [a,b,c]Means without common superscripts are different ($P < 0.05$).

Table 1. Adduction sites observed in beef oxymyoglobin (0.15 mM) incubated with 4-hydroxy-2-nonenal (HNE; 1.0 mM) at pH 5.6 and 4°C in 50 mM sodium citrate buffer for 21 days

		HNE adducted residues and positions			
Peptide position	Peptide sequence	Day 0	Day 7	Day 14	Day 21
17–31	VEADVAGH*GQEVLIR	–	H24	H24	H24
32–42	LFTGH*PETLEK	–	H36	H36	H36
64–77	H*GNTVLTALGGILK	–	–	H64	H64
88–96	HLAESH*ANK	–	H93	H93	H93
103–118	YLEFISDAIIH*VLHAK	–	–	–	H113
148–153	VLGFH*G	–	H152	H152	H152
Total number of HNE adducted residues		**0**	**4**	**5**	**6**

*Adducted residue.

24, 36, 93, and 152 from day 7, HIS 64 was adducted only after day 14. On the other hand, adduction at HIS 113 was detected only on day 21. The representative MS/MS spectrum for the tryptic peptide VEADVAGHGQEVLIR (residues 17–31) of HNE-treated beef Mb (Figure 4) is presented.

Discussion

The HNE-adducted HIS residues exhibited a mass shift of 156 Da, indicating that the adducts were formed via Michael addition (Bolgar and Gaskell, 1996). Although cysteine, lysine, and arginine are also highly susceptible to HNE adduction (Uchida and Stadtman, 1992; Bolgar and Gaskell, 1996), previous studies have also demonstrated that HIS is the only amino acid adducted in beef Mb (Alderton et al., 2003; Suman et al., 2006; Suman et al., 2007; Nair et al., 2014a). Suman et al. (2006) documented 4 sites (HIS 36, 81, 88, and 152) of HNE adduction in beef Mb at meat storage conditions after incubation for 48 h. The greater number of adducts observed in the present study could be attributed to the longer incubation period (21 d) used.

The increase in MMb formation in samples exposed to HNE (Figure 1) could be attributed to the aldehyde's prooxidant capacity. The electrophilic nature of carbon 3 in HNE allows it to covalently bind

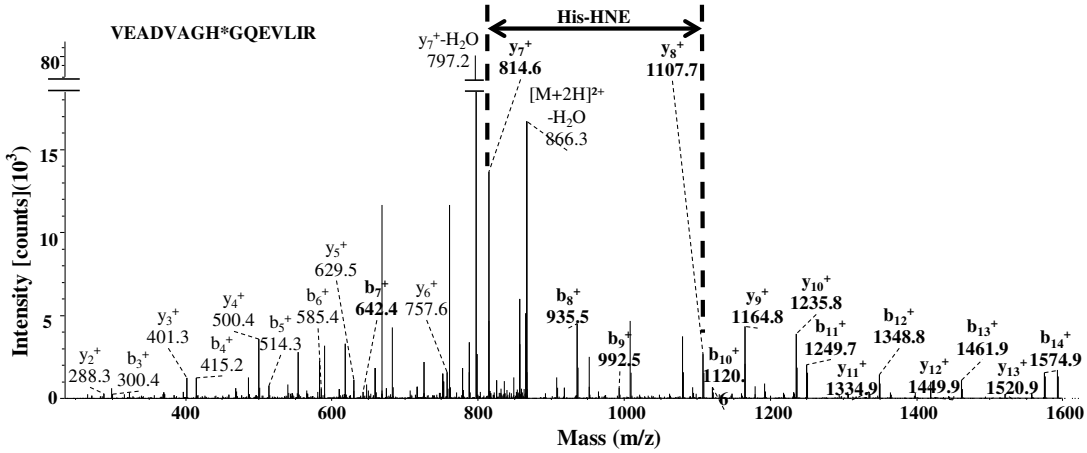

Figure 4. Tandem mass spectrum of the tryptic peptide VEADVAGHGQEVLIR (residues 17–31) from beef oxymyoglobin (0.15 mM) incubated with 4-hydroxy-2-nonenal (HNE; 1.0 mM) at pH 5.6 and 4°C in 50 mM sodium citrate buffer for 21 days.

to nucleophilic sites of proteins (Esterbauer et al., 1991). HNE adduction enhances MMb formation by inducing conformational modifications in the protein tertiary structure (Alderton et al., 2003), which expose the heme pocket to oxidizing agents and increase heme iron release (Tatiyaborworntham et al., 2012).

The increased PMD in the HNE-treated samples (Figure 2) was expected as these samples exhibited greater amount of MMb than their control counterparts (Figure 1). Thermal stability of Mb is influenced by the heme protein's redox state, and the ferric MMb has lower resistance against heat-induced denaturation than the ferrous Mb forms (Machlik, 1965; Hunt et al., 1999; Sepe et al., 2005). In partial agreement, Alderton et al. (2003) reported a decrease in the melting temperature as Mb was destabilized by HNE adduction.

Temperatures above normal living/physiological conditions are considered high for cellular proteins, and thermal stability refers to the protein's resistance to high temperatures (Nurilmala et al., 2018). Numerous factors, including protein hydrophobic internal packing, secondary structure propensity, helix dipole stabilization, and hydrogen bonding, govern the thermal stability of proteins (Vogt et al., 1997). Exposure to high temperatures causes denaturation/unfolding of proteins due to disruption of intermolecular interactions in secondary or tertiary structures. Mb denaturation is characterized by the heme loss and subsequent protein unfolding and ultimately leads to loss of functionality, followed by insolubilization and aggregation (Barrick et al., 1994). High temperatures promote conformational changes in the Mb structure leading to exposure of the heme group (Meersman et al., 2002; Grunwald and Richards, 2006), which results in increased access of prooxidants to heme.

The resistance to heat-induced denaturation is influenced by conformational stability of proteins. The interaction between heme and globin, mainly through proximal (position 93) and distal (position 64) HIS residues, is critical to the stabilization of the Mb (Hargrove et al., 1994). HNE adduction favors the unfolding of Mb helical segments of the secondary structure, which can expose HIS residues that were buried inside the molecule—such as HIS 24 (Friend and Gurd, 1979)—and thus create new adducts with HNE. Moreover, a lower helix content in Mb leads to greater susceptibility to heat denaturation (Ueki et al., 2005).

The increase in PMD during storage could be attributed to the increased ($P < 0.05$) MMb formation throughout the storage observed in both treatments (Figure 1). Previous investigations have documented that beef MMb has lower resistance to heat-induced denaturation than its DMb and OMb counterparts (Machlik, 1965; Sepe et al., 2005). The increase in the PMD difference (Figure 3) during storage suggested that the locations of HNE adducts (Table 1) influenced Mb thermal stability. Adduction of HNE to a greater number of HIS residues over time could have led to destabilization of and conformational changes in Mb resulting in thermal denaturation. The increase in PMD differences on days 14 and 21 could be partially explained by the appearance of HNE adducts at HIS 64 (distal HIS) at these time points. While distal HIS is not bound to the heme group, it is critical to the stability of oxygen bound to the heme iron and thus maintaining Mb in the ferrous redox state (Suman and Joseph, 2013). In partial agreement, previous studies documented a decline in redox stability of beef Mb when HNE adducted HIS 64 at

physiological as well as meat conditions (Alderton et al., 2003; Suman et al., 2006, 2007; Yin et al., 2011). Although Mb redox and thermal stabilities are closely related, the aforementioned studies did not examine thermal stability of beef Mb challenged with HNE.

Modifications or substitutions of amino acid residues, particularly those located in the α-helical segments and heme pocket region, could compromise hydrophobic interactions stabilizing the tertiary structure of Mb (Ueki and Ochiai, 2006). Therefore, due to possible structural destabilization induced by HNE adduction at other HIS residues (positions 24, 36, 152), the HIS residues buried within the hydrophobic pocket, such as proximal HIS and distal HIS, could become more accessible to HNE. In partial agreement, in the present study, HNE adducts were observed in HIS 64 only from day 14, after several other HIS residues were adducted by day 7.

On day 7, HNE adduction was observed at proximal HIS, which is a residue that contributes to maintaining the waterproof environment of the heme pocket and preventing the dissociation of hemin (Musto et al., 2004). Upon HNE alkylation of HIS 93, the heme iron-proximal HIS bond is disrupted and thus the heme affinity is compromised. Mammalian Mb consists of 8 α-helical segments (Nurilmala et al., 2018), and the removal of heme leads to the decrease of helical content, turning the Mb structure into a random coil (Ochiai, 2011) and in turn exposing distal HIS buried in the heme pocket to HNE. Consequently, on day 14, HNE alkylation occurred on distal HIS, which is critical for the Mb redox stability (Suman and Joseph, 2013), and therefore Mb stability is greatly compromised. This could explain why HNE adduction on HIS 64 leads to the increased PMD on days 14 and 21.

Conclusions

The thermal stability of beef OMb was compromised by HNE at meat storage conditions. Mass spectrometric analyses indicated that HNE adduction sites in Mb increased over time, with a concomitant decrease in the heme protein's thermal stability. These results suggested that the direct interactions between reactive products of lipid oxidation and Mb decrease not only redox stability but also thermal stability. The appearance of HNE adducts at HIS 64 corresponded with a decline in Mb thermal stability. While the exact role of each HIS residue adducted by HNE on Mb thermal stability is not clear, the increasing number of HNE adducts contributed to the protein's thermal instability.

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