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Effects of Oxygen Partial Pressure on 4-Hydroxy-2-Nonenal Induced Oxymyoglobin Oxidation

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Abstract: 4-hydroxyl-2-nonenal (HNE) is a lipid oxidation product that can increase oxymyoglobin oxidation. However, limited research has evaluated the role of oxygen partial pressure in HNE-induced metmyoglobin formation. Therefore, the objective of was to compare the effects of atmospheric and high-oxygen partial pressure on HNE-induced oxymyoglobin oxidation in vitro. Oxymyoglobin was incubated with or without HNE at atmospheric (20% O_2) or high-oxygen (80% O_2) partial pressure. Metmyoglobin formation was measured after 0, 48, and 96 h of incubation at 4°C, and mass spectrometry was utilized to characterize the covalent binding of HNE to myoglobin. High-oxygen condition (80% O_2) increased ($P < 0.05$) HNE-induced oxymyoglobin oxidation compared with the atmospheric partial pressure condition (20% O_2). However, HNE was bound to myoglobin at both high-oxygen and atmospheric partial pressure conditions, with no differences ($P > 0.05$) in the extent of adduct formation. These results suggest that high-oxygen conditions had no effect on extent of HNE-binding, but can increase oxymyoglobin oxidation.

Keywords: beef color, 4-hydroxyl-2-nonenal, lipid oxidation, mass spectrometry, myoglobin, oxygen partial pressure
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Introduction

Myoglobin is a water-soluble sarcoplasmic protein that primarily determines meat color. Oxy-, deoxy-, and metmyoglobin are the common myoglobin redox forms seen on freshly cut meat steaks. Greater concentration of oxymyoglobin gives the consumer-preferred, bright cherry-red color. In contrast, oxymyoglobin oxidation results in metmyoglobin formation and discoloration of meat. The redox stability of oxymyoglobin is compromised by various factors, including oxygen partial pressure (George and Stratmann, 1952) and lipid oxidation product promoters. Myoglobin is prone to oxidation at lower oxygen partial pressures, a condition that can occur beneath the meat surface exposed to air (Ledward, 1970). Thus, metmyoglobin formation first occurs beneath the surfaces that are exposed to air, and then the metmyoglobin layer migrates from the interior of a steak to the surface (AMSA, 2012; Ramanathan and Mancini, 2018). Contradictorily, the meat industry utilizes high-oxygen modified packaging (HiOx-MAP; 80% oxygen content) to maximize oxygen partial pressure within a steak. As a result, the average shelf-life of steaks in HiOx-MAP is 10 to 12 d compared to 5 to 7 d in polyvinyl chloride (PVC)/atmospheric oxygen partial pressure (20% oxygen) at 4°C (Cornforth and Hunt, 2008). However, steaks aged for 7 to 14 d and packaged in HiOx-MAP showed less discoloration, but had more lipid oxidation than PVC packaged steaks aged for the same period (English et al., 2016; Wills et al., 2017). This raises a question regarding the potential color stabilizing effects of HiOx-MAP, because high-oxygen content increases lipid oxidation and promote formation of primary and secondary lipid oxidation products. On the other hand, primary and secondary lipid oxidation products can increase myoglobin

oxidation (Faustman et al., 1999; Naveena et al., 2010; Tatiyaborworntham et al., 2012; Elroy et al., 2015).

In the current research, we utilized a 9-carbon aldehyde, 4-hydroxy-2-nonenal (HNE; Esterbauer et al., 1991) as a model lipid oxidation biomarker to study the effects of high-oxygen partial pressure on oxymyoglobin oxidation. 4-hydroxyl-2-nonenal has been extensively utilized to study the effects on myoglobin redox stability (Alderton et al., 2003; Yin et al., 2011). Previous studies investigated the effects of HNE on myoglobin oxidation at atmospheric oxygen partial pressure conditions (20% oxygen; level seen in overwrap or PVC packaging; Suman et al., 2007; Yin et al., 2011; Grunwald et al., 2017). However, it is unknown to what extent high-oxygen (80% oxygen; level seen in high-oxygen modified atmospheric packaging) content might contribute to HNE-induced oxidation. We hypothesized that greater oxygen content in conjunction with HNE binding would increase oxymyoglobin oxidation. Therefore, the objective of this study was to evaluate the effects of high-oxygen content on HNE-induced myoglobin oxidation in vitro and to determine the effect of oxygen levels on the stoichiometry of HNE-myoglobin adduct formation.

Materials and Methods

Materials and chemicals

Equine myoglobin, potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HP_O4), sodium dithionite, urea, tris-hydroxymethyl aminomethane hydrochloride (Tris-HCl), and trypsin were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO). 4-hydroxyl-2-nonenal was obtained from Cayman Chemical Co. (Ann Arbor, MI). The PD-10 columns were obtained from GE Healthcare (Piscataway, NJ). All chemicals were of reagent grade or greater.

Oxymyoglobin preparation

Equine myoglobin shares 88.9% similarity with bovine myoglobin and has been utilized in several in vitro research to elucidate the role of lipid oxidation products in oxymyoglobin oxidation (www.expasy.org). Lyophilized metmyoglobin powder was dissolved in 50 mM phosphate buffer at pH 5.6 to prepare 0.1 mM myoglobin, and the concentration was confirmed by measuring absorbance at 525 nm (assuming an excitation coefficient of 7.6 Mm^{-1} cm^{-1}; Broumad et al., 1958). Oxymyoglobin was prepared via hydrosulfite–mediated reduction of metmyoglobin (0.1 mg sodium hydrosulfite to 1 mg myoglobin; Brown and Mebine, 1969). Residual hydrosulfite was removed by passing through PD-10 columns previously equilibrated with 50 mM phosphate buffer at pH 5.6.

Replacement of ethanol in 4-hydroxy-2-nonenal with water

Commercially available HNE (1 mg/0.1 mL) was dissolved in 100% ethanol. Since ethanol can affect protein functionality, ethanol in HNE vial was evaporated under a gentle stream of high-purity nitrogen gas (Stillwater Steel, Stillwater, OK) according to the method of Grunwald et al. (2017). After 2 min, evaporation of ethanol was confirmed visually. Subsequently, 0.1 mL deionized water saturated with high-purity nitrogen was added to HNE vial. Spectrophotometric confirmation of the dissolution of HNE in deionized water was performed by observing the HNE peak at a wavelength of 221 nm (HNE product technical information, Cayman Chemical Co.).

Reaction of oxymyoglobin with 4-hydroxyl-2-nonenal

Equine oxymyoglobin (0.1 mM, pH 5.6), was reacted with HNE (0.7 mM, dissolved in water) at a ratio of 1:7 (Faustman et al., 1999; Alderton et al., 2003; Suman et al., 2006). Control treatments consisted of oxymyoglobin treated with an equivalent volume of water that was used to deliver HNE. Both HNE-treated and control samples ($n = 5$ each) were incubated under high-oxygen (80% O_2) or atmospheric partial pressure (20% O_2). High-oxygen conditions were achieved by placing 1 mL of control and HNE-treated oxymyoglobin solution in glass test tubes placed inside oxygen impermeable bags (11× 22 cm, 3-mm high barrier Cryovac vacuum bags) that had been flushed with high-oxygen gas (80% O_2 and 20% CO_2; Stillwater Steel). Packaged bags were sealed using an impulse sealer (type AIE-305, watt impulse n850W (impulse), 115V, 60 HZ; American International Electric, City of Industry, CA). The tubes assigned to atmospheric conditions were wrapped with oxygen-permeable PVC film (15,500 to 16,275 cm^3 O_2/m^2/24 h at 23°C, E-Z Wrap Crystal Clear Polyvinyl Chloride Wrapping Film, Koch Supplies, Kansas City, MO) to minimize moisture loss. A headspace analyzer (Bridge 900131 $O_2/CO_2/CO$, Illinois Instruments, Ingleside, IL), was used to determine the percentage of oxygen and carbon dioxide gas compositions. The average gas compositions in the packages were 78.9% oxygen and 19.4% carbon dioxide.

Both atmospheric and high-oxygen partial pressure samples were incubated in a coffin style display case maintained at 2 ± 1°C under continuous fluorescence lighting (Philips Fluorescent lamps; 12 Watts, 48 inches, Philips, Shanghai, China; color temperature = 3,500°K) for 0, 48, and 96 h to simulate retail display. At the end of each incubation period, packages were opened and metmyoglobin formation in control and HNE treated samples were measured spectrophotometrically by scanning absorption from 450 to 650 nm using a Shimadzu UV-2600 UV-Vis spectrophotometer (Shimadzu Co, Kyoto, Japan). The blank contained only phosphate buffer at pH 5.6. Myoglobin oxidation was determined by measuring the wavelength maxima at 503, 557, and 582 nm (Tang et al., 2004).

Determination of dissolved oxygen

After 96 h of incubation, oxymyoglobin incubated under high-oxygen and at atmospheric oxygen conditions were evaluated for dissolved oxygen saturation using a Clark oxygen electrode (polarizing voltage of 0.6 V and an 8 mL incubation chamber). The reaction chamber was maintained at a reaction temperature of 25°C, and stirred with a 10 mm Teflon covered bar at 600 rpm. The electrode was attached to a Rank Brothers digital model 20 oxygen controller (Cambridge, UK) and connected to a personal computer and data logger. The oxygen saturation was measured by suspending oxymyoglobin solution in the reaction chamber for 3 min (Ke et al., 2017). The dissolved oxygen was reported based on a standard oxygen solution in ppm.

Mass spectrometry and intact protein sizing

After determining metmyoglobin content, protein intact sizing was determined according to the method described by Elroy et al. (2015). Samples from both high-oxygen and atmospheric oxygen conditions incubated for 96 h were passed through PD-10 columns calibrated with 50 mM phosphate buffer at pH 7.4 (GE Healthcare) to remove any unbound HNE. The samples were diluted 10-fold into electrospray solvent (water/acetonitrile/formic acid at 50:50:0.1). Individual diluted samples were loaded into metal-tipped capillary infusion tubes (New Objective Econo Tips, Fisher Scientific, Waltham, MA) and ionized via passive infusion using a PV550 ion source (New Objective) by applying 1,700 to 1,900 V to the outside of the metal tips. Protein ions were analyzed within the Orbitrap sector of an LTQ OrbitrapXL mass spectrometer (Thermo Scientific, San Jose, CA). Spectra were collected for 1 min at nominal resolutions of either 15,000 or 60,000. The Qual Browser application (Xcalibur v3.0.63) was used to solve average m/z of protein ions, by averaging approximately 200 spectra acquired at 15,000 resolution. Charge states were solved by averaging approximately 65 spectra acquired at 60,000 resolution, wherein isotopes were well resolved.

Analysis of myoglobin peptides

The HNE-treated and control samples from high-oxygen and atmospheric oxygen partial pressures were diluted 20-fold into 8 M urea, 100 mM Tris HCl, pH 8.5, reduced by 5 mM Tris (2-carboxyethyl) phosphate, at room temperature for 20 min and alkylated with 10 mM iodoacetamide for 15 min in the dark. Alkylated samples were diluted with 4 volumes of 100 mM Tris-HCl, pH 8.5, and digested with 4 µg/mL trypsin/Lys-C (Promega V5072) overnight at 37°C. After overnight digestion, additional protease was added (2 additional µg trypsin per mL of digestion reaction) and digestion was continued for another 8 h. The resultant trypsinolytic peptides were acidified to 1% trifluoroacetic acid, purified by solid-phase extraction with C-18 affinity media tips (Agilent OMIX) and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described by Voruganti et al. (2013). Samples were prepared and analyzed as three independent replicates, and each individual sample was subjected to 2 LC-MS/MS injections (3 replicates × 2 technical replicates).

4-Hydroxyl-2-nonenal peptide identification and quantification

Max Quant (v1.5.3.8) was used to search the raw instrument files against a proteome database of 23,968 equine protein sequences downloaded from UniPort in March 2018. The sequences of common contaminants such as human keratins were included in the searches and were retained in the search results. Search settings included modification of lysine, cysteine, or histidine by HNE as variable modifications. After injecting equal volume of each of the myoglobin digestions onto the LC-MS, the relative abundances of individual peptide ions were calculated using MaxQuant, by using the ion intensities reported in the MaxQuant output file "modified peptides." For peptides of interest, ion intensities reported from technical replicates were averaged, after which the average value for each biological replicate was used to calculate mean values and standard deviations among the biological replicates.

Table 1. Effect of oxygen partial pressure and display time on 4-hydroxyl-2-nonenal (HNE)-induced oxymyoglobin oxidation[1]

	0 h		48 h		96 h	
Item	20% oxygen	80% oxygen	20% oxygen	80% oxygen	20% oxygen	80% oxygen
Control	4.4[a]	4.4[a]	24.6[b]	26.6[bc]	39.4[e]	41.1[f]
HNE	4.5[a]	4.5[a]	27.7[d]	28.7[cd]	41.2[f]	46.1[g]

[1]Standard error = 1.2; *P*-value for the main effects of lipid oxidation product = 0.002; oxygen partial pressure = 0.01; time < 0.001. Only main effects were significant, and interactions were not significant. Number of replications = 5.

[a–g] Least square means with different letters are different ($P < 0.05$).

Statistical analysis

A completely randomized design with factorial arrangement was used to evaluate the combined effects of HNE and oxygen partial pressure on myoglobin oxidation. The fixed effects/factors included lipid oxidation product (HNE), oxygen partial pressures, incubation time, and their interactions. The oxymyoglobin oxidation experiment was replicated 5 times ($n = 5$), while MS/MS study was replicated 3 times ($n = 3$). The data were analyzed using the Mixed Procedure of SAS (vers. 9.4, SAS Inst. Inc., Cary, NC). Least square means were separated using a pairwise *t*-test and were considered significant at $\alpha = 0.05$.

Results and Discussion

The HNE-treated oxymyoglobin incubated for 48 h at 20% oxygen level had greater metmyoglobin ($P < 0.05$) than control oxymyoglobin at the same conditions (Table 1). However, when oxymyoglobin solutions were incubated for 48 h at 80% oxygen level, there were no differences ($P > 0.05$) in metmyoglobin content between HNE-treated oxymyoglobin and control oxymyoglobin. By 96 h of incubation, HNE-treated oxymyoglobin in 80% oxygen content had the greatest % metmyoglobin than other treatments. Additionally, the dissolved oxygen saturation in atmospheric and high-oxygen oxymyoglobin solution were 7.5 and 15.5 ppm, respectively (SE = 0.5; $P < 0.05$). Although previous studies determined the pro-oxidative effects of HNE on bovine, porcine, equine, bison, goat, and avian myoglobins under atmospheric conditions (20% oxygen; Faustman et al., 1999; Suman et al., 2006; Yin et al., 2011; Maheswarappa et al., 2016); to the best of our knowledge, no research has determined the effects of high-oxygen content (80% oxygen) on HNE-induced oxymyoglobin oxidation.

Consistent with our previous findings (Elroy et al., 2015), intact protein sizing by infusion mass spectrometry (Fig. 1) confirmed that HNE increased myoglobin molecular weight by 156.2 Daltons, confirming covalent binding of HNE to myoglobin irrespective of oxygen conditions. Based on tandem mass spectrometry identification, HNE modification occurred at histidine 25, 65, and 120 (Fig. 2 and Table 2). 4-hydroxyl-2-nonenal is an α, β- unsaturated aldehyde derived from the oxidation of ω-6–polyunsaturated fatty acid such as linoleic and arachidonic acids. 4-hydroxyl-2-nonenal is a very stable compound that can diffuse into the sarcoplasm and alter protein functionality (Suman et al., 2007; Catalá, 2009; Patel and Korotchkina, 2002; Chan et al., 1997; Uchida and Stadtman, 1992). 4-hydroxyl-2-nonenal can covalently modify several different amino acid residues, such as ε- amino group of lysine, sulphydryl groups of cysteine, and imidazole moiety of histidine by Michael addition and Schiff base formation (Esterbauer et al., 1991; Chan et al., 1997; Catalá, 2009; Ramanathan et al., 2014). Equine myoglobin lacks cysteine and binding with lysine has not been reported due to greater pKa. Hence, histidine residue makes a potential candidate for HNE modification. Binding of HNE to histidine residue can alter myoglobin tertiary structure and increase oxidation (Faustman et al., 1999; Alderton et al., 2003; Grunwald et al., 2017). Further, histidine 65 is in close proximity to histidine 64, and the distal histidine (64) can influence ligand binding and oxidation (Grunwald et al., 2017).

4-hydroxyl-2-nonenal binding increased myoglobin oxidation both at atmospheric- and high-oxygen-conditions during 96 h of incubation. However, there were no significant differences in the intensity of peptides representing HNE-modified trypsinolytic peptides between atmospheric- and high-oxygen conditions (average intensities of HNE modified peptides: atmospheric condition = 4.79×10^5 and high-oxygen condition = 5.75×105; $P > 0.05$). Further intact protein sizing also showed no significant differences in modification between atmospheric- and high-oxygen conditions (Fig. 2). Together, these "bottom-up" and "top-down" protein mass spectrometry approaches concurred that oxygen levels did not significantly impact rates of HNE

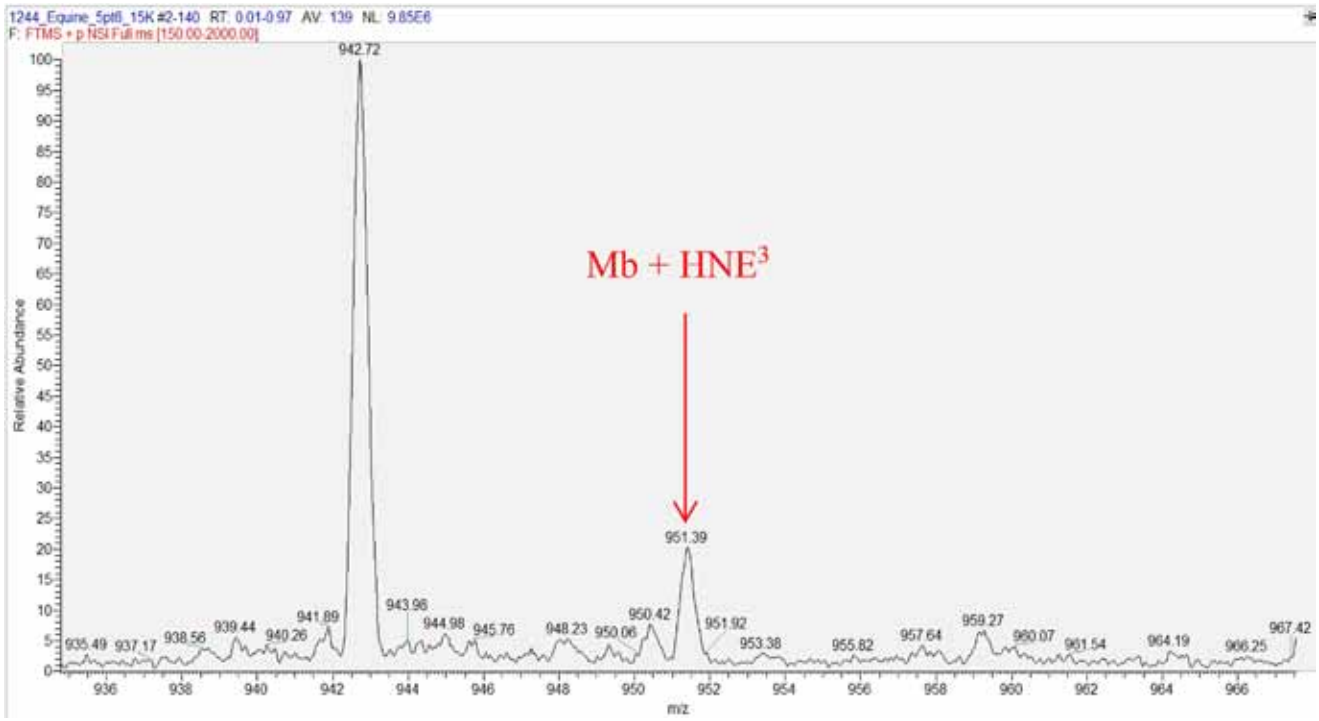

Figure 1. LC-MS/MS spectrum[1] of equine myoglobin and modified myoglobin (Mb +HNE)[2]. [1]X-axis represents mass to charge ratio (m/z) and the y-axis represents relative abundance. [2]Myoglobin ions with an observed mass to charge ratio (m/z) of 942.72 were used to calculate molecular weight, using the equation m/z = ([neutral molecular weight + (number of protons × mass of 1 proton)] / number of protons),where m and z indicate observed mass and charge, respectively. The charge of the precursor ion was 18. Using this value, the experimental molecular weights of equine myoglobin (942.72 × 18 = 16,968.96) and their adducts (951.39 × 18 = 17,125.02) were calculated. The molecular weight of HNE is 156.2. Hence, the observed 156.2-Dalton (17,125.02 – 16,968.96 = 156.06) shift in myoglobin molecular weight demonstrated the covalent binding of HNE to myoglobin molecules. [3]Typically, myoglobin was modified by a single HNE adduct, occurring at 1 of the 3 sites identified in Fig. 2. Further, di- or triple adducts were not identified in the current study. n = 3 with 2 injections/technical replicates per experiment.

adduction to myoglobin under the conditions tested. Hence, the combined effects of high-oxygen conditions and HNE-binding may have accelerated oxymyoglobin oxidation with increased incubation time.

In the current research, greater levels of metmyoglobin in HNE-treated oxymyoglobin kept at 80% oxygen partial pressure by the end of incubation period may be due to the effects of dissolved oxygen level, HNE, and/or display light. The role of oxygen concentration in myoglobin oxidation has been extensively studied (George and Stratmann, 1952; Brantley et al., 1992). High-oxygen packaging can create greater oxygen levels in buffer than atmospheric oxygen, and oxygen solubility is greater at lower temperature. Previous research also noted with a 10°C increase in temperature, dissolved oxygen level was lowered by 4 times (Matthews and Berg, 1997). High-oxygen concentration can favor free radical formation in the presence of a catalyst such as light. For example, in the present study, myoglobin samples were kept under continuous fluorescent lighting to simulate retail display conditions. Therefore, ultra-violet rays in display light, in part, can act as a pro-oxidant and initiate oxymyoglobin oxidation. The superoxide anion radicals (O_2^-) and neutral superoxide react with each other to form H_2O_2 (Cadenas, 1989). Since the pH was acidic, protonation of the O_2^- could have occurred producing reactive hydroxyperoxyl radical species. Further, the acidic condition can favor the protonation of liganded oxygen and leads to rapid autooxidation of myoglobin under high-oxygen conditions (Richards, 2013). Hence, greater metmyoglobin formation was observed in high-oxygen condition (80% O_2) than oxymyoglobin at atmospheric conditions (20% O_2) during 96 h of incubation. The current in vitro system does not involve metmyoglobin reducing activity; hence a balance between antioxidant and pro-oxidant processes in situ may determine meat discoloration.

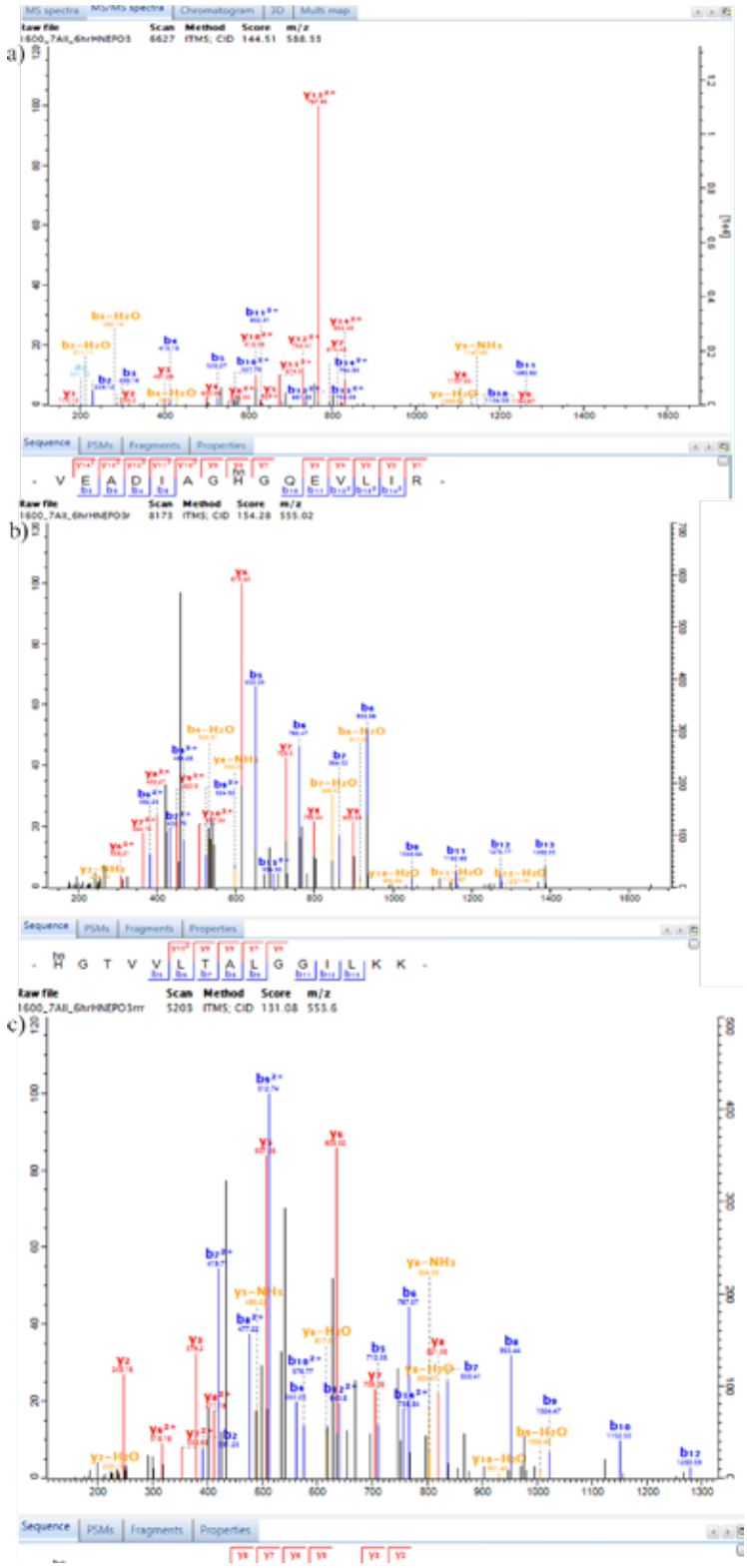

Figure 2. Effect of oxygen partial pressure and display time on HNE binding on 3 (a–c) myoglobin peptides1. (a) V(18)EADIAGH(25)GQEVLIR(32)2; (b) H(65)GTVVLTALGGILKK(79); (c) H(120)PGDFGADAQGAMTK(134).

[1]HNE-modified myoglobin peptides were generated in vitro, and then analyzed by LC-MS/MS as described in methods. The best-scoring peptide-spectrum matches for HNE-modified peptides are presented, showing predicted b (blue) and y (red) peptide fragments. Detailed amino acid weight and modification are included in table 1 as single ion charge. n = 3 with 2 injections/technical replicates per experiment. [2]Histidines modified by HNE are represented as the red font in each peptide sequence.

Table 2. Tabular format of MS/MS spectrum features of 4-hydroxyl-2-nonenal (HNE)-adducted myoglobin peptide in atmospheric- and high-oxygen conditions[1]

Peptide position[2]	Peptide sequence[3]	Modification and mass shift	Precursor m/z	b- and y-ions identified[4]
18–32	VEADIAGHQEVLIR	Unadducted	588.33	b-ions: 100.07 (b_1), 229.12 (b_2), 300.16 (b_3), 415.18 (b_4), 528.27 (b_5), 599.30 (b_6), 656.32 (b_7), 793.38 (b_8), 921.44 (b_9), 1050.48 (b_{10}), 1149.55 (b_{11}), 1262.62 (b_{12}), 1375.72 (b_{13}), 1531.82 (b_{14})
				y-ions: 175.12 (y_1), 288.20(y_2), 401.28 (y_3), 500.35 (y_4), 629.39 (y_5), 757.45 (y_6), 894.51 (y_7), 951.54 (y_8), 1022.57 (y_9), 1135.66 (y_{10}), 1250.68 (y_{11}), 1321.72 (y_{12}), 1450.76 (y_{13}), 1549.83 (y_{14})
18–32	VEADIAG**H*** QEVLIR	HNE, +156.11	588.33	b-ions: 100.07 (b_1), 229.12 (b_2), 300.16 (b_3), 415.18 (b_4), 528.27 (b_5), 599.30 (b_6), 656.32 (b_7), **949.38 (b_8), 1077.55(b_9), 1206.60 (b_{10}), 1305.66 (b_{11}), 1418.75 (b_{12}), 1531.83 (b_{13}), 1687.93 (b_{14})**
				y-ions: 175.12 (y_1), 288.20(y_2), 401.28 (y_3), 500.36 (y_4), 629.40 (y_5), 757.46 (y_6), **1050.63 (y_7), 1107.65 (y_8), 1178.68 (y_9), 1291.77 (y_{10}), 1406.79 (y_{11}), 1477.83 (y_{12}), 1606.88 (y_{13}), 1705.94 (y_{14})**
65–79	HGTVVLTALGGILKK	Unadducted	555.02	b-ions: 138.07 (b_1), 195.09 (b_2), 296.14 (b_3), 395.20(b_4), 494.27(b_5), 607.36 (b_6), 708.40 (b_7), 779.44 (b_8), 892.53 (b_9), 949.55 (b_{10}), 1006.57(b_{11}), 119.65 (b_{12}), 1232.74 (b_{13}), 1360.83(b_{14}), 1488.93(b_{15})
				y-ions: 147.11 (y_1), 275.21(y_2), 388.29(y_3), 501.38(y_4), 558.40(y_5), 615.42 (y_6), 728.50(y_7), 799.54(y_8), 900.59(y_9),1013.67(y_{10}), 1112.74(y_{11}), 1211.81(y_{12}), 1312.86(y_{13}), 1369.88(y_{13}), 1506.95(y_{15})
65–79	**H***GTVVLTALGGILKK	HNE, +156.11	555.02	b-ions: **294.18 (b_1), 351.20(b_2), 452.25 (b_3), 551.31(b_4), 650.38(b_5), 763.47(b_6), 864.51(b_7), 935.55(b_8), 1048.64(b_9), 1105.66(b_{10}), 1162.68(b_{11}), 1275.76 (b_{12}), 1388.85 (b_{13}), 1516.94 (b_{14}), 1645.04(b_{15})**
				y-ions: 147.11(y_1), 275.21(y_2), 388.29 (y_3), 501.38(y_4), 558.40(y_5), 615.42(y_6), 728.50(y_7), 799.54(y_8), 900.59(y_9), 1013.67(y_{10}), 1112.74(y_{11}), 1211.81(y_{12}), 1312.86(y_{13}), 1369.88(y_{14}), **1663.05(y_{15})**
120–134	HPGDFGADAQGAMTK	unadducted	553.6	b-ions: 138.07(b_1), 235.12(b_2), 292.14(b_3), 407.17(b_3), 554.24(b_4), 554.24(b_5), 611.26(b_6), 682.29(b_7), 797.32(b_8), 868.36(b_9), 996.41(b_{10}), 1053.44(b_{11}), 1124.48(b_{12}), 1255.52(b_{13}), 1356.56(b_{14}), 1484.66(b_{15})
				y-ions: 147.11(y_1), 248.16(y_2), 379.20(y_3), 450.23(y_4), 507.26(y_5), 635.31(y_6), 706.36(y_7), 821.38(y_8), 892.41(y_9), 949.44(y_{10}), 1096.51(y_{11}), 1211.54(y_{12}), 1268.56(y_{13}), 1365.61(y_{14}), 1502.67(y_{15})
120–134	**H***PGDFGADAQGAMTK	HNE, +156.11	553.6	b-ions: **294.18(b_1), 391.23(b_2), 448.25(b_3), 563.28(b_4), 710.35(b_5), 767.37(b_6), 838.40(b_7), 953.43(b_8), 1024.47(b_9), 1152.53(b_{10}), 1209.55(b_{11}), 1280.59(b_{12}), 1411.63(b_{13}), 1512.67(b_{14}), 1640.77(b_{15})**
				y-ions: 147.11(y_1), 248.16(y_2), 379.20(y_3), 450.23(y_4), 507.26(y_5), 635.31(y_6), 706.36(y_7), 821.38(y_8), 892.41(y_9), 949.44(y_{10}), 1096.51(y_{11}), 1211.54(y_{12}), 1268.56(y_{13}), 1365.61(y_{14}), **1658.78(y_{15})**

[1]Both normal- and high-oxygen conditions resulted in same location for HNE-modification.

[2]Amino acid positions in the equine myoglobin target peptides.

[3]Amino acid sequence in the myoglobin target peptide.

[4]The peptide bond between amide nitrogen and carbonyl oxygen is cleaved to form a "b-ion" and a "y-ion". A b-ion is the fragment in which the positive charge is retained at the N-terminal portion of the original peptide ion. A y-ion is the fragment in which the charge is retained at the C terminus of the original peptide ion. Observed signals assigned as b- or y-ions are listed. Ions containing an adduct moiety are mass-shifted with respect to the corresponding ions in unmodified peptides and are listed in boldface.

Conclusion

HNE was bound to 3 histidine residues both under atmospheric (20%) and high-oxygen (80%) partial pressure conditions. However, there were no differences between the extent of HNE binding between atmospheric and high-oxygen partial pressure conditions. Greater oxygen levels in combination with HNE-binding increased metmyoglobin formation. Thus, understanding the molecular basis of oxygen partial pressure- and lipid oxidation-dependent effects on oxymyoglobin oxidation will help to enhance our knowledge related to meat discoloration.

Conflict of Interest

The authors declare that they have no conflict of interest.

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