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Myoglobin and haemoglobin-mediated lipid oxidation in washed muscle: Observations on crosslinking, ferryl formation, porphyrin degradation, and haemin loss rate

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ABSTRACT

Reduced trout haemoglobin (Hb) is a mixture of oxy- and deoxy-Hb at pH 6.3. Addition of oxy/deoxyHb to washed muscle resulted in detectable ferryl Hb while adding bovine oxyHb, trout metHb, or bovine metHb did not. Trout metHb promoted lipid oxidation more rapidly than bovine metHb, attributable to lower haemin affinity in fish Hbs. Protoporphyrin IX degradation was prevalent during trout and bovine Hb-mediated lipid oxidation. Caffeic acid prevented porphyrin degradation and lipid oxidation. Crosslinked myoglobin (Mb) promoted lipid oxidation more effectively than metMb. Fish metMb released haemin more readily than mammalian metMb at pH 5.5. These studies suggest haemin dissociation from metHb causes formation of free radicals that degrade protoporphyrin and cause lipid oxidation, and appreciable quantities of deoxyHb are needed to generate ferryl Hb oxidant. Crosslinking appears to facilitate Mb-mediated lipid oxidation in washed muscle yet haemin release can occur from fish metMb at low pH.

1. Introduction

Lipid oxidation mediated by myoglobin (Mb) and haemoglobin (Hb) has received much attention due to the ability of these proteins to cause quality deterioration (e.g. off-odours and off-flavours) in muscle foods (Kanner, 1994). Mb and Hb have also been implicated in various disease states and pathologies (Reeder, Svistunenko, Cooper, & Wilson, 2004). There are numerous forms and derivatives of Mb and Hb that have the potential to promote lipid oxidation (Everse & Hsia, 1997). These include (i) haemin that dissociates from metMb/metHb, (ii) perferryl radical Mb/Hb, (iii) ferryl Mb/Hb, (iv) crosslinked Mb/Hb, and (v) iron atoms that are released upon degradation of the porphyrin moiety (Potor et al., 2013; Richards, 2013). Haem and haemin are the terms used to describe the protoporphyrin IX moiety containing a Fe^{2+} and Fe^{3+} atom, respectively. Perferryl Mb/Hb and ferryl Mb/Hb indicates the iron atom within the porphyrin that is bound to the globin is in the +4 state.

The addition of Mb and Hb to washed muscle has been used to assess various aspects of Mb and Hb-mediated lipid oxidation. One finding was that enhancing iron release from Mb, using Mb mutants susceptible to porphyrin degradation, decreased lipid oxidation rates in washed cod (Grunwald & Richards, 2006). This suggested that released iron does not contribute to Mb-mediated lipid oxidation. Another finding was that Hb promoted lipid oxidation much more effectively compared to Mb in washed muscle which was attributed to the much lower haemin affinity of Hb (Thiansilakul, Benjakul, Park, & Richards, 2012). Assessment of the other oxidative forms and derivatives of Mb and Hb requires further attention to have a more complete understanding of Hb and Mb-mediated lipid oxidation.

The purpose of this work was to better understand the primary mechanism of Mb and Hb-mediated lipid oxidation using the washed muscle model system. The tools employed include the use of acid–acetone to quantify degradation of the protoporphyrin IX moiety, sodium sulfide to detect ferryl Mb/Hb formation during storage, a haemin capturing reagent to measure haemin dissociation from Mb/Hb, and investigating crosslinked Mb as a reactant in washed cod muscle. The use of fish and mammalian Mbs and Hbs were also examined to further understand how the types of Hb and Mb in different muscle foods can differentially influence lipid oxidation reactions.

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2. Materials and methods

2.1. Chemicals

Horse heart myoglobin, potassium ferricyanide, streptomycin sulphate, sodium sulphide nonahydrate, and thiobarbituric acid were obtained from Sigma–Aldrich (St. Louis, MO). All other chemicals were reagent grade.

2.2. Preparation of trout and bovine Hb

Bovine and trout haemoglobin were prepared from freshly drawn blood in heparin anticoagulant (30 Units/ml blood) as described previously (Sannaveerappa, Undeland, & Sandberg, 2007).

2.3. Preparation of metHb and metMb

Four mol of potassium ferricyanide were added per mol of Hb (on a haem basis) or Mb and mixed. After incubation on ice for 1–2 h, ferricyanide was removed using 10 DG desalting columns (Bio-Rad, Hercules, CA). The millimolar extinction coefficients of 179 mM^{-1} cm^{-1} at 405 nm for metHb (haem basis) and 188 mM^{-1} cm^{-1} at 408 nm for metMb were used (Antoni & Brunoni, 1971).

2.4. Preparation of ferryl Hb and ferryl Hb treated with Na_2S

Trout metHb (25 μM on haem basis) was reacted with H_2O_2 (75 μM) in 50 mM sodium phosphate buffer (pH 7.4) at room temperature for 0.5 min. Then Na_2S was added (2 mM final concentration) and immediately scanned for optical density from 700 to 400 nm using a Shimadzu double-beam absorbance spectrophotometer. A peak near 620 nm of the Na_2S treated samples is representative of ferryl Hb (Arduini, Eddy, & Hochstein, 1990).

2.5. Detection of ferryl Hb in washed cod muscle

Washed cod (pH 6.3) containing added Hb (0.5 g sample) was centrifuged at 16,100*g* for 10 min at 4 °C in a polypropylene microfuge tube at each time point of interest. One hundred microlitres of the supernatant were then mixed with 900 μL of 50 mM phosphate buffer (pH 7.4), scanned for optical density from 700 to 400 nm, followed by addition of 70 μL Na_2S (2 mM final concentration) and scanned immediately after mixing. A peak near 620 nm of the Na_2S treated samples is representative of ferryl Hb (Arduini et al., 1990). Sampling of supernatants from washed cod containing added Hbs was done at 1, 15, 25, 39, 48, 71, 113, 182, 234, and 279 h. A millimolar extinction coefficient of 10.5 mM^{-1} cm^{-1} at 620 nm was used to quantify the concentration of ferryl Hb present on a haem basis.

2.6. Preparation of crosslinked horse Mb and Mb from carp dark muscle

Crosslinked horse Mb was provided by Professor Yoichi Osawa (University of Michigan). A millimolar extinction coefficient of 76 mM^{-1} cm^{-1} at 406 nm was used to quantify crosslinked Mb concentration (Vuletich, Osawa, & Aviram, 2000). Bighead carp Mb was prepared as described previously (Thiansilakul et al., 2012).

2.7. Measurement of haemin loss from metMbs

MetMb (10 μM) was incubated with apoH64Y/V68F (40 μM) at 4 °C for 72 h. Haemin released from metMb is transferred to apo to form holoH64Y/V68F (containing bound haemin). HoloH64Y/V68F has high optical density at 600 nm and is used to measure haemin loss rate (Hargrove et al., 1994).

2.8. Storage studies of added Hb and caffeic acid in washed cod muscle

Washed cod (pH 6.3) was prepared as described previously (Sannaveerappa et al., 2007). Added Hb and Mb were examined at 44–58 μM (haem basis). HCl or NaOH (1 N) were used to adjust the pH when necessary. Final pH values of 6.3 for Hb trials and 5.7 for Mb trials were used. Streptomycin sulphate (200 ppm) was added as an antimicrobial. Final moisture content was adjusted to 90%. Caffeic acid was examined as an antioxidant at 100 μM.

2.9. Measurement of intact protoporphyrin IX during storage

The amount of intact protoporphyrin IX extracted from washed cod containing added Hb using acidic acetone (Hornsey, 1956) was quantified spectrophotometrically according to Thiansilakul, Benjakul, and Richards (2010) with slight modifications. Protoporphyrin IX in washed muscle samples (0.5 g) was extracted with 4.5 mL acid acetone reagent (acetone:H_2O:12 M HCl = 90:8:2, by volume) using a glass rod for 1.5 min. The mixture was incubated at room temperature in the absence of light for 1 h followed by centrifugation at 2000*g* for 10 min (4 °C). The acetone phase was scanned from 350 to 700 nm against blank (0.45 mL of milliQ water instead of the washed cod muscle containing added Hb). The difference between the absorbance values at 640 (A640) and 700 nm (A700) was used for calculating the concentration of protoporphyrin IX in the samples using a millimolar extinction coefficient (4.80 mM^{-1} cm^{-1}).

2.10. Thiobarbituric acid reactive substances (TBARS)

TBARS were determined according to the modified method of Buege and Aust (1978). On the day of analysis, a solution of 50% trichloroacetic acid (TCA) with 1.3% thiobarbituric acid (TBA) was prepared by mixing and heating on a hot plate (setting 5) until soluble. Then, 80–120 mg of sample was added to 1.2 ml of the reagent. After heating at 65 °C for 60 min, the samples were cooled at 4 °C for 60 min. Samples were then centrifuged at 16,000*g* for 5 min. Optical density in the supernatant was measured at 532 nm minus 650 nm. A standard curve was constructed using tetraethoxypropane and concentrations of TBARS in samples were expressed as μmol TBARS/kg washed muscle.

2.11. Statistical evaluations

A MIXED procedure of the SAS system was used to analyse data from the storage studies (Littell, Henry, & Ammerman, 1998). Means were separated using the *p*-diff test. For each treatment, two or three separate reactions were examined at each time point during storage. Since a subsample was removed from each reaction vessel at each time point, repeated measures were used. Significance was determined at $p < 0.05$.

3. Results

3.1. Effect of caffeic acid on lipid oxidation and protoporphyrin IX degradation in washed cod containing added Hb

Caffeic acid effectively inhibited trout Hb-mediated lipid oxidation during iced storage ($p < 0.05$) at pH 6.3 (Fig. 1A). In the absence of caffeic acid, Hb-mediated lipid oxidation reached maximal levels of 140 μmol/kg tissue based on TBARS at 20 h of storage, whereas in the presence of caffeic acid TBARS values were