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Effects of hemopexin on hemin and hemoglobin-mediated lipid oxidation in washed fish muscle

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ABSTRACT

Hemopexin (Hx) was isolated from pig blood using hemin-agarose chromatography. The effect of addition of Hx on hemin and hemoglobin (Hb) mediated lipid oxidation in washed cod muscle was investigated during iced storage at pH 5.5. At a 1:1 ratio of Hx to hemin, lipid oxidation as measured by development of thiobarbituric acid reactive substances (TBARS) was not significantly inhibited ($p = 0.12$). This may be attributed to a lack of hemin binding by Hx due to influence of pH and hemin precipitation. At a 1:2 ratio of Hx to Hb on a heme basis, TBARS development was significantly inhibited ($p < 0.01$) but not prevented. Equimolar addition of bovine serum albumin (BSA) in place of Hx did not inhibit TBARS development ($p = 0.43$). Hemin release from porphyrin-containing Hx, i.e. holoHx, and ferric sperm whale myoglobin (Mb) was measured at 37 °C, pH 5.7. Hemin release rates of 0.27 h^{-1} and 0.05 h^{-1}, were calculated for holoHx and Mb, respectively. While the hemin affinity of Hx was greater than published values for trout Hb, the relatively low value measured for Hx compared to Mb may be caused by a combination of low pH and the absence of NaCl in the assays.

1. Introduction

Free hemin and hemoglobin (Hb) have been shown to be responsible for promoting lipid oxidation processes that lead to chronic diseases such as atherosclerosis and inflammation (Miller, Felikman, & Shaklai, 1996). A growing body of evidence suggests that the acute phase heme binding protein hemopexin (Hx) can reduce or completely inhibit the oxidative effects of hemin and Hb in model systems of lipid oxidation (Grinshtein, Bamm, Tsemakhovich, & Shaklai, 2003; Gutteridge & Smith, 1988; Miller, Smith, Morgan, & Shaklai, 1996; Vincent, Grady, Shaklai, Snider, & Muller-Eberhard, 1988).

In addition to a role in chronic diseases, the mechanisms of lipid oxidation are of critical importance in the muscle foods industry (Pearson, Love, & Shorland, 1977). Oxidation of unsaturated fatty acids has been shown to be responsible for the development of undesirable characteristics in muscle foods such as reduced nutritional value, off-flavors, and discoloration (Kanner, 1994; Morrissey, Sheehy, Galvin, Kerry, & Buckley, 1998). Hemin and heme proteins such as Hb and myoglobin (Mb) have been shown to promote lipid oxidation in washed fish muscle (Grunwald & Richards, 2006a, 2006b; Richards & Hultin, 2002, 2003). Historically, chilling or freezing have been preferred methods to control lipid oxidation in raw muscle foods. Additionally, techniques using synthetic antioxidants such as butylated hydroxytoluene have been investigated (Torres-Arreola, Soto-Valdez, Peralta, Cárdenas-López, & Ezquerra-Brauer, 2007). However, with the limited efficacy of low temperature treatment alone, and a growing demand for foods containing fewer synthetic additives, additional methods to control lipid oxidation in raw muscle foods are needed. To date, no study of the effects of Hx on the oxidative processes in muscle food systems has been reported. To that end, the goal of this work was to investigate a possible role for Hx in the inhibition of hemin or Hb induced lipid oxidation in a model system of washed muscle.

2. Materials and methods

2.1. Chemicals

Hemin chloride was obtained from Sigma Chemical A/S (St. Louis, MO). Methyl ethyl ketone, and tris [hydroxymethyl] aminomethane (tris) were obtained from Fisher Scientific (Pittsburgh, PA). Phenylmethylsulphonyl fluoride (PMSF) was obtained from Acros Organics (Geel, Belgium). All other chemicals used were analytical grade. Distilled, deionized water was used for the preparation of all solutions, substrates, and experiments.

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2.2. Preparation of hemopexin

The method of Muller-Eberhard (1988) was adapted. Blood was collected from 5 to 7 month old male or female pigs (1/2 duroc, 1/4 landrace, 1/4 large white) by drainage from carotid arteries or jugular veins at the University of Wisconsin–Madison Meat Science and Muscle Biology Laboratory. A 1.5 L volume of blood was drained into a beaker containing 0.5 L of 5 g/100 g sodium citrate for a final concentration of 1.250 g/100 g citrate to inhibit coagulation. Immediately after collection, the blood was centrifuged at 650× *g* for 10 min at 4 °C and the slightly pink plasma was aspirated. Aprotinin dissolved in water and PMSF dissolved in ethanol were added to the plasma at 0.025 g/L and 0.001 mol/L final concentrations, respectively. The plasma was stored by placing in plastic bottles in a −20 °C freezer. For purification of Hx, two 0.4 L volumes of the plasma were thawed in a 55 °C water bath for 15 min. Protease inhibitors were re-added in the same final concentrations and the plasma was cooled on ice for 60 min. Insoluble material was removed from the plasma by centrifugation at 14,000× *g* for 60 min at 4 °C. Supernatant was diluted in half with a solution of 0.020 mol/L sodium phosphate, 0.200 mol/L sodium chloride, pH 7.5. A 0.020 L volume of hemin-agarose resin prepared by method of Tsutsui (1986) was rinsed with 0.1 L of 0.010 mol/L sodium phosphate, 0.100 mol/L sodium chloride, pH 7.5, added to the supernatant, and mixed overnight at 4 °C in the dark (Tsutsui, 1986). Next day, resin containing bound Hx was collected from the slurry by centrifugation in a clinical centrifuge and packed into an empty Econo-Pac® 10DG desalting column (Bio-Rad Laboratories, Hercules, CA). The resin was washed with 0.120 L of 0.010 mol/L sodium phosphate, 0.500 mol/L sodium chloride, pH 7.5, containing 1.0×10^{-4} mol/L PMSF, and 0.025 g/L aprotinin. Bound Hx was eluted by washing the resin with 0.1 L of 0.200 mol/L sodium citrate, pH 4.0. Eluent was concentrated to ≤0.010 L with Amicon Ultra-15 10000 MWCO centrifugal concentrators (Millipore Corporation, Bedford, MA). The pinkish-peach colored Hx preparation was frozen in liquid nitrogen and stored at −80 °C. It was suspected that the pinkish-peach color was evidence that some fraction of the Hx in the preparation contained bound porphyrin(s). Protein concentration of the preparation was determined using the BCA method (Pierce Biotechnology, Inc., Rockford, IL).

2.3. Analysis of Hx preparation

Hx preparation was analyzed by SDS-PAGE according to the method of Laemmli (1970). Gel was run at 0.200 V and 0.020 A for 2 h. To determine the relative quantities of proteins in the preparation, a digital photograph was made of the Coomassie stained gel and densitometry was performed using NIH Image 1.62. The proteins in gel bands were characterized at the mass spectrometry facility in the University of Wisconsin Biotechnology Center by excision of bands from gel, tryptic digestion, MALDI TOF/TOF mass spectrometry, and comparison of protein fragments to the United States National Center for Biotechnology Information Protein Database (National Center for Biotechnology Information Protein Database, 2011).

2.4. Preparation of washed cod muscle

Cod fish (*Gadus morhua*) fillets, determined to be fresh based on odor and appearance, were obtained from The Seafood Center (Madison, WI). Washed cod muscle (WCM) was prepared from the fillets and frozen at −80 °C according to the method of Grunwald and Richards (2006a), except that cold 0.050 mol/L sodium phosphate buffer at pH 6.3 was used for all three washing steps (Grunwald & Richards, 2006a).

2.5. Preparation of trout hemolysate

Rainbow trout (*Onchorhynchus mykiss*) housed at the University of Wisconsin–Madison Water Science and Engineering Laboratory were used. Hemolysate was purified from trout blood by the method of Richards, Modra, and Li (2002).

2.6. Addition of hemopexin, trout Hb, bovine serum albumin, and hemin to washed cod muscle

Frozen WCM was thawed overnight at 4 °C, mixed on ice for 10 min, and ground three times for 3 s in a Hamilton Beach Custom Grind Type CM04 coffee grinder (Hamilton Beach/Proctor Silex, Inc., Southern Pines, North Carolina). The pH of the WCM was adjusted to 5.6 by addition and mixing of 1.00 mol/L HCL or 1.00 mol/L NaOH on ice for 10 min. WCM was then weighed into amber reaction vials. For experiments investigating Hx and hemin, solutions were prepared in order to allow hemin to associate with Hx prior to distribution of the reactants into the WCM. This was done by combining volumes of stock 4.5×10^{-3} mol/L hemin (in 0.100 mol/L sodium hydroxide) with aliquots of the Hx preparation and incubating on ice 45 min in proportions such that the final concentrations of Hx and hemin when the solutions were added to the WCM would both be 3.5×10^{-5} mol/kg WCM. Hemin-only solutions were prepared using 0.010 mol/L sodium phosphate, pH 7.2 in place of the Hx preparation. The pH values of the incubated Hx-hemin and control hemin solutions were determined to be 5.6 and 7.2, respectively. During incubation, water and streptomycin sulfate were mixed into the WCM in vials such that the final concentrations would be 900 g/kg WCM and 0.200 g/kg WCM, respectively. After incubation, the solutions were mixed into the WCM. Reaction vials were stored on ice for the duration of the experiment. The pH values of the WCM reactions were determined to be 5.5. For experiments investigating Hx and trout hemolysate, volumes of 2.1×10^{-3} mol/L Hb (in 0.001 mol/L tris, 0.032 mol/L sodium chloride, pH 8.0) were combined with aliquots of the Hx preparation that had been buffer exchanged into 0.025 mol/L sodium phosphate, 2.5×10^{-3} mol/L tris, pH 6.0, such that the final concentrations of Hx and Hb when the solutions were added to the WCM would be 17.5×10^{-6} and 3.5×10^{-5} mol/kg WCM, respectively. Hb only solutions were prepared using 0.025 mol/L sodium phosphate, 2.5×10^{-3} mol/L tris, pH 6.0 in place of the Hx preparation. Bovine serum albumin (BSA)-Hb solutions were prepared such that the final concentrations of BSA and Hb when added to the WCM would both be 3.5×10^{-5} mol/kg WCM. It was determined that the pH values of the incubated solutions were 5.6. The solutions were incubated on ice 45 min and combined with the WCM (water and streptomycin sulfate added) in vials and handled as described above. The pH values of the WCM reactions were determined to be 5.5.

2.7. Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined according to the method of Grunwald and Richards (2006b). A standard curve was constructed using tetraethoxypropane and concentrations of TBARS in samples were expressed as μmol TBARS/kg washed cod muscle.

2.8. Exposure of Hx preparation to hemin and further purification of Hx

A 12.5×10^{-4} L volume of the Hx preparation was combined with 0.300 g hemin and 5.0×10^{-4} L of 0.1 mol/L sodium hydroxide, and mixture was incubated on ice. The mixture was buffer