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Novel interactions of caffeic acid with different hemoglobins: Effects on discoloration and lipid oxidation in different washed muscles

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ABSTRACT

Caffeic acid (CA) accelerated methemoglobin (Hb) formation at pH 5.8 and 25 °C. This was attributed to electron donation from CA to liganded O_2 in Hb. CA inhibited hemin dissociation from metHb. Pig Hb remained mostly as oxyHb and did not promote lipid oxidation in washed cod muscle (WCM) nor washed turkey muscle (WTM) during iced storage at pH 5.8. Conversely, perch Hb rapidly was converted to metHb and readily promoted lipid oxidation based on lipid peroxide and hexanal formation. CA strongly inhibited perch Hb-mediated lipid oxidation during storage. Once metHb formation occurred in WCM, CA appeared to maintain the heme protein as metHb during the remainder of iced storage. CA may have become bound to perch Hb based on filtration analysis. CA facilitated the transfer of perch Hb (but not pig Hb) from the aqueous phase to the insoluble components of WCM. Collectively, these results suggest that CA inhibited Hb-mediated lipid oxidation by various mechanisms not related to inhibition of metHb formation.

1. Introduction

Lipid oxidation is a major cause of quality deterioration in various meat products, mainly due to incurring off-flavors and discoloration. Hemoglobin (Hb) and myoglobin (Mb) are present in post mortem muscle tissue and have been shown to promote lipid oxidation in model lipid systems (Tichivangana & Morrissey, 1985; Undeland, Kristinsson, & Hultin, 2004). Yet studies examining the relative ability of Hb and Mb to promote lipid oxidation are lacking. A recent study showed that Hb was much more effective at promoting lipid oxidation compared to Mb in washed muscle at pH 6.0 (Thiansilakul, Benjakul, Park, & Richards, 2012). Residual Hb levels are substantial in muscles from bled animals that include pork, poultry, fish, and beef (Richards, 2010). The much greater ability of Hb to promote lipid oxidation compared to Mb warrants investigation of Hb as a catalyst of lipid oxidation and the ability of antioxidants to counteract Hb reactivity.

One mechanism proposed for Hb-mediated lipid oxidation involves formation of peroxyl and alkoxyl radicals via decomposition of lipid hydroperoxides by hemin that is released from α and β chains of Hb (Sevanian & Hochstein, 1985). Alternatively, iron released from hemin can catalyze the generation of hydroxyl radicals that initiate lipid oxidation (Welch, Davis, Van Eden, & Aust, 2002). It is challenging to determine the primary mechanism of lipid oxidation due to Hb because complicated phenomenon such as Hb autooxidation, hemin dissociation, ferryl Hb radical formation, and iron release upon destruction of the prophyrin ring can occur simultaneously and continually in a short time frame (Everse & Hsia, 1997).

Fish Hbs showed extremely high autooxidation and hemin loss rates as compared to bovine Hb which was attributed to amino acid differences at key sites near and within the heme crevice that weakened heme-globin linkages, displaced ligands, and enhanced solvent channels in the fish Hbs (Aranda et al., 2009). This likely explained the much elevated ability of fish Hbs to promote lipid oxidation compared to bovine Hb (Richards, Modra, & Li, 2002). The relative ability of pig Hb to promote lipid oxidation in washed muscle has not been examined.

The ability of various phenolics to inhibit lipid oxidation in muscle systems has recently been undertaken (Kristinova, Mozuraityte, Storro, & Rustad, 2009; Medina, Gallardo, Gonzalez, Lois, & Hedges, 2007; Pazos, Lois, Torres, & Medina, 2006). Phenolics including phenolic acids, anthocyanins and flavonoids, are secondary metabolites in numerous plants, fruits, spices, tea, coffee, seeds, vegetables and grains. Caffeic acid (CA) contains hydroxyl group in the 3- and 4-position of cinnamic acid and has been observed to have free radical scavenging and metal chelating properties (Huang, Smart, Wong, & Conney, 1988; Rice-Evans, Miller, & Paganga, 1997).

One objective of this study was to compare the pro-oxidative characteristic of perch and pig Hbs to better understand the mechanisms by which heme proteins accelerate lipid oxidation in muscle food systems. Furthermore, we have examined various aspects involving effects of CA on Hb-mediated lipid oxidation beyond free radical scavenging and metal chelating capacities.

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2. Materials and methods

2.1. Chemical reagents

Ferric chloride, streptomycin sulfate, ferrous sulfate, barium chloride, ammonium thiocyanate, and sucrose were obtained from Sigma Chemical A/S (St. Louis, MO). Chloroform, methanol, methyl ethyl ketone, and tris-(hydroxymethyl)-aminomethane (Tris) were obtained from Fisher Scientific (Pittsburgh, PA). All other chemicals used were analytical grade.

2.2. Preparation of perch and pig Hbs

Four volumes of blood from perch and swine, both of which were obtained from campus sources (University of Wisconsin–Madison), were well mixed with 1 volume of anticoagulant containing 150 mM NaCl and sodium heparin (120 units/ml). To remove plasma, washing buffer (4 volumes of 1.7% NaCl in 1 mM Tris, pH 8.0) was added to heparinized blood and centrifuged (700 *g* for 10 min at 4 °C) in a Beckman J-6B centrifuge (Beckman Instruments Inc., Palo Alto, CA). The red blood cells were washed three times more using 10 volumes of same washing buffer (Fyhn et al., 1979). After which 3 volumes of stock solution (1 mM Tris, pH 8.0) were added to lyse cells for 30 min. One-tenth volume of 1 M NaCl was then added to aid in stromal removal, and they were centrifuged (28,000 *g* for 15 min at 4 °C) in a Beckman L8-70M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). Hemolysates were then stored at −80 °C until use.

2.3. Quantification of Hb concentration

To measure Hb concentration, perch and pig Hbs were diluted in 1 mM Tris, pH 8.0. The diluted Hbs were scanned from 700 to 400 nm in a model UV-2401 dual-beam spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD) using 1 mM Tris, pH 8.0 as a reference. Hb concentrations were calculated using the peak absorbance occurring near 578 nm (Zijlstra & Buursma, 1977).

2.4. Preparations of washed cod muscle (WCM) and washed turkey muscle (WTM)

Cod fish (*Gadus morhua*) fillets and turkey breast muscle were obtained from The Seafood Center (Madison, WI) and Kraft-Oscar Mayer (Newberry, SC), respectively. Freshness test of cod fillets was based on their odor and appearance. To remove all bones, dark tissue and remaining blood, cod fillets and turkey muscles were trimmed and cut into small pieces. The pieces were ground using a Kitchen Aid, Inc. (St. Joseph, MI) KSM90WW household mixer equipped with grinding apparatus (5 mm plate diameter). Ground cod muscles were washed 3 times using 50 mM sodium phosphate buffer (pH 6.3) in 1:3 ratio. In each washing time, cod muscles were mixed with phosphate buffer for 2 min with a glass rod, soaked for 15 min and then dewatered using a fiberglass screen. Ground turkey breast were washed 6 times using 50 mM sodium citrate buffer (pH 5.6), and washing procedures were same as above. After the final soak, muscle slurry was homogenized for approximately 3 min with a Polytron Type PT 10/35 probe (Brinkmann Instruments, Westbury, NY), and then were centrifuged at 15,263 ×*g* for 25 min (Sorvall, RC5C-PLUS). Collected pellets were stored at −80 °C in vacuum-sealed plastic bags. All washing, dewatering, and centrifugation steps were performed at 4 °C.

2.5. Addition of Hb and CA to WCM and WTM

The pH of WCM and WTM was adjusted to 5.8 by addition of 1 N HCl or 1 N NaOH. WCM or WTM were then added to an amber bottle (30 ml capacity). Water and streptomycin sulfate (200 ppm) were added to adjust final moisture content of 90% and to inhibit microbial growth, respectively, and they were mixed well using a plastic spatula for 2 min. Ethanolic CA was then added to the mixture and mixed for 4 min using a plastic spatula. A concentration of 555 μM CA (100 ppm) was typically examined since this was shown to inhibit cod Hb-mediated lipid oxidation in washed muscle while 83 μM CA (15 ppm) was less effective (Larsson & Undeland, 2010). Pure ethanol were added to treatments without CA and mixed in same conditions. The perch or pig Hb (10 μM) was then added to the mixture and mixed for 2 min using a plastic spatula. Samples were stored at 2 °C for 4 days. The pH values of washed muscle systems were measured.

2.6. Determination of lipid peroxides

Samples (0.3 g) were homogenized with 5 ml of chloroform: methanol (1:1) for 20 s using a Polytron Type PTA 20/2W probe (Brinkmann Instruments, Westbury, NY) and homogenate was transferred to a screw-cap glass centrifuge tube. The Polytron probe was then rinsed for 20 s with another 5 ml of the solvent, which was also added to the centrifuge tube. A 0.5% NaCl (3.08 ml) was added to the centrifuge tube, and then vortexed for 30 s. The mixture was centrifuged at 1800 *g* for 6 min at 4 °C using a Beckman J-6B centrifuge (Beckman Instruments Inc., Palo Alto, CA). A 2 ml of the lower chloroform layer was removed and transferred to a clean glass screw-cap tube. A 1.33 ml of chloroform:methanol (1:1), 25 μl of 30% ammonium thiocyanate and 25 μl of 18 mM iron(II) chloride were added to the tube. The samples were incubated for 20 min at room temperature and absorbance read at 500 nm (Shantha & Decker, 1994). A standard curve was constructed using cumene hydroperoxide.

2.7. Hexanal determination

Hexanal from WCM and WTM was extracted using solid phase microextraction (SPME) technique. Samples (1 g) and internal standard (0.2 μl, 200 ppm chlorobenzene in methanol, Supelco, Bellefonte, USA) were transferred into a 10 ml vial with 20 mm clear crimp and the vial was sealed with a hole metal cap with PTFE/silicone septa (MicroLiter Analytical Supplies Inc., Suwanee, USA). Before extraction of hexanal, fiber (65 μm, polydimethylsiloxane-divinylbenzene, Supelco, Bellefonte, USA) was conditioned by heating in a gas chromatograph (GC, HP 6890, Hewlett-Packard, Palo Alto, USA) injection port equipped with capillary column (DB-5, 30 m length × 0.25 mm i.d. × 0.1 μm film thickness) and flame ionization detector (FID) at 260 °C for 30 min. Vials containing samples were preheated for equilibration at 40 °C for 5 min, and SPME fiber for extraction of hexanal was exposed to the headspace above the sample at 40 °C for 10 min. After injection of SPME fiber into the GC/FID injection port, hexanal extracted from samples was isolated from SPME fiber at 250 °C for 5 min. Helium as a carrier gas and splitless mode were used. Flow rate of carrier gas was 1 ml/min. Inlet and detector temperatures were 250 and 270 °C, respectively. The oven temperature was programmed at 40 °C for 5 min with a 10 °C/min ramp rate until 90 °C. Hexanal was identified by comparison of retention time of hexanal standard (Sigma-Aldrich, Steinheim, Germany) in GC/FID. The quantity of hexanal in sample was calculated using area of the internal standard.

2.8. MetHb formation

MetHb formation was calculated from the absorbance changes at 540, 560 and 576 nm as described previously (Benesch, Benesch, & Yung, 1973). Hb was buffered with 50 mM sodium phosphate (pH 5.8). Three millimolars of superoxide dismutase and catalase per M of heme were added to the mixture to remove any superoxide and hydrogen peroxide formation. Ethanol was added to treatments that did not contain ethanolic CA. Absorbance values at 540, 560, and 576 nm were also used to calculate percentages of oxyHb and deoxyHb.