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Effects of Released Iron, Lipid Peroxides, and Ascorbate in Trout Hemoglobin-Mediated Lipid Oxidation of Washed Cod Muscle

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Approximately 7% of the iron associated with hemoglobin was released from the heme protein during 2 °C storage in washed cod muscle. EDTA (2.2 mM) neither accelerated nor inhibited hemoglobin-mediated lipid oxidation based on the formation of lipid peroxides and TBARS. This suggested that low molecular weight iron was a minor contributor to hemoglobin-mediated lipid oxidation in washed cod muscle. Ascorbate (2.2 mM) was a modest to highly effective inhibitor of hemoglobin-mediated lipid oxidation depending on which washed cod preparation was assessed. Experimental evidence suggested that the ability of residual ascorbate to breakdown accumulating lipid hydroperoxides to reactive lipid radicals can explain the shift of ascorbate from an antioxidant to a pro-oxidant. Increasing the lipid peroxide content in washed cod muscle accelerated hemoglobin-mediated lipid oxidation and decreased the ability of ascorbate to inhibit lipid oxidation. Preformed lipid peroxide content in cod muscle was highly variable from fish to fish.

KEYWORDS: Heme proteins; chelators; blood, role in quality; cumene hydroperoxide; ascorbic acid; antioxidants; muscle foods; iron determination

INTRODUCTION

Lipid oxidation is a major cause of quality deterioration in muscle foods. Off-odors and off-flavors that result from lipid oxidation effectively end shelf life. Hemoglobin, myoglobin, copper, and iron have the potential to promote lipid oxidation in muscle foods (*1*). Since iron can be released from hemoglobin during storage, it is difficult to ascertain whether the intact heme protein, dissociated heme or released iron is responsible for the bulk of lipid oxidation that occurs during storage. The term low molecular weight iron is used instead of free iron since iron binds to other low molecular weight compounds to have solubility and hence potential reactivity (*2*). Metal chelators can be used as tools to investigate the role of low molecular weight (LMW) iron in lipid oxidation processes. Phosphates, citrate, ADP, and ethylenediamine tetraacetic acid (EDTA) are commonly used chelators that can either inhibit or promote lipid oxidation processes. EDTA will shift from a pro-oxidant to an antioxidant depending on its concentration (*3*). A high ratio of EDTA to iron inhibits lipid oxidation while a 1:1 ratio promotes lipid oxidation (*3*). The 1:1 ratio promotes lipid oxidation because it increases solubility of iron for reaction and facilitates redox cycling of iron. Ferrous and ferric forms of iron can promote lipid oxidation processes (*4*, *5*). The higher ratios of EDTA to iron will prevent reactivity of iron with reactants such as hydrogen peroxide and lipid peroxides. It should be kept in mind that another metal chelator, desferrioxamine, inhibited lipid oxidation by an electron-donating mechanism (*6*). Thus, it may not be prudent to strictly associate inhibition of lipid oxidation by "metal chelators" with only their metal chelating properties. The concentration of LMW iron relative to heme protein catalyst must also be considered when assessing the impact of released iron relative to other forms of hemoglobin.

Assessing mechanisms of lipid oxidation and exogenously added antioxidants in muscle tissue is difficult due to the multitude of endogenous antioxidants and pro-oxidants that are present. The approach of these studies was to use washed cod muscle as the lipid substrate. Washing removes aqueous antioxidants and pro-oxidants. The remaining washed tissue contains myofibrillar proteins and membrane phospholipids. Trout hemoglobin is used to stimulate oxidation of the membrane phospholipids. This is because hemoglobins from other species such as bovine and avian animals were found to be weaker catalysts of lipid oxidation in washed cod (*7*). Further, cod muscle has a low mitochondria content. Mitochondria are a source of reactive oxygen species that could confound lipid oxidation reactions due to added hemoglobin.

Loss of redness is an indicator that lipid oxidation processes mediated by hemoglobin (Hb) are progressing. Just after death Hb in muscle tissue is primarily in the reduced state (e.g. oxyHb and deoxyHb). The mixture of oxyHb and deoxyHb possesses a red color. With increased postmortem aging this reduced Hb autooxidizes to metHb, a brown pigment. MetHb is considered

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more pro-oxidative than reduced Hb due to its less tightly bound heme group and the reactivity of metHb with hydrogen peroxide and lipid peroxides to form hypervalent Hb catalysts (*8*).

Ascorbate can have antioxidative and pro-oxidative effects depending on factors that include the presence of low molecular weight metals and lipid hydroperoxides, as well as ascorbate concentration (*9*, *10*). The ability of ascorbate to inhibit lipid oxidation is attributable to regenerating tocopherol radicals, reducing hypervalent forms of hemoglobin, and scavenging of lipid radicals that have the ability to abstract a hydrogen atom from polyunsaturated fatty acids and hence initiate lipid oxidation processes (*11*). Pro-oxidant effects of ascorbate include the ability of ascorbate to reduced ferric iron to the more pro-oxidative ferrous Hb (*12*). These studies were undertaken to examine effectors and mechanisms of hemoglobin-mediated lipid oxidation. More efficient strategies to improve the shelf life of muscle foods can be achieved as the basic mechanisms of lipid oxidation processes mediated by heme proteins are made clearer.

MATERIALS AND METHODS

Materials. Cumene hydroperoxide, disodium ethylenediamine tetraacetate, sodium ascorbate, sodium dithionite, bovine hemoglobin, and tetraethoxypropane were obtained from Sigma (St. Louis, MO). Chloroform stabilized with ethanol was used (Fisher, Pittsburgh, PA). All other chemicals were reagent grade. Rainbow trout (*Onchorhynchus mykiss*) (25−40 cm) were maintained in tanks at the University of Wisconsin, Water Science and Engineering Laboratory (Madison, WI). Codfish (*Gadus morhua*) were obtained from a local fish market or delivered overnight via air transport from Boston, MA. The postmortem age ranged from 12 to 72 h, and gutted fish or fillets were transported with cold packs. Fillets used were considered of excellent quality based on odors that ranged from sea-like (very fresh) to minimal overall odor.

Preparation of Washed Cod Muscle. Washed cod was prepared from 3 to 4 fillets as soon as fish arrived at the laboratory. All dark muscle was removed. The rest of the fillets were ground in a KS M90 mincer (Kitchen Aid Inc., St Joseph, MI) (plate diameter 5 mm). The mince was washed once in distilled deionized water at a 1:3 mince-to-water ratio (w:w) by stirring with a plastic rod for 2 min. Subsequently, the mixture was allowed to stand for 15 min before dewatering with 2 layers of cotton cheesecloth. Mince was then mixed with 50 mM sodium phosphate buffer (pH 6.3) at the same 1:3 ratio. This phosphate wash was repeated and the mince-buffer mixture was homogenized (setting 1) using a Polytron Type PT 10/35 (Brinkmann Instruments, Westbury, NY). It was allowed to stand for 15 min and finally centrifuged (15,000 *g* for 20 min at 4 °C) using a Beckman L8−70M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The resulting pellet was then used as the washed cod muscle. The final moisture content of the washed cod preparations was between 85.7 and 86.7%, indicating fairly consistent yields.

Preparation of Trout Hemoglobin and Quantifying Hb Concentration. Blood was removed from the caudal vein via syringe after exposure of rainbow trout to aminobenzoic acid ethyl ester anesthetic (0.5 g/L water). Four volumes of ice cold 1.7% NaCl in 1 mM Tris, pH 8.0, were 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). After removal of the plasma, the red blood cells were washed by suspending three times in 10 volumes of the above buffer (*13*). Cells were lysed in 3 volumes of 1 mM Tris, pH 8.0 for 1 h. One-tenth volume of 1 M NaCl was then added to aid in stromal removal before ultracentrifugation (28,000 *g* for 15 min at 4 °C) using a Beckman L8−70M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). Hemoglobin solutions were stored at −80 °C prior to use. The method of Brown (*14*) was used to quantify the hemoglobin concentration. This involves reduction of the diluted Hb solution in 50 mM Tris, pH 8.0 with a few crystals of dithionite followed by bubbling with carbon monoxide gas (99.3% purity). The peak absorbance between 440 and 400 nm is then recorded. A standard curve was prepared using bovine Hb. SDS−PAGE electrophoresis indicated the only detectable bands were in the range of hemoglobin polypeptides (data not shown).

Addition of Trout Hemoglobin to Washed Cod Muscle. An appropriate volume of the hemoglobin stock (around 400 μM Hb) was added to washed cod muscle so that a final concentration of 13.3 μM Hb was obtained. Samples were stirred with a plastic spatula for 3 min to distribute the heme protein. This level of hemoglobin was selected since it is near the range of hemoglobin levels found in light muscle of trout (*15*). Streptomycin sulfate (200 ppm) was added to inhibit microbial growth during storage. The pH was adjusted if necessary by addition of 1 M NaOH or 1M HCl. To measure pH, around 0.5 g of sample was diluted in 10 vol of distilled deionized water, homogenized, and readings were recorded using an Accumet AR50 pH meter (Fisher Scientific, Pittsburgh, PA). The final pH and moisture content of the samples was pH 6.3 and 90%, respectively. It is critical to maintain or adjust all samples at a uniform pH since trout Hb-mediated lipid oxidation in washed cod is sensitive to pH (*16*).

Addition of Ascorbate, EDTA, and Cumene Hydroperoxide to Washed Cod. A concentrated stock solution of ascorbate and EDTA was prepared in distilled deionized water. These solutions were added just prior to the addition of trout hemoglobin so that the final concentrations of ascorbate (20 μM, 200 μM, or 2.2 mM) and EDTA (2.2 mM) were obtained. Cumene hydroperoxide was dissolved in ethanol so that the percentage of ethanol in the stored samples was 1%. Control samples not containing cumene hydroperoxide contained an equivalent percentage of ethanol. Mixing with a plastic spatula for 2 to 3 min was done to obtain a homogeneous distribution of added solutions. Samples were stored in 30 mL light-resistant amber glass bottles with screw-cap lids. The thickness of samples was 4 to 6 mm in each container. The storage condition of the samples was 2 °C for up to 8 days.

Low Molecular Weight Iron Assay. The amount of free iron in the washed cod muscle model system was measured based on a modified ferrozine method (*17*, *18*). Around 1 g of washed cod muscle was homogenized using a Polytron PT10−35 (Brinkmann Instruments, Westbury, NY) in three volumes of 0.1M citrate and 0.1M phosphate, pH 5.5. Homogenate was then centrifuged (Beckman J−6B, Fullerton, CA) at 1800 g for 10 min to remove the muscle solid. One ml of the supernatant was mixed with freshly prepared 2% ascorbic acid (0.5 mL) in 0.2N HCl and incubated at room temperature for 5 min. Then, 0.5 mL of 11.3% trichloroacetic acid was added, mixed thoroughly, and the mixture was centrifuged at 13,400 rpm in an Eppendorf 5415D microfuge (Brinkmann Instruments, Westbury, NY) for 10 min. The supernatant (1 mL) was mixed with 10% ammonium acetate (0.4 mL) and ferroin color reagent (0.1 mL). Absorbance was read at 562 nm against a blank. All assays were completed in either disposable polyethylene test tubes or disposable microfuge tubes to minimize possible iron contamination.

Determination of Thiobarbituric Acid Reactive Substances (TBARS). TBARS were determined according to a modified procedure of Buege and Aust (*19*). To dissolve the TBA, 50% trichloroacetic acid (TCA) containing 1.3% thiobarbituric acid (TBA) was heated to 65 °C on the day of use. Approximately 150 mg of sample was added to 1.2 mL of the TCA−TBA mixture and incubated for 1 h at 65 °C. After centrifugation (2500 *g* for 10 min.), the absorbance of the supernatant at 532 nm was determined. A standard curve was constructed using tetraethoxypropane.

Determination of Lipid Content in Cod Muscle. Minced muscle was homogenized in 10 volumes of chloroform:methanol (1:1) using a Polytron PT10−35 (Brinkmann Instruments, Westbury, NY). The slurry was then added to a separatory funnel. Two volumes of 0.5% NaCl were added and swirled for 30 s. The separatory funnel was capped and allowed to stand for 1−6 h at room temperature or for 24 h in the cold room. The bottom chloroform layer was collected into a glass graduated cylinder and the volume was recorded. The chloroform layer was then added into a preweighed glass tube (using a scale that records to the 1/10,000th decimal place). Nitrogen gas (99.995% purity) was then bubbled into the sample to remove chloroform. The mass of the oil was then recorded.

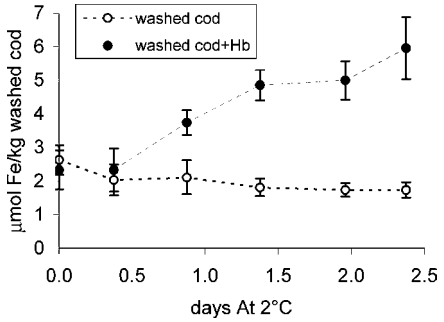

Figure 1. Changes in low molecular weight (LMW) iron concentration in washed cod muscle during 2 °C storage in the presence and absence of added trout hemoglobin. Hemoglobin concentration was 13.3 μM (hemoglobin basis).

Determination of Lipid Peroxides. Washed cod muscle was homogenized in 10 volumes of chloroform/methanol (1:1) (w:v) for 30 s using a Polytron Type PT 10/35 (Brinkmann Instruments, Westbury, NY). Subsequently, the polytron was rinsed for 30 s with 10 volumes of solvent. The homogenate and wash solution were then combined. Six volumes of 0.5% NaCl were added and the mixture was mixed for 30 s with a Vortex before centrifugation for 10 min (4 °C and 700 g) to separate the mixture into two phases. Then, 1.33 mL of ice cold chloroform/methanol (1:1) was added to 2 mL of the lower phase and mixed briefly. Before the absorbances at 500 nm were determined, 25 μL ammonium thiocyanate (4.38 M) and 25 μL iron(II) chloride (18 mM) were added to the assay for lipid hydroperoxides (*20*) and the samples were incubated for 20 min at room temperature. A standard curve was prepared using cumene hydroperoxide. The chloroform used contained ethanol as a preservative to eliminate high blank readings (*21*).

Color Measurements. The *a** values were measured with a Minolta CR-200 Chroma Meter (Minolta Camera Co., Osaka, Japan). The aperture size was 1 cm. Illuminant C was used. A white calibration plate supplied with the unit was used to calibrate the instrument. The *a** value of the white plate was −0.14.

Statistical Evaluations. Analysis of variance with a MIXED procedure of the SAS system was used to evaluate data from storage studies (*22*). Triplicate extractions were obtained per sample in each study. The study replication was two. The analytical replication was in triplicate. Probability was based on differences at the $\alpha = 0.05$ significance level. Sources of variation were number of extractions per sample, time, and study. The student t-test was used to obtain significant differences in samples analyzed at a single time point.

RESULTS

The level of low molecular weight (LMW) iron determined in washed cod muscle without added hemoglobin *did not increase* during 2.5 days of iced storage (**Figure 1**). Mean LMW iron values ranged from 1.74 to 2.03 μM during storage. In the presence of Hb, LMW iron levels significantly increased ($p < 0.05$) during the storage period. Mean LMW iron values increased from 2.33 μM at 0-time to 5.96 μM at the end of the storage period (**Figure 1**). Thus, approximately 7% of the iron initially bound to hemoglobin was released during 2.5 days of 2 °C storage. This is based on the findings that around 2 μM LMW iron was determined to be in washed cod with no added Hb, and LMW iron in washed cod containing added Hb reached 6 μM (**Figure 1**). This indicates that 4 μM iron was released from an initial pool of 53.2 μM iron bound to hemoglobin. The value of 53.2 μM iron is based on the Hb concentration (13.3 μM) and the fact that each of the four heme groups in each hemoglobin molecule contain an iron atom.

The ability of hemoglobin to stimulate lipid oxidation in washed cod muscle was determined in two separate trials using thiobarbituric acid reactive substances (TBARS) as an indicator

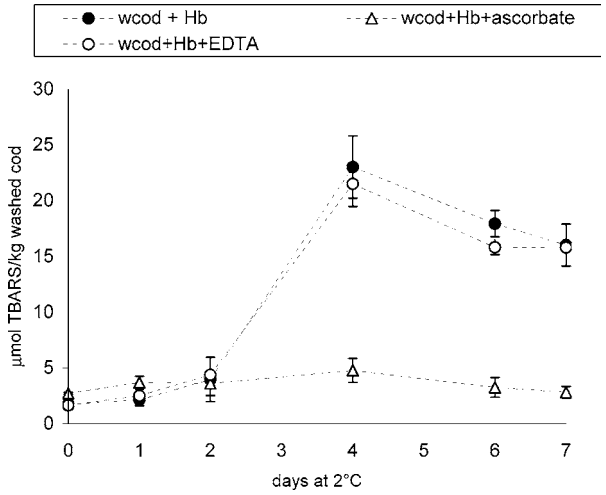

Figure 2. Effect of ascorbate (2.2 mM) and EDTA (2.2 mM) on hemoglobin-mediated TBARS formation in washed cod muscle (preparation 1) during 2 °C storage. The pH was 6.3. Hemoglobin concentration was 13.3 μM (hemoglobin basis).

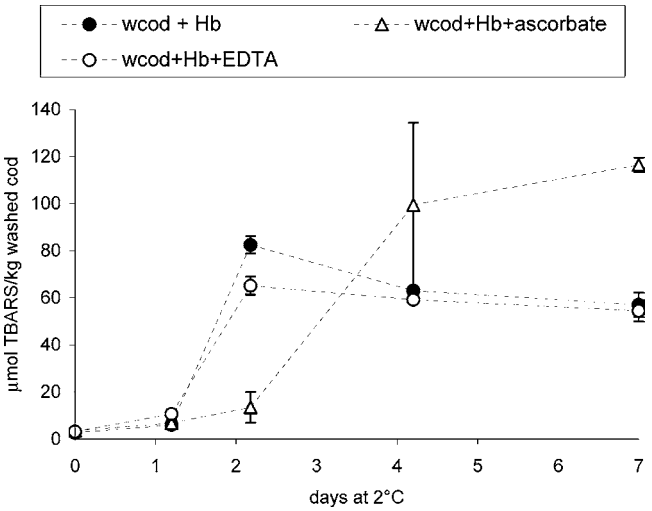

Figure 3. Effect of ascorbate (2.2 mM) and EDTA (2.2 mM) on hemoglobin-mediated TBARS formation in washed cod muscle (preparation 2) during 2 °C storage. The pH was 6.3. Hemoglobin concentration was 13.3 μM (hemoglobin basis).

of lipid oxidation. The difference between trial 1 and 2 was that fillets from different codfish were used in the preparation of the second batch of washed cod; the washing procedure was identical. Adding hemoglobin to washed cod muscle effectively stimulated lipid oxidation based on TBARS development in both trials (**Figures 2** and **3**). However, in Trial 1 there was a greater lag phase prior to the onset of TBARS compared to Trial 2 and the extent of TBARS formation was greater in Trial 2 (**Figures 2** and **3**).

EDTA (2.2 mM) was added to washed cod muscle containing hemoglobin catalyst to determine the effect of this metal chelator on rates of lipid oxidation. The rate and extent of TBARS development was similar when control samples (washed cod with added Hb) were compared to those samples containing added Hb and EDTA; this was the case both in Trial 1 (**Figure 2**) and Trial 2 (**Figure 3**).

The ability of ascorbate (2.2 mM) to inhibit TBARS formation varied considerably between trials. In Trial 1, ascorbate effectively inhibited TBARS formation during the entire 7-day storage period (**Figure 2**), whereas in Trial 2 those samples containing ascorbate attained elevated TBARS values on the

Table 1. Initial Lipid Peroxide Levels in Different Cod Muscle Procurements and TBARS Values in the Resulting Washed Cod Muscle Containing Added Hemoglobin after 1 day at 2 °C[a]

procurement	lipid peroxides in unwashed muscle (mmol/kg lipid)	TBARS (μmol/kg washed cod) in washed cod containing added Hb
1	0.56 ± 0.06x	4.2 ± 2.3x
2	0.85 ± 0.03y	6.6 ± 2.0x
3	2.43 ± 0.57z	54.8 ± 11.5y

[a] Trout hemoglobin was used in all trials (12 μmol/kg washed cod). The pH was standardized between 6.1 and 6.2 in lipid oxidation trials. Washed cod was stored at −80 °C no more than 45 days prior to lipid oxidation trial. Lipid peroxides were obtained within 1 week of obtaining fillets. Cod muscle was stored at −80 °C in the interim period. Letters in column with same letter are not significantly different ($p < 0.05$). Hb, trout hemoglobin.

4th day of storage (**Figure 3**). Further, at day 7, ascorbate-containing samples in Trial 2 attained TBARS values of 117 μmol/kg washed cod while washed cod containing only hemoglobin catalyst attained values no greater than 85 μmol/kg washed cod during storage.

The only difference between Trial 1 and 2 was that the two washed cod preparations were each prepared from different fish. Determining the preformed lipid peroxide content in the muscle used in Trial 1 and 2 was desired but the fillets were not retained. Three different procurements of cod fillets were examined to investigate if variation in the preformed lipid peroxide content of the fillets was observed. Preformed lipid peroxide values of 0.56, 0.85, and 2.43 mmol/kg lipid were measured (**Table 1**). Lipid oxidation induced by hemoglobin based on TBARS values in the resulting washed cod muscle preparations was then measured after 1 day of 2 °C storage. A trend can be observed that those washed cod preparations prepared from fish muscle with elevated peroxide values were more susceptible to hemoglobin-mediated lipid oxidation (**Table 1**). Preformed lipid peroxides determined in ordinary muscle of yellow fin tuna immediately after death were around 120 μmol/kg tissue (6.67 mmol/kg lipid) (*23*).

Loss of redness was also assessed in washed cod muscle containing added hemoglobin and either EDTA (2.2 mM) or ascorbate (2.2 mM). These data are from the same samples that were used to generate TBARS values in **Figure 3**. Loss of redness was rapid and similar in rate when comparing control- and EDTA-containing samples. Loss of redness was slowest in ascorbate-containing samples (**Figure 4**).

Lipid peroxides were also measured to provide a second indicator of lipid oxidation products. These data are from the same samples that were used to generate TBARS values in **Figure 3**. EDTA had little effect on the rate of hemoglobin-mediated lipid peroxide formation (**Figure 5**). Both the control samples and those supplemented with EDTA had extensive lipid peroxide formation at day 2 of storage. Ascorbate-containing samples had lower lipid peroxide values at day 2 and 4, and by day 7 had attained lipid peroxide values similar to the control- and EDTA-containing samples (**Figure 5**).

Cumene hydroperoxide (222 μM) was added to washed cod muscle to further examine the ability of lipid hydroperoxides to affect both hemoglobin-mediated lipid oxidation and its inhibition by ascorbate. A lag phase of around 1 day was observed prior to TBARS formation in washed cod containing added hemoglobin while no detectable lag phase was observed in washed cod containing hemoglobin and cumene hydroperoxide (**Figure 6**). Ascorbate (2.2 mM) in the absence of added cumene peroxide inhibited hemoglobin-mediated lipid oxidation

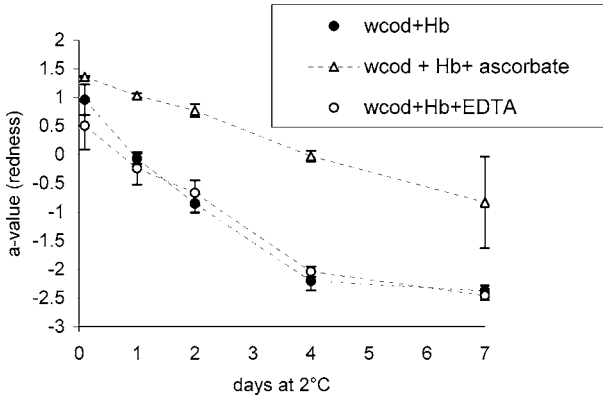

Figure 4. Effect of ascorbate (2.2 mM) and EDTA (2.2 mM) on *a*-values (redness) in washed cod muscle (preparation 2) containing hemoglobin during 2 °C storage. The pH was 6.3. Hemoglobin concentration was 13.3 μM (hemoglobin basis).

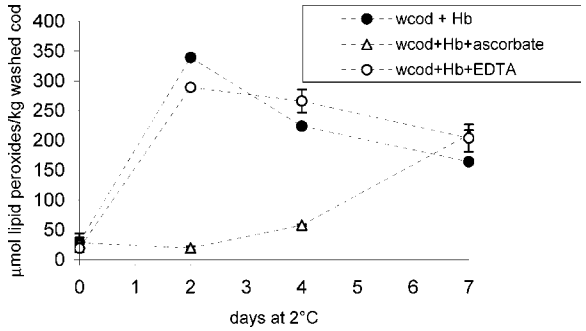

Figure 5. Effect of ascorbate (2.2 mM) and EDTA (2.2 mM) on lipid peroxide formation in washed cod muscle (preparation 2) containing hemoglobin during 2 °C storage. The pH was 6.3. Hemoglobin concentration was 13.3 μM (hemoglobin basis).

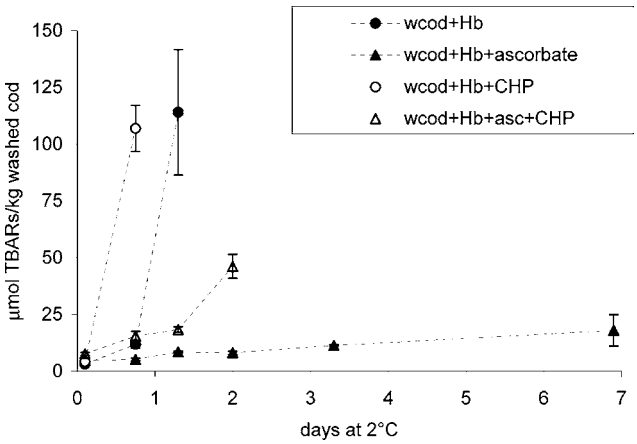

Figure 6. Effect of cumene hydroperoxide (200 μmol/kg washed cod) on TBARS values in hemoglobin-mediated lipid oxidation of washed cod muscle during 2 °C storage in the presence and absence of ascorbate (2.2 mM). The pH was 6.3. Hemoglobin concentration was 13.3 μM (hemoglobin basis).

during the entire 7-day storage period, yet when cumene hydroperoxide and ascorbate were present elevated TBARS values were determined as early as day 2 of storage (**Figure 6**). In a second trial, ascorbate was slightly less effective at inhibiting Hb-mediated lipid oxidation as evidenced by the higher TBARS values during 5 days of iced storage compared to ascorbate-containing samples in **Figure 6**, and all inhibitory efficacy of ascorbate was negated when cumene hydroperoxide was also added (**Figure 7**).

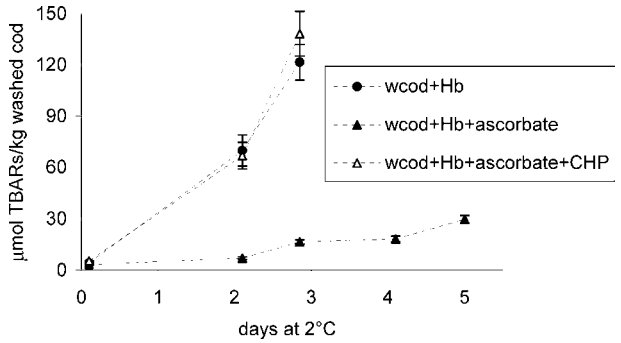

Figure 7. Effect of ascorbate (2.2 mM) on TBARS values in hemoglobin-mediated lipid oxidation of washed cod muscle during 2 °C storage in the presence and absence of cumene hyroperoxide (200 μmol/kg washed cod). The pH was 6.3. Hemoglobin concentration was 13.3 μM (hemoglobin basis).

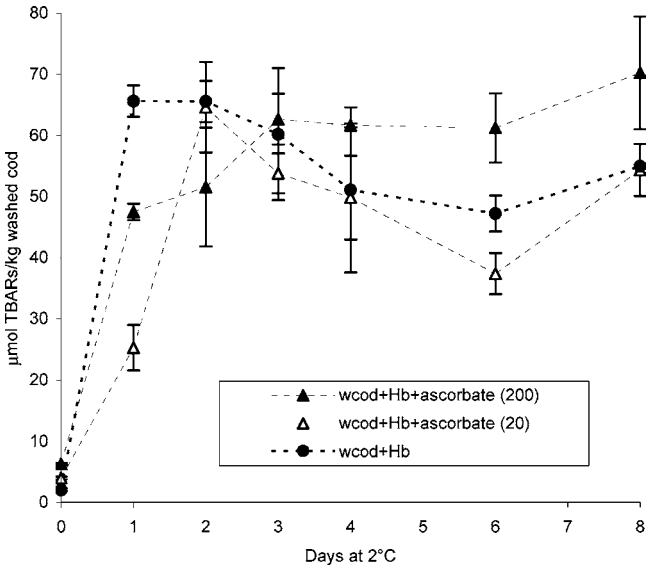

Figure 8. Effect of ascorbate (20 and 200 μM) on TBARS values in hemoglobin-mediated lipid oxidation of washed cod muscle during 2 °C storage. The pH was 6.3. Hemoglobin concentration was 13.3 μM (hemoglobin basis).

The effect of ascorbate at concentrations of 20 and 200 μM in washed cod containing Hb was also examined. The extension in the lag phase prior to TBARS development was less than 1 day for both concentrations of ascorbate compared to control samples containing Hb but without added ascorbate (**Figure 8**). The extent of TBARS development was similar in control samples compared to those containing added ascorbate. Control samples had a maximal TBARS value of 65.7 at day 1 while samples containing ascorbate at 20 and 200 μM had maximal TBARS values of 64.6 and 70.3 at day 2 and 8, respectively.

DISCUSSION

A key reaction in the promotion of lipid oxidation processes is the breakdown of preformed lipid hydroperoxides. The lipid radicals that result from the breakdown (e.g. alkoxyl, peroxyl, and heme radicals) have the ability to abstract a hydrogen atom from polyunsaturated fatty acids, which propagates further lipid peroxidation (*1*). The ability of metHb, heme, Fe^{2+}, and Fe^{3+} to decompose linoleic acid hydroperoxides was measured on an equimolar iron basis and the relative rates were 38, 198, 4.8, and 0.46 mol/min x 10^{-4}, respectively (*24*). This may explain the inability of released iron from hemoglobin to stimulate lipid oxidation in washed cod, which was based on the inability of excess EDTA, an iron chelator, to decrease the rate or extent of Hb-mediated lipid oxidation (**Figures 2**, **3**, and **5**). MetHb and heme appear to be superior in their ability to breakdown lipid hydroperoxides compared to Fe^{2+} and Fe^{3+}. In addition, iron bound to heme was present in much greater concentration than LMW iron in the period in which extensive Hb-mediated lipid oxidation occurred, which was the first 2.5 days of storage (**Figures 2**−**8**). The ratio of heme-bound iron to LMW iron ranged from 27 at day 0 to 8 after 2.5 days (**Figure 1**).

Previously, 15 μM $FeCl_3$ in the presence of histidine, ADP, and NADH did not stimulate TBARS development or rancidity in washed cod muscle (*25*). NADH is added to facilitate reduction of Fe^{3+} and Fe^{2+} through a membrane-bound reductase in the washed cod. Our results are further evidence of the inability of LMW iron to stimulate lipid oxidation in washed cod because no greater than 6 μM iron was determined in washed cod samples containing added Hb during 2.5 days of storage (**Figure 1**).

Ascorbate at a concentration of 2.2 mM inhibited lipid oxidation in washed cod containing hemoglobin in all trials (**Figures 2**−**7**). The modes of inhibition include the free radical scavenging ability of ascorbate and its ability to reduce hypervalent forms of hemoglobin (*26*). Ascorbate has a favorable electron reduction potential to mediate scavenging of alkoxyl, peroxyl, and tocopheroxyl radicals (*11*).

However, a pro-oxidant effect of ascorbate at a concentration of 2.2 mM late in storage (day 7) was observed compared to control samples containing only added Hb (**Figure 3**). This pro-oxidant effect late in storage has at least two possible explanations. One is that the small but significant amount of released iron that accumulated with storage could be reduced to its catalytic ferrous (Fe^{2+}) form by residual ascorbate (*12*). Alternatively, by the time lipid peroxides reach a threshold concentration due to Hb-mediated lipid oxidation, those lipid peroxides can be broken down by residual ascorbate to form lipid radicals capable of propagating lipid oxidation. The ability of ascorbate to decompose linoleic acid hydroperoxides to alkoxyl radicals has been shown (*27*). The latter mechanism seems more likely. First, it has been shown that ascorbate (25 μM to 1 mM) continued to inhibit lipid oxidation in freshly prepared human plasma even when the model system was supplemented with hydrogen peroxide (200 μM or 1 mM) and Fe (50 μM to 1 mM) (*28*). This indicates that even high concentrations of iron that were reduced by ascorbate did not promote lipid oxidation. Further, the ability of ascorbate to inhibit lipid oxidation even in the presence of iron and hydrogen peroxide may have been due to the very low levels of lipid hydroperoxides that contaminate fresh plasma (*29*). Second, it can be seen in **Figure 5** that lipid peroxide levels were lower in ascorbate-containing samples (2.2 mM) at day 4 compared to the corresponding TBARS values at day 4 (**Figure 3**). Since TBARS result from lipid peroxide breakdown, this indicates that lipid hydroperoxides were being broken down much faster than they were being formed in ascorbate-containing samples at day 4. This is consistent with residual ascorbate breaking down lipid hydroperoxides to alkoxyl radicals, which results in an observable pro-oxidant effect on day 7. Lipid peroxide values were always greater compared to corresponding TBARS values at all time points in samples lacking added ascorbate (**Figures 3** and **5**).

Ascorbate at 20 and 200 μM had a small inhibitory effect on hemoglobin-mediated lipid oxidation in washed cod but no pro-

oxidant effect later in storage (**Figure 8**). Ascorbate at these lower concentrations was likely oxidized to negligible levels by the time lipid hydroperoxides reached concentrations that have the potential to react with ascorbate and promote lipid oxidation processes. In slices of mackerel dark muscle, initial ascorbate concentrations of around 85 μM were reduced to 40% of their original value after 2 days of 2 °C storage (30). TBARS values in these slices increased from 2 to 12 μmol/kg tissue in the 2-day period of 2 °C storage, indicating a moderately oxidizing system (*30*). Our washed cod system containing added hemoglobin is a more potent oxidation system compared to the slices of mackerel dark muscle based on TBARS values reaching 67 to 114 μmol/kg tissue during a 2-day storage period (**Figures 3**, **6**, **7** and **8**). Thus, it is likely that 20 μM and 200 μM ascorbate were consumed rapidly, preventing a pro-oxidant effect later in storage as was observed when ascorbate was present at 2.2 mM.

The ability of ascorbate at 2.2 mM to inhibit Hb-mediated lipid oxidation varied substantially when comparing different washed cod preparations (**Figures 2** and **3**). It was suspected that variation in preformed lipid peroxide content among the cod fillets used to prepare the washed cod muscle could partially explain the variation in ascorbate efficacy. The lipid peroxide contents in the cod muscle used to generate **Figures 2** and **3** were not determined. However, the preformed lipid peroxide content in three different cod muscle procurements was measured (**Table 1**). The different batches of cod muscle had widely different preformed lipid peroxide contents (**Table 1**). Further, the cod muscles that had elevated levels of preformed lipid peroxides were more sensitive to hemoglobin-mediated lipid oxidation in the resulting washed cod than cod muscles that had lower levels of preformed lipid peroxides (**Table 1**). This suggests that variation in preformed lipid hydroperoxides among the different fish muscles that were used in the preparation of washed cod muscle substrates may have contributed to the variation in the ability of ascorbate to inhibit Hb-mediated lipid oxidation (**Figures 2** and **3**).

The effect of added lipid hydroperoxides was tested by adding cumene hydroperoxide to washed cod muscle. Cumene hydroperoxide (222 μM) decreased the ability of ascorbate (2.2 mM) to inhibit hemoglobin-mediated lipid oxidation (**Figures 6** and **7**). Other researchers have found that incorporating lipid hydroperoxides into lipid model systems negated the ability of ascorbate to inhibit lipid oxidation through a mechanism by which ascorbate reacts with lipid hydroperoxides to form free radicals capable of propagating further lipid oxidation (*31*).The release of approximately 10 μM from human Hb (58 μM iron bound to hemoglobin initially) was caused by *tert*-butyl hydroperoxide (250 μM) (*4*). These concentrations of Hb and organic peroxide are similar to those used in our experimental conditions. Thus, the potential of cumene hydroperoxide to increase the pool of LMW iron might be considered as part of the mechanism by which cumene hydroperoxide accelerated Hb-mediated lipid oxidation and decreased the ability of ascorbate to be inhibitory. However, the fact that dissociated heme was found to breakdown lipid hydroperoxides 40 and 430 times more rapidly than Fe^{2+} and Fe^{3+}, respectively, and metHb was 8 and 83 times more efficient than Fe^{2+} and Fe^{3+}, respectively, should be kept in mind (*24*).

In conclusion, these studies suggest that iron released from trout hemoglobin during 2 °C storage contributed little to hemoglobin-mediated lipid oxidation in washed cod muscle. The ability of residual ascorbate to breakdown accumulating lipid hydroperoxides to reactive lipid radicals can explain the shift of ascorbate from an antioxidant to a pro-oxidant. Ascorbate was an effective inhibitor at low levels of lipid hydroperoxides but not at higher levels of lipid hydroperoxides. Future work should differentiate between the ability of released heme and intact hemoglobin to promote lipid oxidation in washed cod muscle and other animal tissues.

ABBREVIATIONS USED

LMW, low molecular weight; TBARS, thiobarbituric acid reactive substances; ADP, adenosine diphosphate; EDTA, disodium ethylenediamine tetraacetate; NADH, nicotinamide adenine dinucleotide, reduced; Hb, hemoglobin.

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