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Characterization of Aqueous Components in Chicken Breast Muscle as Inhibitors of Hemoglobin-Mediated Lipid Oxidation

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Unheated press juice (PJ) obtained from chicken breast muscle was a potent inhibitor of hemoglobin-mediated lipid oxidation in washed cod muscle. The <1 kDa fraction had a negligible effect on the rate of lipid oxidation. The high-molecular-weight (HMW) fraction was mildly inhibitory when added alone and highly inhibitory in the presence of <1 kDa components. Proteins of the HMW fraction were further fractionated by ammonium sulfate precipitation. Proteins in the 80% fraction were most inhibitory compared with other precipitated fractions on an equal protein basis. Inhibition by PJ was substantially decreased due to treatment with ascorbate oxidase. Adding ascorbate to the HMW fraction did not increase its inhibition, which suggested the presence of a complex ascorbate-reducing system in PJ consisting of HMW and low-molecular-weight (LMW) components. The ability of added ceruloplasmin to inhibit lipid oxidation was remarkably enhanced by addition of ascorbate or the <1 kDa fraction. Heated and centrifuged PJ had 8 times more LMW iron compared to unheated PJ. Adding heated PJ to washed cod containing hemoglobin slightly increased the rate and extent of lipid oxidation.

KEYWORDS: hemoglobin; lipid oxidation; press juice; antioxidants; ascorbate; ceruloplasmin; antioxidant enzymes; cytosol; protein inhibitors

INTRODUCTION

Quality deterioration due to lipid oxidation is a major concern in the muscle food industry. Lipid oxidation processes result in rancidity, color defects, nutritive loss, and formation of toxic compounds (*1*). Hemoglobin (Hb) is an abundant iron-containing protein in muscle foods and a potent catalyst of lipid oxidation (*2*). There are numerous potential mechanisms of Hb-mediated lipid oxidation in muscle foods. Hb autoxidizes to met Hb, which reacts with H_2O_2 or lipid peroxides to generate ferryl Hb, which can abstract a hydrogen atom from polyunsaturated fatty acids and, hence, initiate lipid oxidation (*3*). Alternatively, displaced heme or released iron can stimulate lipid oxidation (*4, 5*). The search continues for economical and natural antioxidants that effectively inhibit lipid oxidation processes in muscle foods.

Aqueous components (press juice) from mackerel muscle were potent catalysts of lipid oxidation when added to liposomes prepared from phosphatidylcholine, indicating an imbalance of pro-oxidants and antioxidants (*6, 7*)**.** On the other hand, press juice from chicken breast muscle and cod light muscle had a strong antioxidative effect in minced washed cod muscle containing added hemoglobin as the catalyst of lipid oxidation (*8*). Inhibitory candidates in the aqueous phase of chicken breast muscle include low-molecular-weight (LMW) components such as ascorbate, urate, glutathione, bilirubin, and histidine-containing dipeptides (*9, 10*). High-molecular-weight (HMW) candidates include glutathione peroxidase, superoxide dismutase, catalase, transferrin, haptoglobin, albumin, ceruloplasmin, and hemopexin (*11, 12*)

Erickson and Hultin (*13*) reported that both the LMW (<10 kDa) and HMW (>6−8 kDa) cytosol fractions from flounder tissue inhibited iron-mediated lipid oxidation in flounder sarcoplasmic reticulum and that the effect was believed to be due to the binding of iron. Han and Liston (*14*) examined the ability of rainbow trout cytosol to inhibit iron-mediated lipid oxidation in fish muscle microsomes and determined that both iron chelating agents and antioxidative defense enzymes were effective. Slabyj and Hultin (*15*) found that both HMW and LMW components in herring cytosol inhibited iron-mediated lipid oxidation in microsomes. Our objective was to further evaluate the capacity of chicken PJ to inhibit hemoglobin-mediated lipid oxidation.

MATERIALS AND METHODS

Materials. Rainbow trout (*Onchorhynchus mykiss*) (20−30 cm) and chicken broilers (6−9 weeks) (Cornish Rock) were maintained at campus facilities. Cupric sulfate, Chelex 100, sodium heparin, streptomycin sulfate, 2-thiobarbituric acid, sodium ascorbate, ammonium

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persulfate, dithiothreitol, ethyl 3-aminobenzoate, ceruloplasmin, transferrin, bovine serum albumin, and ascorbate oxidase were obtained from Sigma Chemical A/S (St. Louis, MO). Trichloroacetic acid, acrylamide, bis-acrylamide, glycine, Tris [hydroxymethyl] aminomethane, sodium dodecyl sulfate, urea, and thiourea were obtained from Fisher Scientific (Fairlawn, NJ). Ammonium sulfate was purchased from ICN Biomedicals (Aurora, OH). Tetramethylethylenediamine (TEMED) and prestained broad-range SDS-PAGE standard were obtained from Biorad (Hercules, CA). All other chemicals and reagents were analytical grade.

Preparation of Washed Minced Cod Muscle. Cod fish (*Gadus morhua*) were delivered overnight from Gloucester, MA or obtained from a local seafood store that utilizes air shipment from Boston, MA. The postmortem age of the fish ranged from 12 to 72 h. Fillets used were considered of excellent quality on the basis of odors that ranged from sea-like (very fresh) to minimal overall odor. Sensory panelists familiar with seafood deterioration made this assessment. All dark muscle was removed. White muscle was ground in a KS M90 mincer (KitchenAid, Inc., St Joseph, MI) (plate diameter 5 mm). The mince was washed twice in distilled, deionized water at a 1:3 mince-to-water ratio (w/w) for 2 min. Subsequently, the mixture was allowed to stand for 15 min before dewatering with fiberglass screen. Mince was then mixed with three volumes of 50 mM sodium chloride (pH 5.5) and homogenized using a Polytron Type PT 10/35 (Brinkmann Instruments, Westbury, NY). It was finally centrifuged (15 000 g for 25 min at 4 °C) using a Beckman L8-70M ultracentrifuge (Beckman Instruments, Inc.) and stored in a −80 °C freezer until use. The pH and moisture content of the washed cod preparations ranged from 4.8 to 5.8 and from 75% to 82%.

Preparation of Trout Hemoglobin. Anesthetized trout (0.5 g ethyl 3-aminobenzoate/L of water) were bled from the caudal vein into syringes (25G, 1 in. needle) preloaded with saline heparin solution (150 units/mL). 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 being suspended three times in 10 volumes of the above buffer (*16*). Cells were lysed in 3 volumes of 1 mM Tris, pH 8.0 for 1 h. One-tenth of the 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. SDS-gel electrophoresis indicated that the only detectable polypeptide in the hemoglobin preparation were in the range of hemoglobin subunits (data not shown). The method of Brown (*17*) was used to quantify hemoglobin concentration.

Chicken Press Juice (PJ) and Heated PJ Preparation. Chickens were killed by exposure to carbon dioxide gas. Thirty grams of minced chicken breast muscle was packed in 50 mL polypropylene centrifuge tubes and centrifuged at 22 000 *g* for 15 h at 4 °C. After centrifugation an aqueous phase, oil phase (top of tube) and muscle fibers (bottom of tube) were obtained. The aqueous phase was removed with a glass pipet and designated as press juice. The pH of chicken PJ was around 5.8. To obtain heated PJ, 15 mL of press juice was poured into polycarbonate tubes and held in a boiling water bath for 10 min. After cooling on ice for 5 min, the coagulated PJ was centrifuged at 45 900 *g* for 10 min at 4 °C. The coagulated mass was then removed by filtration utilizing Whatman filter paper (no. 4). The filtrate obtained was designated as heated PJ.

Determination of LMW Iron in PJ. LMW iron in samples was measured on the basis of a modification of the Ferrozine method (*18, 19*). PJ or heated PJ was ultrafiltrated by Amicon Centriplus YM10 centrifugal device (10 kDa molecular weight cutoff) (Millipore Corporation, Bedford, MA) at 3 000× *g* prior to analysis. The filtrate was analyzed because all HMW iron is retained in the retentate. One-half milliliter of the filtrate was mixed with freshly prepared 2% ascorbic acid (0.5 mL) in 0.2 N HCl and incubated at room temperature for 5 min. Trichloroacetic acid (11.3%) (0.5 mL) was added and mixed thoroughly, and the mixture was centrifuged at 10 000 *g* for 10 min. One milliliter of the clear supernatant was mixed with 0.4 mL of 10% ammonium acetate buffer and 0.1 mL of ferroin color reagent (*18*). Absorbance was read at 562 nm. A standard curve was constructed using ferrous ammonium sulfate. Chelex 100 was used to remove iron from heated press juice. Chelex was loaded into 20 mL capacity disposable columns (1 cm diameter) (Bio-Rad, Hercules, CA). Heated press juice was passed through the column once.

Preparation of <1 kDa and >6−8 kDa Fraction. Chicken PJ was filtered through a 1 kDa ultrafiltration membrane (Millipore Corporation, Bedford, MA) using a 250 mL Amicon ultrafiltration cell (model 8200, Amicon Corporation, Danvers, MA). Nitrogen gas at 40 psi was used to facilitate filtration. To obtain a HMW fraction, whole PJ was dialyzed against 100× volume of 50 mM phosphate buffer (pH 7) at 4 °C for 48 h using dialysis tubing with a molecular weight cutoff of 6−8 kDa (Spectrum Medical Industries, Los Angeles, CA). The buffer was changed once during this time.

Preparation of Ammonium Sulfate Precipitated Fractions. One volume of PJ was diluted five times with 50 mM phosphate buffer at pH 7 and then subjected to ammonium sulfate precipitation. An appropriate amount of ammonium sulfate to bring the diluted PJ to 20% saturation was added slowly while stirring (*20*). The mixture was stirred for 30 min in a cold room until the solution reached equilibrium and then was centrifuged at 10 000 *g* for 20 min. The supernatant was decanted and subsequently fractioned while the precipitate was dialyzed. Precipitates were resuspended in one-half the original volume with 50 mM phosphate buffer pH 7 and then dialyzed against 100× volume of the same buffer. Ammonium sulfate was added in increments as above in order to obtain the 40%, 60%, and 80% cut of PJ. The dialysis buffer was changed four times at increased time intervals (5, 15, 25, 37, and 61 h). Fractions obtained were utilized immediately or freeze-dried and resuspended in 50 mM phosphate buffer pH 7 upon usage. The protein concentration and character of the fractions were analyzed by Biuret assay and SDS-PAGE (see below).

Oxidation of Ascorbate in PJ and <1 kDa Fraction by Ascorbate Oxidase. The activity of ascorbate oxidase was determined using the spectrophotometric stop rate method with L-ascorbic acid standard (*21*). Then, a stock solution of ascorbate oxidase was prepared in 8 mM sodium phosphate buffer (pH 5.6). The stock solution (8.2 units/mL) was then added to PJ and the <1 kDa fraction and incubated at room temperature for 10 min. The ascorbate oxidase-fortified samples were then added to washed cod, giving rise to 0.53 units ascorbate oxidase/g washed cod.

Protein Levels in PJ Fractions. Protein concentration in original PJ and in each fraction was measured by Biuret assay. Biuret reagent was prepared by mixing 0.15% cupric sulfate and 0.6% sodium thiocyanate with 3% NaOH. A 1 mL sample was mixed with 4 mL of the Biuret reagent and incubated for 30 min at room temperature prior to reading the absorbance at 540 nm. Albumin was utilized to generate the standard curve.

SDS-PAGE Electrophoresis. Proteins in whole and fractionated PJ were characterized by the electrophoresis procedure described by Laemelli (*22*). The protein samples were diluted appropriately (5−10 μg protein/lane) with premade sample buffer (8 M urea, 2 M thiourea, 0.05 M Tris pH 6.8, 75 mM DTT, 3% SDS, and 0.05% bromophenol blue). Samples were then heated at 100 °C for 3.5 min and loaded into 12% w/v acrylamide resolving gels in a Pharmacia Biotech (San Francisco, CA) SE250 Mighty Small SDS-PAGE unit. SDS gels were run at 20 mA until the dye front reached the bottom of the glass plate. The gel was then stained in 50% methanol, 10% acetic acid, and 0.05% Coomassie blue R-250 for 30 min and destained in 10% methanol and 7.5% acetic acid overnight. Bio-rad prestained broad range standard (Hercules, CA) was applied to gels for estimation of molecular weights of PJ peptides. Densitometry of electrophoresis scans was done using NIH Image 1.62 software.

Preparation of Samples Prior to Iced Storage. Previously frozen washed cod muscle (−80 °C) was partially thawed at room temperature and blended using a Waring blender Model 33BL79 (Waring Commercial, New Hartford, CT). Blending was continued until a uniform consistency was obtained with intermittent breaks between cycles of blending to prevent the build-up of heat in the sample. Streptomycin sulfate (200 ppm) was then added to inhibit microbial growth during storage followed by addition of PJ fractions. Various commercially available chemicals were also added at this stage to washed cod. The stock solution of ascorbate was prepared in water; urate, glutathione,

NADH, and NADPH in 50 mM sodium phosphate (pH 7.0); transferrin and bovine serum albumin in phosphate buffered saline; and ceruloplasmin in 0.25 M NaCl, 0.05 M sodium acetate (pH 7.0). The pH of the samples was then adjusted to 6.3 with 1 M NaOH or 1M HCl if necessary. Final moisture content was adjusted to 90% by addition of distilled, deionized water and finally the trout hemoglobin solution. Trout Hb was added to the washed cod at a final concentration of 12 μmol Hb/kg washed cod. Mixing with a plastic spatula for 2−3 min was done to obtain a homogeneous distribution of added solutions. Samples were stored in 30 mL glass bottles or 125 mL flasks equipped with screw-cap lids on ice for 8−10 days. A final thickness of 4−6 mm of sample in each container was used.

Determination of Lipid Peroxides. Lipid peroxides were determined according to the method of Shantha and Decker (*23*) as modified by Undeland et al. (*24*). A standard curve was determined with cumene hydroperoxide.

Thiobarbituric Acid Reactive Substances (TBARS). TBARS were determined according to a modified procedure of Buege and Aust (*25*). Fifty percent trichloroacetic acid (TCA) containing 1.3% TBA was heated to 65 °C on the day of use to dissolve the TBA. The TCA−TBA mixture was added to samples at a 1:10 weight/volume ratio and incubated for 1 h at 65 °C. After being cooled in a cold room for 1 h and centrifugation (10 000 *g* for 3.5 min), the absorbance of the supernatant at 532 nm was measured. A standard curve was constructed using tetraethoxypropane. The lag phase was defined as the time it took for TBARS values to reach 20 μmol/kg washed cod during iced storage.

Sensory Analysis. Three to four trained panelists sniffed the headspace above the samples that were held in 125 mL flasks during ice storage to detect the onset and development of painty odor. A break time of approximately 5 min between analyses by each panelist was employed to allow for equilibration of volatiles between the washed cod and headspace. A scale of 0−10 with 10 being the strongest was utilized. Slightly rancid references were provided by blending heavily oxidized menhaden oil with vegetable oil (mostly odorless) (*26*). This reference was used to train the panelist. Temperature-abused, mechanically separated turkey was also used to familiarize panelists with strong rancid odors.

Statistics. A general linear model procedure of the SAS system was used to evaluate data from storage studies using data from replicated experiments (*27*). Analysis of variance was employed to examine the difference among treatments, and in addition, the Tukey test was used for adjustment of multiple comparisons.

RESULTS

Effect of Unheated and Heated PJ on Hb-Mediated Lipid Oxidation in Washed Cod Muscle. Trout hemoglobin (Hb) was added to washed cod muscle to stimulate formation of lipid oxidation products. Previous studies indicated that storing washed cod muscle at 2 °C in the absence of Hb resulted in undetectable lipid oxidation (*24*). There was a lag phase prior to lipid oxidation of around 1 day in washed cod containing added Hb based on formation of thiobarbituric acid reactive substances (TBARS) (**Figure 1**). Unheated PJ was added on the basis of protein content (45.8 mg PJ protein/g washed cod). This amount of added PJ incurred a 3.1-fold dilution of PJ into the other aqueous components of the samples. Unheated chicken press juice (PJ) was a potent inhibitor of Hb-mediated lipid oxidation based on formation of TBARS ($p < 0.01$) (Figure 1). Even after 10 days at 2°C, TBARS were negligible in samples containing added unheated PJ (**Figure 1**). Addition of heated PJ on the other hand at a 3.1-fold dilution accelerated Hb-mediated lipid oxidation based on TBARS formation during the initial 2 days of storage ($p < 0.01$) (**Figure 1**). Higher TBARS values were also observed at day 2 through day 10 in samples that contained heated PJ compared to control samples ($p < 0.01$).

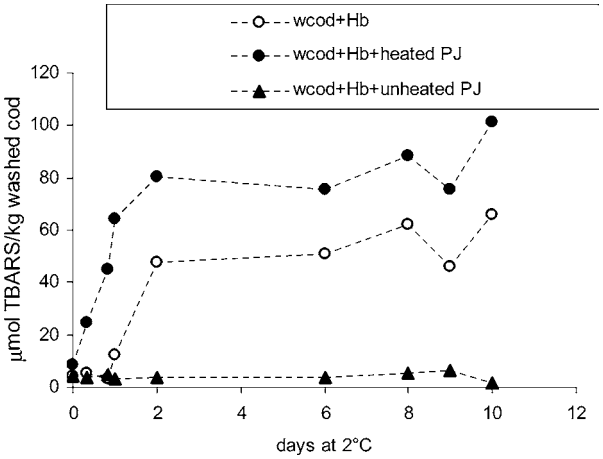

Figure 1. Effect of unheated and heated/centrifuged chicken PJ on Hb-mediated lipid oxidation in washed cod during 2 °C storage based on formation of thiobarbituric acid reactive substances (TBARS). PJ was present at a 3.1-fold dilution (45.8 mg of PJ protein/g of sample). An equivalent volume of heated/centrifuged PJ was added. The Hb level was 12 μmol/kg washed cod, and the moisture content was adjusted to 90%. The final pH of the model system was 6.15 ± 0.05.

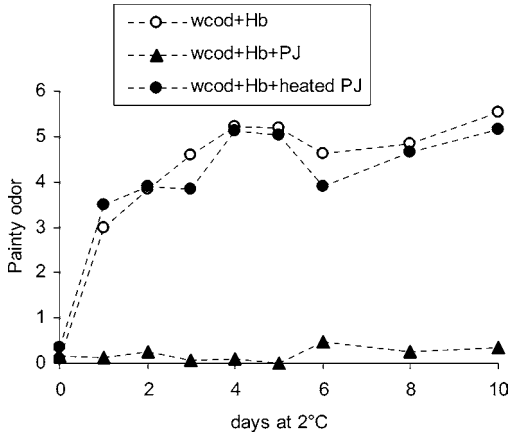

Figure 2. Effect of unheated and heated/centrifuged chicken PJ on Hb-mediated lipid oxidation based on painty odor development. A painty score of 1.5 indicated slight rancidity; the scale was 0–10 with 10 being strong rancidity. For experimental conditions, see Figure 1.

Painty odor development during 2 °C storage was also assessed in washed cod containing added Hb with or without addition of PJ. A score of 1.5 indicated slight rancidity, while a strongly rancid sample received a score of ten. Painty odor was not detected in samples containing unheated PJ during 10 days of storage (**Figure 2**). Moderate levels of rancidity were detected by day 1 of storage in washed cod containing Hb or Hb with added heated PJ (**Figure 2**).

Formation of lipid peroxides served as a second chemical indicator of lipid oxidation. Unheated PJ inhibited Hb-mediated lipid peroxide formation during the entire storage period (**Figure 3**). Lipid peroxides increased rapidly in washed cod containing added Hb or Hb with added heated PJ by day 1 of storage; however, the samples containing heated PJ accumulated lower levels of lipid peroxides between day 1 and 10 ($p < 0.05$) (**Figure 3**).

LMW iron levels determined in unheated PJ and heated PJ were 1.69 and 13.46 μmol/kg PJ, respectively (**Table 1**). Chelex 100 was used to decrease the amount of free iron in heated PJ. Treatment of heated PJ with Chelex 100 decreased the detectable free iron level from 13.68 to 4.88 μmol Fe/kg PJ (**Table 1**).

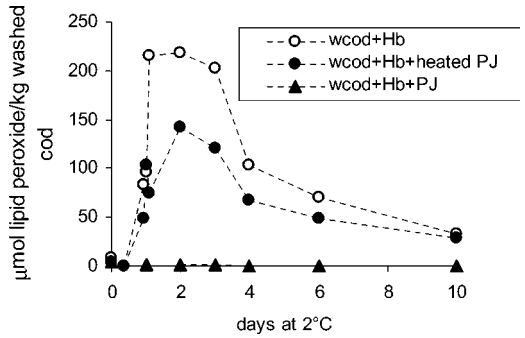

Figure 3. Effect of unheated and heated/centrifuged chicken PJ on Hb-mediated lipid oxidation based on formation of lipid peroxides. For experimental conditions, see Figure 1.

Table 1. Low Molecular Weight Iron in Unheated PJ, Heated PJ, and Heated PJ Exposed to Chelex 100 Resin[a]

sample	μmol LMW iron/kg PJ
unheated PJ	1.69 ± 0.01
heated PJ	13.46 ± 0.64
heated PJ	13.68 ± 0.03
stripped heated PJ	4.88 ± 0.03

[a] Heating treatment was exposure to 100 °C for 10 min followed by cooling on ice and 10 min centrifugation at 45 900 *g*.

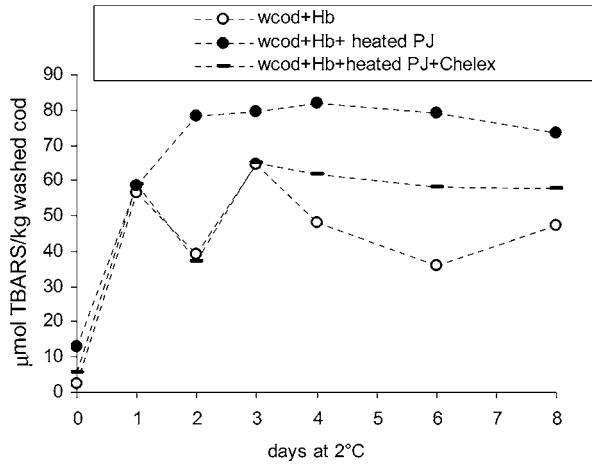

Figure 4. Effect of heated PJ and Chelex-treated heated PJ on Hb-mediated lipid oxidation in washed cod based on TBARS values. LMW iron levels from the press juices were 3.94 and 1.40 μmol/kg washed cod, respectively. The Hb level was 12 μmol/kg washed cod, and the moisture content was adjusted to 90% in all samples. The final pH of the model system was 6.3 ± 0.1.

This decrease in concentration of LMW iron had a small effect on hemoglobin-mediated lipid oxidation between day 0 and 1, with rapid lipid oxidation observed in all samples (**Figure 4**). However, the extent of TBARS values was around 23% lower in samples containing heated PJ treated with Chelex compared to untreated samples containing heated PJ at days 3 through 8 (**Figure 4**).

The protein content in unheated PJ was determined to be 140−150 mg/mL. The protein content in heated chicken PJ was previously determined to be minute on the basis of gel electrophoresis (*8*), and our determination was 3.3 mg/mL. The fact that heating followed by removal of heat-precipitated proteins negated the inhibitory efficacy observed from unheated PJ suggested that proteins of PJ were responsible for at least part of the inhibition.

Table 2. Effect of <1 kDa Fraction on Inhibition of Hb-Mediated Lipid Oxidation by PJ Proteins Precipitated at 20%, 40%, 60%, and 80% Ammonium Sulfate Saturation

	lag phase[b] prior to onset of lipid oxidation during 2 °C storage (days)	
sample[a]	no added <1 kDa fraction	added <1 kDa fraction[c]
wcod + Hb	1.1	1.1
wcod + Hb + 20% cut	0.2	1.8
wcod + Hb + 40% cut	0.4	1.5
wcod + Hb + 60% cut	1.0	2.5
wcod + Hb + 80% cut	1.2	4.1

[a] Thiobarbituric acid reactive substances (TBARS) were used as the indicator of lipid oxidation. Each ammonium sulfate precipitated PJ fraction (with or without <1 kDa) was added at 9.2 mg protein/g washed cod (15.5-fold dilution). The Hb level was 12 μmol/kg washed cod, and the moisture content was adjusted to 90% in all samples. The final pH of the model system was 6.3 ± 0.05. [b] Lag phase was defined as the extrapolated time until TBARS reached the level of 20 μmol/kg washed cod. [c] The amount of <1 kDa fraction added was 0.058 mL/g washed cod.

Effect of Protein Fractions from PJ on Hb-Mediated Lipid Oxidation in Washed Cod Muscle. The 20%, 40%, 60%, and 80% fractions were added at 0.14, 3.4, 10, and 24 mg protein/g washed cod muscle, respectively. Each fraction was added at a protein concentration that reflects the concentration of that fraction in the whole PJ sample. Around 16% of the protein in the original PJ was lost during isolation of the fractions. The 20% and 40% fractions provided no to little inhibition of lipid oxidation compared to the control which was washed cod containing added Hb (data not shown). The 60% fraction increased the lag phase by 0.6 days compared to the control while the 80% fraction increased the lag phase by 1.4 days.

Effect of the <1 kDa Fraction on the Ability of Protein Fractions To Inhibit Hb-Mediated Lipid Oxidation. Each ammonium sulfate precipitated fraction was added on an equivalent protein basis to washed cod containing Hb (9.2 mg PJ protein/g washed cod). At this concentration, none of the fractions delayed the onset of lipid oxidation in washed cod (**Table 2**). However, adding the <1 kDa fraction (3.1-fold dilution) increased the inhibitory efficacy of the 20, 40, 60, and 80% fraction (**Table 2**). The <1 kDa fraction in the presence of the 20, 40, or 60% fraction delayed TBARS formation approximately 1.5 days compared to the control, and in the presence of the 80% fraction, the extension of the lag phase was around 3 days.

The <1 kDa fraction and the pooled ammonium sulfate precipitated protein fractions of PJ were examined separately and in combination to determine if a synergistic inhibition of lipid oxidation occurred between the fractions. When the <1 kDa fraction or the 20−80% ammonium sulfate precipitated protein fraction were added separately at a 15.5-fold dilution, little effect on Hb-mediated lipid oxidation was observed (**Figure 5**). Combining these two fractions extended the lag phase considerably and was nearly as effective as whole PJ at the 15.5-fold dilution (**Figure 5**). At a 3.1-fold dilution, the 20−80% protein fraction alone was moderately inhibitory to lipid oxidation, but this fraction had to be combined with the <1 kDa fraction to obtain inhibition similar to whole PJ (data not shown).

Effect of Candidate LMW Components on the Ability of Different PJ Fractions To Inhibit Hb-Mediated Lipid Oxidation. Whole PJ and the <1 kDa fraction were separately pretreated with ascorbate oxidase to convert ascorbate to

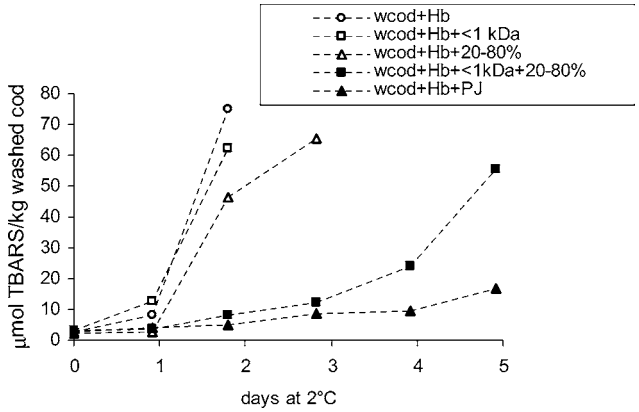

Figure 5. Effect of the <1 kDa fraction, the pooled 20–80% ammonium sulfate precipitated protein fraction, a combination of the <1 kDa fraction with the 20–80% fraction, and unheated PJ (all at 15.5-fold dilution) on Hb-mediated lipid oxidation. The Hb level was 12 μmol/kg washed cod, and the moisture content was adjusted to 90% in all samples. Final pH of the model system was 6.3 ± 0.1.

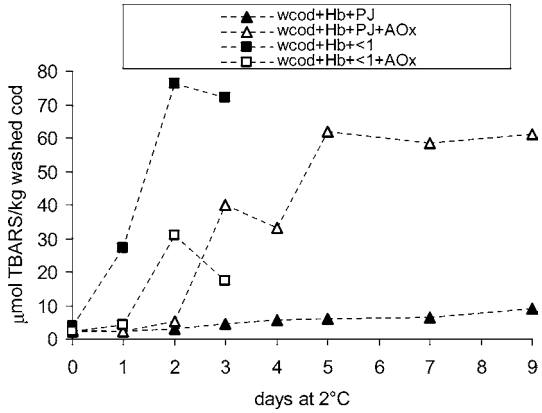

Figure 6. Effect of treating unheated PJ or the <1 kDa fraction with ascorbate oxidase on Hb-mediated lipid oxidation based on TBARS values. The amount of PJ added was 9.2 mg protein/g washed cod (15.5-fold dilution). Ascorbate oxidase was first mixed with PJ (8.2 units/mL) or the <1 kDa fraction (8.2 units/mL) and incubated at room temperature for 10 min. The ascorbate oxidase-fortified samples were then added to washed cod giving rise to 0.53 units ascorbate oxidase/g washed cod. The Hb level was 12 μmol/kg washed cod, and the moisture content was adjusted to 90% in all samples. The final pH of the model system was 6.3 ± 0.1.

dehydroascorbate. Ascorbate oxidase treatment improved the ability of the <1 kDa fraction to inhibit Hb-mediated lipid oxidation on the basis of TBARS ($p < 0.01$) (1 day extension) (**Figure 6**). Ascorbate oxidase treatment of unheated PJ decreased the ability of unheated PJ to inhibit Hb-mediated TBARS formation by at least 7 days (**Figure 6**).

Combining ascorbate (200 μmol/kg washed cod) with the >6−8 kDa fraction was not more effective at inhibiting TBARS formation than the >6−8 kDa fraction alone (**Figure 7**). In fact, a pro-oxidative effect was observed up to day 2 ($p < 0.01$). Ascorbate at 20, 50, and 100 μmol ascorbate/kg washed cod was also not effective at extending the ability of the >6−8 kDa fraction to inhibit Hb-mediated lipid oxidation (data not shown). Glutathione, urate, NADH, and NADPH, each at 100 μM, in combination with the >6−8 kDa fraction did not inhibit Hb-mediated lipid oxidation to any measurable extent (data not shown).

Effect of Candidate Proteins on Hb-Mediated Lipid Oxidation in the Absence and Presence of Ascorbate and the <1 kDa Fraction. In the presence of 200 μmol ascorbate/kg washed cod, 0.3 mg ceruloplasmin/g washed cod inhibited Hb-mediated lipid oxidation for at least 9 days, while the ceruloplasmin alone did not have any significant effect (**Table 3**). Adding the <1 kDa fraction with ceruloplasmin inhibited lipid oxidation as effectively as adding ascorbic acid (**Table 3**). Addition of ceruloplasmin at 0.06 mg and 0.03 mg/g washed cod was also examined. These amounts of ceruloplasmin extended the lag phase around 6 days in the presence of the <1 kDa fraction compared to the control, while ascorbate and ceruloplasmin at 0.06 mg/g washed cod was not inhibitory (data not shown).

The plasma proteins bovine serum albumin and transferrin were also examined (20 and 3 mg/g washed cod, respectively). Bovine serum albumin and transferrin had little effect on Hb-mediated lipid oxidation (**Table 3**). However, combining the <1 kDa fraction with bovine serum albumin and transferrin delayed lipid oxidation approximately 2 days in each case (**Table 3**).

Electrophoresis of Chicken PJ, Chicken Plasma, Ammonium Sulfate Precipitated Fractions, and Selected Com-

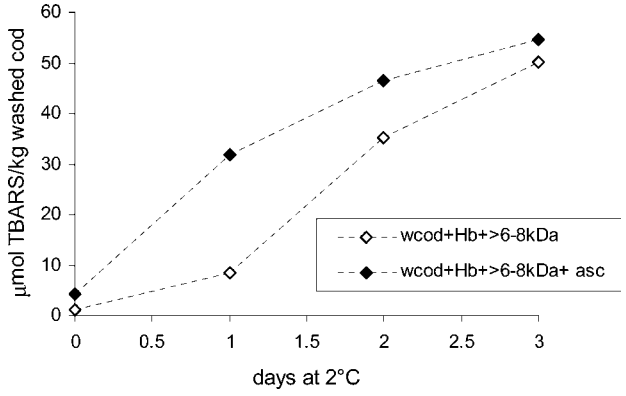

Figure 7. Effect of ascorbate on the ability of the >6–8 kDa fraction of PJ to inhibit Hb-mediated lipid oxidation in washed cod during 2 °C storage based on TBARS values. The amount of >6–8 kDa (9.2 mg protein/g washed cod) was based on a 15.5-fold dilution of original PJ. Ascorbate was added at 200 μM. The Hb level was 12 μmol/kg washed cod, and the moisture content was adjusted to 90% in all samples. The final pH of the model system was 6.2 ± 0.05.

Table 3. Effect of Ceruloplasmin, Bovine Serum Albumin, Transferrin with or without Ascorbate, and <1 kDa Fraction of PJ on Hb-Mediated Lipid Oxidation in Washed Cod Muscle

	lag phase[b] prior to onset of lipid oxidation during 2 °C storage (days)		
sample[a]	no Asc or <1	+Asc (200 μmol/kg)	+<1 kDa fraction[c]
wcod + Hb	0.9	0.4	0.6
wcod + Hb + Ceruloplasmin (0.3 mg/g washed cod)	1.2	≥9	≥9
wcod + Hb + BSA (20 mg/g washed cod)	0.9	ND[d]	3.4
wcod + Hb + Transferrin (3 mg/g washed cod)	1.3	ND[d]	3.2

[a] Thiobarbituric acid reactive substances (TBARS) were used as the indicator of lipid oxidation. The Hb level was 12 μmol/kg washed cod, and the moisture content was adjusted to 90% in all samples. The final pH of the model system was 6.2 ± 0.1. [b] Lag phase was defined as the extrapolated time until TBARS reached the level of 20 μmol/kg washed cod. [c] The volume of <1 kDa fraction added to ceruloplasmin and transferrin was 0.29 mL/g washed cod, which is equivalent to the volume of whole PJ that was added at the 3.1-fold dilution. In the case of BSA, <1 kDa fraction was added based on a 15.5-fold dilution level (0.058 mL/g washed cod). [d] ND = not determined.

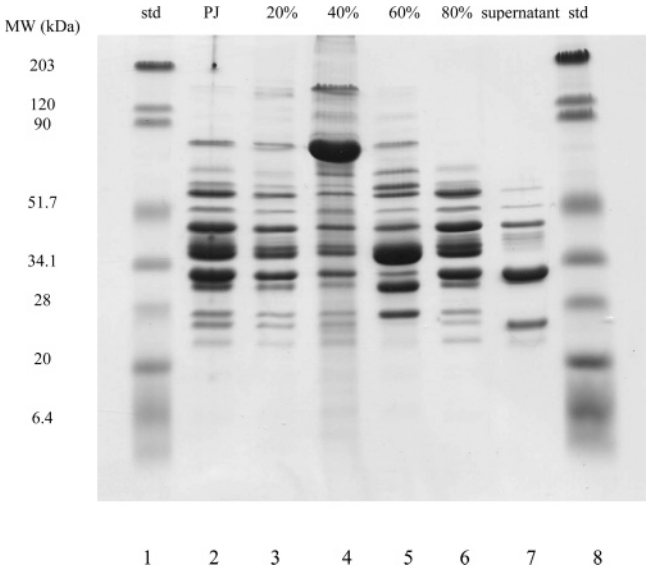

Figure 8. 12% SDS-PAGE of chicken PJ and ammonium sulfate precipitated fractions. Lane 1 and 8 Bio-Rad prestained molecular weight standards. Lane 2, PJ (15.8 μg); Lane 3, 20% fraction (8.1 μg); Lane 4, 40% fraction (19.8 μg); Lane 5, 60% fraction (11.5 μg); Lane 6, 80% fraction (9.3 μg); Lane 7, supernatant (3.6 μg).

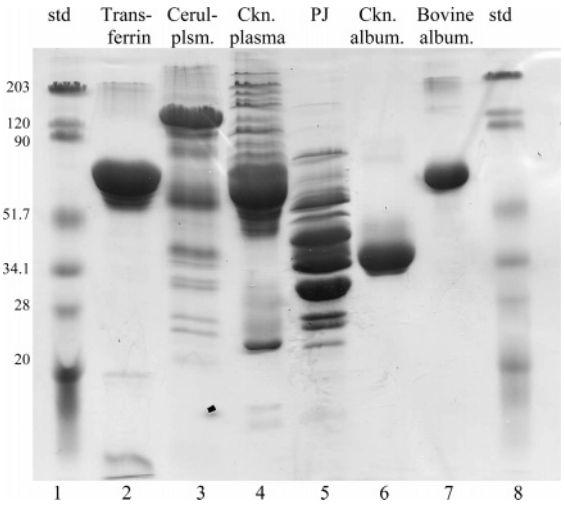

Figure 9. 12% SDS-PAGE of chicken plasma, chicken PJ, ceruloplasmin, albumin, and transferrin. Lane 1 and 8, Bio-Rad prestained broad range standard; Lane 2 bovine transferrin (10 μg); Lane 3, bovine ceruloplasmin (10 μg); Lane 4, chicken plasma (20 μg); Lane 5, chicken PJ (15 μg); Lane 6, chicken albumin (10 μg); Lane 7, bovine albumin, (6.7 μg).

mercially Available Proteins. The majority of the polypeptides from chicken PJ exposed to SDS-PAGE were 30−50 kDa (**Figure 8**, Lane 2). The 20% fraction had a similar distribution of polypeptides as whole PJ (Lane 3), while the 40% cut had a much higher concentration of a polypeptide at around 85 and 160 kDa (Lane 4). The 60% and 80% cuts presented different polypeptide patterns compared to whole PJ (Lane 5 and Lane 6). In the 60% cut, polypeptides of 34, 30, and 26 kDa were enriched. The 80% cut was void of polypeptides greater than 65 kDa. The supernatant contained significant amounts of a 30 kDa polypeptide.

The distribution of polypeptides in chicken plasma was quite different compared to chicken PJ (**Figure 9**, Lanes 4 and 5). Chicken plasma possessed numerous polypeptides in the 70−200 kDa range, whereas PJ was mostly void of bands in this range. Multiple polypeptides were present in commercially available bovine ceruloplasmin (Lane 3). The purity of commercially available tranferrin and albumin (Lanes 2, 5, and 6) were higher than that of bovine ceruloplasmin. The apparent molecular weight of chicken albumin was very different from bovine albumin, 34 vs 65 kDa.

DISCUSSION

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 utilize 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 was used as the promoter of lipid oxidation. This is because hemoglobins from other species such as bovine and avian animals were weaker promoters of lipid oxidation in washed cod (*28*). When trout hemoglobin is added to washed cod, substantial amounts of lipid oxidation products are formed after about 1 day of ice storage, which allows the magnitude of different potential antioxidant fractions to be assessed in a reasonable timeframe. In these studies, the ability of aqueous components in PJ of chicken breast muscle to inhibit hemoglobin-mediated lipid oxidation was examined. This PJ was investigated because previous studies indicated strong inhibition of painty odor development by unheated chicken PJ in washed cod containing trout hemoglobin (*8*). Our studies confirmed that the unheated PJ was a potent inhibitor of Hb-mediated lipid oxidation in washed cod on the basis of TBARS, lipid peroxides, and painty odor development (**Figures 1**, **2**, and **3**).

Heating and centrifugation of PJ resulted in a complete loss of the strong inhibition of Hb-mediated lipid oxidation that was observed from unheated PJ (**Figure 1**). LMW iron levels increased around 8-fold in PJ after heating (**Table 1**). This addition of LMW iron may explain the fact that TBARS were around 35% higher at day 2−10 in samples with heated PJ compared to controls with only Hb added (**Figure 1**). Compared to the controls, addition of heated PJ to washed cod containing Hb also accelerated formation of TBARS and lipid peroxides by around 1 day on the basis of lag times prior to TBARS reaching a value of 20 μmo/kg washed cod (**Figure 1**). Decreasing the LMW iron content in heated PJ approximately 65% with Chelex 100 decreased the extent of TBARS formation around 23% (**Figure 4**). Collectively, these data suggest that LMW iron from heated PJ had a small but detectable role in the rate and extent of lipid oxidation of washed cod in the presence of hemoglobin.

Previous work showed that addition of 15 μM $FeCl_3$ in the presence of ADP, histidine, and NADH failed to initiate lipid oxidation in washed cod muscle during ice storage (*24*). Levels of LMW iron contributed from raw PJ, heated and stripped PJ, and heated PJ in washed cod containing added Hb were 0.53, 1.58, and 4.38 μM. The fact that heated PJ had a slight pro-oxidant effect in our studies on the basis of TBARS (**Figure 1**) suggests that a favorable chelator and reducing system is present in heated PJ to facilitate LMW-iron-mediated lipid oxidation that was not present in the aforementioned study containing 15 μM $FeCl_3$, ADP, NADH, and histidine. Alternatively, the ability of Hb to effectively stimulate lipid oxidation could cause formation of a threshold level of lipid hydroperoxides that are enough substrate for reaction with LMW iron that results in enhanced levels of lipid oxidation products.

Addition of heated PJ caused TBARS values to be higher during storage compared to control samples, yet heated PJ

decreased lipid peroxide values compared to controls (**Figures 1** and **3**). This may be due to the fact that some component in heated PJ such as LMW iron was adept at breaking down lipid hydroperoxides as they formed, keeping in mind that TBARS formation results from the breakdown of lipid hydroperoxides.

Proteins denatured and coagulated upon heating of PJ. The insoluble mass that resulted from the heat treatment was removed prior to adding heated PJ to washed cod containing Hb. Since the heated PJ had no inhibitory efficacy, this suggested that proteins played a major role in the ability of unheated PJ to inhibit hemoglobin-mediated lipid oxidation.

HMW and LMW components of unheated PJ had to be combined to obtain the inhibition similar to that which was observed from whole PJ (**Figure 5**). There are a multitude of LMW and HMW components in PJ that may contribute to this synergistic inhibition. Adding components such as glutathione, NADH, NADPH, and urate to the >6−8 kDa fraction were not inhibitory toward Hb-mediated lipid oxidation (data not shown). This indicated that the mechanism by which the <1 kDa fraction complements the HMW fraction in inhibiting Hb-mediated lipid oxidation is not straightforward and likely requires multiple components for inhibition to be observed.

Adding ascorbate oxidase to PJ is one way to assess the role of ascorbate in PJ on Hb-mediated lipid oxidation. Treatment of PJ with ascorbate oxidase was effective at removing most of the inhibitory efficacy of whole PJ (**Figure 6**). This suggests that ascorbate contributed substantially to the inhibitory efficacy of unheated PJ. However, adding ascorbate to the >6−8 kDa fractions at concentrations likely to be in PJ (20−200 μM) was not antioxidative (**Figure 7**). It is curious that ascorbate appears to have such a large inhibitory role in the experiments that utilized ascorbate oxidase in PJ and at the same time adding back ascorbate to the HMW fraction was not antioxidative. To explain this, the fact that LMW components in PJ are removed in the preparation of the >6−8 kDa fraction should be considered. This is because glutathione, one such LMW component has been found to regenerate ascorbate in erythrocytes (*29*). Glutathione reductase, which reduces oxidized glutathione, requires NADH and NADPH as cofactors (*30*). Thioltransferase and protein disulfide isomerase each catalyze reduction of dehydroascorbate by glutathione (*31*). The above scenario implicates no less than three HMW components and three LMW components in the recycling of ascorbate reducing equivalents. These findings suggest the presence of a complex ascorbate-reducing system consisting of HMW and LMW components that contributes to the inhibitory efficacy of unheated PJ. Further, ascorbate has been found to reduce urate radicals back to urate, a LMW antioxidant (*32*).

One-electron reduction potentials of various components that exist in either reduced, semioxidized, or fully oxidized states can be used to predict the likelihood of certain reactions occurring in biological systems (*33*). Whether antioxidative or pro-oxidative effects result from ascorbate will depend on the pool of other components that are present. Tocopherol (Toc-H) imbedded in the phospholipid membranes of washed cod has the ability to scavenge pro-oxidative lipid alkoxyl radicals to produce relatively harmless lipid alcohols and oxidized tocopherol (Toc$^{\bullet}$) (reaction 1). Fully reduced ascorbate (Asc-H) reacts with Toc$^{\bullet}$, resulting in formation of the semidehydroascorbyl radical (Asc$^{-\bullet}$) and regenerated Toc-H antioxidant (reaction 2). An undesired yet thermodynamically favorable reaction is that of Asc$^{-\bullet}$ reacting with Fe^{3+} complexes to produce pro-oxidative Fe^{2+} and dehydroascorbate (DHA) (reaction 3). The presence of glutathione would limit reaction 3 by reducing Asc$^{-\bullet}$ to asc-H (reaction 4):

$$LO^{\bullet} + \text{Toc-H} \rightarrow LOH + \text{Toc}^{\bullet} \quad (1)$$

$$\text{Toc}^{\bullet} + \text{Asc-H} \rightarrow \text{Asc}^{-\bullet} + \text{Toc-H} \quad (2)$$

$$Fe^{3+}\text{-ADP} + \text{Asc}^{-\bullet} \rightarrow Fe^{2+}\text{-ADP} + \text{DHA} \quad (3)$$

$$\text{GSH} + \text{Asc}^{-\bullet} \rightarrow \text{Asc-H} + \text{GS}^{\bullet} \quad (4)$$

A strong correlation between endogenous ascorbate concentration and rancidity in mackerel muscle was previously observed (*34*). Other researchers have found that lipid oxidation in blood plasma did not occur until ascorbate was depleted (*35*).

The <1 kDa fraction alone had virtually no effect on Hb-mediated lipid oxidation; however, ascorbate oxidase treatment of the <1 kDa fraction induced an antioxidant effect from the <1 kDa fraction (**Figure 6**). This may be due to enzymatic removal of Asc$^{-\bullet}$ so that Asc$^{-\bullet}$ cannot reduce ferric iron to its pro-oxidative ferrous form (reaction 3) (*36*). Reaction 3 will be limited by PJ components that either inactivate iron by inhibitory chelation or effectively limit pools of Asc$^{-\bullet}$.

Ceruloplasmin did not function as an antioxidant when added alone to washed cod containing Hb, but ceruloplasmin greatly extended the lag phase prior to lipid oxidation in the presence of ascorbate or the <1 kDa fraction (**Table 3**). Antioxidant functions of ceruloplasmin include metal chelation and ferroxidase activity, which is the oxidation of reactive LMW Fe^{2+} to the less reactive Fe^{3+} form (*37*). One explanation for the synergy of ascorbate with ceruloplasmin is that ceruloplasmin inactivates LMW iron catalyst via chelation or ferroxidase activity. This will allow antioxidant properties of ascorbate to be manifested (reaction 2) including the ability of ascorbate to convert ferryl-Hb catalyst to met-Hb and maintain low levels of lipid hydroperoxides without producing lipid radicals through the reaction cycle shown below (*38*):

$$\text{ferryl}(+4)\text{Hb} + \text{Asc-H} \rightarrow \text{metHb}(+3) + H_2O + \text{Asc}^{-\bullet} \quad (5)$$

$$\text{met-Hb}(3+) + \text{LOOH} \rightarrow \text{ferryl}(+4)\text{Hb} + LO^{-} \quad (6)$$

A reason for concern in interpreting data from added ceruloplasmin is the multitude of polypeptides obtained when commercially obtained ceruloplasmin was analyzed using gel electrophoresis (**Figure 9**). Since mammalian ceruloplasmin is a monomeric protein (~132 kDa), our densitometry scan of the bovine ceruloplasmin indicated purity of around 45%.

The statistical approach was to combine data from two or more replicated studies and to analyze the data using a general linear model (GLM) over the time span of interest. The lag phase prior to onset of lipid oxidation within a treatment in some cases was variable between studies. For example in studies I, II, and III, the mean TBARS value at day 1 in samples containing washed cod and added Hb was 3.25, 7.88, and 25.9, respectively. Because of this type of variation, means from the multiple studies are reported without error bars that would obscure some of the data from other treatments. The range of TBARS values at day 1 in studies I, II, and III may be due to variation in the preformed lipid peroxide content among different procurements of cod muscle substrate (*39*).

In conclusion, these studies indicated that HMW and LMW interactions of chicken PJ are required to provide maximal inhibition of Hb-mediated lipid oxidation. Ascorbate oxidase removed a large percentage of the inhibitory efficacy of PJ

which suggests a key role for ascorbate in the ability of PJ to be inhibitory. The fact that adding ascorbate to the HMW fraction was not inhibitory suggests a complex ascorbate reducing system that requires multiple LMW components. Future work should determine concentrations of reduced and oxidized forms of ascorbate relative to other redox active components such as sulfhydryl groups of proteins, glutathione, urate, and iron during storage of washed cod containing Hb catalyst and chicken PJ fractions. The remarkable ability of commercially available ceruloplasmin in combination with ascorbate or <1 kDa components of chicken PJ to inhibit Hb-mediated lipid oxidation also warrants further attention.

ABBREVIATIONS USED

hemoglobin, Hb; washed cod muscle, wcod; press juice, PJ; thiobarbituric acid reactive substances, TBARS; high molecular weight, HMW; low molecular weight, LMW; sodium ascorbate, asc; ascorbate oxidase, AOx; less than 1 kDa fraction of press juice, <1 kDa; greater than 6−8 kDa fraction of press juice, >6−8 kDa.

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