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Aqueous Extracts from Some Muscles Inhibit Hemoglobin-Mediated Oxidation of Cod Muscle Membrane Lipids

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It was evaluated whether trout hemoglobin (Hb)-mediated oxidation of minced washed cod muscle lipids could be prevented by an aqueous isolate from cod and some other muscle sources. Lipid hydroperoxides and painty odor developed ∼4 days faster in washed than unwashed cod mince. When adding back an aqueous fraction (press juice) isolated from unwashed mince to washed mince at 2−6-fold dilutions, development of hydroperoxides and painty odor was either delayed or completely prevented. The inhibitory substances were heat stable, and their effect was slightly reduced at reduced pH. The <1 kDa fractions of whole and heated press juices were as inhibitory as the unfractionated press juices. Inhibition by the unheated, heated, and ultrafiltered (30 kDa) press juices was lost after dialysis. These findings implied the presence of one or more highly effective aqueous low molecular weight antioxidants in cod muscle press juice. The same antioxidative properties were found in heated haddock, dab, and winter flounder muscle press juices but not in heated herring and chicken muscle press juices. Unheated chicken press juice was however highly inhibitory.

KEYWORDS: Cod; trout; hemoglobin; Hb; membrane; lipid oxidation; aqueous; cytosol; inhibition; antioxidant; lipid hydroperoxide

INTRODUCTION

Rancidity development resulting from lipid oxidation often limits the shelf life of pelagic dark muscle fish during cold storage. A reason behind the susceptibility of these fish toward lipid oxidation is their abundance of highly prooxidative heme proteins, particularly Hb (*1*−*3*). It was recently found that Hb's from mackerel, menhaden (*4*), and herring (*3*) were better catalysts of lipid oxidation in a washed minced cod model system than were Hb's from less active fish such as flounder and trout.

Many traditional antioxidative strategies for muscle foods were developed under the assumption that the rate of oxidation is related to the size and composition of the triacylglycerol fraction, i.e., the depot fat. They are therefore often focused on preventing autoxidation as it occurs in bulk oils. A recent study showed that adding up to 15% triacylglycerols in the form of menhaden oil did not alter the rate or intensity of Hb-catalyzed oxidation of washed cod mince containing about 0.7% membrane lipids. This occured even though the level of preformed lipid hydroperoxides was around 100 times greater in samples containing added oil (*5*). Instead, the maximum oxidation intensity was determined by the Hb concentration. Thus, only very low levels of lipids (only membrane lipids) and preformed hydroperoxides are needed for rancidity to develop if Hb is present. It is therefore suggested that any antioxidative strategy that can inhibit the reaction between membrane lipids/lipid hydroperoxides and Hb would be valuable in preventing rancidity in food products based on dark muscle fish.

During frozen storage of minced herring (*6*), samples washed once (1:5, w/w) oxidized faster than unwashed samples. Similarly, minced cod that had been washed three times (1:3, w/w) oxidized faster than unwashed minced cod at 2 °C in the presence of 5.8 μM Hb (*7*). Both observations suggested that the aqueous fraction of fish muscle contains strong antioxidative systems that are lost/diluted in the washing process. In support of this are scattered studies showing that the cytosolic aqueous fraction of fish muscle can inhibit oxidation of isolated membrane systems in vitro, flounder SR (*8*, *9*), herring microsomes (*10*), and trout microsomes (*11*). However, so far, there is no evidence that the aqueous fraction of fish muscle can prevent oxidation in a muscle system in situ.

The aim of this study was to evaluate whether addition of an aqueous fraction (press juice) of cod muscle could prevent Hb-

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catalyzed oxidation of the lipids of washed cod mince during ice storage. The antioxidative components were also to be characterized in terms of their Mw range, heat stability, and pH dependence. It was also desired to compare the effect of the soluble fraction of cod with the soluble fractions from other muscle sources.

MATERIALS AND METHODS

Fish and Chicken. Fresh cod (*Gadhus morhua*), haddock (*Melanogrammus aeglefinus*), American dab (*Hippoglossoides plattessoides*), and winter flounder (*Pseudopleuronectes americanus*) were obtained from Steve Connoly's Seafood Inc. (Gloucester, MA). Herring (*Clupea harengus*) was obtained from D&B Bait (Gloucester, MA). The white muscle from these fish was manually removed and ground using a kitchen grinder (Ultra Power model: KS M90, Kitchen Aid Inc., St Joseph, MI). Live chickens were obtained from Longwood Farm (Reading, MA). The bird was sacrificed by carbon dioxide asphyxiation. The skin around the breast muscle was immediately cut open, and the breast muscles were removed and ground as above. Fresh skin-on chicken breasts (Perdue) were obtained from Shaw's (Gloucester, MA). The skin was removed, and the muscle was ground as above.

Washed Minced Cod Muscle Model System. To allow large additions of press juice to the washed mince without exceeding physiological moisture contents (~82%), a washing procedure was developed to minimize the moisture content (~70−75% moisture) of the washed mince. A 600 g amount of minced cod muscle was washed once with 3 volumes of distilled water at natural pH (~ pH 7) and twice with 3 volumes of 50 mM NaCl at pH 5.5. Salt and low pH helped to minimize the water binding capacity of the cod muscle proteins. In the first two washes, mince and washing solution were stirred manually for 1 min and then leached for 15 min at 4 °C. Washed mince was collected via filtering the muscle suspension through glass fiber screen. In the third wash, the mince and washing solution were homogenized (1 min, speed 4) with a Kinematica Gmb H Polytron (Type PT 10/35, PCU 1, Brinkman Instruments, Westbury, NY) and then centrifuged for 20 min (15 000*g*, 4 °C) as above. The washed mince was packed in plastic bags and was frozen at −80 °C. Upon use, the mince was thawed in the plastic bag under cold running water. Excess water was squeezed out using cheesecloth. The final moisture was 70 or 75%.

A slightly modified procedure was used to prepare a washed mince that could be compared directly with an unwashed mince control. Here, the 50 mM NaCl was replaced by 50 mM phosphate buffer (pH 6.65). Also, after the second wash, the drained mince was chopped for 2 × 30 s in a stainless steel chopper (model R 301 Ultra, Robot coupe USA Inc., Ridgeland, MS) after which the buffer was stirred with the mince for 6 min by hand. After it was leached for 15 min, the washed mince was collected via centrifugation (20 min, 15 000*g*, 4 °C) in a Beckman Ultracentrifuge L8-55M (Beckman Instruments Inc., Palo Alto, CA). The unwashed control was prepared by subjecting minced cod to an identical 2 × 30 s chopping as that included in the washing process. Half of this control sample was kept at its original moisture (82%), and half was adjusted to the moisture of the mince washed this way (90.6%) using ice-cold DDW. Both unwashed and washed minces were used immediately.

Preparation of Press Juices. Minced muscles from cod, haddock, dab sole, winter flounder, herring, and chicken were packed in 200 mL polypropylene centrifuge bottles (200 g in each) and centrifuged at 22 000*g* for 15 h at 4 °C. The press juice obtained was filtered through four layers of cheesecloth and used immediately or after short-term frozen storage at −80 °C.

Twenty milliliters of each press juice was poured into 70 mL polycarbonate centrifuge bottles and held in a boiling water bath for 10 min. During this treatment, the press juice reached 88 °C. After it cooled for 30 min on ice, the coagulated press juices were centrifuged at 17 800*g* (20 min, 4 °C). The supernatants were then filtered through a No. 1 Whatman filter, and the filtrate was frozen at −80 °C.

Using a stirred 50 mL Amicon ultra filtration cell (model 52, Amicon Corporation, Danvers, MA), 50 mL of either unheated or heated cod press juice was filtered through a 1 kDa ultrafiltration membrane (Millipore Corporation, Bedford, MA) at 50 psi. For the unheated press juice, a 30 kDa ultrafiltration membrane (Millipore Corporation) was also used. The first 25 mL of filtrate was collected and frozen at −80 °C.

Ten milliliters of the unheated cod press juice, heated cod press juice, and <30 kDa fraction of unheated cod press juice was each dialyzed against 4000 mL of 50 mM phosphate buffer (pH 7) at 4 °C for 48 h. The buffer was changed once during this period. The cutoff molecular mass of the dialysis tubing was 3.5 kDa (Fisher Scientific, Fair Lawn, NJ). The dialysis retentates were frozen at −80 °C.

Bleeding of Fish, Preparation of Hemolysate, and Analysis of Hb. Farmed rainbow trout (*Onchorhynchus mykiss*) were bled as described by Rowley (*12*). Hemolysate was prepared from the whole blood according to Fyhn et al. (*13*) by washing the red blood cells four times in 1 mM Tris (pH 8) containing 290 mM NaCl and then lysing them in 1 mM Tris (pH 8). To quantify the Hb levels in the hemolysate and press juices, the method of Brown (*14*) was adapted as described by Richards and Hultin (*15*).

Preparation of Oxidation System. To compare washed and unwashed minces under equal conditions of tissue disruption, moisture content, and pH, 25 g of chopped unwashed mince (with or without adjustment of moisture to 90.6%) as well as 25 g of chopped washed mince (moisture = 90.6%) were mixed with streptomycin sulfate (Sigma, St. Louis, MO) to a final concentration of 200 ppm. The mixing was done by hand (2 min, ~160 turns/min) using a stainless steel spatula. The pH of the washed mince sample was adjusted to that of unwashed mince (7.3) by dropwise addition of 0.5−2 N NaOH. Trout hemolysate, with a final concentration of 6 μM Hb, or a corresponding amount of water, was then stirred in by hand as described above. The samples were flattened out with an L-shaped stainless steel spatula in the bottom of 125 mL screw-capped glass Erlenmeyer flasks resulting in a sample thickness of ~6−7 mm.

In the following experiments, 5−15 g portions of the thawed and dewatered "low moisture" washed mince were mixed for 2 min by hand with 200 ppm streptomycin sulfate and enough press juice to bring the moisture of the model system to ~82%. Typically, 5 g of a washed mince with ~70% moisture was fortified with 3.4 mL of press juice or 15 g of washed mince system with ~75% moisture was fortified with 6 mL of press juice. This brought about 2-fold and 3.2-fold dilutions, respectively, of the added press juices. Trout hemolysate to a final concentration of 6 μM Hb was then stirred in for 2 min by hand. In control samples, the press juice was replaced by the same amount of either 50 mM sodium phosphate buffer (pH 6.6) or DDW. The pH of the samples was adjusted to 6.6 ± 0.1. All samples were flattened out in bottles or Erlenmeyer flasks to give a final thickness of ~4−5 mm. Samples were stored on ice until bacterial growth became sensorically evident, typically after 7−10 days.

Tentative effector compounds in press juice were evaluated for their antioxidative capacity. All compounds were dissolved/diluted in DDW and then added to the low moisture washed cod to bring the moisture up to 82%. The tested compounds and their final concentrations in the aqueous phase of the model system included potassium dihydrogen phosphate (Pi) (0.2 and 40 mM) (ACS certified, Fisher Scientific), sodium tripolyphosphate (STPP) (0.2%) (pentasodium salt, 90−95%, practical grade, Sigma), pyrophosphate (15 μM) (anhydrous sodium salt, Sigma), 2,3-diphopsphoglycerate (5.8 and 58 μM) (Sigma), calcium chloride (70 mM) (Baker analyzed reagent, JT Baker, Phillipsburg, NJ), trimethylamine oxide (TMAO) (100 μM and 100 mM) (Sigma), and spermine (61.2 and 612 μM) (Sigma).

Analysis of Moisture Content, pH, and Total Proteins. The moisture content of unwashed and washed minces was measured using a Cenco infrared moisture balance (CSC Scientific Co., Inc.) or by heating the samples at 105 °C overnight. The pH was recorded with an Orion combination epoxy Ross Sure-Flow Electrode (Orion Research Inc., Beverly, MA) in conjunction with a pH meter (Orion Research Inc., Boston, MA). In muscle mince samples, the pH was measured after manually stirring one part mince with nine parts DDW. Total proteins were measured according to Lowry et al. (*16*) as modified by Markwell et al. (*17*).

Electrophoresis. Proteins in the whole and fractionated press juices were separated according to the electrophoresis procedure

described by Laemelli (*18*) using precast mini linear gels 10−20% (ICN Biomedicals Inc., Aurora, OH) on a vertical PAGE Mini Device (Daiichi Scientific, Tokyo, Japan) with a constant current of 30 mA per gel. The protein samples were diluted twice in a premade sample buffer (Sigma) and heated for 1 min at 100 °C. Protein bands were fixed using a 1 h incubation in 12% trichloroacetic acid, followed by overnight staining using Pro-Blue (Owl Separation Systems, Portsmouth, NH). Scanning of the stained gels was accomplished using a Hoefer Scanning Densitometer (model GS 300, Hoefer Sci., San Fransisco, CA) in the transmittance mode with model 365W Densitometer Analysis software for protein quantification. A standard curve was constructed using wide range Mw sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) standards (Sigma) on a linear gradient as described by Hames (*19*).

Sensory Analysis. At regular intervals, 3−4 trained panelists (*20*) sniffed the headspace present above the samples by uncapping the bottles or flasks. Panelists concentrated on detecting stale, fishy, and painty odors using a scale of 0−10, with 10 being the strongest. Reference samples were prepared by blending different amounts of heavily oxidized menhaden oil with nearly odorless vegetable oil (*20*). The lag phase for development of the different odors is defined as the time elapsing until an intensity of 1 is reached.

Analyses of Lipid Hydroperoxides. At regular intervals during storage, two 0.5−0.8 g sample "plugs" were taken out from each sample for analyses of lipid hydroperoxides. The plugs were removed using a plastic cylinder (diameter 1 cm) and, thus, had constant surface-to-volume ratios. Each sample plug was wrapped in aluminum foil and put in a zip-lock plastic bag. All samples were stored at −80 °C until the day of analysis. Lipid hydroperoxides were determined with a modified version of the ferric thiocyanate method (*21*). Total lipids were extracted from the mince with 9 mL of chloroform:methanol (1:1). Sample and solvents were homogenized with a biohomogenizer mixer (ESGE, M133/1281-0, Biospec Products Inc., Bartlesville, OK). Sodium chloride, 2.46 mL (0.5%), was added, and the sample was vortexed for 30 s. Phase separation was achieved after 10 min of centrifugation at 2000*g* in a tabletop centrifuge. Two milliliters of the lower chloroform layer was removed using a 5 mL glass syringe equipped with a 20 gauge 6 in. stainless steel needle and diluted with 1.33 mL of ice-cold chloroform:methanol (1:1). Ammonium thiocyanate (4.38 M) and iron(II) chloride (9 mM) (33.4 mL of each) were added with 2−4 s of vortexing between each addition. The sample was incubated for 20 min at room temperature, and the absorbance was read at 500 nm. A standard curve was prepared using cumene hydroperoxide (80%, Sigma). The results are expressed as μmol lipid hydroperoxide/kg of sample. Blanks were prepared according to the described procedure by replacing the muscle by 0.6 mL of ice-cold distilled water.

Trout Hb Deoxygenation and Autoxidation. An aqueous test system consisting of 2 mL of phosphate buffer (50 mM, pH 6.6), 1 mL of press juice, 200 ppm streptomycin, and trout Hb to a final concentration of 6 μM was prepared to mimic the washed minced model system. The final pH of the test system was adjusted to 6.6. Control samples were prepared at pH 6.0 and pH 6.6 by replacing the press juice with phosphate buffer. For each sample, a Hb free reference sample was prepared. The samples with their corresponding references were stored on ice in 15 mL glass test tubes for up to 12 days. Every day, the samples were scanned between 600 and 500 nm against the Hb free reference sample.

Deoxygenation of trout Hb was measured at time 0 as the difference in absorbance between the peak at 575 nm and the valley at 560 nm (*22*). To estimate if the presence of press juice had an effect on Hb deoxygenation, the data for samples with press juice added were divided by the values for the control sample (pH 6.6) without press juice. The reason for not reporting Hb deoxygenation data over time (**Table 4**) was that storage-induced autoxidation of the heme protein was interfering with the deoxy-Hb measurements.

Hb autoxidation was measured as a reduction in the absorbance peak at 575 nm using the following formula: $-\ln(A_{575}\text{ (stored sample)}/A_{575}\text{ (unstored sample)})$ (*23*). As this formula yields 0 for unstored samples, the Hb autoxidation data (y) are only reported as changes over storage time (x). The latter was done using linear regression models ($y = kx + m$). Similarly to above, the effect from adding press juice was estimated by relating the rate of change (k) for samples with press juice added to the k values for the control sample (pH 6.6) without press juice.

Statistics. The use of sensory analysis limited the possible number of sample replicates within each experiment to ~6. To compensate for this, the entire experiment was repeated at least twice using different batches of washed mince, press juice, and hemolysate. Lipid hydroperoxide analyses supported the sensory analyses during one of these experiments. Lipid hydroperoxide analyses were repeated twice on the lipid extract obtained from each sample. The analytical variation was established by calculating standard deviations (SD) from these two analyses.

RESULTS

Hb-Mediated Lipid Oxidation in Unwashed Mince, Washed Mince, and Washed Mince with Added Cod Press Juice. When unwashed and washed minces were stored on ice at the same pH (6.7), moisture content (82%), and Hb level (6 μM), the lag phase for painty odor development was 6 and 2 days, respectively (**Figure 1a**). It was ascertained that the difference in oxidative stability did not arise in the different degrees of muscle disruption of these samples. When unwashed and washed minces were compared after subjection to equal chopping treatments, painty odor was still 4 days slower in unwashed than washed mince (data not shown). Addition of cod press juice to washed mince at a 3.2-fold dilution level delayed the Hb-mediated development of painty odor by 4 days (**Figure 1a**). Thus, washed mince containing press juice obtained the same oxidation lag phase as unwashed mince, 6 days. Similar kinetics, but with a lower intensity, were detected for the fishy odor development.

The level of lipid hydroperoxides was determined in the same samples on which sensory analyses were conducted (**Figure 1b**). The data obtained reflected sensory scores in that lipid hydroperoxides started developing 3 days faster in washed mince as compared to unwashed mince and washed mince with added press juice. The hydroperoxide level started declining at day 3 in the washed mince and at day 8 in the unwashed mince and in press juice-fortified mince. At the point of decline, the unwashed mince had reached 1.8 times and 5 times higher hydroperoxide levels than the washed mince and press juice-fortified mince, respectively. A possible reason could be the presence of catalytic systems other than Hb or to more stable lipid hydroperoxides in the unwashed mince.

The degree of diluting the cod press juice added to washed mince was reflected in the length of the paintiness lag phase obtained after addition of Hb (**Table 1**). At 2-, 3.2-, and 6-fold dilution levels, the average lag phases were ≥8 days ($n = 4$), 6.7 ± 1.8 days ($n = 4$), and 4.1 ± 1.3, days ($n = 2$), respectively. In samples of unwashed mince where no press juice was added but where the endogenous aqueous phase had been diluted in situ, the paintiness lag phase was also shortened as compared to that obtained without dilution. At 2.2-fold and 70-fold dilutions, the latter being the result of the washing procedure, the lag phases were shortened from ≥11 days in the unwashed mince to 8 and 1.8 ± 0.2 days ($n = 11$), respectively (**Table 1**).

Effect of Cod Press Juice on Lipid Oxidation in Washed Mince When Changing the Hb Concentration and pH. When increasing the Hb concentration of the washed mince from 6 to 10 μM, the development of painty odor was still completely prevented in the presence of cod press juice at a 2-fold dilution level (data not shown). In controls, without press juice added, the paintiness lag phase did not change, but the intensity became 1.7 times higher at 10 as compared to 6 μM Hb.

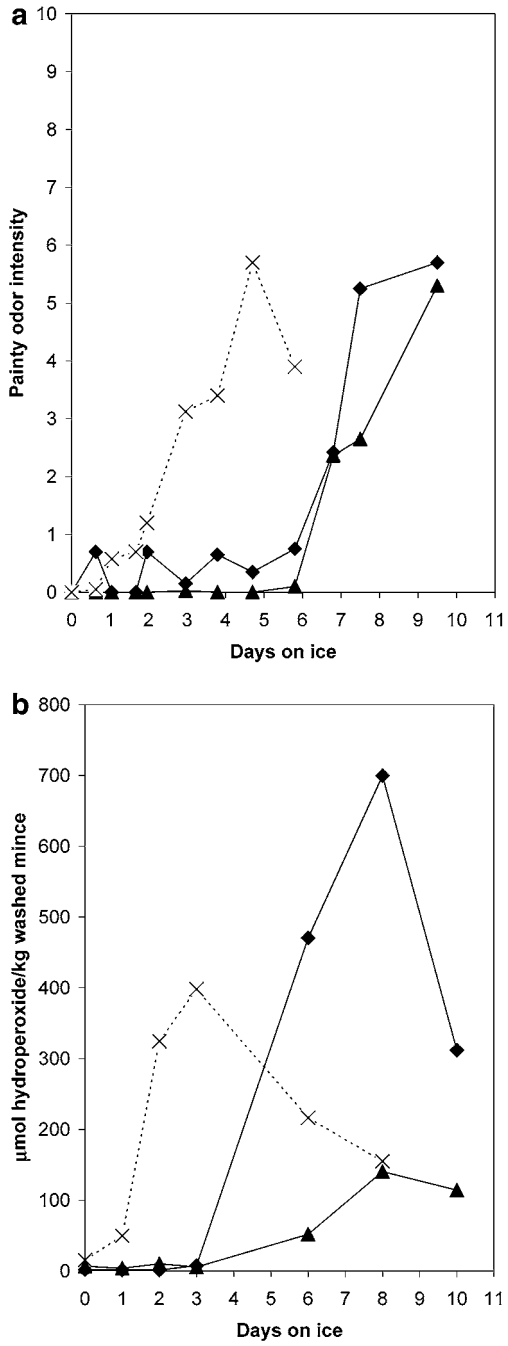

Figure 1. Hb-induced development of (a) painty odor and (b) lipid hydroperoxides in unwashed mince (♦), washed mince with added cod press juice (▲), and washed mince with added DDW (control) (×). Press juice and DDW were added at levels raising the moisture content of the "extra dry" washed mince (75%) to the same value as that of the unwashed mince (82%). This diluted the added press juice 3.2 times. Oxidation was initiated with 6 μmol trout Hb/kg mince. Details about Hb and press juice preparations are given in **Table 1**. The final pH of the whole and washed mince model systems was 6.55–6.7.

When decreasing the pH of the washed mince from pH 6.6 to 6.0, the lag phase for Hb-mediated paintiness development decreased from ~1.5 to ~0.7 days (**Table 2**). In the presence of cod press juice diluted 3.2 times, the lag phases at pH 6.0 and 6.6 became 2.7 and 6.5 days, respectively. Thus, the presence of cod press juice extended the lag phase by 2 days at pH 6.0 and by 5 days at pH 6.6. However, at both pH values, the relative extension of the lag phase caused by adding press juice was ~4-fold.

Table 1. Effect from Cod Press Juice[a] on Hb-Mediated[b] Development of Painty Odor[c] in Unwashed and Washed Minces[d] after Different Degrees of Dilution[e]

sample	painty odor lag phase (days)
unwashed minced cod (dilution 1)	≥11
cod press juice added/present at dilution 2	≥8
cod press juice added at dilution 3.2	6.7 ± 1.8
cod press juice added at dilution 6	4.1 ± 1.3
washed mince without press juice added (dilution 70)	1.8 ± 0.2

[a] Minced cod muscle was subjected to centrifugation (22 000*g*, 15 h, 4 °C). The press juice obtained was filtered through four layers of cheesecloth and then frozen at −80 °C. [b] Hb (6 μM) was added as trout hemolysate prepared from whole trout blood using the method of Fyhn et al. (*13*). [c] The lag phase for development of painty odor was defined as time elapsing until an intensity of one was reached. [d] Minced cod was washed once with distilled water (1:3) at pH 7 and twice with 50 mM NaCl (1:3) at pH 5.5. In the last wash, muscle and 50 mM NaCl were homogenized followed by centrifugation. The washed mince was then frozen at −80 °C. When thawed, excess water was manually squeezed out to reduce the moisture content down to 70 or 75%. [e] Two-fold and 3.2-fold dilutions were obtained when cod press juice was added to extra dry washed mince to raise the moisture content from 70 and 75%, respectively, to 82%. In one experiment, the endogenous aqueous phase was diluted twice directly in situ by adding distilled water to minced cod. For the 6-fold dilution, the added press juice was diluted with distilled water prior to adding it to washed mince. The 70-fold dilution of the endogenous aqueous phase arose from preparation of the extra dry washed mince model system. Experiments were repeated 4, 4, 2, and 11 times at the 2-, 3.2-, 6-, and 70-fold dilution levels, respectively. The final pH in the samples ranged between 6.55 and 6.7.

Table 2. Effect from pH on Hb-Mediated[a] Development of Painty Odor[b] in Washed Mince[c] with or without Added Cod Press Juice[d]

sample	painty odor lag phase (days)
control (DDW) pH 6.0	0.7
control (DDW) pH 6.6	1.5
cod press juice pH 6.0	2.7
cod press juice pH 6.6	6.5

[a] For trout hemolysate preparation, see **Table 1**. The final Hb level was 6 μmol/kg washed mince. [b] For lag phase definition, see **Table 1**. [c] Washed mince was prepared as described in **Table 1**. [d] Press juices were prepared as described in **Table 1** and added at a 3.2-fold dilution level. In the control samples, press juice was replaced by DDW.

Effect of Heating, Dialysis, and Ultrafiltration of Cod Press Juice on Its Ability to Prevent Hb-Mediated Lipid Oxidation in Washed Mince. As shown both by odor (**Figure 2a**) and hydroperoxide analyses (**Figure 2b**), the antioxidative properties of the cod press juice remained after 10 min of heating in a 100 °C water bath followed by centrifugation to remove coagulated proteins. The antioxidative properties of unheated and heat-treated press juices were however lost when the <3.5 kDa fraction was removed by dialysis (48 h, 4 °C) (**Figure 2a**,**b**). To evaluate how the time period during which the dialysis was conducted affected the antioxidative properties of unheated and heat-treated press juices, control samples were stored for 48 h at 4 °C before adding them to washed mince. This storage period did not affect the inhibitory properties of the unheated cod press juice control, while the heated press juice control lost its ability to prolong the oxidation lag phase. However, the stored heated press juice still reduced the maximum intensity of painty odor and hydroperoxides by ~50%.

Unheated and heated cod press juices were subjected to ultrafiltration (1 and 30 kDa membranes) after which the filtrates were tested for their ability to inhibit Hb-mediated oxidation at

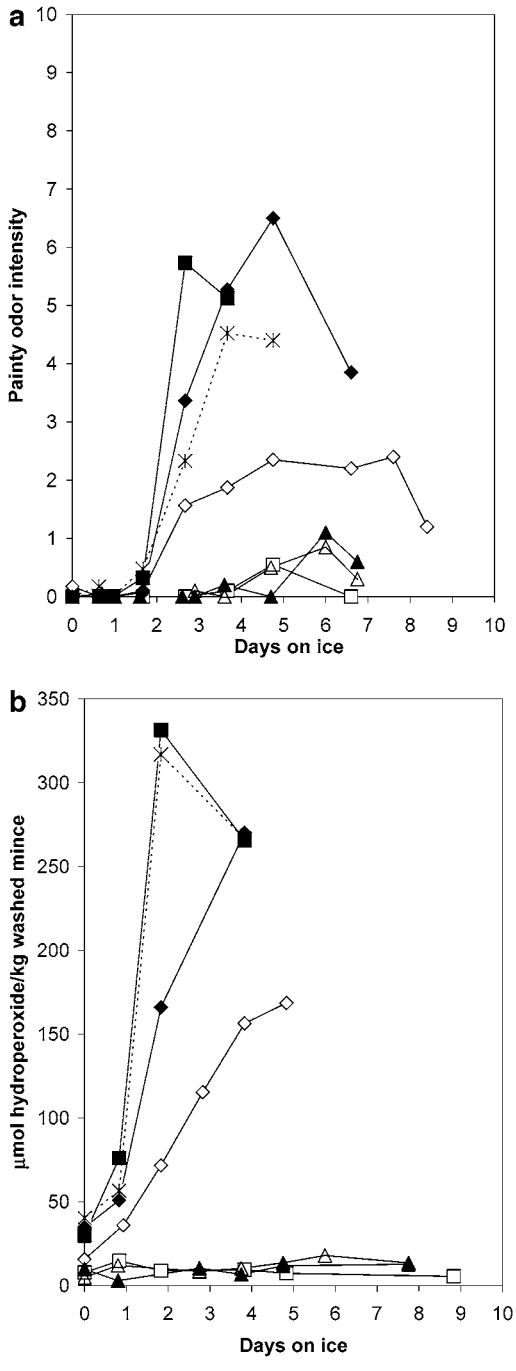

Figure 2. Hb-induced development of (a) painty odor and (b) lipid hydroperoxides in washed mince with added 50 mM phosphate buffer (control) (*), unheated cod press juice (▲), heated cod press juice (△), unheated cod press juice stored for 48 h at 4 °C (□), unheated cod press juice dialyzed for 48 h at 4 °C against 50 mM phosphate buffer (pH 7) (3.5 kDa molecular mass cut off) (■), heated cod press juice stored for 48 h at 4 °C (◇), and heated cod press juice dialyzed for 48 h against 50 mM phosphate buffer (pH 7) (3.5 kDa molecular mass cut off) (♦). Buffer and press juices were added at levels raising the moisture content of the extra dry washed mince (75%) to the same value as that of the unwashed mince (82%). This diluted the press juice 3.2 times. Oxidation was initiated with 6 μmol trout Hb/kg washed mince. Details about Hb and press juice preparations are given in **Table 1.** The final pH of the model system was 6.55–6.65.

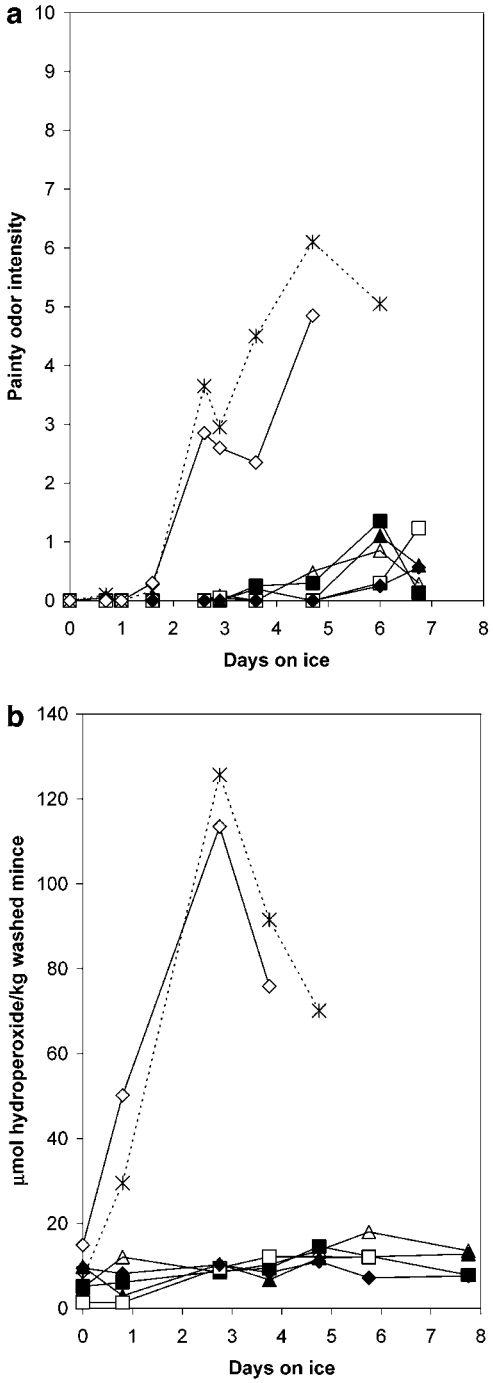

Figure 3. Hb-induced development of (a) painty odor and (b) lipid hydroperoxides in washed mince with added 50 mM phosphate buffer (control) (*), unheated cod press juice (▲), <1 kDa fraction of unheated cod press juice (■), <30 kDa fraction of unheated cod press juice (♦), <30 kDa fraction of dialyzed unheated cod press juice (◇), heated cod press juice (△), and <1 kDa fraction of heated cod press juice (□). Buffer and press juices were added at levels raising the moisture content of the extra dry washed mince (75%) to 82%, which diluted the press juice 3.2 times. Oxidation was initiated with 6 μmol trout Hb/kg washed mince. Details about Hb and press juice preparations are given in **Table 1**. The final pH of the model system was 6.55–6.65.

3.2-fold dilution (**Figure 3a**,**b**). All antioxidative capacities of unheated and heated press juices remained in the <1 kDa fractions. The <30 kDa fraction of unheated cod press juice showed the same inhibitory properties as did the <1 kDa fraction. However, after the <30 kDa fraction was dialyzed, the inhibitory properties were lost (**Figure 3a**,**b**). The 48 h at 4 °C during which dialysis was conducted did not affect the antioxidative properties of the <30 kDa fraction.

Table 3. Hb-Mediated[a] Development of Painty Odor[b] in Washed Mince[c] after Adding Heated Press Juices[d] from Fish and Chicken[e]

sample	painty odor lag phase (days)	protein content[f] in press juice before/after the heating (mg/mL)
control (DDW)	1.5	
cod	≥7	51/3.7
haddock	≥7	85/2.5
dab sole	≥7	73/7.6
black back	≥7	67/7.9
herring	2	99/7.6
chicken	2	150/7

[a] For trout hemolysate preparation, see **Table 1**. The final Hb level was 6 μmol/kg washed mince. [b] For lag phase definition, see **Table 1**. [c] Washed mince was prepared as described in **Table 1**. [d] Press juices were prepared as described in **Table 1**. The heating was done by holding 20 mL of press juice in a boiling water bath for 10 min. After it cooled for 30 min on ice, the coagulated press juices were centrifuged. The supernatants were filtered through a No. 1 Whatman filter, and the filtrate was frozen at −80 °C. Press juices were added at a 2-fold dilution level. In control samples, the press juice was replaced by DDW. The final pH in the samples ranged between 6.5 and 6.65. [e] The table also includes protein data on the different press juices. [f] Total proteins were determined by the Lowry method (*16*) as modified by Markwell et al. (*17*).

Hb-Mediated Lipid Oxidation after Adding Heated Press Juice from Additional Fish Species and from Chicken to Washed Minced Cod Muscle. Table 3 shows the ability of heated press juices from cod, haddock, dab, winter flounder, herring, and chicken to prevent Hb-mediated paintiness development in washed cod at 2-fold dilutions. Heated press juices from all of the white fish species gave rise to paintiness lag phases of ≥7 day. Heated herring and chicken press juices did not extend the paintiness lag phase as compared to the control (**Table 3**) but reduced the maximum paintiness intensity by ∼50%. Unheated chicken press juice however totally inhibited development of oxidation during the studied storage period.

Protein Composition of the Evaluated Press Juices. The unheated cod press juice contained 51 mg total proteins/mL, which was reduced to 3.7 mg/mL during the heating and centrifugation treatment. The dialysis procedure removed 18 and 30% of the total proteins from the unheated and heated cod press juices, respectively. The <30, 3.5−30, and <1 kDa fractions of cod press juice only contained traces of proteins, 0.4, 0.17, and 0.25 mg/mL ($n = 2$), respectively. The latter was true for the <1 kDa fraction of both unheated and heated press juice. The protein concentrations in the heated press juices from additional fish species and from chicken ranged from 2.5 to 7.6 mg/mL, which was the result of removing 88−97% of the proteins in the heating−centrifugation procedure (**Table 3**).

Figure 4 shows the polypeptide pattern of whole cod press juice (lane 2), heated cod press juice (lane 3), the <30 kDa fraction (lane 4), <1 kDa fraction (lane 5), and the press dialysis retentate (lane 6) of whole cod press juice. The unheated and heated chicken press juices are also included (**Figure 4**, lanes 7 and 8) to exemplify a press juice in which the antioxidative activity was not resistant to heat. It can be seen how the majority, 86%, of the heated cod press juice polypeptides was located in the 8 kDa region (**Figure 4**, lane 3). The same accumulation of 8 kDa polypeptides was seen in heated extracts from all of the other fish species that efficiently inhibited paintiness development: haddock, dab, and winter flounder. Heated chicken press juice, on the other hand, only had 3% of its total polypeptides in the 8 kDa region (**Figure 4,** lane 8). On the basis of these observations, the hypothesis was raised that the 8 kDa peptides were an additional source of antioxidative activity in fish press

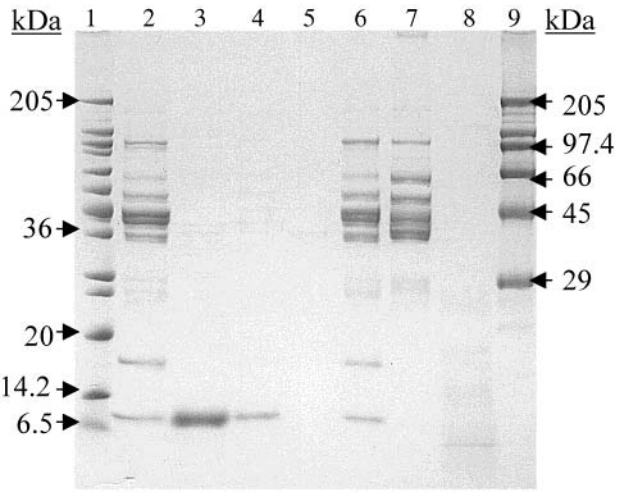

Figure 4. SDS–PAGE (10–20%, linear gradient) analysis of cod press juice before/after heating and fractionation. Lane 1, wide range Mw standards; lane 2, whole cod press juice; lane 3, heated cod press juice; lane 4, <30 kDa fraction of whole cod press juice; lane 5, <1 kDa fraction of whole cod press juice; lane 6 whole cod press juice dialysis retentate; lane 7**,** chicken press juice; lane 8, heated chicken press juice; and lane 9, high Mw standards. Protein was applied to all lanes at 7 μg/lane. For details on preparation and heating of press juices, see **Tables 1** and **3**, respectively. Fifty milliliters of crude or heated press juice was subjected to ultrafiltration using a stirred 50 mL Amicon ultrafiltration cell equipped with 1 or 30 kDa membranes. The first 25 mL of filtrate was collected and frozen at −80 °C. Ten milliliters of crude, heated, or ultrafiltered press juice was dialyzed against 2 × 2 L 50 mM phosphate buffer (pH 7) for 48 h using dialysis tubing with a 3.5 kDa molecular mass cut off. The dialysis retentate was frozen at −80 °C.

juice. However, the hypothesis was ruled out by the findings that the >3.5 kDa fraction of boiled cod press juice (**Figure 2a**,**b**) as well as the 3.5−30 kDa (**Figure 3a**,**b**) and the 3.5−10 kDa fraction (data not shown) of unheated cod press juice did not inhibit oxidation when added at the same volume as the unfractionated heated and unheated cod press juices.

Evaluation of Antioxidative Mechanism and Tentative Antioxidative Candidates. Using an aqueous test system, it was tested whether deoxy-Hb content and/or autoxidation of trout Hb was affected by the presence of fractionated cod press juice and by reducing the pH to 6.0. In **Table 4**, the levels of deoxy-Hb formed immediately after mixing Hb with the press juice fractions/HCl are shown together with the Hb autoxidation changes during storage. Both zero time deoxygenation data and the data on met-Hb formation rates (*k* values) were divided with data for the control sample without added press juice. Increased levels of deoxy-Hb as compared to the control are denoted by ratios <1 while increased rates of met-Hb formation are indicated by ratios >1. The zero time level of oxy-Hb was immediately reduced by ∼40 and 20%, respectively, in the presence of the dilayzed cod press juice and when reducing the pH of a control sample to 6.0. Adding the 3.5−30 kDa fraction, a 20% increase in the oxy-Hb level was obtained. The Hb autoxidation rate was moderately accelerated by the <30 kDa fraction and by pH reduction to 6.0, while the >3.5 kDa fraction accelerated Hb autoxidation by an order of magnitude.

Tentative LMW antioxidant candidates were added to washed mince in order to test if they could inhibit Hb-catalyzed paintiness development to the same extent as the white fish press juices. Physiological levels of inorganic phosphate (40 mM), chloride (70 mM), 2,3-diphosphoglycerate (2,3-DPG) (5.8 μM), TMAO (100 mM), and spermine (61.2 μM) did not inhibit Hb-

Table 4. Effect of Heated and/or Fractionated Press Juices[a] on Deoxygenation and Autoxidation[b] of Trout Hb[c]

	Hb deoxygenation		Hb autoxidation	
sample	ΔA ($A_{575} - A_{560}$)	ΔA (sample)/ΔA (control)	fitness of model (R^2)	k (sample)/k (control)
control (pH 6.6)	0.0358	1	1	1
control (pH 6.0)	0.030	0.83	0.90	1.9
heated press juice	0.0376	1.05	0.99	1
press juice, <1 kDa fraction	0.0386	1.08	0.98	1.2
heated press juice, <1 kDa fraction	0.037	1.03	0.99	1.1
press juice, <30 kDa fraction	0.038	1.06	1*	1.6
press juice, 3.5–30 kDa fraction	0.0438	1.2	0.96	0.8
press juice, >3.5 kDa fraction	0.0208	0.58	0.90	12.6
heated chicken press juice	0.044	1.24	0.91*	0.25
heated herring press juice	0.039	1.09	0.65*	2

[a] Press juices were prepared, heated, and fractionated as described in **Tables 1** and **3** and **Figure 4**, respectively. [b] The test system consisted of 2 mL of phosphate buffer (50 mM, pH 6.6), 1 mL of press juice, 200 ppm streptomycin, and 6 μM trout Hb (final pH 6.6). Control samples were prepared at pH 6.0 and pH 6.6 by replacing the press juice with phosphate buffer. For each sample, a Hb free reference sample was prepared. Samples and references were stored for 12 days on ice in 15 mL glass test tubes. The samples were scanned daily between 600 and 500 nm against the Hb free reference. Hb deoxygenation was only measured at time 0 as A_{575nm}–A_{560nm}. Hb autoxidation was measured over time as changes in A_{575nm}. To estimate the rate of change, linear regression models ($y = kx + m$) relating Hb autoxidation (y) to storage time (x) were calculated. Both zero time deoxygenation data and data from regression analysis of the met-Hb formation (k values) were divided by the values for the control sample without added press juice (pH 6.6). Data marked with * are based on results from 9 days of storage. The other models cover the period 0–12 days.

catalyzed lipid oxidation as compared to control samples with DDW or phosphate buffer. The same was true for 200 μM potassium phosphate, 15 μM pyrophosphate, 0.2% STPP, 100 μM TMAO, and 58 μM 2,3-DPG (5.8 μM). A slight lag phase extension (from 2 to 3.5 days) was obtained in the presence of 612 μM spermine.

DISCUSSION

Odor and lipid hydroperoxide analyses showed that added Hb effectively catalyzed oxidation of membrane lipids in unwashed mince and washed mince during ice storage (**Figures 1**−**3**). The odor and lipid hydroperoxide data followed each other well during the storage. Both measures were inhibited by the presence of cod muscle press juice, while in its absence, both increased and then declined during storage. The hydroperoxide level increased and declined earlier than did the sensory scores, which is in line with the need for lipid hydroperoxide breakdown in order for volatiles to form. Sensory analysis is the only measure reflecting how lipid oxidation is perceived by humans. Because there was a strong link established between the painty odor and the lipid hydroperoxide development, painty odor was chosen as the primary tool to investigate the antioxidative properties of cod muscle press juice in this study.

Antioxidative Capacity in the Aqueous Fractions of Fish and Chicken Muscle. There have been previous observations of faster lipid oxidation in washed than unwashed minced fish muscle during storage (*6*, *7*, *24*, *25*). The reasons for these findings have been difficult to identify as washed and unwashed samples have differed both in their lipid, pro-, and antioxidant composition. At almost the same lipid content (0.7 and 1%) and after matching initial differences in moisture content, pH, degree of muscle disruption, and prooxidant concentration, this study showed that oxidation in washed minced cod was still faster than in unwashed minced cod (**Figure 1**, **Table 1**). It was suggested that water soluble oxidation inhibitor(s) were removed/diluted during the washing. This theory was confirmed by the inhibition of Hb-mediated lipid oxidation of washed cod membrane lipids following addition of cod press juice at different dilutions (**Figure 1**, **Table 1**). The present results showed that the press juice could not be diluted excessively for complete inhibition of Hb-mediated rancidity in washed mince. In unwashed mince, a 2.2-fold dilution of the endogenous aqueous phase by addition of water shortened the pantiness lag phase from >11 to ∼8 days (**Table 1**). A 70-fold dilution of the endogenous aqueous phase, which was the result of washing the mince, shortened the lag phase from >11 days to ∼1.8 days (**Table 1**). When adding back press juice to washed mince, a 2-fold dilution as compared to the amount of press juice naturally present in unwashed mince was needed to prevent painty odors from limiting shelf life (**Table 1**). Already at 3.2- and 6-fold dilutions of the press juice, painty odors started developing prior to odors that were regarded as "microbial spoilage". However, as compared to a washed mince sample with no added press juice, press juice at 3.2- and 6-fold dilutions still prolonged the painty odor lag phase by 5 and 2.5 days, respectively (**Table 3**). The responses to dilutions could indicate the presence of multiple antioxidants in press juice and/or a qualitative change in activity, e.g., from anti- to prooxidative, upon a change in concentration. It is well-known that the activity of both pro- and antioxidants is highly concentration-dependent and that some compounds, e.g., ascorbate, change from being inhibitory to prooxidative upon dilution (*26*). Upon increased dilution of mackerel press juice from 5-fold to 7-fold, the lag phase of soybean PC oxidation decreased from >48 to 6 h (*27*).

The <1 kDa fraction of cod press juice gave the same inhibition as did nonfractionated cod press juice (**Figure 3a**,**b**). The inhibitory properties of cod press juice also remained after heating and centrifugation (**Figure 2a**,**b**), while dialysis of the inhibitory press juice fractions brought about a loss of the antioxidative properties (**Figure 2a**,**b**). These findings implied that the observed inhibitior was not a protein or any other biopolymer. It is therefore unlikely that cytosolic antioxidative enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-px) were responsible for the inhibition. Rather, nonenzymic LMW compounds targeting, e.g., free radicals, membrane surfaces, or Hb, might be involved. A few possible candidates (phosphates, spermine, TMAO, inorganic phosphate, chloride, and 2,3-DPG) were tested but ruled out at the studied levels. The inability of phosphates to prevent oxidation to the same degree as the press juices was different from in previous studies. Inorganic phosphate largely contributed to the capacity of a heat resistant <10 kDa fraction of fish muscle cytosol to prevent Fe-catalyzed oxidation of isolated membrane fractons (*8*, *9*, *11*). This might reflect the low significance of LMW iron for lipid oxidation in our model system. Richards (*7*) showed that LMW Fe added at a 23.2 μM

level (i.e., the level provided by 5.8 μM Hb) did not induce oxidation of washed minced cod lipids.

Aging and pH reduction decreased the inhibitory properties of heated and unheated cod press juice, respectively (**Figure 2**, **Table 2**). The former could indicate the involvement of unstable nucleotides and/or reducing agents in the inhibition. That the whole press juice was resistant to aging could be the result from compounds stabilizing such LMW compounds, e.g., via recycling. The smaller extension of the painty odor lag phase at pH 6.0 than 6.6 (**Table 2**) could indicate the involvement of ionic interactions in the contact between the inhibitor and the target molecule. It is also possible that Hb did not respond to the inhibitor because of molecular changes brought about at pH 6.0, e.g., dissociation (*28*).

That the dialysis retentate of cod press juice (i.e., the >3.5 kDa fraction) slightly accelerated Hb-mediated painty odor development (**Figure 2a**) suggests that crude cod press juice is a complex mixture of anti- and prooxidants, with the latter dominating in the >3.5 kDa fraction. One high molecular weight prooxidant in press juices was Hb. The unheated cod and chicken press juices contained 1.8 and 3.2 μM Hb, respectively. That unheated cod and chicken press juices showed a net antioxidative activity despite their content of Hb could indicate that any LMW inhibitor(s) was effective against the prooxidative activity of Hb. Takama (*29*) found that after dialysis of an aqueous dorsal trout muscle extract, oxidation of the proteins in the extract (e.g., oxy-Hb) was accelerated as compared to that in the nondialyzed extract.

One prooxidative role of Hb in washed cod membrane lipid oxidation is cleavage of preexisting lipid hydroperoxides into volatile compounds and free radicals (*1*, *5*). Even at very low levels of preformed hydroperoxides, Hb has proven limiting in this breakdown reaction (*2*, *5*). It has been hypothesized that deoxy-Hb and met-Hb are more active prooxidants than oxy-Hb (*15*). A possible mechanism for the press juice inhibition could be prevention of Hb from being deoxygenated and/or oxidized. However, the press juice fractions that slowed painty odor and hydroperoxide development in washed mince did not markedly reduce Hb deoxygenation or Hb autoxidation in an aqueous test system, which implied other mechanisms. On the other hand, the test conditions that accelerated oxidation in washed mince, pH reduction, and addition of the dialysis retentate also accelerated Hb deoxygenation and Hb autoxidation (**Figure 2**, **Tables 2** and **4**). Acid catalysis of Hb deoxygenation (*15*) and Hb autoxidation (*23*) has been reported previously.

Heated chicken and herring press juices were less inhibitory than the heated white fish press juices (**Table 4**). One reason could be that the unheated chicken and herring press juices had higher Hb levels than the unheated white fish press juices (3.2 and 11.6 μM vs 1.8 μM in cod). Heat can release free heme and/or LMW iron from Hb (*30*), and at high levels, these prooxidants could possibly have counteracted the effect from tentative LMW antioxidants in heated chicken and herring press juices. Furthermore, the finding that unheated chicken press juice was highly antioxidative could indicate that heat labile proteins such as SOD, catalase, and ceruloplasmin play more important roles in the aqueous antioxidative defense system of a warm-blooded animal such as chicken than do LMW compounds. It is interesting that there was a high protein content in the chicken press juice, 150 mg/mL as compared to 51 mg/mL in cod press juice. It must be pointed out, though, that chicken is only one species of a warm-blooded animal, a bird, and that it is not known what the situation would be in other types of muscle tissues used for food. At ~4-fold dilutions of unheated mackerel light muscle press juice, a proteinaceous antioxidant (>5 kDa) overcame the ability of LMW prooxidants in press juice to mediate TBARS production in a phosphatidyl choline emulsion (*27*).

Practical Relevance. Several features found in relation to inhibition of Hb-catalyzed membrane lipid oxidation by aqueous fractions of certain muscles are of practical relevance. First, extraction of the aqueous fraction could easily be tied into a surimi process, e.g., by utlilizing the wash water residue. Mincing and centrifugation of various fish byproducts (e.g., fish frames and heads) is another pathway. The LMW nature of the active component(s) (**Figures 2** and **3**) offers the possibility to produce a virtually protein free antioxidant concentrate/powder from both water extracts and press juice. A concentrate/powder could then be added to more unstable washed materials such as surimi from dark muscle fatty fish. The heat tolerance of the inhibition makes it possible to use a simple heating/centrifugation/filtration/decanting procedure as a first isolation step. Heating/centrifugation removed ~94% of the proteins from cod press juice and 88−97% of the proteins from other fish press juices. The heat tolerance of the inhibitor(s) also facilitates for drying and possibly allows for prevention of heat-accelerated rancidity in muscle foods, so-called "warmed over flavor" (WOF). It is believed that membrane lipids and hemoproteins have pronounced roles in WOF development (*31*).

ABBREVIATIONS USED

DDW, double distilled water; Hb, hemoglobin; LMW, low molecular weight; Mw, molecular weight; SR, sarcoplasmatic reticulum.

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