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Antioxidative Properties of Press Juice From Herring (*Clupea harengus*) Against Hemoglobin (Hb) Mediated Oxidation of Washed Cod Mince

THIPPESWAMY SANNAVEERAPPA,* NILS-GUNNAR CARLSSON, ANN-SOFIE SANDBERG, AND INGRID UNDELAND

Department of Chemical and Biological Engineering, Food Science, Chalmers University of Technology, SE 412 96, Göteborg, Sweden

The antioxidative effect of herring (*Clupea harengus*) light muscle press juice (PJ) against hemoglobin-(Hb-) mediated oxidation of washed cod mince during ice storage was tested. The PJ was fractionated into low-molecular-weight (LWM; >1 kDa) and high-molecular-weight (HMW; >1, >3.5, and > 50 kDa) fractions; it was preheated (10 min, 100 °C) and tested with or without removing heat coagulated proteins. Its antioxidative effect was compared with that given by endogenous levels of two tentative antioxidant candidates: ascorbic acid and uric acid. Oxidation was followed by determining rancid odor, peroxide value, and redness. Whole herring PJ and the LMW-PJ fraction significantly ($p < 0.001$) extended the oxidation lag phase of controls, from 2 up to 8 and 7 days, respectively. The HWM-PJ fractions were significantly ($p < 0.05$) less efficient than the whole and LMW-PJ samples, giving only 3.5–4.5 days of lag phase. Heat-treated PJ, with and without the heat-coagulated proteins, gave 7 and 5 days of oxidation lag phase, respectively. Heating different batches of the LMW-PJ fraction grouped the results into two categories: one where heating almost fully destroyed the antioxidative activity (fractions prepared from spring-caught herring) and another where heating had no or a minor effect (fractions prepared from fall-caught herring). The spring LMW-PJ had low ascorbic acid levels (18-42.6 μM), and 50-100% were destroyed by the heating. In fall LMW-PJ, the levels were 76.2-137.6 μM, and only 43-51% were destroyed. Ascorbic acid fortification of heated spring LMW-PJ to reach the levels found in the corresponding unheated spring LMW-PJ sample and the heated fall LMW-PJ gave back most of the antioxidative activity, which proved an important role of ascorbic acid for the antioxidative activity of LMW-herring PJ. This conclusion is drawn despite the fact that pure solutions with endogenous levels of ascorbic acid (giving 8.4–19.6 μM in final model) only very slightly delayed Hb-mediated oxidation of the washed cod mince.

KEYWORDS: Herring (*Clupea harengus*); press juice; hemoglobin; lipid oxidation; washed cod mince; ascorbic acid; antioxidant

INTRODUCTION

Lipid oxidation in food results in the development of off flavor, undesired color, and a loss of nutrients (*1*). Heme proteins (hemoglobin, Hb, and myoglobin, Mb), heme-derived transition metals, and enzymes are normal components of muscle foods, which may accelerate lipid oxidation (*2*). In fish, Hb has been identified as one of the most potent pro-oxidants and can start oxidation according to several mechanisms (*3, 4*). In seafood processing, blood may not be removed prior to, for example, filleting and mincing. So, in postmortem fish, Hb can react with the muscle lipids and accelerate lipid oxidation (*5*); therefore, its rapid removal or inhibition is important to prevent the onset of lipid oxidation.

* Author to whom correspondence should be addressed. Phone: +46-31-772 38 13. Fax: +46-31-772 38 30. E-mail: swamy@chalmers.se.

Fish muscle also contains a multicomponent antioxidant defense system to combat the damaging effects of pro-oxidants. The function of these antioxidants, which are both lipid- and water-soluble, is to scavenge free radicals, inactivate reactive oxygen species, chelate transition metals, and so forth. Fish-derived water-soluble antioxidants have been a matter of focus in recent years. In a previous study, it was reported that an isolated aqueous fraction (press juice, PJ) from white muscle fish like cod, haddock, dab, and winter flounder had strong inhibitory activity against Hb-mediated oxidation of washed cod mince membranes (*6*). This finding raised the question whether fish muscle PJ isolated also from underutilized species could be a future natural tool in preventing lipid oxidation during the post-mortem storage and processing of muscle foods.

Herring (*Clupea herangus*) is an abundant species in Scandinavian waters, and there is a large desire to better utilize it

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for human consumption. Currently, a major percentage of the catch goes for nonhuman consumption. Not much information is available about antioxidative components of herring muscle, but it could be speculated that the antioxidative system should be well-developed to counteract the high pro-oxidative activity of the herring muscle. Some indications of this were given in a previous study by Slabyj and Hultin (*7*) in which herring light muscle cytosol was shown to be antioxidative.

The aim of this study was to evaluate the antioxidative properties of herring light muscle PJ against the Hb-mediated oxidation of washed cod mince. This was to help the understanding of how the aqueous endogenous antioxidative system of herring is built up and to look into possible better utilization of herring and herring processing byproducts (e.g., frames and surimi wash water) by preparing their PJ. The antioxidative components of herring PJ were characterized in terms of their molecular size (<1, >1, >3.5, and >50 kDa) and their heat stability (exposure to 100 °C for 10 min). To search for antioxidative candidates in herring PJ, ascorbic acid and uric acid were tested for their individual activity, their synergistic effects, and their heat stability at levels endogenous to the herring PJ.

MATERIALS AND METHODS

Fish and Blood Supply. Fresh whole cod (*Gadus morhua*) weighing between 5 and 7 kg were obtained iced from Leröy Allt i Fisk AB (Göteborg, Sweden), and 5–6 kg of whole herring (*Clupea harengus*) weighing 150–200 g each were collected iced from Paul Mattsson AB (Ellös, Sweden). Herring and cod caught in different seasons between 2003 and 2007 were used in this study. The post-mortem age of both cod and herring was 36–72 h. The light muscle was manually removed and was minced in a kitchen grinder (Ultra Power, model KSM90, KitchenAid, St. Joseph, MI) with a plate hole diameter of 5 mm. The cod light muscle was used for model system preparation, and the herring light muscle was used for PJ preparation. Fish blood for hemolysate preparation was obtained from five to six rainbow trout (*Onchorhynchus mykiss*) weighing about 375–500 g each, which were maintained at the Department of Zoology (Göteborg University, Sweden).

Chemicals. Ammonium thiocyanate, barium chloride, cumene hydroperoxide, 3-aminobenzoic acid ethyl ester, heparin (sodium salt), sodium hydrosulfite, bovine hemoglobin, streptomycin sulfate, Folin and Ciocalteu's phenol reagent, bovine serum albumin (BSA), and uric acid were obtained from Sigma (St. Louis, MO). Ascorbic acid was obtained from Fluka (Sigma Aldrich, Buchs, Switzerland). Milk protein concentrate (MPC), Alapro 4560, was purchased from NZMP, Rellingen, Germany. DL-α-tocopherol was purchased from Calbiochem (an affiliate of Merck KGaA, Darmstadt, Germany). Cupric sulfate was obtained from Fisher Scientfic Company, Fair Lawn, NJ. All other chemicals used were of analytical grade. Ready electrophoresis gels (4–20% and 10–20%), tris HCl, buffers, and staining and destaining solutions were purchased from Bio-Rad (Bio-Rad Laboratories, CA).

Washed Cod Mince Model System. A low-moisture washed cod mince model system (65–75% moisture) was prepared by washing mince in three volumes, once with Milli Q water and twice with 50 mM NaCl (pH 5.5) according the method described by Sannaveerappa et al. (*8*). The washed mince was immediately stored frozen at –80 °C.

Bleeding of Fish and Preparation and Analysis of Hemoglobin (Hb). Rainbow trout (*Onchorhynchus mykiss*) were bled according to the method of Rowley (*9*). Hemolysate was prepared by repeated washes of the erythrocytes in a 1 mM Tris buffer (pH 8, 1.7% NaCl) as described by Fyhn et al. (*10*). The Hb content in the hemolysate was determined spectrometrically after the conversion of Hb to the CO form by the addition of sodium dithionate and bubbling with CO gas (*11*). Hemoglobin was expressed as micromolar Hb. The number of sample replicates (*n*) was ≥ 3, and an analytical replicate (*a*) was 3.

Preparation of Herring PJ. Minced herring light muscle was packed in 200 mL polypropylene centrifuge bottles (200 g in each) and centrifuged at 18300*g* for 2 h at 4 °C in a Sorval Superspeed centrifuge (model RC-5C Plus, Kendro Laboratory Products, Stockholm, Sweden) using an SLA-1500 rotor. The resulting supernatant (i.e., the PJ), which on a weight basis normally contributed to 14–17% of the initial mince, was filtered through Munktell's filter paper no. 1003 (typical pore size of 12–15 μm, Munktell filter AB, Grycksbo, Sweden) and was stored frozen at –80 °C until use. Also, some samples of the pellet were taken for basic compositional analyses.

Preparation of Different Fractions of Herring PJ. The low-molecular-weight (LMW) fraction below 1 kDa was obtained by filtering herring PJ through a 1 kDa filter membrane (Millipore, Bedford, MA) in a stirred ultrafiltration cell of 50 mL capacity (Model 8050, Millipore Corporation, Bedford, MA). Filtration was carried out on an ice bath under 35–40 psi of nitrogen pressure. When 50% of the original volume (50 mL) was filtered, the filtration was stopped, and the filtrate was frozen and stored at –80 °C until use. The high-molecular-weight (HMW) fractions above 1, 3.5, and 50 kDa were obtained by dialyzing herring PJ in dialysis tubes with cutoffs of 1, 3.5, and 50 kDa (Spectrum Laboratories Inc., Rancho Dominguez, CA) against 100 volumes of a 50 mM phosphate buffer with saline (0.9%) at pH 6.6–6.8 and 4 °C for 24 h. During dialysis, the buffer was changed three times at regular intervals.

Preparation of Antioxidant Candidate Solutions. Ascorbic acid (19.2, 25, 50, 62, and 106 μM) was made in a 50 mM phosphate buffer, pH 6.3. Uric acid (5.5, 9.3, 25, and 50 μM) was made by dissolving 15.40 mg in 2 mL of 0.2 M NaOH followed by dilution with a 50 mM phosphate buffer, pH 6.3. A bovine serum albumin solution (94 and 47 mg/mL) was prepared in a cold 50 mM phosphate buffer, pH 6.3. A milk protein concentrate solution (94 mg/mL) was prepared by dissolving in a 50 mM phosphate buffer, pH 6.3.

Heat Treatment of PJ and Antioxidant Solutions. A total of 20 mL of native PJ, the LMW-PJ fraction, as well as selected ascorbic acid and uric acid solutions were poured into 50 mL polycarbonate centrifuge tubes and held in a boiling water bath for 10 min. During this treatment, the solutions reached 87–89 °C, after which they were cooled for 30 min on ice. The heated native PJ was either added directly into the washed cod or first centrifuged at 17800*g*(20 min, 4 °C) to remove coagulated proteins. Then, the supernatant was filtered through filter paper (Munktell's 00H, Munktell Filter AB, Grycksbo, Sweden) prior to addition to the washed cod.

Preparation of Oxidation System. Frozen washed cod mince was thawed in a plastic bag under running cold water. Excess water was then squeezed out manually using a cotton towel. The moisture content of the model was thereby lowered down to 65–75%. This lowering was done to be able to add PJ without increasing the moisture in the model above physiological levels, ~81%. Squeezed mince was chopped in a small precooled mixer (Hugin, model MC-851, Coop Electro AB, Upplands Väsby, Sweden) for 30–60 s. The larger the quantity of mince, the longer was the chopping time. To start oxidation trials, the procedure described by Sannaveerappa et al. (*8*) was followed. In brief, the cod model was manually mixed with the different PJ samples (see **Figure 1**), antioxidant candidate solutions, or a buffer to raise the moisture up to 81%. The added test solutions hereby got diluted ~2.5 times in the model. In the Results section, the final concentrations of the antioxidant candidates after this diluion are given. To prevent microbial growth, 200 ppm streptomycin was also mixed in. The pH was then adjusted to 6.3 ± 0.05, and hemolysate, to reach a final Hb level of 15 μM, was mixed for 2 min using a spatula. A total of 20 g of the model substance was then flattened out in the bottom of 250 mL Erlenmeyer flasks (E-flasks), which were capped and stored on ice for approximately 10 days. Samples were exposed to 900 ± 50 lx of light at an average 3–4 h per day. The storage trial was stopped when the sensory panel detected a putrid smell which indicated bacterial growth.

Measurement of Redness (a*). Changes in redness (a*) in the bottom of the E-flasks were followed during the storage of models with a colorimeter (Minolta Chroma Meter CR-300, Minolta Corp., Ramsey, NJ) using a CIE Laboratory color scale. The procedure followed for color measurement has been described by Sannaveerappa et al. (*8*). The number of analytical replicates (*a*) was 5.

Determination of Peroxide Value (PV). To follow the development of the peroxide value (PV), 1–2 g sample plugs were taken by piercing

a hollow plastic tube (∅ = 10 mm) on a flattened surface in the E-flask during ice storage. The sample plugs were frozen and stored at –80 °C until they were analyzed. Lipids were extracted from the plugs with chloroform and methanol (1:1) according to the method of Lee et al. (*12*), and PV determination was done (a = 1–2) in the chloroform extract using the ferric thiocyanate method of Shantha and Decker (*13*) as modified by Undeland et al. (*14*). Cumene hydroperoxide was used as the standard, and PV was expressed as micromoles of hydroperoxides per kilogram of the sample.

Sensory Analysis. The head space of the E-flasks was sniffed by a small internal panel (3–4 people) during ice storage of the cod model sample (*5*). Attention was given to recognize the intensity of rancid odor development (*15*), which was marked on a scale from 0 to 100. On this scale, 0 indicated no smell, 10 slightly rancid, 50 medium rancid, and 100 maximum rancid. The internal panel was trained on both fish and fish oil samples at various stages of oxidation to learn to quantify rancid odor on the 100 mm scale.

α-Tocopherol Analysis. α-Tocopherol was analyzed in the cod mince that was used for preparing the model system (n = 2). Extractions were done with methanol. Separation as well as quantification was conducted in duplicate (a = 2) according to Sannaveerappa et al. (*8*) using reverse-phase high-performance liquid chromatography (HPLC) on a C18 column (Chromacil, EKA Chemicals, Bohus, Sweden). The mobile phase was 98% methanol–water. Peaks were detected with a spectroflurometric detector (Shimadzu RF-551, Kyoto, Japan), and α-tocopherol was expressed as milligrams per kilogram of cod mince (dry weight, dw basis). The relative standard deviation (RSD) of α-tocopherol analysis was 1.77%.

Compositional Analyses of Herring PJ and the Pellet Obtained During PJ Preparation. *Moisture Content, pH, and Conductivity.* The moisture contents of PJ, its fractions, and the pellet from PJ preparation were measured using a HA300 Moisture Balance (Precisa balance 310M, Zurich, Switzerland; n = 3 and a = 1, where n represents the number of sample replicates and a the number of analytical replicates). The moisture results were expressed as a percentage of wet weight. A Hamilton double-pore electrode (Hamilton Double Pore, Bonaduz, Switzerland) in conjunction with a pH meter (Radiometer analytical PHM210, Villeurbanne Cedex, France) was used for pH measurement both in solutions and in cod model systems. The conductivity of PJ samples was measured with a conductivity meter (CDM 210, Meter Laboratory, Radiometer Analy SAS, Villeurbanne Cedex, France).

Protein Content. Total protein measurements of herring PJ, PJ fractions, and the PJ pellet were done according to Lowry et al. (*16*) using BSA as a protein standard (n = 3 and a = 2). The protein content was expressed as milligrams per milliliter or as a percentage of the sample.

Total Lipids. The method described by Lee et al. (*12*) was used to determine the total lipids in PJ and its fractions using chloroform and methanol (1:1) as the extraction solvent (n = 3 and a = 1). The lipid content of the PJ pellet was determined using a 2:1 ratio of chloroform to methanol during the extraction.

Ascorbic Acid and Uric Acid. The ascorbic acid and uric acid of the PJ samples were analyzed with HPLC using an electrochemical detector (*17, 18*), with modifications according to Gunnarsson et al. (*19*) (n = 2–8 and a = 2–3). Both ascorbic acid and uric acid results are expressed as micromolar. The repeatability expressed as relative standard deviation, RSD%, of the method for ascorbic acid and uric acid was 2.6% and 1.2% RSD (n = 1 and a = 6).

Amino Acids. Total and free amino acids in PJ samples were analyzed with HPLC according to Fontaine et al. (*20*) (n = 2 and a = 1). Amino acids were expressed as grams per kilogram of the sample.

Mineral Content. Minerals (Fe, Cu, and Zn) were determined in PJ samples using ion chromatography after microwave digestion (*21*) (n = 2–3 and a = 2–3). Minerals were expressed as micrograms per gram of the sample.

Electrophoresis. The polypeptide patterns of native herring PJ, the HMW-PJ (>3.5 kDa) fraction, and the heated plus centrifuged PJ were analyzed with SDS-PAGE according to Laemmli (*22*) using a Ready gel vertical cell unit (n = 3 and a = 1) and slab ready gels (4–20%, tris-HCl) at a constant voltage of 200 V. The purity of the LMW-PJ fraction was confirmed using the same SDS-PAGE setup, but with 10–20% tris-HCl gels. The gels were stained for 30 min and destained for 2 h with 2–3 changes of the destaining solution on a shaking platform to facilitate both staining and destaining.

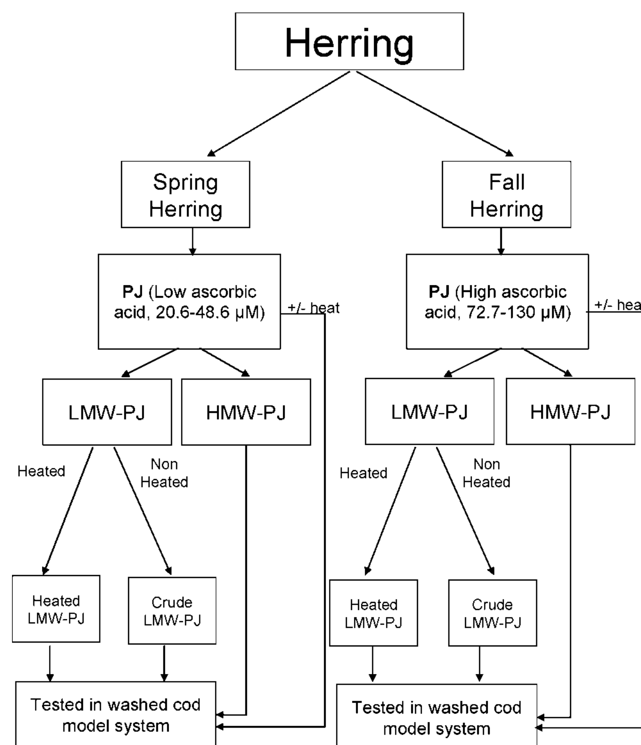

Figure 1. Overview of the experimental design used for testing the capacity of crude, fractionated, and heated herring PJ obtained from various batches of herring (grouped into spring and fall) to prevent hemoglobin-mediated lipid oxidation of washed cod mince. PJ stands for press juice;LMW-PJ and HMW-PJ stand for low-molecular-weight PJ and high-molecular-weight PJ, respectively. Heated crude PJ was used before and after the removal of heat-coagulated proteins.

Statistics. Each sample type was replicated between 1 and 10 times (thus, n = 1–10) mostly with the replicates distributed over independent storage experiments using different batches of washed cod mince and hemolysate. The sensory analysis limited the possible number of sample replicates within each storage experiment. Attempts were made to avoid merging results from different storage experiments into a single graph in order not to dilute valuable information on oxidation differences caused by different sample enrichements into the variation caused by batch-to-batch differences in the raw material. That the latter variations existed was shown by the time it took for the control to oxidize in the different trials. Because of this, error bars showing the variation between different storage experiments are not shown in the graphs. In the tables, results are expressed as mean ± standard deviations (SD) in cases were $n \geq 3$, while where $n = 2$, mean values ± (max – min)/2 are given. In order to significantly differentiate between oxidation data obtained from different storage points or from different sample types, data were compared with a t test. Differences among mean values obtained from the rancid odor lag phases and rancid odor intensity were tested by ANOVA, and significance levels were obtained with Tukey's HSD multiple-range test. The software used was SPSS (version 13.0 for Windows). Differences are regarded as being significant when $p \leq 0.05$.

RESULTS

Evaluation of Antioxidative Property of Native and Fractionated Herring PJ Against Hb-Mediated Oxidation of Washed Cod Mince. Figure 1 shows an overview of the study design used for testing the different herring PJs and its fractions in the washed cod mince model system. The antioxidative activity of herring PJ and its different fractions was evaluated mainly by following the development of rancid odor,

PV, and the loss of redness (a*) due to met-Hb formation in a washed cod mince model system. Rancid odor results are shown in **Figures 2a**, **3a**, and **4a**; PV results are shown in **Figures 2b**, **3b**, and **4b**, and redness data in **Figures 2c**, **3c**, and **4c**. An oxidation lag phase defined as the time it took for the rancid odor to enter its exponential increase phase was considered to assess the antioxidative capacity of herring PJ and its fractions. To be consistent in this asesseement, a rancid odor value > 10 was defined as the end of the lag phase. In this text, we mostly discuss the extension of the oxidation lag phase compared to a control sample.

When native unfractionated PJ was added to the cod model, it extended the rancid odor lag phase by ≥ 6 days over the control, the latter having ~2 days of lag phase (**Figure 2a**). After 4 days of storage, the rancid odor was significantly lower ($p < 0.001$) in the PJ sample compared to the control. To study the PJ in detail, it was fractionated into three HMW fractions (>1, > 3.5, and > 50 kDa) and one LMW fraction (<1 kDa). SDS-PAGE analyses of the native PJ and the > 3.5 kDa and the < 1 kDa fractions (**Figure 5**) revealed that there were no qualitative polypeptide differences between the native PJ and its HMW-PJ fraction (lanes 2–3), while in the LMW-PJ fraction, no polypeptides could be detected (lane 4). Upon adding these fractions to the cod model, the LMW-PJ fraction gave ≥ 4 days of lag phase extension over the control, whereas the HMW-PJ fractions only gave 1–2 days of extension over the control (**Figure 2a**). After 4 days of storage, the rancid odor was significantly lower ($p < 0.001$) in the LMW-PJ sample compared to the control. As can be seen from **Figure 2b**, inhibition of the rancid odor by PJ and LMW-PJ was clearly supported by the prevention of PV development. However, the distinction between the active PJ samples (native PJ and its LMW fraction) and nonactive PJ samples (HMW fractions) was even clearer. That the sample with the >1 kDa fraction reached much higher PVs than the other samples was due to it being run in another cod model system in which all PV results were high. The results of redness loss are shown in **Figure 2c**. It can be seen that the rate of redness loss was significantly ($p > 0.001$) slower in cod models containing native PJ and the LMW-PJ fraction than in the control.

Evaluation of Tentative Candidates Responsible for the Antioxidative Effect of Herring PJ. Since almost all activity of the PJ seemed to be located in the LMW-PJ fraction, there was a great interest in trying to identify the active LMW-PJ components responsible for this activity. The first step was to analyze the concentration of two well-known water-soluble LMW antioxidants (ascorbic acid and uric acid) in the PJ and its fractions. Ascorbic acid and uric acid were then added individually to the washed cod mince at the same levels as were found in the LMW fraction of herring PJ. To elucidate possible synergistic effects, the two components were also added together. Results on ascorbic acid and uric acid levels in PJ and its LMW fraction are shown in **Table 1**. The HMW fraction was deficient in these compounds. It was noted that the batches of herring PJ made from spring-caught herring had significantly ($p < 0.001$) lower levels of ascorbic acid than the PJ made from fall-caught herring; that is why spring and fall results are shown separately. The variations in uric acid due to season were much smaller. On the basis of the data in **Table 1**, solutions with the following concentrations of ascorbic acid were tested in the washed cod mince: 19.2, 25, 50, 62, and 106 μM. This gives the following final concentrations in the cod model: 8.4, 8.9, 19.6, 25.6, and 44.0 μM on the basis of the moisture content of the model. Regarding uric acid, solutions with 5.5, 9.3, 25,

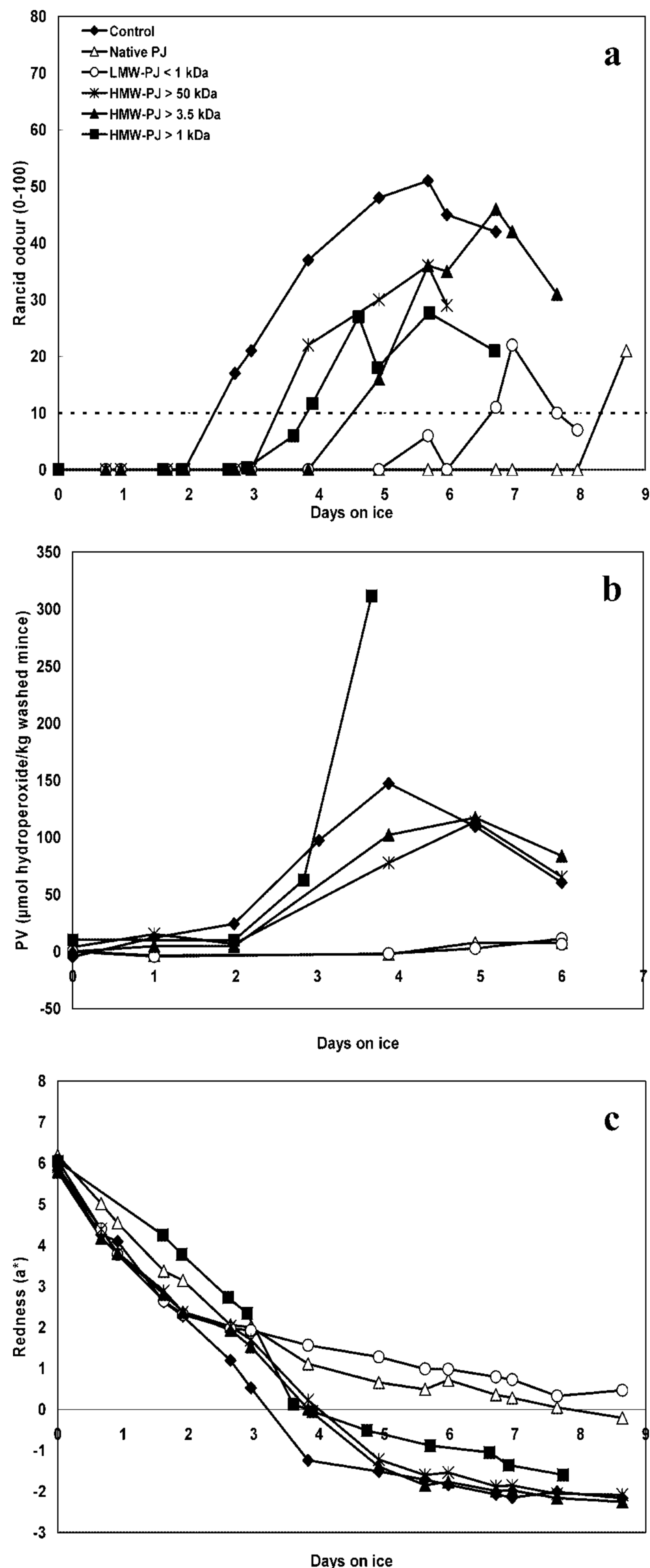

Figure 2. Development of rancid odor (**a**), lipid hydroperoxides (**b**), and redness (a*) loss (**c**) in washed cod mince fortified with a 50 mM phosphate buffer (control) or different fractions of herring PJ: native PJ, LMW-PJ fraction (<1 kDa), HMW-PJ fraction (>1 kDa, >3.5 kDa, and >50 kDa). Oxidation was catalyzed by adding 15 μM trout Hb. The final pH and moisture of the models were 6.3 and 81%, respectively. The dotted line in **a** indicates a rancid odor intensity of 10 out of 100, which we used to define the rancid odor lag phase.

and 50 μM were tested (in the model, the final concentrations were 2.4, 8.9, 9.3, and 19.6 μM, respectively). To evaluate

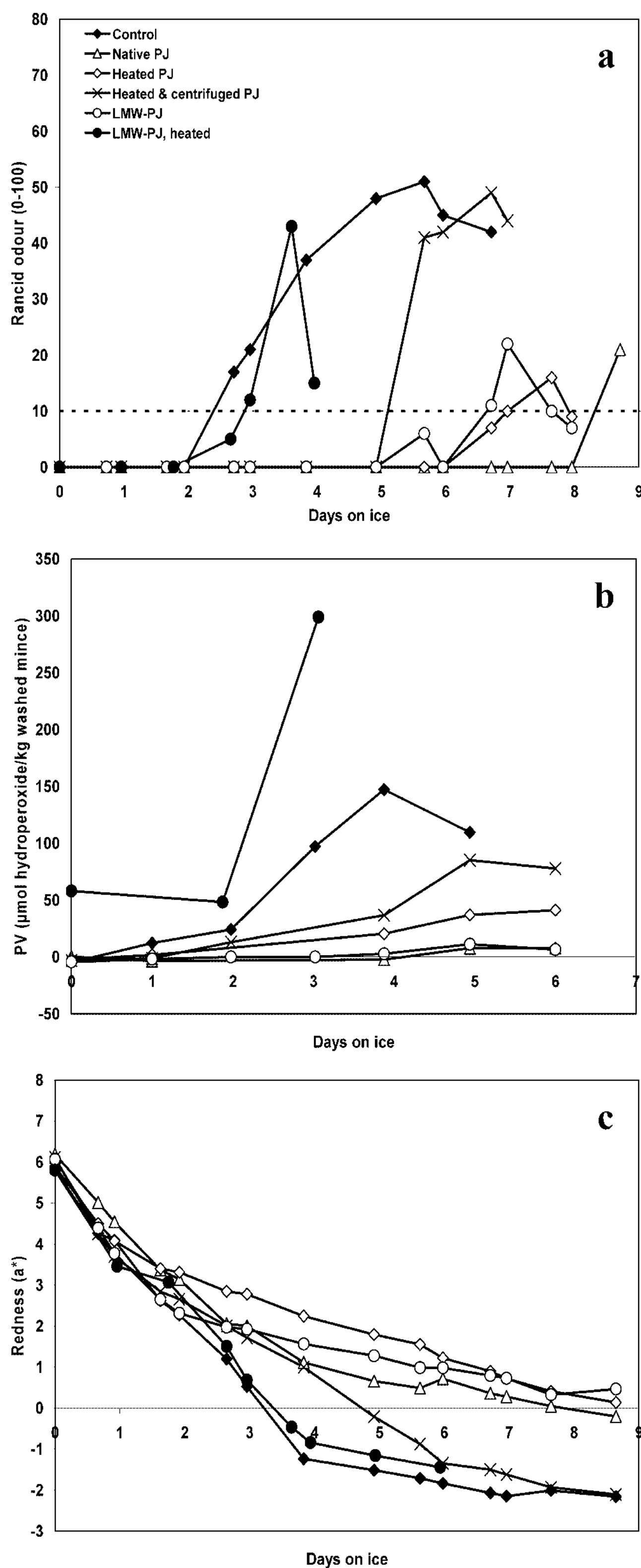

Figure 3. Development of rancid odor (**a**), lipid hydroperoxides (**b**), and redness (a*) loss (**c**) in washed cod mince fortified with a 50 mM phosphate buffer (control) or different fractions of herring PJ: native PJ, heated PJ, heated and centrifuged PJ, LMW-PJ fraction (<1 kDa), and heated LMW-PJ fraction (<1 kDa). Oxidation was catalyzed by adding 15 μM trout Hb based on the moisture of the model. The final pH and moisture of the models were 6.3 and 81%, respectively. The dotted line in **a** indicates a rancid odor intensity of 10 out of 100, which we used to define the rancid odor lag phase.

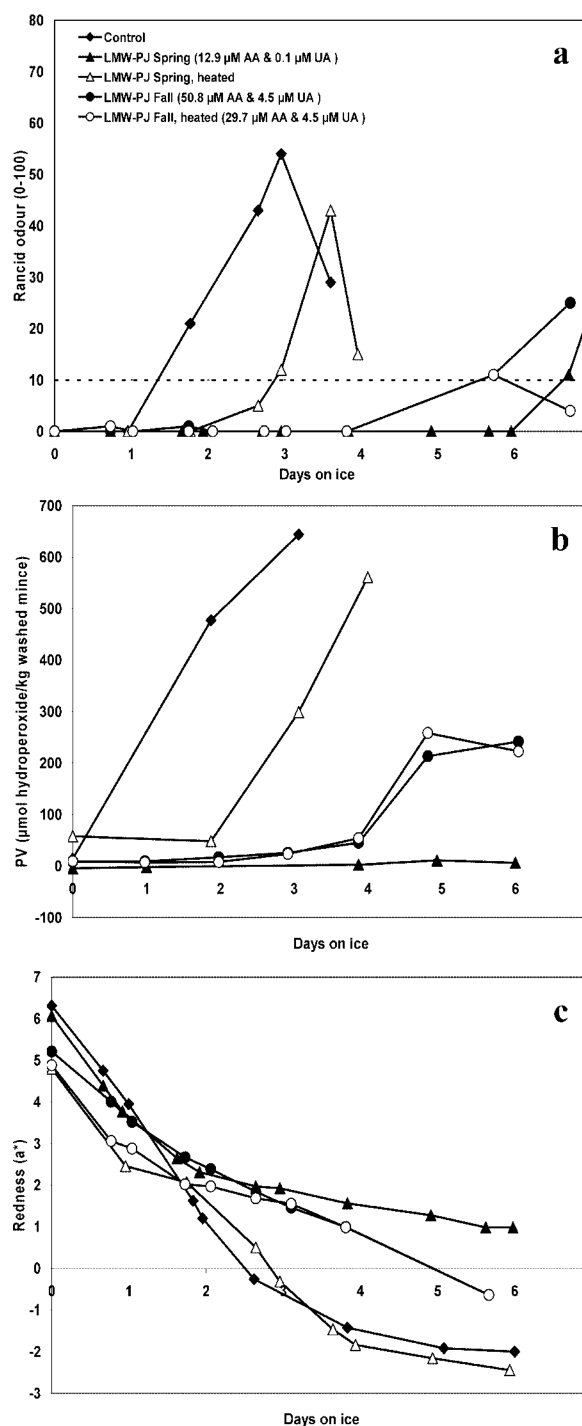

Figure 4. Development of rancid odor (**a**), lipid hydroperoxides (**b**), and redness (a*) loss (**c**) in washed cod mince fortified with a (♦) 50 mM phosphate buffer (control) or different fractions of herring PJ: (▲) Spring LMW-PJ fraction (<1 kDa) in which the concentration of ascorbic acid, AA, and uric acid, UA, were 32.2 and 0.23 μM, respectively, giving 12.9 and 0.1 μM in the model; (△) heated spring LMW-PJ fraction (<1 kDa); (●) fall LMW-PJ fraction (<1 kDa) in which the concentration of AA and UA were 106 and 9.3 μM, respectively, giving 50.8 and 4.5 μM in the model; (○) heated fall LMW-PJ fraction (<1 kDa), in which the concentration of AA and UA were 62 and 9.3 μM, respectively, giving 29.7 and 4.5 μM in the model. Oxidation was catalyzed by adding 15 μM trout Hb. The final pH and moisture of the models were 6.3 and 81%, respectively. The dotted line in **a** indicates a rancid odor intensity of 10 out of 100, which we used to define the rancid odor lag phase.

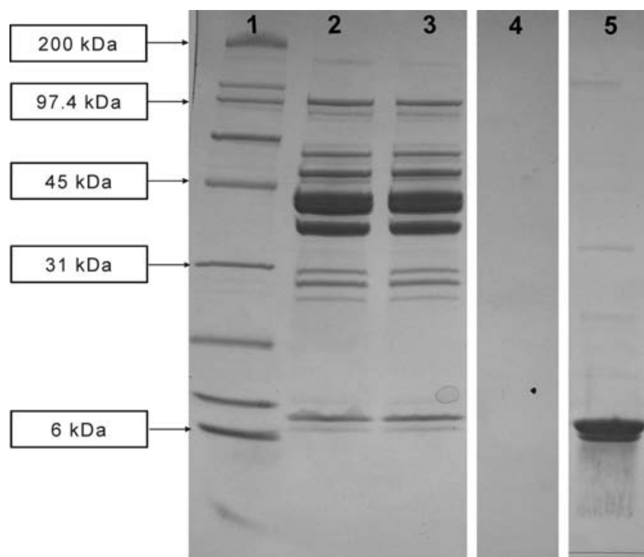

Figure 5. SDS-PAGE of native herring PJ and its fractions. The gels were precast linear minigels (4–20%), and a 20 μL protein/lane was loaded. Lane 1 shows standards, lane 2 shows native herring PJ, lane 3 shows the HMW-PJ fraction (>3.5 k Da), lane 4 shows the LMW-PJ fraction (<1 k Da), and lane 5 shows heated and centrifuged PJ. The gel used for lane 4 was precast linear minigels (10–20%). Lanes 1–4 are reproduced from Gunnarson et al. (*19*).

synergetic effects, a solution mimicking the ascorbic acid and uric acid levels found in one of the LMW-PJ fractions from spring-caught herring PJ was prepared (19.2 μM ascorbic acid and 5.5 μM uric acid final levels in the model: 8.4 and 2.4, respectively) and also one solution with equimolar levels of ascorbic acid and uric acid (25 μM each). It has to be kept in mind that all the antioxidant solutions were diluted ~2.5 times after addition to the models, with the exact dilution factor being dependent of the starting moisture content of the models. In **Table 2**, where results from the oxidation trials are shown, the final concentrations of antioxidants in the washed cod model are also shown.

In **Table 2**, data on the rancid odor lag phase obtained in the presence of ascorbic acid and uric acid are given. Uric acid alone (2.4, 8.9, and 19.6 μM in the final model) only very slightly inhibited rancid odor development, which was also supported by the PV development (**Figure 6**). Results on ascorbic acid alone in the washed cod mince were harder to interpret since the variations in the responses were large. However, the general trend was that levels of around 8–9 μM in the model extended the rancid odor lag phase by ~1 day, which was also reflected in the PV results (**Figure 6**). In some trials, solutions with both ascorbic acid and uric acid pointed at a synergy between the two components (**Table 2**). As an example, 8.9 μM (concentration in model) for each compound gave an average lag phase extention of 3.3 days. However, in some trials, the lag phase was extended even up to 5 days (**Table 2**). At 19.6 μM for each compound in the model, the lag phase extension was 3.5 days, which was more than the average lag phase given by 19.6 μM ascorbic acid alone (1.5 days). However, as can be seen in **Table 2**, the data variation at 19.6 μM ascorbic acid was large, and in some trials, a 3-day lag phase extension was given. Similarly, the solution made to mimic the LMW-PJ fraction from spring-caught herring PJ (final levels in the model of 8.4 and 2.4 μM) extended the lag phase by 2 days, while alone, these levels gave 1 and 0.5 days of lag phase extension, respectively. There appeared to be a loss of antioxidative activity at higher ascorbic acid levels. As examples, at 25.6 and 44 μM ascorbic acid + 3.8 μM uric acid in the model tested (to mimic the addition of the LMW-PJ fractions from fall-caught herring), no antioxidative activity was seen (**Table 2**).

To test whether the weak antioxidative effect of the HMW-PJ fraction (**Figure 2**) was due to some general radical scavengeing effect of sarcoplasmic proteins, solutions having the same protein content as the native PJ used in this trial (94 mg/mL) were prepared using BSA and MPC. A weaker solution of BSA was also tested (47 mg/mL), since a solution of higher concentration (94 mg/mL) presented an interfering odor. When added to the cod model (final BSA level of 16.6 mg/mL in the model), this weaker BSA solution only gave 0.5 days of extension of the rancid odor lag phase (data not shown). Milk proteins at 94 mg/mL (final level of 37.7 mg/mL in the model) had no effect on rancid odor extension and the prevention of PV development.

Evaluation of the Heat Sensitivity of Herring PJ and its Subfractions. In order to get further information on the identity of the components contributing to the strong antioxidative activity of native herring PJ and its fractions, these samples were heat-treated (10 min, 100 °C), after which they were added to the cod model together with the corresponding nonheated PJ samples. The heat-treated native PJ was added to the washed cod both before and after removal of the heat-coagulated proteins. Removal lowered the protein content from 95.6 mg/mL to 5.5 mg/mL, and only polypeptides around 4–6 kDa remained (**Figure 5**, lane 5). The results on Hb-mediated oxidation in the presence of these samples are given in **Figure 3a–c**. Without removing the coagulated proteins, the heat-treated PJ could extend the lag phase up to 4–5 days, whereas the fraction without coagulated proteins only gave 2.5 days of lag phase extension (**Figure 3a**). The development of PV and loss of redness also showed the same trends (**Figure 3b** and **c**). Again, the higher PVs in the presence of the heated <1 kDa fraction seen were due to the use of another model.

From six repeats of the experiment of adding a heated and nonheated LMW-PJ fraction into washed cod, it was obvious that the results differed largely and that they could be clearly grouped into two categories. In one category, heating decreased the antioxidative effect of the LMW-PJ fraction ($p < 0.05$), and in other, it did not. In **Figure 4a–c**, two extreme cases are shown, together with the season at which the herring raw material was caught. The reason for showing the latter was that, without exceptions, LMW-PJ fractions prepared from spring-caught herring lost their activity upon heating, while samples from fall-caught herring did not. On the basis of this observation, and the observation that the ascorbic acid levels were significantly lower ($p < 0.001$) in PJ from spring herring than those from fall herring, the ascorbic acid levels were analyzed also in a few heated LMW-PJ fractions (**Table 1**). As expected (*23*), all samples lost ascorbic acid during the heat treatment, but the relative losses were larger in LMW-PJ from spring herring (up to 100%) than from fall herring (43–51%), which was linked to the lower starting levels in the spring LMW-PJ (**Table 1**). Uric acid was not heat-sensitive. Also, some tests were done in which pure ascorbic acid solutions, 36 and 106 μM (+ 9 and 5 μM uric acid, respectively), were heated in the same way as the PJ samples. This was to test if the ascorbic acid was protected better against heat when present in the LMW-PJ than when alone. The results indicated a slight protection of ascorbic acid in the LMW-PJ as the pure solutions went down to 0–10% of initial levels after heating. A 25 μM solution of ascorbic acid and uric acid on average extended the oxidation lag phase by

Table 1. Ranges and Average Values of Ascorbic Acid (AA) and Uric Acid (UA) Contents of Herring PJ and Its LMW Fraction Prepared from Herring Caught in Different Seasons of the Year[a]

PJ fractions	AA (μM), range	AA (μM), average	UA (μM), range	UA (μM), average
spring PJ ($n = 5$)	20.6–48.6	34.9 ± 13.1 a	0.4–3.9	2.3 ± 1.3 a
spring LMW-PJ ($n = 5$)	18.0–42.6	33.3 ± 9.4 a	0.2–5.5	2.0 ± 1.9 a
spring LMW-PJ, heated ($n = 3$)	0.0–21.0	10.2 ± 10.5 a	1.5–5.6	3.5 ± 2.12 ab
fall PJ ($n = 5$)	72.7–130	112.9 ± 34.7 b	0.6–10.5	6.0 ± 3.8 ab
fall LMW-PJ ($n = 8$)	76.2–137.6	111.4 ± 17.7 b	0.8–9.3	4.4 ± 3.2 ab
fall LMW-PJ, heated ($n = 2$)	31.6–62.0	46.8 ± 15.1 a	6.1–12.0	9.1 ± 3.0 b

[a] The number of PJ batches used is indicated by n. Changes in AA and UA after heat treatement of the LMW-PJ fractions are also given. Where $n = 2$, the mean of duplicate samples ± (max – min)/2 is shown. Where $n \geq 3$, the mean of triplicate samples ± standard deviation is shown. Figures in the same column followed by different letters are significantly different ($p < 0.05$).

Table 2. Extension of Rancid Odor Lag Phase by Ascorbic Acid (AA) and Uric Acid (UA) during Hb-Mediated Oxidation of Washed Cod Mince[a]

candidates	concentration in solution (μM)	concentration in the model (μM)	rancid odor lag phase extension over control (days)
AA ($n = 3$)	19.2	8.4	1.4 ± 1.4
AA ($n = 2$)	25	8.9	1.3 ± 0.3
AA ($n = 2$)	50	19.6	1.5 ± 1.5
UA ($n = 3$)	5.5	2.4	0.5 ± 0.5
UA ($n = 2$)	25	8.9	1.0 ± 0.0
UA ($n = 1$)	50	19.6	0.0
AA + UA ($n = 3$)	19.2 + 5.5	8.4 + 2.4	2.3 ± 1.0
AA + UA ($n = 2$)	25 + 25	8.9 + 8.9	3.3 ± 1.3
AA + UA ($n = 2$)	50 + 50	19.6 + 19.6	3.5 ± 0.5
AA + UA[b] ($n = 1$)	106 + 9.3	44.0 + 3.8	0.0
AA + UA[c] ($n = 1$)	62 + 9.3	25.6 + 3.8	0.0

[a] Where $n = 2$, the mean of duplicate samples ± (max – min)/2 is shown. Where $n = 3$, the mean of triplicate samples ± standard deviation is shown. A detailed preparation protocol for the oxidation models in which these candidates were tested is given in the Materials and Methods section. [b] Simillar to the concentration seen in LMW-PJ obtained from herring caught in fall. [c] Simillar to the concentration seen after heating LMW-PJ obtained from herring caught in fall.

about 3.3 days (**Table 2**) but lost all of its activity when heated, probably due to a heat-induced loss of ascorbic acid (data not shown).

On the basis of these results, the hypothesis was set up that ascorbic acid may still play a key role for the antioxidative activity of native PJ and LMW-PJ, although it did not prove to be very active alone. There are numerous compounds of the LMW-PJ fraction with which it may act in synergy, and thus, its loss due to heat can be an important reason for reduced antioxidative activity. To test this hypothesis, a series of experiments were conducted where heated LMW-PJ samples from spring herring were fortified with extra ascorbic acid to test whether they gained back their initial antioxidative activity. **Figure 7a–c** shows that, when fortifying heated LMW-PJ from spring herring so that it contained the same ascorbic acid level as its corresponding nonheated sample (thus, a raise of the final concentration in the model from 8.7 to 15.8 μM), then indeed its antioxidative activity was increased (2 days of lag phase extension as compared to 0.5 day for the heated sample). This improvement was more clearly shown on the basis of the PV data (**Figure 7b**). Here, the original antioxidative activity of nonheated spring LMW-PJ was almost regained. If fortifying the heated spring LMW-PJ sample even more, so that it contained the same ascorbic acid level (62 μM) as heated LMW-PJ from fall herring (final concentration in the model of 25.6 μM), the same antioxidative activity as for 15.8 μM in the model was gained, but not more. Thus, again, there was no clear dose-response effect for ascorbic acid.

Additional Compositional Characteristics of PJ, its Subfractions, and the Pellet Obtained During PJ Preparation.

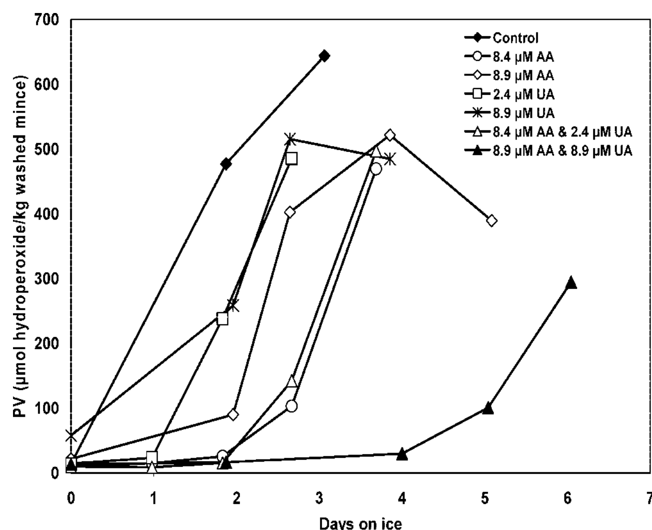

Figure 6. Development of PV in washed cod mince fortified with (♦) a 50 mM phosphate buffer (control) or (○) a 19.2 μM ascorbic acid, AA solution (8.4 μM in the model); (◇) a 25 μM AA solution (8.9 μM in the model); (□) a 5.5 μM uric acid, UA solution (2.4 μM in the model); (*) 25 μM UA solution (8.9 μM in the model); (△) a combination of 19.2 μM AA and 5.5 μM UA solution (8.4 μM AA and 2.4 μM UA in the model); and (▲) a combination of 25 μM each of AA and UA solution (8.9 μM AA and 8.9 μM UA in the model). Oxidation was catalyzed by adding 15 μM trout Hb. The final pH and moisture of the models were 6.3 and 81%, respectively.

Table 3 summarizes the basic composition of native PJ and its LMW-PJ and HMW-PJ (>3.5 kDa) fractions. The dry matter percentages of native PJ, LMW-PJ, and HMW-PJ fractions were 11.6%, 3.2%, and 7.8%, respectively. That the HMW-PJ fraction had less dry matter than native PJ was due to a slight dilution during the dialysis step. This was also reflected in the protein content, which was significantly ($p < 0.001$) lower in the HMW-PJ sample than in native PJ (75.3 vs 94 mg/mL). The protein content of native herring PJ varied quite a lot throughout this study (between 94 and 125 mg/mL). Both native and fractionated herring PJ had a pH around 6.7. Conductivities of the native PJ, and the LMW- and HMW-PJ fractions were 13.4, 12.9, and 16.0 mS/cm, respectively. The native PJ, LMW-PJ, and HMW-PJ fractions contained 11.5, 0, and 10.5 μM Hb. Herring PJ and its fractions were also analyzed for Fe, Cu, and Zn. These metals were totally absent in the LMW-PJ fraction, while they were present at 2.6, 0.17, and 3.5 μg/g, respectively, in native PJ and at 1.3, 0, and 2.4 μg/g, respectively, in the HMW-PJ fraction. Total amino acid analysis (**Table 4**) revealed that aspartic acid, glutamic acid, and lysine were the dominant amino acids in native PJ and the HMW-PJ fraction with levels of ~10 g/kg. In the LMW-PJ fraction, glycine and histidine were dominating at ~1 g/kg (**Table 4**). Regarding free amino acids

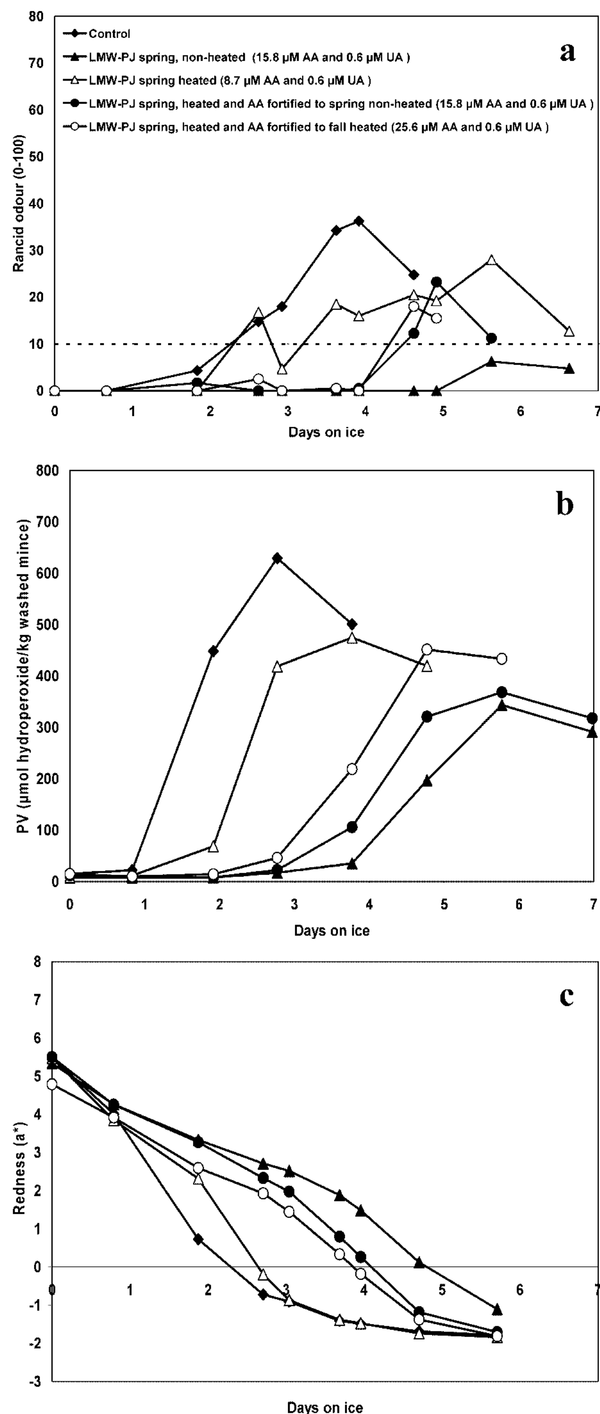

Figure 7. Development of rancid odor (**a**), lipid hydroperoxides (**b**), and redness (a*) loss (**c**) in washed cod mince fortified with (♦) a 50 mM phosphate buffer (control) or different fractions of herring PJ: (▲) nonheated LMW-PJ (<1 kDa) from spring herring in which the concentrations of ascorbic acid, AA, and uric acid, U,A was 38.3 and 1.4 μM, respectively, which gave 15.8 and 0.6 μM in the model; (△) heated LMW-PJ (<1 kDa) from spring herring in which the concentrations of AA and UA were 21 and 1.4 μM, respectively, which gave 8.7 and 0.6 μM in the model; (●) heated LMW-PJ (<1 kDa) from spring herring fortified with AA to reach the same AA concentration as nonheated LMW-PJ (<1 kDa) from spring herring (see ▲); and (○) heated LMW-PJ (<1 kDa) from spring herring fortified with AA to reach the same AA concentration as heated LMW-PJ from fall herring, which had concentrations of AA and UA of 62 and 9.3 μM, respectively, giving 25.6 and 0.6 μM in the model. Oxidation was catalyzed by adding 15 μM trout Hb. The final pH and moisture of the models were 6.3 and 81%, respectively. The dotted line in **a** indicates a rancid odor intensity of 10 out of 100, which we used to define the rancid odor lag phase.

Table 3. Compositional Data of Native Herring Light Muscle PJ, the LMW-PJ (<1 kDa), and the HMW-PJ (>3.5 kDa) Fraction[a]

measurements	native PJ	LMW-PJ (<1 kDa) fraction	HMW-PJ (>3.5 kDa) fraction
dry matter % ($n = 2$)	11.6 ± 0.03 a	3.22 ± 00 b	7.76 ± 0.01 c
pH ($n = 3$)	6.70 ± 0.03 a	6.72 ± 0.01 a	6.73 ± 0.02 a
conductivity (mS/cm) ($n = 3$)	13.38 ± 0.02 a	12.90 ± 0.04 b	16.05 ± 0.04 c
total lipids % ($n = 3$)	nil	nil	nil
total protein (mg/mL) ($n = 3$)	94 ± 0.93 a	0.70 ± 0.02 b	75.30 ± 2.5 c
total hemoglobin (μM) ($n = 2$)	11.47 ± 1.65 a	nil	10.49 ± 0.37 a
Fe (μg/g) ($n = 3$)	2.59 ± 0.09 a	nd	1.3 ± 0.06 b
Cu (μg/g) ($n = 3$)	0.17 ± 0.08	nd	nd
Zn (μg/g) ($n = 3$)	3.53 ± 0.10 a	nd	2.39 ± 0.10 b
total amino acids (g/kg) ($n = 2$)	96 ± 1.25 a	3.05 ± 0.20 b	71.8 ± 1.65 c

[a] Where $n = 2$, the mean of duplicate samples ± (maximum – minimum)/2 is shown. Where $n = 3$, the mean of triplicate samples ± standard deviation is shown. Figures in the same row followed by different letters are significantly different ($p < 0.05$). nd stands for not detected.

Table 4. Composition of Total Amino Acids in Native PJ, the LMW (<1 kDa) PJ Fraction, and the HMW (>3.5 kDa) PJ Fraction[a]

	native PJ	LMW-PJ (<1 kDa) fraction	HMW-PJ (>3.5 kDa) fraction
cysteine	2.6 ± 1.3	<0.1	1.05 ± 0.05
methionine	2.85 ± 0.05	<0.1	2.05 ± 0.05
aspartic acid	10.55 ± 0.25	<0.1	8.2 ± 0.2
threonine	4.1 ± 0.5	<0.1	3.55 ± 0.05
serine	3.35 ± 0.85	<0.1	3.15 ± 0.05
glutamic acid	9.4 ± 0.6	0.1 ± 0.0	7.05 ± 0.55
proline	3.45 ± 0.15	<0.1	2.6 ± 0.1
glycine	5.8 ± 0.1	1.05 ± 0.05	3.65 ± 0.05
alanine	6.7 ± 0.1	0.5 ± 0.0	4.95 ± 0.05
valine	6.55 ± 0.05	0.1 ± 0.0	5.15 ± 0.05
isoleucine	5.3 ± 0.1	<0.1	4.15 ± 0.05
leucine	7.8 ± 0.0	<0.1	6 ± 0.1
tyrosine	3.25 ± 0.05	<0.1	2.5 ± 0.1
phenyl alanine	5.15 ± 0.05	<0.1	4.05 ± 0.05
histidine	4.55 ± 0.15	1 ± 0.0	2.6 ± 0.1
ornithine	<0.1	<0.1	<0.1
lysine	9.55 ± 0.05	0.3 ± 00	7.3 ± 0.1
arginine	5.05 ± 0.05	<0.1	3.8 ± 0.1
hydroxyproline	<0.1	<0.1	<0.1
sum	96 ± 1.25	3.05 ± 0.2	71.8 ± 1.65

[a] All results are shown as average results from duplicate samples ± (maximum – minimum/2) expressed as g/kg of PJ. This table is reproduced from Gunnarson et al. (*19*); however, additional statistical treatment is included.

(**Table 5**), taurine and histidine dominated in the native PJ and LMW-PJ fractions at 0.9 g/kg, follwed by glycine, alanine, and lysine at ~0.4 g/kg. The HMW-PJ fraction was totally deficient in free amino acids. Basic analyses of the pellet from PJ production showed that it consisted of 75.5% moisture, 20.7 ± 0.5% protein, and 3.3 ± 0.4% total lipid content. It, further, had a pH of 6.79 ± 0.05.

DISCUSSION

PJ is basically the intra- and intercellular fluids of muscle tissue recovered during centrifugation of muscle mince. Thus, it has a highly complex composition and contains a multitude of both anti- and pro-oxidants. Interestingly enough, a series of previous studies has shown that, despite the presence of pro-oxidants, muscle PJ can be highly antioxidative against Hb-mediated oxidation of washed cod mince (*6, 24*) and against

Table 5. Composition of Free Amino Acids in Native PJ, the LMW (<1 kDa) PJ Fraction, and the HMW (>3.5 kDa) PJ Fraction[a]

	native PJ	LMW-PJ (<1 kDa)	HMW-PJ (>3.5 kDa)
phosphoserine	<0.02	<0.02	<0.02
taurine	0.93 ± 0.00	0.995 ± 0.035	<0.02
phosphoethanolamine	<0.02	<0.02	<0.02
urea	<0.02	<0.02	<0.02
aspartic acid	<0.02	<0.02	<0.02
threonine	0.07 ± 0.0	0.075 ± 0.005	<0.02
serine	0.08 ± 0.0	0.08 ± 0.0	<0.02
aspargine	<0.02	<0.02	<0.02
glutamic acid	0.105 ± 0.005	0.105 ± 0.005	<0.02
sarcosine	<0.02	<0.02	<0.02
α-aminoadipitic acid	<0.02	<0.02	<0.02
proline	0.035 ± 0.005	0.035 ± 0.005	<0.02
glycine	0.4 ± 0.0	0.435 ± 0.005	<0.02
alanine	0.43 ± 0.0	0.455 ± 0.005	<0.02
citrulline	<0.02	<0.02	<0.02
α-amino-*n*-butyric acid	<0.02	<0.02	<0.02
valine	0.065 ± 0.005	0.18 ± 0.13	<0.02
cysteine	<0.02	<0.02	<0.02
methonine	<0.02	<0.02	<0.02
cystathionine	<0.02	<0.02	<0.02
isoleucine	0.035 ± 0.005	0.025 ± 0.005	<0.02
leucine	0.065 ± 0.005	0.06 ± 0.0	<0.02
tyrosine	0.02 ± 0.0	0.02 ± 0.0	<0.02
β-alanine	<0.02	<0.02	<0.02
phenyl alanine	<0.02	<0.02	<0.02
β-aminoisobutyric acid	<0.02	<0.02	<0.02
L-homocystine	<0.02	<0.02	<0.02
γ-amino-*n*-butyric acid	<0.02	<0.02	<0.02
ethanolamine	<0.02	<0.02	<0.02
ammonia	0.135 ± 0.005	0.12 ± 0.02	<0.02
γ-hydroxilysine	<0.02	<0.02	<0.02
ornithine	0.02 ± 0.0	0.02 ± 0.0	<0.02
lysine	0.3 ± 0.0	0.29 ± 0.0	<0.02
1-methylhistidine	<0.02	<0.02	<0.02
histidine	0.905 ± 0.015	0.915 ± 0.0	<0.02
3-metylhistidine	<0.02	<0.02	<0.02
anserine	<0.02	<0.02	<0.02
carnosine	<0.02	<0.02	<0.02
arginine	0.02 ± 0.0	<0.02	<0.02
hydroxiproline	<0.02	<0.02	<0.02
glutamine	<0.02	<0.02	<0.02

[a] All results are shown as average results from duplicate samples ± (maximum – minimum/2) expressed as g/kg of PJ. This table is reproduced from Gunnarson et al. (*19*); however, additional statistical treatment is included.

Fe-mediated oxidation of flounder sarcoplasmic reticulum (*25*). In white fish PJ (cod, haddock, dab sole, and black back), most of the antioxidative activity was located in the LMW-PJ fraction (*6*), while in chicken PJ, the HMW-PJ components were more active (*24*).

Herring is a pelagic, dark muscle, fatty fish which is highly susceptible to lipid oxidation during post-mortem handling due to its high levels of Hb and transition metals together with its rapid post-mortem drop in pH. However, since muscle tissues which are susceptible to oxidation are known to be equipped with an array of antioxidants (*26*), it was expected that herring PJ could be very rich in antioxidants.

As it can be seen from **Figure 2**, native herring PJ indeed had very good antioxidative properties towards Hb-mediated oxidation of washed cod mince. The reasons for this could be attributable to both LMW-PJ compounds and various antioxidative enzymes (superoxide dismutase, SOD; catalase; peroxidases; etc.). SDS-PAGE analyses showed the presence of a wide range of sarcoplasmic proteins between 6 and 95 kDa in the PJ (**Figure 5**). The higher activity seen in the LMW-PJ compared to that in the HMW-PJ fraction (**Figure 1**) however indicates a minor role of antioxidative enzymes or other proteins in our system. Enzymes in the HMW fraction could be dependent on cofactors in the LMW fraction for possessing full activity. These results agree with previous observations from white fish PJ (*6*) and are supported by the fact that BSA and milk proteins did not inhibit oxidation in our model. Furthermore, the fact that heating native PJ did not destroy its antioxidative activity (**Figure 3**) diminishes the possible role of enzymes, unless they are very heat resistant. Another reason could be due to a nonspecific antioxidative activity of proteins through the H-donating capacity of SH groups, possibly improved by the heating itself. Raghunath et al. (*27*) reported an increased exposure of reactive SH groups during the heating of fish up to 60 °C. A factor that may have lowered the impact of enzymes in this system could be the reduced pH (6.3) that was used to mimic post-mortem fish. Serum proteins were found to have antioxidative properties at physiological pH, but they reduced their activity at pH 6.0–6.6 (*28*). It must also be stressed that among the proteins of the HMW-PJ fraction was also a pretty large quantity of Hb 10.5 μM (**Table 3**). In the absence of LMW-PJ compounds, the pro-oxidative nature of Hb may have become more dominating. Similarly, since the LMW-PJ fraction was deficient in both Hb and trace elements (**Table 3**), its antioxidative power probably became more enhanced. It was reported (*29*) that, after the dialysis of an aqueous trout dorsal muscle extract, oxidation of the proteins in the extract (e.g., oxy-Hb) was accelerated as compared to that in a nondialyzed extract. This is in line with the observed loss of antioxidative activity when the HMW-PJ fraction was separated from the LMW-PJ compounds.

The LMW-PJ fraction consists of an array of known and tentative antioxidative compounds that could be responsible for its capacity to delay the Hb-mediated oxidation of washed cod mince. Examples are reducing agents (e.g., glutathione, GSH, and ascorbic acid), radical scavengers (e.g., uric acid, ascorbic acid, and some free amino acids), metal chelators (e.g., small peptides, free amino acids, and inorganic phosphate), and Hb-modulating compounds (e.g., 2,3-diphosphoglycerate, DPG). In previous studies, the effect from several of these compounds on Hb-mediated oxidation of washed cod mince was tested. Undeland et al. (*6*) reported that there was no significant role of spermine (61.2 μM); phosphates (40 mM); trimethyl amine oxide, TMAO (100 mM); and 2,3-DPG (5.8 μM) on the Hb-mediated oxidation of washed cod mince. Wetterskog and Undeland (*30*) tested taurine (7.5 μM), histidine (154 μM), carnosine (154 μM), anserine (5.9 mM), GSH (115 and 1150 μM), ascorbic acid (35 and 350 μM), and uric acid (73.3 and 220 μM). They found a strong effect from both levels of uric acid and from 1150 μM GSH. A small inhibitory effect was seen from 35 μM ascorbic acid, and from 115 μM GSH. The other compounds and levels were inefficient. Most levels selected in the above studies were based on literature data regarding concentrations in fish tissue or on measured levels in cod muscle PJ. Some were also picked for theoretical considerations, for example, dose-response testing. Li et al. (*24*) treated chicken PJ with ascorbate oxidase and found that it lost its antioxidative properties. This result indicated that ascorbic acid might be a tentative antioxidative compound of the LMW-PJ fraction.

On the basis of the above, the focus of this study was to investigate ascorbic acid, and we got some information about the role of ascorbic acid for the antioxidative activity of LMW-PJ by a series of heating and fortification experiments (**Figures 3**, **4**, and **7**). Adding endogenous levels of pure ascorbic acid and uric acid into washed cod showed a weak effect of the

former and no to a very small effect of the latter. Together, they showed some signs of improved activity. At higher ascorbic acid levels, 25.6–50.8 μM, in the model +/− 3.8–4.5 uric acid, there appeared a ceasing or reduction of the antioxidative activity, which is in line with the results of Wetteskog and Undeland (*30*). Thus, from these trials alone, we would not ascribe ascorbic acid and uric acid major antioxidative roles in LMW-PJ. However, the striking differentiation between how heating affected LMW-PJ with low and high ascorbic acid levels (**Figure 4**) gives reasons to hypothesize that ascorbic acid significantly contributes to the antioxidative activity of LMW-PJ, most likely by interaction with other compounds. That such interactions exist was indicated both by the weak synergy with uric acid and by the fact that ascorbic acid was somewhat more stable towards heat when present in the LMW-PJ fraction than when alone in solution. When we heated fall LMW-PJ with 106 μM ascorbic acid, 43% was lost, whereas a pure solution of 106 μM ascorbic acid lost 91%. Further evidence for an important role of ascorbic acid for the LWM-PJ fraction effect was given by the trials in which heated LMW-PJ was fortified with extra ascorbic acid, upon which its original activity was almost gained back. At endogenous concentrations, ascorbic acid is an important reducing agent and is capable of inhibiting the formation and activity of the pro-oxidative met and ferryl forms of myoglobin and Hb (*31*). Petillo et al. (*32*) reported a strong correlation between endogenous ascorbate concentration and the development of rancidity in mackerel fillets. Ascorbate can also help in regenerating α-tocopherol (*24*). This could be an important mechanism in our system as α-tocopherol is present in the washed cod model membranes (*8*). The mechanism of how ascorbate can regenerate α-tocopherol is explained by Li et al. (*24*). It has been found that the oxidative role of ascorbic acid in muscle tissue is very concentration-dependent, with the location of the shift in mechanism being linked, for example, to the tocopherol content (*33*) or Fe content (*2*). Kunert and Ederer (*33*) found an ascorbic acid to tocopherol ratio of < 1 to render ascorbic acid more pro-oxidative in plant tissues. In our system, the ascorbic acid to tocopherol ratio varied from 0.7 to 12 in the presence of the pure candidate solutions as well as LMW-PJ fractions. In samples where a higher level of ascorbic acid was added, and where a shift in activity was noted, the ratio of ascorbic acid and tocopherol was around 7.5. The variations in antioxidative responses of ascorbic acid at a particular concentration could also be due to the presence of its different forms like fully protonated, monoanionic, and dianinonic ascorbate (*34*). The very weak antioxidative effect from uric acid at endogenous levels could be linked to its radical scavengeing, reducing, or chelating properties (*35*). The weak synergy between ascorbic acid and uric acid could be due to the fact that ascorbate can reduce urate radicals back to urate (*36*). The formation of urate–Fe^{3+} complexes was shown to dramatically inhibit Fe^{3+}-catalyzed ascorbate oxidation in the liposome system (*35*).

Although not proven active in previous studies of Hb-mediated oxidation in washed cod mince (*30*), it cannot be ruled out that histidine or taurine contributed to the LMW antioxiative power of herring PJ. Both of these amino acids were present in high quantities (**Table 5**), and the levels obtained in our model (2.4 and 1.9 mM) were higher than those previously tested, 154 and 7.5 μM, respectivley. Taurine has been suggested to be a radical scavenger; however, the exact mechanism seems unclear (*37*). Histidine has been suggested to be a scavenger, for example, of hydroxyl radicals (*38*).

Conclusion. This study concluded that native herring light muscle PJ has a strong antioxidative activity towards the Hb-mediated oxidation of washed cod mince. A major part of the activity lies in the LMW-PJ (<1 kDa) fraction, and it is suggested that ascorbic acid is one of the antioxidative actors, probably through interations with other LMW-PJ compounds.

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