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Added Triacylglycerols Do Not Hasten Hemoglobin-Mediated Lipid Oxidation in Washed Minced Cod Muscle

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Hemoglobin-mediated lipid oxidation in washed, minced cod muscle was related to the triacylglycerol to membrane lipid ratio. The same rapid development of thiobarbituric acid reactive substances (TBARS) and painty odor occurred with and without the presence of up to 15% menhaden oil. Without hemoglobin, development of TBARS and painty odor was slow, despite a high amount of hydroperoxides in samples with oil added (1135 μmol/kg muscle). This suggested that hemoglobin reacted by cleaving preformed hydroperoxides into secondary oxidation products. Nearly doubling the hemoglobin concentration approximately doubled the extent of lipid oxidation with and without added oil. This indicated that hemoglobin was limiting for the oxidation reaction. The noneffect of added oil suggests that membrane lipids and/or preformed membrane lipid hydroperoxides provided sufficient substrate in hemoglobin-catalyzed oxidation of washed minced cod muscle. Fe^{2+}−ADP did not induce any oxidation of washed minced cod with/without added oil. Results suggest that lipid oxidation in fatty fish may be more related to the quantity and type of the aqueous pro-oxidant and the membrane lipids than to variations in total fat contents.

KEYWORDS: Triacylglycerols; oil; membrane lipids; hemoglobin; oxidation; TBARS; sensory; cod

INTRODUCTION

Lipid oxidation negatively affects flavor, odor, color, texture, and nutritional value of muscle foods during storage. Some possible endogenous catalysts of fish muscle lipid oxidation are hemoglobin (Hb), myoglobin, low molecular weight (LMW) transition metal complexes, lipoxygenases, microsomal enzymes, and mitochondrial enzymes. When tested in a washed cod muscle model system, a crude hemoglobin preparation was highly effective in catalyzing lipid oxidation (*1*).

The two major groups of muscle lipids are the phospholipids and triacylglycerols. The phospholipids give structure and fluidity to membranes and are found at relatively constant levels, in white muscle at ∼0.5−1% (w/w) (*2*) and in dark muscle at slightly higher levels due to the presence of more mitochondria (*3*). The triacylglycerols are found both in adipose tissues and integrated into muscle tissue. In the muscle tissue, the triacylglycerols are located in the endomysial area outside the muscle cell and in the interior of muscle cells; the latter is particularly true for fatty species (*4*). The intracellular triacylglycerols are surrounded by a phospholipid monolayer (*5*). In contrast to membrane lipids, triacylglycerol levels vary widely with species and environmental conditions; for example, the total fat content of herring can vary from 0.5−30% depending for example on season and geographic location (*6*).

Within the meat research field, it has been stated that membrane lipids are the main substrates in muscle lipid oxidation (*7*). Although indications are given in the literature (*8*,*9*), no such statements have yet been made regarding fish, possibly since a substantial part of the fish triacylglycerols are integrated within muscle cells. Instead, rapid oxidation in the muscle of fatty fish species is often attributed to its high total lipid content, that is, triacylglycerol level (*10*,*11*). However, recent research using a reduced lipid (∼0.1%), washed cod muscle system revealed that a strong rancid odor developed in the presence of whole blood (*12*). The rate and extent of rancidity were not increased by the presence of >6 times more membrane lipids. Slabyj and Hultin (*13*) found that NADH-driven, iron-catalyzed enzymic lipid oxidation of herring membrane lipids (microsomes) accelerated oxidation of herring triacylglycerols emulsified in the microsomal suspension.

Currently, there is a growing interest in producing food from fatty pelagic fish species, that is, fast swimming, migratory cold-water species from the pelagic zone. There is also an increasing interest in incorporating oil from such fish into muscle protein isolates such as surimi to produce n-3 fatty acid enriched muscle-based functional food products (*14*). Within both areas, it is of

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utmost importance to know how the triacylglycerols quantitatively affect product storage stability, particularly the development of rancidity. Manufacturing of refrigerated fish muscle foods is constantly increasing in popularity, and although bacterial growth is not suppressed at such temperatures, rancidity development from lipid oxidation can limit the shelf life (*15*). The objective of the present study was to investigate how added triacylglycerols in the form of menhaden oil chemically and sensorially affected the progress of Hb-initiated oxidation of membrane lipids in minced, water-washed cod muscle during ice storage. Such knowledge would allow for optimization of storage/process conditions and antioxidative strategies according to seasonal and geographical fat variations.

MATERIALS AND METHODS

Chemicals. In the microsomal enzyme stimulating system, the $FeCl_3 \cdot 6H_20$, L-histidine monohydrochloride/monohydrate (min 98%), NADH, and ADP sodium salt (from a bacterial source) were all from Sigma (St. Louis, MO). The potassium chloride was from JT Baker Inc. (Phillipsburg, NJ). All other chemicals were reagent grade

Bleeding of Fish and Preparation of Hemolysate. Farmed rainbow trout (*Onchorhynchus mykiss*) were bled as described by Rowley (*16*). Hemolysate was prepared from the whole blood according to Fyhn et al. (*17*) 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 hemoglobin (Hb) levels in the hemolysate, the method of Brown (*18*) was adapted as described by Richards and Hultin (*1*).

Washed Cod Muscle Model System. Whole cod, obtained from local fishermen in Gloucester, MA, was hand filleted and skinned. All dark muscle and any traces of blood were removed. The white muscle was then minced using a grinder (Ultra power model KS M90, Kitchen Aid Inc., St Joseph, MI), and the mince was washed twice (1:3, w/w) with water and once (1:3) with 50 mM sodium phosphate buffer, pH 6.7, as described by Richards and Hultin (*1*). The final washed cod was frozen at −80 °C. Upon use, the muscle was thawed in a sealed plastic bag under running cold water, and excess water was manually squeezed out using cheesecloth. Moisture was determined with a moisture balance (CSC Scientific Co. Inc., USA), and the pH was measured using an Orion pH meter (model 420 A, Boston, MA) equipped with a Ross Sure-Flow Electrode (model no. 8165 combination epoxy electrode, Orion, Beverly, MA) after manual mixing of 1 part washed cod with 9 parts of distilled water. Adjustment of the pH to ∼6.7 or 7.2 was carried out by dropwise addition of 2 N HCL/2 N NaOH into the washed cod followed by severe manual stirring with a stainless steel spatula. To make sure the acid and base had been evenly distributed into the mince, the pH was analyzed in several samples taken at different locations of the washed cod mince. Different levels (7.5 or 15%) of unstabilized refined menhaden oil (Omega Pure, lot no. AD0599, Omega Protein Inc., Reedville, MA) or 10% of oil isolated by centrifugation of minced mackerel fillets (4 °C, 105 000*g*, 30 min) was manually stirred in for 2 min (∼160 stirs/min) using a 250 mL plastic beaker (bottom diameter, 60 mm) and a stainless steel spatula. Streptomycin (200 ppm) was added to prevent bacterial growth. Lipid oxidation was started by adding rainbow trout hemolysate to final Hb levels of 3.0 or 5.8 μmol per kg washed cod or by adding $FeCl_3$ (15 μM), ADP (100 μM), NADH (100 μM), L-histidine (5 mM), and KCl to a final ionic strength of 120 mM (*13*) in order to stimulate microsomal enzymic lipid oxidation. Antimicrobial agent and prooxidants were manually stirred in during 2.5 min. In controls, the hemolysate/enzymic reagents were replaced by distilled water. The samples, which had a total weight of either 20 or 25 g, were flattened out in the bottom of 125 or 225 mL screw-capped Erlenmeyer flasks (bottom diameter, 60 and 75 mm, respectively) giving rise to a sample thickness of ∼6.3 or ∼5 mm, respectively. The capped sample bottles were stored on ice for up to 7−10 days. Within each experiment, the sizes of the samples and flasks were kept constant to allow for direct comparisons.

Filter Paper and Cellulose Model Systems. Four grams of fibrous cellulose powder (CF1, Whatman International Ltd., Maidstone, U.K.) or 3 × 3 mm pieces of Whatman no. 1 filter paper were hydrated from the initial 3.5% moisture to 80% moisture with 50 mM phosphate buffer (pH 6.7). Fifteen percent (w/w) menhaden oil was manually stirred into half of the hydrated cellulose/filter paper paste for 2 min. Pastes with and without oil were then stirred manually for 2.5 min with 200 ppm streptomycin and 5.8 μM Hb. In controls, hemolysate was replaced by distilled water. The samples (total weight of 25 g) were flattened out in the bottom of 225 mL screw-capped Erlenmeyer flasks (bottom diameter, 75 mm) giving a sample thickness of ∼5 mm. Samples were stored on ice for up to 14 days.

Peroxide Value (PV) Analyses. Prior to storage, samples were taken from the oil and the washed cod model system for PV analyses. PV was determined with a modified version of the ferric thiocyanate method (*19*). Total lipids were extracted from 1 g of the muscle with 10 mL of chloroform/methanol (1:1). Sample and solvents were homogenized with a Kinematica Gmb H Polytron (type PT 10/35, PCU 1, Brinkman Instruments, Westbury, NY) connected to a Variable autotransformer (type 3PN 1010, Staco Energy Products Co., Dayton, OH) in 25 × 150 mm glass test tubes for 30 s on speed 30. Sodium chloride (3.08 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 (IEC Clinical centrifuge equipped with a 809 fixed angle 45°Rotor, International Equipment Co., Needham, MA). Two mL of the lower chloroform layer was removed using a 5 mL glass syringe (model 5016, Popper and Sons Inc., New Hyde Park, NY) equipped with a 20 gauge, 6 inch stainless steel needle (Popper and Sons Inc.). Oil samples were prepared by diluting the oil directly in chloroform to a concentration of 30 mg/mL. Chloroform/methanol (1:1, 1.33 mL) was added to the 2 mL lipid-in-chloroform solution followed by brief vortexing. Ammonium thiocyanate (4.38 M) and iron-(II) chloride (9 mM) (16.7 μL of each) were added with 2−4 s 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. PV is expressed as μmol lipid hydroperoxide/kg of tissue or as μmol lipid hydroperoxide/kg of oil. Blanks were prepared according to the described procedure by replacing the 1 g of muscle by 0.8 mL of ice-cold distilled water.

Thiobarbituric Acid Reactive Substances (TBARS) Analyses. At regular intervals, 1 g sample "plugs" were taken out from the washed cod model system for TBARS analyses. The plugs were removed using a plastic cylinder (∅, 1 cm) and thus had a constant surface-to-volume ratio. The samples were stored in aluminum foil at −80 °C until the day of analyses. TBARS were analyzed after extraction of the muscle with TCA (*20*). Results are expressed as μmol malondialdehyde (MDA)/kg tissue.

Sensory Analysis. At regular intervals during storage, three to four trained panelists (*21*) sniffed the headspace above the samples by uncapping the 125 or 225 mL screw-capped Erlenmeyer flasks. Panelists concentrated on detecting stale, fishy, and painty odors using a scale of 0 to 10, with 10 being the strongest. Reference samples were prepared according to Richards et al. (*21*).

Fatty Acid Pattern Analyses. The fatty acid pattern of the menhaden oil was analyzed with gas chromatography according to the Americal Oil Chemists Society official method (*22*).

Statistics. The use of sensory analysis limited the possible number of sample replicates within each experiment. To compensate for this, the entire experiment was repeated four times using different batches of raw materials and different storage conditions. In the first experiment (Figure 1a,b), all of the five sample groups were prepared and stored in duplicate. To estimate the variation spans within each group, standard deviations were calculated using Excel 2000 (Microsoft Corp., Seattle, WA). In the second experiment, two of the six sample groups in which large changes were expected to take place during storage (15% oil + Hb and 15% oil + the microsomal enzyme stimulating system) were prepared and stored in duplicate. The other four groups were prepared and stored as singles. In the third (Figure 3) and fourth (reported only in text) experiments, single samples were used. When applied, the chemical analyses were made twice on each sample to estimate the analytical variation. Average values from these two analyses were then used when calculating standard deviations for each sample group.

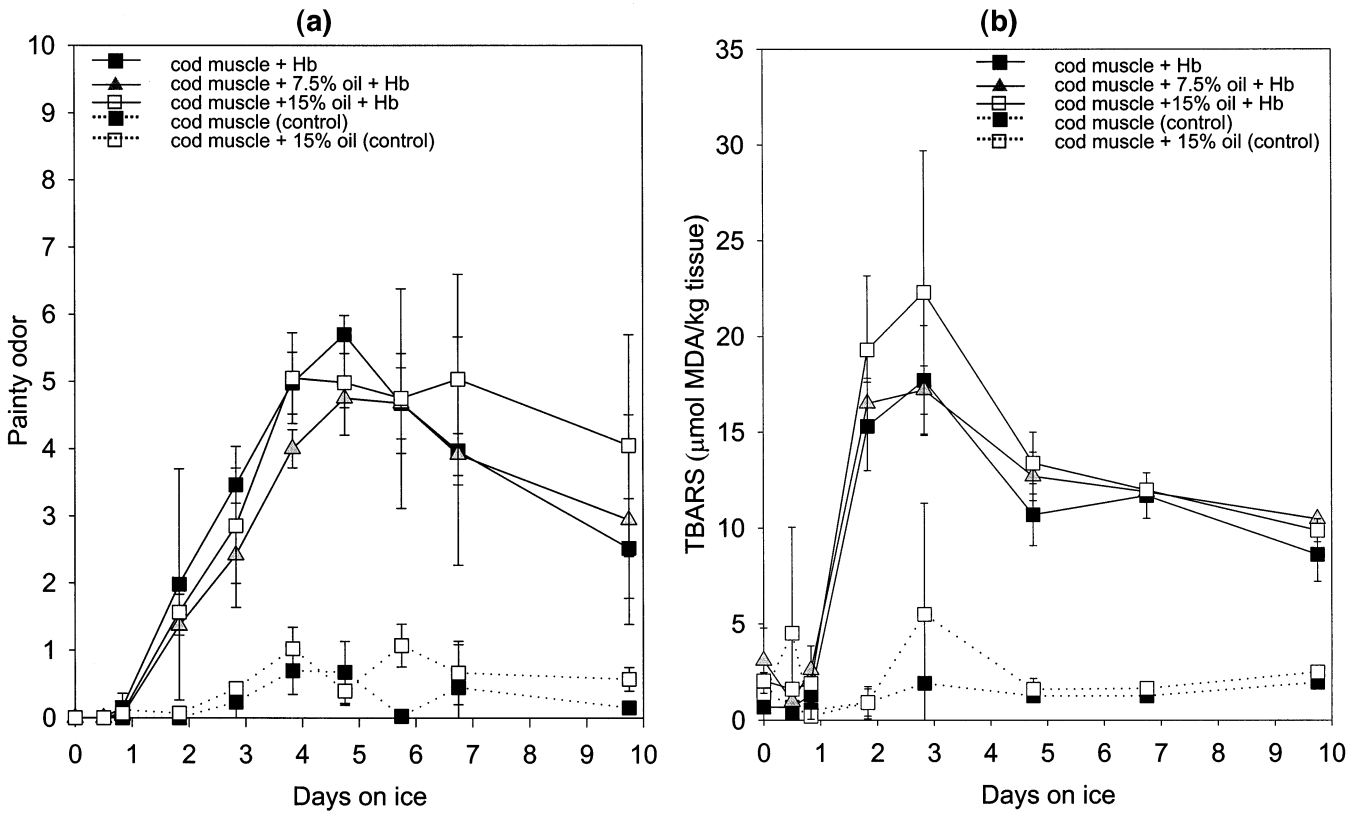

Figure 1. Development of (a) painty odor and (b) TBARS of washed cod muscle without oil and with 7.5% or 15% added commercial menhanden oil. On a wet weight basis, the lipid content of the washed cod was 0.7%. The sensory scale used ranged from 0 to 10, with 10 representing the strongest intensity. Oxidation was initiated with 3 μmol trout Hb/kg washed cod. The final pH was 6.7, and moisture was 86%. Samples were prepared and stored in duplicate ($n = 2$). Error bars show the variations between these two samples in terms of standard deviations (SD). TBARS analyses were performed twice on each sample ($a = 2$), and the average values from these two analyses were used in SD calculations.

Table 1. Fatty Acid Pattern[a] of the Menhaden Oil Added to Washed, Minced Cod

saturates	% of total fatty acids	monoenes	% of total fatty acids	polyenes	% of total fatty acids
C12	0.1	C14:1	0.1	C18:2	1.4
C14	7.1	C16:1	10.6	C20:2	0.2
C15	0.6	C17.1	0.8	C18:3	2
C16	18.1	C18:1	13.6	C18:4	2.9
C17	2.1	C20:1	1.8	C20:4	2.3
C18	3.3	C22:1	0.2	C20.5	11
C20	0.2	C24:1	0.3	C22:5	2.2
C22	0.1	**total**	**27.4**	C22:6	9.5
C24	0.1			**total**	**18.3**
total	**31.7**				

[a] The fatty acid pattern of the oil analyzed with gas chromatography (*20*). Fatty acids other than saturates, monoenes, and polyenes contributed to 9.3% (w/w).

RESULTS

The washed minced cod muscle contained 0.7% lipids (w/w), essentially all as endogenous membrane phospholipids. Unstabilized commercial menhaden oil was added to washed cod mince at 7.5 or 15% of the sample weight. The fatty acid pattern of the oil is shown in **Table 1**. In **Table 2**, it is shown how a substantial amount of lipid hydroperoxides was initially present both in the oil and in the washed cod membrane lipids. At time 0, the samples containing 0, 7.5, and 15% oil thereby contained 14, 570, and 1135 μmol lipid hydroperoxide per kg muscle tissue, respectively. During storage on ice, no significant odor changes were detected in the absence of added Hb (Figures 1a and 2a). When 3 μM Hb was added, moderate to strong painty odors were detected during ice storage in samples both with and without added oil. In all samples, there was a lag phase of ~1 day and the intensity started declining at ca. day 5. Similar kinetics, but with a lower intensity, was detected for the fishy odor development (data not shown). Thus, the rate and extent of rancidity development were not altered by the addition of either 7.5 or 15% oil to washed cod mince.

TBARS values were determined in the same samples on which sensory analyses were conducted (Figure 1b). TBARS reflected sensory scores in that TBARS development occurred to a greater extent in samples containing added Hb and the rate and extent of TBARS formation were altered little by the addition of added oil. The TBARS values started declining at day 3.

To investigate whether the noneffect of added oil was exclusive to Hb-catalyzed lipid oxidation, an oxidation system consisting of $FeCl_3$, ADP, and NADH was utilized to stimulate microsomal enzyme reduction of ferric iron. Both catalysts were added to washed cod with/without 15% oil. The initial PV of the washed cod membrane lipids was 1860 μmol per kg lipid, which resulted in 13 and 1135 μmol lipid hydroperoxide per kg muscle tissue in the 0 and 15% oil samples, respectively. It can be seen in Figure 2 that Hb was a much more effective catalyst of lipid oxidation than the LMW iron system. In this experiment, the addition of 15% oil may have slightly depressed Hb-catalyzed rancidity development compared to the sample without added oil.

The sensory part of the experiment was repeated at pH 7.2 in order to prolong the oxidation lag phase (*1*) and thereby increase the chances for capturing possible kinetic differences

Table 2. Levels of Lipid Hydroperoxides[a] in Oil, Washed Minced Cod Muscle, and Final Samples Used in Experiments 1–3 (Figures 1–3)

experiment no.	pure oil (μmol/kg oil)	washed cod (μmol/kg membrane lipid)	washed cod + 0% oil (μmol/kg final sample)	washed cod + 7.5% oil (μmol/kg final sample)	washed cod + model 15% oil (μmol/kg final sample)
1	7500	2100	14	570	1135
2 & 3	7500	1860	13		1135

[a] Lipid hydroperoxides measured in a chloroform extract using the ferric thiocyanate method (*17*). In experiment 1 (Figure 1a,b), three levels of oil were compared (0, 7.5, and 15%), while in experiments 2 (Figure 2a,b) and 3 (Figure 3), only 0% and 15% oil were used.

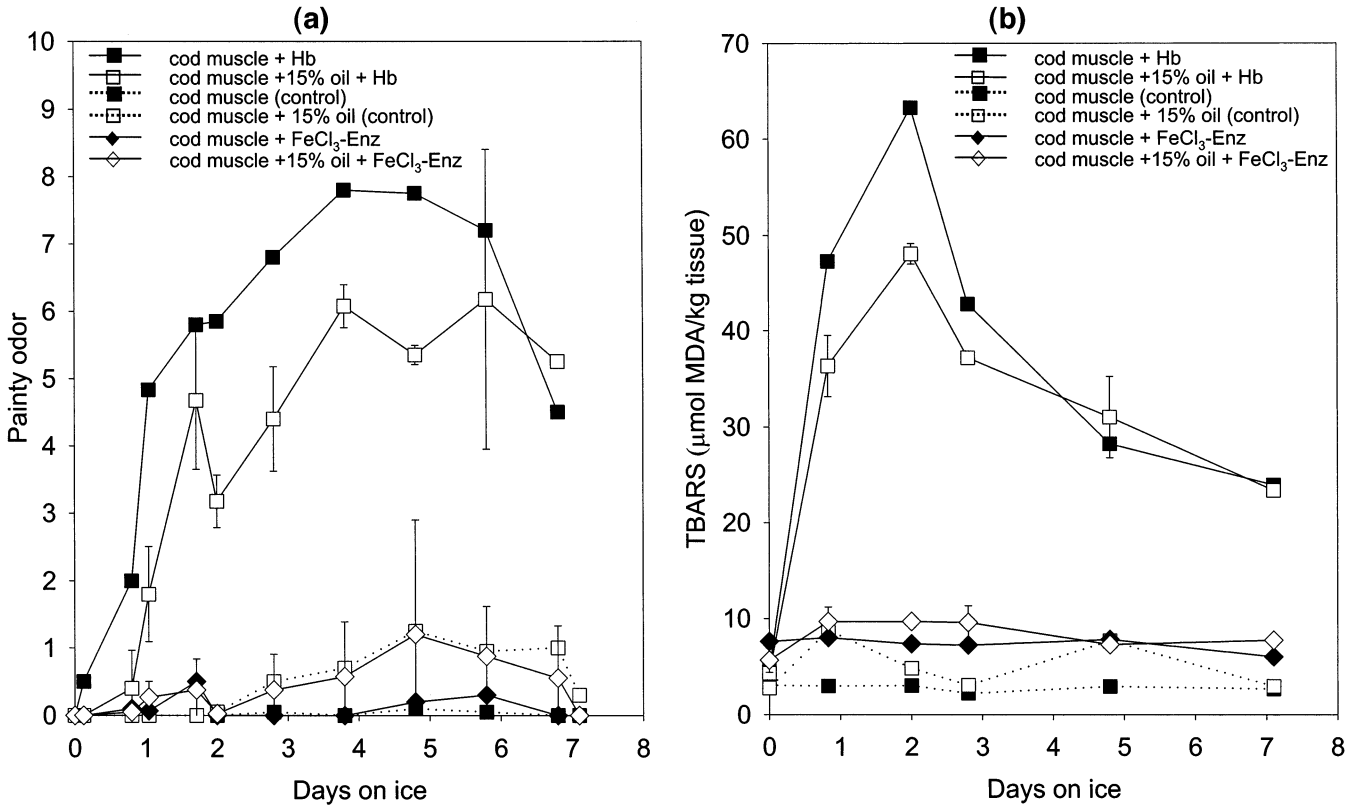

Figure 2. Development of (a) painty odor and (b) TBARS of washed minced cod muscle without oil and with 15% commercial menhaden oil added. On a wet weight basis, the lipid content of the washed cod was 0.7%. The sensory scale used ranged from 0 to 10, with 10 representing the strongest intensity. Oxidation was initiated with 5.8 μmol trout Hb/kg washed cod or with a microsomal enzyme stimulating system consisting of NADH, ADP, $FeCl_3$, KCl, and histidine. The final pH was 6.75, and the final moisture was 90%. Two samples (□/◇) were prepared and stored in duplicate, the others as singles. Error bars show the variations between these two samples in terms of SD. TBARS analyses were performed twice on each sample ($a = 2$), and the average values from these two analyses were used in SD calculations.

between samples. Figure 3 shows that the paintiness lag phase was increased to 5 days at pH 7.2. There was still no difference whether or not 15% oil was present.

The sensory part of the experiment was also repeated using samples with and without 10% added mackerel oil. This oil had been isolated by cold centrifugation of minced mackerel from fish that had been frozen in rigor at −80 °C. There was still no difference in the development of painty odor between samples with and without added oil, showing that the results above were not exclusive to the commercially produced menhaden oil.

To evaluate the ability of Hb to induce painty odors via breakdown of oil hydroperoxides in the absence of membrane lipids, 15% oil was added to a hydrated fibrous cellulose matrix and a filter paper matrix followed by addition of 5.8 μM Hb (Figure 4). Strong painty odors developed during the first 1.5 days in the presence of Hb. In the filter paper and cellulose controls, autoxidation of the oil started at days 7 and 13, respectively. In samples with only hemolysate added, no paintiness was detected throughout the entire storage period.

DISCUSSION

The use of washed, minced cod muscle as a model system provides a matrix that has the structure of muscle, that is, with intact myofibrillar proteins and membranes but virtually free of endogenous triacylglycerols and pro- and antioxidants. Controlled physiological levels of triacylglycerols and catalysts can then be added and studied in relation to lipid oxidation. Here, menhaden oil was used to mimic fish muscle triacylglycerols.

Odor and TBARS data showed that added Hb effectively catalyzed oxidation of membrane lipids in washed cod muscle (Figure 1a,b). Both sensory scores and TBARS increased and then declined during storage. TBARS declined earlier than did the sensory scores. This could indicate that TBA-reactive volatiles are only part of the volatiles yielding rancidity, that some odor volatiles are present at supersaturating levels, or that the TBARS lost did not significantly contribute to the odor. Well-documented reactions of aldehydic secondary oxidation products in muscle are those with free amino groups, for

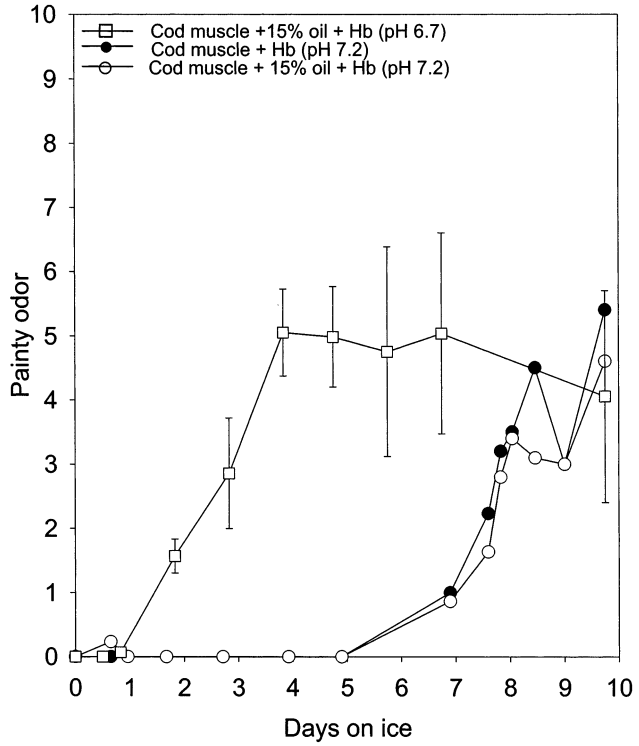

Figure 3. Development of painty odor at pH 7.2 in washed cod muscle without oil and with 15% commercial menhaden oil added. For comparative purposes, painty odor development in washed cod at pH 6.7 with 15% menhaden oil added is also included (from Figure 1a). The sensory scale used ranged from 0 to 10, with 10 representing the strongest intensity. Oxidation was initiated with 5.8 μmol trout Hb/kg washed cod in all samples. The final moisture was 87.6%.

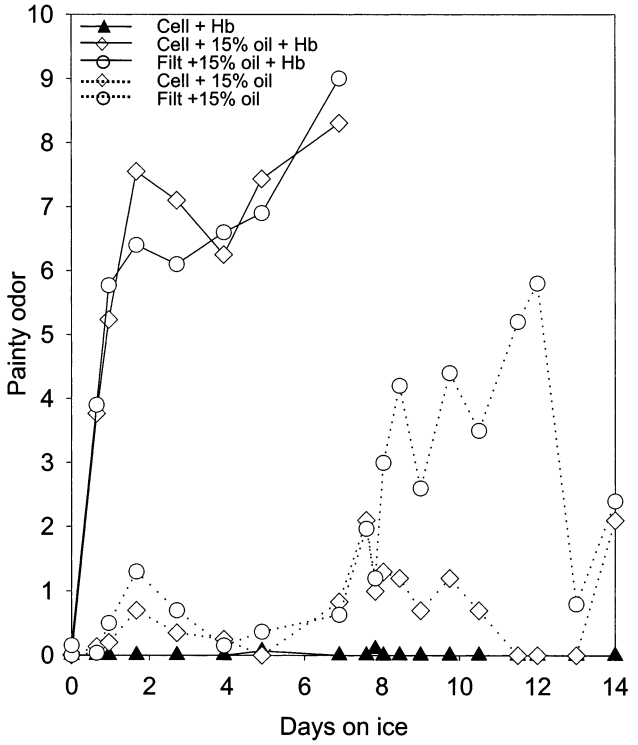

Figure 4. Development of painty odor of a cellulose matrix (Cell) without oil, a cellulose matrix with 15% commercial menhaden oil added, and a filter paper matrix (Filt) with 15% commercial menhaden oil added. The sensory scale used ranged from 0 to 10, with 10 representing the strongest intensity. Oxidation was initiated with 5.8 μmol trout Hb/kg matrix. The final pH was 6.7, and moisture was 79.8%.

example, in membrane lipids, proteins, and peptides, to form Schiff's bases (*23*) and with active methylene compounds such as in histidine to form Michael addition products (*24*,*25*).

Although there was a high initial amount of lipid hydroperoxides present in washed cod muscle containing 15% added oil, rancid odors did not develop in the absence of Hb (Figures 1a and 2a). Thus, Hb was necessary for the production of hydroperoxide breakdown products associated with rancidity. It was seen that nearly doubling the Hb concentration increased the maximum paint intensity by ~30% (Figures 1a and 2a) and approximately doubled the maximum intensity of TBARS (Figures 1b and 2b) that occurred in the washed cod muscle. This was the case whether the lipid substrate was 0.7% membrane lipids or 0.7% membrane lipids plus 15% oil. These results indicate that Hb limited the extent of the oxidation reaction, suggesting that Hb acted as a reactant rather than as a catalyst. In support of this theory are recent findings showing that increasing Hb levels between 0.06 and 5.8 μmol/kg (*26*) almost proportionally increased the maximum oxidation intensities in washed cod. In several separate studies (*1*,*12*,*26*), a strikingly constant ratio was obtained between maximum TBARS values and the Hb levels added to washed cod: 13.6 $\pm$ 4.8 μmol MDA/μmol Hb tetramer (range 6.7−24) (n = 12).

Thus, it is suggested that the amount of hydroperoxides initially present in the membrane lipid fraction (~14 μmol/kg washed cod), together with new hydroperoxides that were tentatively formed by alkoxyl and/or peroxyl free radicals emerging from the membrane lipid hydroperoxide breakdown reaction (*27*), was sufficient substrate for hydroperoxide breakdown by 3 or 5.8 μM Hb. The presence of another 1120 μmol lipid hydroperoxide/kg washed cod from the addition of 15% oil did not increase the rates or intensities of paintiness and TBARS nor keep up a steady-state level of the oxidation products responding to these measurements (Figures 1−3). This indicates that Hb was not regenerated after performing its hydroperoxide cleaving action or that the oil hydroperoxides were not accessible to the Hb. In accordance with this, it was previously found that minced muscle samples from catfish that had 2.6, 3.5, and 4.4% lipid developed the same amount of "oxidized oil odor" during frozen storage (*28*). In the study of Kolakowska et al. (*29*), it was even seen that rancidity became evident at *lower* PVs when the membrane to neutral lipid ratio increased. In other words, lean spawning herring (4% total lipids, 20 mequiv peroxide/kg lipid) were found to be more susceptible to rancidity compared to fattier autumn herring (11% total lipids, 40 mequiv peroxide/kg lipid). The results in Figure 2 show a similar tendency whereby the samples containing less total lipids and lipid hydroperoxides appeared more susceptible to painty odor development. This brings up the question of whether membrane lipid hydroperoxides were the preferred substrates for Hb in samples both without and with added oil or whether Hb reacted unselectively with both lipid classes. Preferential breakdown of membrane lipid hydroperoxides by Hb has several possible explanations. Membranes exist as phospholipid bilayers, which is why the surface area that the membrane lipids expose to the aqueous phase is ~50−100 times greater than the oil droplet surface areas on a weight basis (*30*). In addition, the higher polarity of the membrane lipids as compared to that of oil may allow for better interaction with a water-soluble prooxidant such as Hb (*31*). There are previous indications of preferential oxidation of membrane lipids over oil in fish model systems. Along with extensive MDA formation in minced herring tissue during storage at −20 °C, there was a significant

decrease in the 22:6/16:0 ratio in the sarcoplasmatic reticulum lipids but not in the total lipids (*8*). This indicates that membrane lipids were more susceptible to lipid oxidation than oil/triacylglycerols. In another study, 70% of the membrane lipid fraction of cod flesh was lost after ~200 days at −20 °C, whereas the triacylglycerol fraction was intact (*2*). Similarly, only the membrane lipids oxidized during frozen storage of cod and haddock (*11*).

To test the hypothesis of whether the lower degree of surface area, unsaturation, and polarity of oil droplets prevents oil and/or oil hydroperoxides from being reactants in Hb-catalyzed oxidation, oil and Hb were added to two solid lipid-free matrixes, hydrated filter paper and cellulose. That oil and Hb interactions gave rise to rapid and extensive rancidity in these systems (Figure 4) implied either that Hb unselectively reacts with membrane lipids and oil *or* that the noneffect of oil in washed cod may be due to the ability of proteins to protect the oil droplets from oxidation. Neutral fish lipid was found to interact more strongly with actin than did polar fish lipid (*32*). Tong et al. (*33*) suggested that muscle proteins can prevent oil droplets from oxidizing by binding metals that would otherwise attach to the oil droplet surface or by acting as free radical scavengers. Okazaki et al. (*34*) reported that oxidation of oil was limited due to protective emulsification by proteins. After emulsification of sardine oil into Alaska pollack surimi using various protein emulsifiers, lipid peroxide development in the oil was prevented the more finely the oil was emulsified.

Unlike Hb, a combination of NADH, ADP, Fe^{3+}, and histidine was ineffective at catalyzing lipid oxidation in the washed cod muscle matrix (Figure 2a,b). This was in spite of the fact that the Fe level (15 μM) was 20 and 4 times higher than the amount of low molecular weight Fe that was found in mackerel light and dark muscle, respectively (*35*), while the Hb level added was in the range found in trout light muscle (*36*). Possible explanations for the ineffectiveness of low molecular weight iron as a catalyst include (i) inadequate reduction of Fe^{3+} by the NADH-requiring enzymic reduction system of the membranes of the washed cod or (ii) competition for iron between ADP and other chelators that can inactivate iron. It is possible that the globin part of Hb prevents the heme-iron from such inactivating chelation, which could explain the higher pro-oxidative effect of Hb as compared to those of NADH, ADP, and Fe^{3+}. The results by Richards (*37*) support this theory. He compared oxidation of washed cod membrane lipids in the presence of Hb and Fe^{2+}−ADP−ascorbate at equimolar iron levels (23.2 μmol/kg) and obtained oxidation only with Hb-bound iron.

Altogether, these findings imply that the high fat content of pelagic fish species may have little to do with the rate and extent of lipid oxidation that occurs with storage of the muscle. Rather, the difference in susceptibility to lipid oxidation, for example, between pelagic fish species and white fish species seems more pertinent to the type and quantity of the aqueous catalysts than the quantity of the lipid substrate.

ABBREVIATIONS USED

Hb, hemoglobin; LMW, low molecular weight; MDA, malondialdehyde; PV, peroxide value; TBARS, thiobarbituric acid reactive substances.

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