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Involvement of Methemoglobin (MetHb) Formation and Hemin Loss in the Pro-oxidant Activity of Fish Hemoglobins

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The capacity of Atlantic pollock (*Pollachius pollachius*), seabass (*Dicentrarchus labrax*), and horse mackerel (*Trachurus trachurus*) hemoglobin (Hb) to promote lipid oxidation has been evaluated in liposomes and washed minced horse mackerel muscle. The pro-oxidant ability of fish Hbs was related with their vulnerability to suffer oxidation to metHb and release hemin either in spontaneous situation or in the presence of two representative lipid oxidation products, linolein hydroperoxides and *trans*-2-pentenal. The results indicated similar effectiveness to promote lipid oxidation in liposomes and washed fish muscle: pollock Hb > horse mackerel Hb > seabass Hb. Pollock Hb showed a more elevated autoxidation rate and spontaneous hemin loss and also faster oxidation to metHb in the presence of hydroperoxides and *trans*-2-pentenal. The autoxidation and spontaneous hemin loss rates were intermediate for horse mackerel Hb, whereas seabass Hb exhibited the highest stability. The isoelectrofocusing (IEF) pattern of pollock Hb revealed the presence of isoforms with elevated anionic character, which are known to have poor oxygen affinity at the pH values found in fish muscle (pH 7.0−5.5). In agreement with the IEF patterns, pollock Hb was less oxygenated at pH 6.8, and seabass Hb exhibited more oxygenation than did horse mackerel. MetHb forms were significantly more effective in catalyzing lipid oxidation than the corresponding reduced fish Hbs and free hemin. The present investigation highlights a direct correlation between the pro-oxidant capacity of fish Hbs and their susceptibility to undergo metHb formation and hemin loss and also suggests a potential role of lipid oxidation byproducts in activating the pro-oxidative action of hemoglobin.

KEYWORDS: Fish hemoglobin; pro-oxidant; autoxidation; methemoglobin; hemin loss; hydroperoxides; *trans*-2-pentenal; isoelectrofocusing

INTRODUCTION

Lipid oxidation is a major cause of quality deterioration in muscle-based foods where flavor, color, texture, and nutritional value can be drastically affected (*1*). Fish muscle is especially susceptible to develop damaging oxidative processes as a consequence of a lipid composition rich in long-chain n-3 polyunsaturated fatty acids (n-3 PUFA), essentially eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3). The vulnerability of the allylic and bisallylic C−H bonds of PUFA to form carbon-centered free radicals is essentially involved in either initiation or propagation chain reactions of lipid oxidation (*1*, *2*). Among the catalysts of lipid oxidation, heme pigments such as hemoglobin and myoglobin are considered to play a fundamental role in the oxidative deterioration of fish muscle. Several investigations have expressed that fish hemoglobins catalyze lipid oxidation more intensively than those from terrestrial animals such as beef, chicken, and turkey (*3*, *4*) and that catalytic amounts of fish hemoglobin are able to initiate extensively lipid oxidation in a model system of washed minced fish muscle (*5*, *6*).

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Hemoglobin has been reported to be the prevalent heme pigment in mackerel light muscle (6 μmol of Hb/kg) and whole rainbow trout muscle (11 μmol of Hb/kg), whereas dark muscle from mackerel contained roughly equal amounts of hemoglobin (Hb) and myoglobin (Mb) on a weight basis (*5*). Hemoglobin is composed of four polypeptide chains with each chain containing one heme group, whereas myoglobin is a monomeric molecule with a single polypeptide chain holding a heme group. The prosthetic heme moiety consists of an iron atom coordinated in the center to a porphyrin ring. Two main pathways are considered to explain the pro-oxidative capacity of Hb/Mb. One includes the ability of heme proteins to stimulate the generation of free radicals via cleavage of preformed lipid hydroperoxides. O'Brien (*7*) has shown that oxyHb cleaves hydroperoxides significantly more rapidly than free ferrous iron and free ferric iron. Accordingly, we have reported faster kinetics of hydroperoxide decomposition in fish membranes triggered by horse mackerel Hb than in those activated by ferrous iron (*8*). In addition, free hemin, which designates to the ferric porphyrin ring, has demonstrated higher aptitude to generate free radicals through decomposition of preformed hydroperoxides in conditions of low hydroperoxide concentration, whereas either reduced or oxidized forms from rainbow trout Hb were more effective

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under more elevated hydroperoxide concentration or successive episodes to hydroperoxide exposure (*9*). These results draw attention to a central role of the free hemin and the affinity of hemoglobin by hemin during the initial stages of lipid oxidation. The formation of the hypervalent ferryl Hb radical [•HbFe(IV)═O], which can initiate lipid peroxidation via abstraction of a hydrogen atom of a PUFA, is also proposed as a principal pathway for the pro-oxidative activity of Hb/Mb (*10*). Ferryl Hb radical species are generated by reaction of metHb with hydrogen peroxide or preformed lipid hydroperoxides. Consequently, the propensity of Hb to be oxidized to metHb appears to necessarily influence the contribution of this preceding mechanism to hemoglobin-catalyzed lipid oxidation. However, more research is needed to understand completely the real contribution of all these mechanisms on the pro-oxidative activity of Hb.

The purpose of the present study was to characterize the pro-oxidative activity of hemoglobins from three commercial fish species (Atlantic pollock, horse mackerel, and seabass) and to estimate the most decisive factors involved in such activity. For such scope, the activity of fish hemoglobins to promote lipid oxidation was studied in liposomes and washed minced fish muscle, which can essentially mimic membranes and fish muscle. The pro-oxidant activity was then related with the oxygenation state of such hemoglobins and their vulnerability to undergo metHb formation and hemin loss. The pro-oxidative capacity of fish Hb (mainly found in reduced form) was compared with that found for free hemin (oxidized form of heme) and the corresponding metHb species. Furthermore, the diverse isoforms of fish hemoglobins were characterized by native isoelectrofocusing (IEF) electrophoresis.

MATERIALS AND METHODS

Materials. Atlantic pollock (*Pollachius pollachius*), seabass (*Dicentrarchus labrax*), and horse mackerel (*Trachurus trachurus*) were acquired from a local market. Bovine hemoglobin, hemin chloride, ammonium persulfate, sodium heparin, tris[hydroxymethyl]aminomethane (Tris), sodium dithionate, dibasic sodium phosphate ($Na_2HPO_4 \cdot 2H_2O$), monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$), lecithin from soybean with 30% of L-α-phosphatidylcholine, thiobarbituric acid, trichloroacetic acid, 1,1,3,3-tetraethoxypropane (TEP), $FeCl_2 \cdot 4H_2O$, $FeCl_3 \cdot 6H_2O$, streptomycin sulfate, butylated hydroxytoluene (BHT), *trans*-2-pentenal, and 5-sulfosalicylic acid were purchased from Sigma (Steinheim, Germany). Carbon monoxide (99.97%) was provided by Air Liquide (Porriño, Spain). Linolein hydroperoxides (>98%) were purchased from Cayman Europe (Tallin, Estonia). IEF polyacrylamide gels were obtained from GE Healthcare (Uppsala, Sweden). All chemicals and solvents used were of either analytical or HPLC grade (Merck, Darmstadt, Germany).

Extraction of Fish Hemoglobin. Blood was taken from the caudal vein after cutting off the tail of individual fresh fish in the rigor state. Blood was collected with a transfer glass pipet rinsed with 150 mM NaCl and sodium heparin solution (30 units/mL), and it was immediately mixed with approximately 1 volume of the saline sodium heparin solution. Hemolysate was then prepared according to the method of Richards and Hultin (*11*). The visible spectrum at pH 8.6 showed that fish Hb was mostly reduced and oxygenated because of the appearance of two absorption peaks at 540 and 570 mn, which are characteristic of fully oxygenated Hb (OxyHb). Hemolysate was stored at −80 °C for no longer than 2 weeks and thawed just before used.

Preparation of Methemoglobin (MetHb). MetHb was obtained by oxidation of the corresponding fish Hb by modification of the procedure of Svistunenko et al. (*12*). Briefly, Hb was incubated with 4 mM ammonium persulfate for 1 h at room temperature, and then passed through a PD-10 desalting column containing 8.3 mL of Shephadex G-25 medium (GE Healthcare). Following recommendations of the manufacturer, 1 mL of metHb solution was loaded on the column and then eluted with 3.5 mL of a 1 mM Tris solution, pH 8. MetHb was stored at −80 °C and thawed just before used.

Determination of the Total Hemoglobin Concentration. Hemoglobin was quantified in the hemolysate and in the metHb solutions according to the method of Brown (*13*). Briefly, samples diluted to 0.2−1.2 μM Hb in 50 mM Tris, pH 8.6, were mixed with around 1 mg of sodium dithionite and bubbled with carbon monoxide gas for 20 s. The samples were then scanned from 400 to 480 nm against a blank that contained only Tris buffer. The peak absorbance was recorded, and the absorbance at the peak (≈416 nm) minus the absorbance at the valley (465 nm) was related with the hemoglobin concentration. A standard curve was built with commercial hemoglobin from bovine blood.

Determination of the Relative Oxygenation of Fish Hb. Fish Hb was diluted in a 50 mM phosphate buffer at pH 6.8 to a concentration of 5 μM, and then the visible spectrum ranging from 500 to 640 nm was acquired. Two well-defined peaks faintly overlapped are characteristic in this area of the spectrum for oxyHb (*14*), and the difference in absorbance between the peak (574 nm) and the valley (560 nm) was used to estimate their initial relative state of oxygenation as previously employed by Richards and Hultin (*11*).

Hb Autoxidation and MetHb Formation in the Presence of Lipid Oxidation Byproducts. The experiments were performed by incubating 5 μM fish Hb in 50 mM phosphate buffer (pH 6.8) at 4 °C in the absence (autoxidation) or presence of primary and secondary lipid oxidation products, linolein hydroperoxides and *trans*-2-pentenal, respectively. Linolein hydroperoxides were added in ethanolic solution on an empty cuvette and dried under a flow of nitrogen gas before the incorporation of Hb solution in phosphate buffer. *trans*-2-Pentenal was directly incorporated in aqueous solution. The final concentrations of linolein hydroperoxides and *trans*-2-pentenal were 125 and 1000 μM, respectively. The formation of metHb was evaluated at different incubation times by adaptation of Winterboun's equation (*14*) that estimates the concentration of metHb in micromolar (on hemoglobin basis) considering the absorbance at 576 and 630 nm:

$$[\text{MetHb}] = \left(\frac{279A_{630} - 3A_{576}}{4}\right)$$

Determination of Hemin Loss from Fish Hb. The method has been developed in our laboratory and is derived from the differential polarities of hemoglobin (polar) and free hemin (apolar) and the capacity of solid phase extraction (SPE) by C18 cartridges to retain compounds with low polarity. ISOLUTE C18 100 mg/1 mL cartridges (Biotage, Uppsala, Sweden) were previously preconditioned with 2 mL of a water/methanol (1:1) solution, and then 1 mL of a 5 μM Hb solution was passed through. Later, cartridges were washed with 4 mL of water and dried for 15 min by passing through a flow of air by action of a vacuum pump. Free hemin was eluted with 1 mL of methanol, and the absorbance peak of hemin in the visible spectrum (350−450 nm) was acquired. The standard curves were built up with known concentrations of commercial hemin chloride subjected to the entire procedure, providing good linearity ($R^2 > 0.99$) in the range of hemin concentration of 1−12 μM. The method showed good repeatability with variation coefficients ranging from 2 to 6%.

Experiments in Liposomes. Liposomes were daily prepared according to the procedure described by Huang and Frankel (*15*). Briefly, lecithin at a concentration of 0.8% (w/w) was dissolved in 50 mM phosphate buffer at pH 6.8 by magnetic stirring and subsequently sonicated for 10 min with a water bath ultrasonicator (P-Selecta, Barcelona, Spain). Liposomes were then filtered through a paper filter and introduced into 50 mL Erlenmeyer flasks. Liposomes with Tris buffer or Hb were incubated at 30 °C in a thermostatic water bath (P-Selecta), and the oxidative stability of liposomes was evaluated by monitoring conjugated dienes and TBARS at different incubation times. Fish Hb and hemin were incorporated at 3 μM on a hemoglobin basis.

Experiments in Washed Minced Fish Muscle. Washed minced muscle was prepared from light muscle of horse mackerel by modification of the procedure previously described by Richards and Hultin (*5*). Briefly, fish mince was washed twice in distilled water at a 1:3 mince-to-water ratio (w/w) by stirring with a plastic rod for 2 min. The mixture was allowed to stand for 15 min and then dewatered on a stainless strainer. Washed minced muscle was finally mixed with a 50 mM sodium phosphate buffer, pH 6.8, and streptomycin sulfate (200 ppm) was added to inhibit microbial growth. Washed fish muscle was supplemented with Hb or hemin at

3 μmol/kg of muscle (on Hb basis), whereas the corresponding volume of 1 mM Tris buffer, pH 8.0, was added in control samples. The final moisture of the washed fish muscle was 84.2±0.8%. The samples were incubated at 4 °C, and peroxide value and TBARS were measured every day during the storage period.

Conjugated Dienes. Liposomes (100 mg) were dissolved in 3 mL of methanol, and the absorbance was measured at 234 nm (UV−vis spectrophotometer Perkin-Elmer) and calculated as millimoles of dienes per kilogram of phosphatidylcholine (PC) as previously described by Frankel and co-workers (*16*).

Peroxide Value (PV). PV was measured in washed minced fish muscle using the procedure by Buege and Aust (*17*), which is based on a lipid extraction with 5 mL of CH_2Cl_2/MeOH (2:1) and analysis by using the ferric thiocyanate method (*18*). PV was expressed in milliequivalents (mequiv) of oxygen per kilogram of lipid.

Thiobarbituric Acid Reactive Substances (TBARS) Analyses. TBARS were determined in liposomes and washed minced fish muscle according to the method of McDonald and Hultin (*19*). 1,1,3,3-Tetraethoxypropane was used as standard. The results are expressed in terms of milligrams of malonaldehyde (MDA) per kilogram of phosphatidylcholine (PC) or muscle in liposomes and washed fish muscle, respectively.

Native IEF Pattern of Fish Hb. Native IEF was done using precast polyacrylamide gels (Ampholine PAGplate IEF, 250×110×1 mm, pH 3.5−9.5, GE Healthcare). Sixty micrograms of total proteins was applied near a cathode using pieces of sample application paper (GE Healthcare). One line with markers of isoelectric point (p*I*) in the range of pH 3.5−9.5 (Broad pI calibration Kit, GE Healthcare) was included. The IEF gels were run at 10 °C in a Multiphor II electrophoresis unit (Amersham Biosciences, Uppsala, Sweden) with a thermostatic water-circulator unit. The running conditions were as follows: 30 W constant (1500 V maximum) for 1.5 h, with 15 min prefocusing. Gels were maintained for 30 min in fixed solution [sulfosalicylic acid 5% (w/v) and trichloroacetic acid 10% (w/v)] and then stained with Coomassie brilliant blue R250 (GE Healthcare).

Statistical Analysis. The experiments were performed twice, and data are reported as mean±standard deviation of three replicates ($n = 3$). The data were analyzed by one-way analysis of variance (ANOVA) and the least-squares difference method. Statistical analyses were performed with the software Statistica 6.0 (StatSoft, Tulsa, OK).

RESULTS

Pro-oxidant Capacity of Atlantic Pollock, Seabass, and Horse Mackerel Hemoglobins in Liposomes and Washed Minced Fish Muscle. The capacity of each fish hemoglobin to catalyze lipid oxidation was evaluated in two different systems, liposomes and washed minced muscle from horse mackerel. In liposomes, all fish hemoglobins at a concentration of 3 μM produced an extensive generation of both primary and secondary lipid oxidation products, represented, respectively, by conjugated dienes and TBARS, after 3 h of incubation at 30 °C (**Figure 1**). In contrast, control liposomes without hemoglobin supplemented did not exhibit a significant increment of those lipid oxidation products during the same incubation time, thus demonstrating the capacity of the studied hemoglobins to promote lipid oxidation in liposomes. From the comparison of the different fish hemoglobins, pollock Hb was found to be significantly the strongest promoter of conjugated dienes ($p < 0.05$), followed in decreasing order by horse mackerel Hb and seabass Hb (**Figure 1A**). TBARS formation also indicated a lower pro-oxidative activity for seabass Hb than for pollock and mackerel hemoglobins (**Figure 1B**). Moreover, pollock Hb produced a more elevated amount of TBARS during the initial propagation stages of lipid oxidation (20−40 min) than horse mackerel Hb. However, the pro-oxidative activities of both hemoglobins were similar in terms of TBARS values after longer incubation times. In summary, fish hemoglobins exhibited different pro-oxidative effectiveness in liposomes, and the relative order of activity was found to be pollock Hb > horse mackerel Hb > seabass Hb.

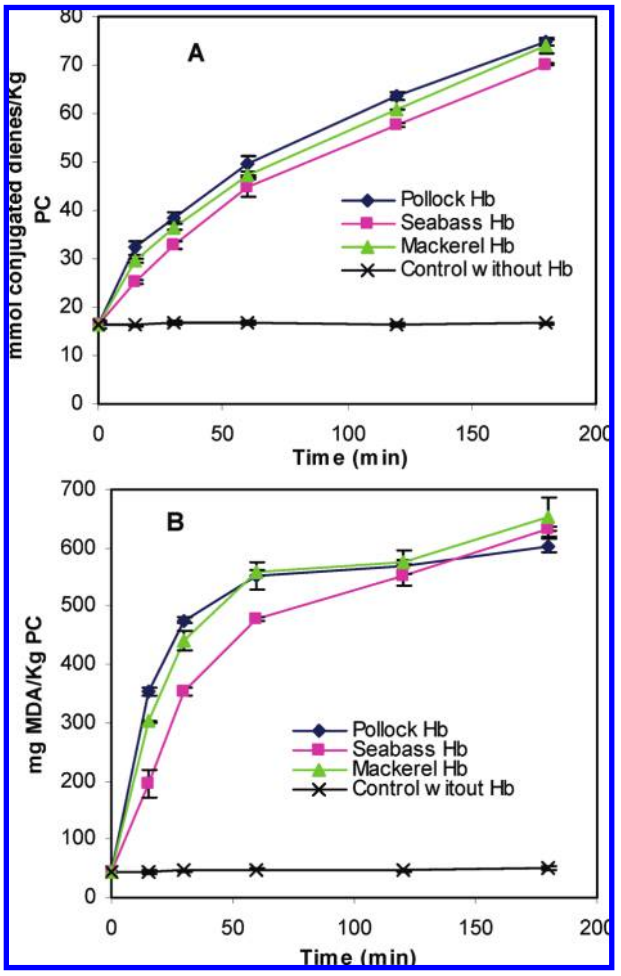

Figure 1. Pro-oxidative capacity of Atlantic pollock, seabass, and horse mackerel Hb in liposomes by monitoring the formation of conjugated dienes (**A**) and TBARS (**B**). Liposomes were prepared in 50 mM phosphate buffer at pH 6.8, supplemented with fish Hb at 3 μM and incubated at 30 °C.

Washed minced fish muscle provided an appropriate model system to investigate the activity of hemoglobin in relation to lipid oxidation under controlled physiological levels. It provides a matrix that has the structure of fish muscle, that is, with intact myofibril proteins and membrane lipids, but virtually free of heme pigments (*20*). Washed minced muscle from horse mackerel light muscle with lipid content and moisture of, respectively, 0.67±0.05 and 84.2±0.8%, was supplemented with 3 μmol of hemoglobin/kg of muscle. The addition of hemoglobin produced an extensive generation of lipid peroxides and TBARS during 5 days of storage at 4 °C (**Figure 2**). Conversely, control washed muscle without supplemented fish hemoglobin did not exhibit a significant formation of lipid oxidation products. Supplementation with pollock Hb produced a severe increment of lipid peroxides at day 2, which decreased subsequently after day 3. Mackerel Hb and seabass Hb were found to be less active in promoting the formation of lipid peroxides because both formation rate and maximum value of lipid peroxides were significantly lower than those corresponding to pollock Hb (**Figure 2A**). Seabass Hb was less active than mackerel Hb in the promotion of peroxides during the propagation phase of lipid oxidation (2−4 days). The pro-oxidative pattern found for TBARS values was similar to that observed for peroxides: pollock Hb > horse mackerel Hb > seabass Hb (**Figure 2B**).

Relative Oxygenation and Rates of Autoxidation and Spontaneous Hemin Loss for Atlantic Pollock, Horse Mackerel, and Seabass Hemoglobins. The relative affinity of hemoglobins to

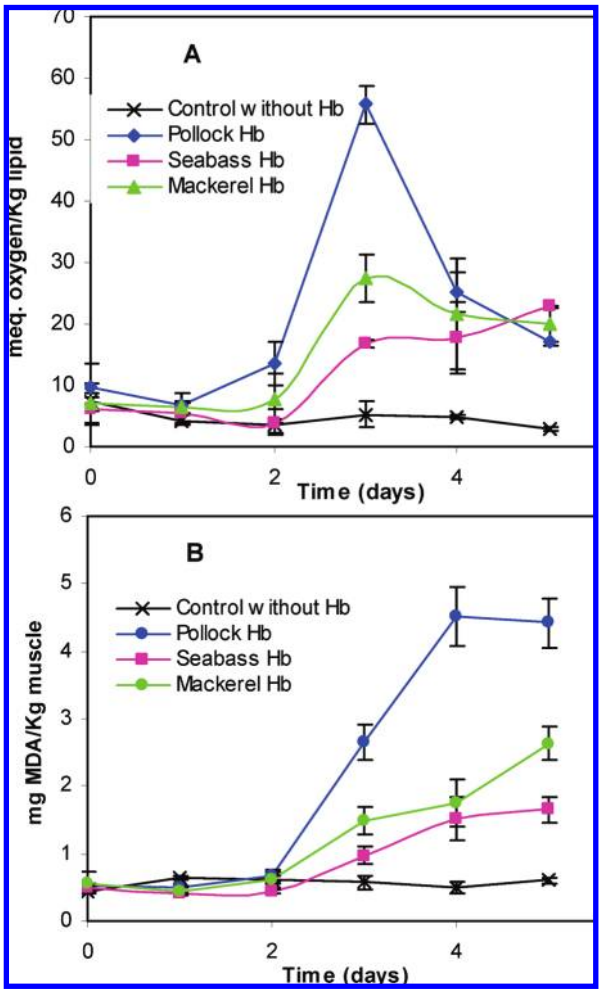

Figure 2. Pro-oxidative capacity of Atlantic pollock, seabass, and horse mackerel Hb in washed minced horse mackerel muscle by monitoring the formation of lipid peroxides (**A**) and TBARS (**B**). Washed minced muscle was supplemented with fish Hb at 3 μmol/kg of muscle and incubated at 4 °C.

bind oxygen and their rates of autoxidation and hemin loss were evaluated at pH 6.8 and 4 °C, conditions in which the lipid oxidation experiments were conducted. There were significant differences in the relative oxygen-binding affinity of hemoglobins, showing pollock Hb with lower initial oxygenation, followed by horse mackerel Hb and then seabass Hb, which presented the strongest relative oxygenation state (**Table 1**).

Concerning the spontaneous oxidation of hemoglobin to metHb, the results clearly indicated a net formation of 1.7 μM metHb for pollack Hb after 1 day of incubation, followed by a reduction of metHb levels between days 4 and 8 (**Figure 3A**). Seabass and mackerel hemoglobins exhibited higher stability, and so, the levels of metHb remained practically constant for both hemoglobins during the entire period of incubation, 9 days. A significant feature was found to be the lower initial metHb levels for seabass Hb (0.5 μM) in comparison with those from horse mackerel and pollack, the initial values of metHb of which were found to be 1.5 and 1.7 μM, respectively. The higher initial metHb levels for horse mackerel Hb than for seabass Hb suggested a higher susceptibility of horse mackerel Hb to be oxidized to metHb.

The ability of fish hemoglobins to lose hemin spontaneously was also investigated in those samples employed to study autoxidation. The results showed that pollock Hb released an amount

Table 1. Relative Oxygenation State of Atlantic Pollock, Seabass, and Horse Mackerel Hb in 50 mM Phosphate Buffer, pH 6.8[a]

	relative oxygenation, absorbance (peak 575 nm − valley 560 nm)×1000
pollock	30.2 ± 2.1
seabass	86.7 ± 0.8
horse mackerel	64.6 ± 0.8

[a]The difference in absorbance between the peak (574 nm) and the valley (560 nm) was used to estimate the relative oxygenation.

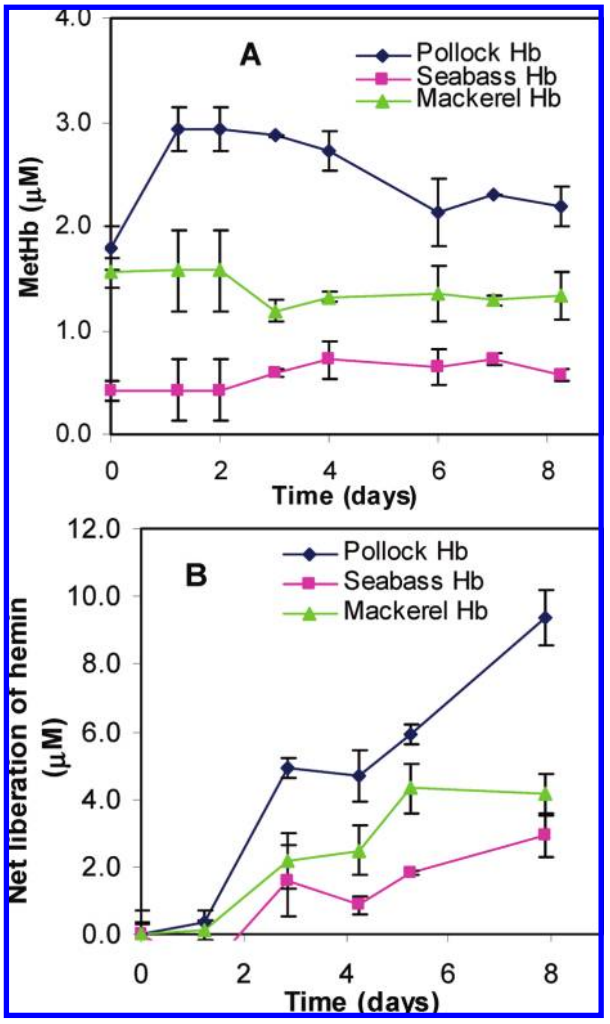

Figure 3. Autoxidation (**A**) and spontaneous hemin loss (**B**) of Atlantic pollock, seabass, and horse mackerel Hb. Fish Hb was diluted to 5 μM in 50 mM phosphate buffer, pH 6.8, and incubated at 4 °C.

higher than 4 μM hemin between days 1 and 3, whereas approximately 5 μM hemin was discharged between days 4 and 8 (**Figure 3B**). Horse mackerel and seabass hemoglobins showed higher hemin affinity considering the lower spontaneous hemin loss in comparison to pollack Hb. There were also differences between hemin affinities of seabass and horse mackerel Hb, making horse mackerel Hb more vulnerable to loss hemin. In conclusion, the spontaneous loss of hemin was found to be faster for pollack Hb > horse mackerel Hb > seabass Hb.

MetHb Formation and Hemin Loss in the Presence of Linolein Hydroperoxides and *trans*-2-Pentenal. Fish hemoglobins were incubated with a primary and secondary lipid oxidation product, linolein hydroperoxides and *trans*-2-pentenal, respectively, to evaluate the vulnerability of hemoglobins to undergo metHb formation and hemin loss during the development of lipid

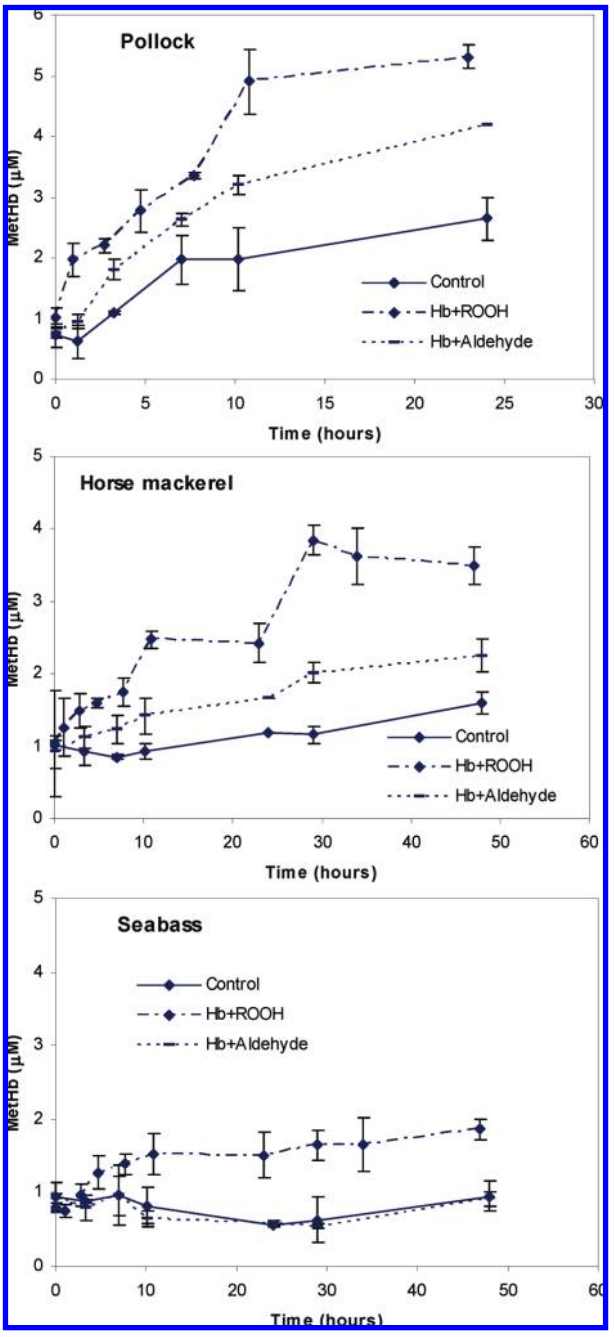

Figure 4. Oxidation to metHb of Atlantic pollock, horse mackerel, and seabass Hb by incubation with linolein hydroperoxides and *trans*-2-pentenal. Fish Hb was diluted to 5 μM in 50 mM phosphate buffer, pH 6.8, and incubated at 4 °C. The final concentrations of linolein hydroperoxides and *trans*-2-pentenal were 125 and 1000 μM, respectively.

oxidation reactions. The incubation of 5 μM hemoglobin with 125 μM linolein hydroperoxides accelerated notably the generation of metHb for all hemoglogins. The relative susceptibility to undergo oxidation to metHb was similar to that found spontaneously in the absence of hydroperoxides: pollock Hb > horse mackerel Hb > seabass Hb (**Figure 4**). The interaction with *trans*-2-pentenal (1000 μM) also raised the rate of metHb formation in the case of pollock Hb and, to a lower extent, for horse mackerel Hb. On the contrary, seabass Hb was not significantly affected by *trans*-2-pentenal in terms of metHb formation, and therefore, seabass Hb did not produce a significant generation of metHb even in the presence of *trans*-2-pentenal. Consequently, the susceptibility of fish hemoglobins to generate metHb in the presence of *trans*-2-pentenal had a result similar to that found in the presence of linolein hydroperoxides: pollock Hb > horse mackerel Hb > seabass Hb (**Figure 4**). It is a relevant fact that linolein hydroperoxides were more effective than *trans*-2-pentenal in inducing the generation of metHb, even when *trans*-2-pentenal was employed in a concentration 8-fold higher compared to hydroperoxides.

With regard to the effect of linolein hydroperoxides and *trans*-2-pentenal on the release of hemin by hemoglobins, the aldehyde was significantly more active than linolein hydroperoxides in promoting the generation of free hemin (**Figure 5**). However, these results should be considered carefully because hemin can be easily destroyed by interaction with preformed lipid hydroperoxides (*9*) and because linolein hydroperoxides originated a higher formation of metHb, which possesses a weaker affinity by hemin than the corresponding fish hemoglobin (conclusion inferred from the observation of the spontaneous hemin loss of metHb and fish Hb).

The interaction with linolein hydroperoxides (125 μM) increased the amount of free hemin for seabass Hb in comparison with control hemoglobin not supplemented with hydroperoxide and, to a lower degree, raised free hemin for horse mackerel Hb. On the contrary, linolein hydroperoxides apparently did not have an effect on the release of hemin by pollock Hb. Therefore, linolein hydroperoxides produced apparently a stronger release of hemin for seabass Hb, which has exhibited the lower spontaneous hemin loss, whereas hemoglobin with the fastest spontaneous hemin loss (pollock Hb) did not raise the free hemin content by interaction with hydroperoxides. These results seem to point to an important destruction of hemin by hydroperoxide-derived free radicals in the case of those fish hemoglobin with lower hemin affinity.

All fish hemoglobins increased the amount of free hemin by incubation with *trans*-2-pentenal, but still pollock Hb originated the most rapid generation of free hemin (**Figure 5**). With regard to hemoglobins from horse mackerel and seabass, both showed a similar pattern for the release of hemin by interaction with *trans*-2-pentenal.

Prooxidant Capacity of Pollock, Horse Mackerel, and Seabass MetHb in Liposomes and Washed Minced Fish Muscle. The capacity of the metHb forms to promote lipid oxidation was compared with that found for free hemin and fish Hbs, mainly found in reduced state. All hemoglobin species and hemin were tested at the same heme molar concentration. In liposomes, metHb from pollock and horse mackerel catalyzed to a higher extent the formation of conjugated dienes than metHb from seabass (**Figure 6A**). Pollock metHb was notably the most effective in promoting the formation of secondary oxidation products (TBARS), whereas horse mackerel metHb showed an intermediate activity and metHb from seabass was found to be the less active in generating TBARS (**Figure 6B**). Pollock metHb exhibited a more elevated pro-oxidant activity in liposomes than pollock Hb (**Figure 6**), and the same behavior with stronger pro-oxidative capacity for metHb compared to the corresponding oxyHb was obtained for either horse mackerel or seabass Hb (data not shown). The results also demonstrated that free hemin is a weaker catalyst of lipid oxidation in liposomes than metHb species and pollack Hb (**Figure 6**).

The formation of primary and secondary lipid oxidation products, peroxides and TBARS, respectively, was monitored in washed minced fish muscle during incubation at 4 °C. The results showed a more elevated capacity of pollock metHb to promote either the formation of peroxides or TBARS in comparison with reduced pollock Hb (**Figure 7**). This tendency was also observed for horse mackerel and seabass Hb, and therefore, their

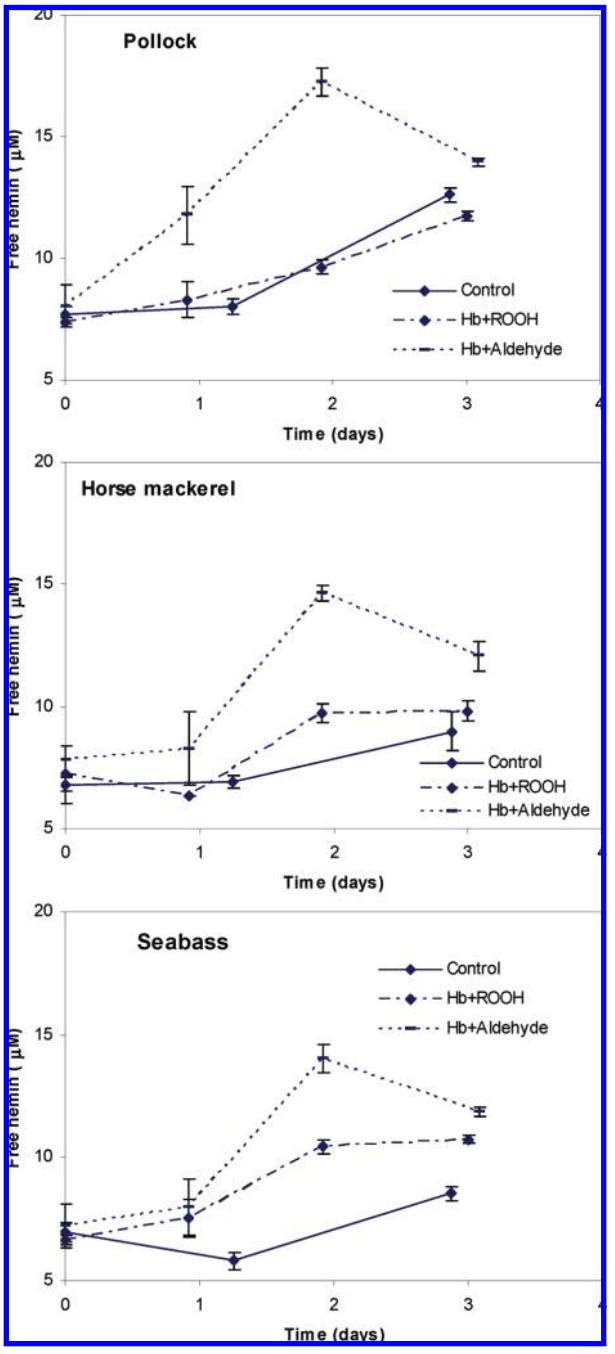

Figure 5. Hemin loss of Atlantic pollock, horse mackerel, and seabass Hb by incubation with linolein hydroperoxides and *trans*-2-pentenal. Fish Hb was diluted to 5 μM in 50 mM phosphate buffer, pH 6.8, and incubated at 4 °C. The final concentrations of linolein hydroperoxides and *trans*-2-pentenal were 125 and 1000 μM, respectively.

metHb forms were found to be more pro-oxidant in washed fish muscle than the corresponding oxyHb (data not shown). The relative pro-oxidant capacity of the fish metHb species was found to be similar to that achieved for the reduced hemoglobin forms in washed fish muscle, and so, pollack metHb was able to promote lipid oxidation to a higher extent, horse mackerel metHb showed an intermediate activity, and seabass metHb was found to be the weakest promoter of lipid oxidation in washed fish muscle (**Figure 7**). As a general rule, all metHb species were able to catalyze lipid oxidation to a higher extent than free hemin given that the formation of peroxides and TBARS was significantly slower in the case of free hemin. From the comparison with the

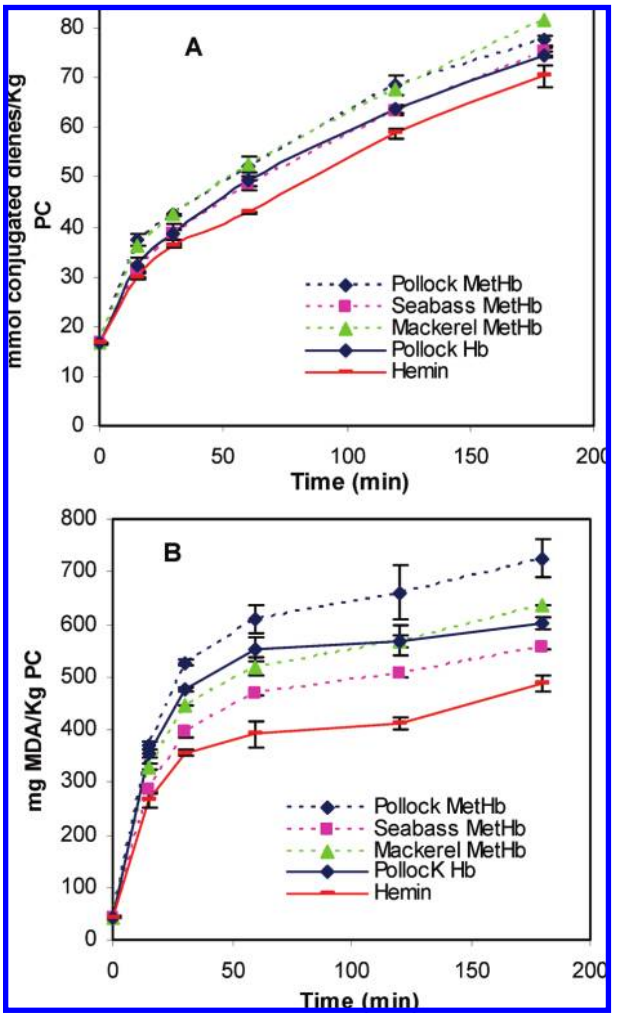

Figure 6. Pro-oxidative capacity of free hemin and metHb from Atlantic pollock, seabass, and horse mackerel in liposomes by monitoring the formation of conjugated dienes (**A**) and TBARS (**B**). Atlantic Pollock Hb was introduced as reference. Liposomes were prepared in 50 mM phosphate buffer at pH 6.8, supplemented with free hemin or metHb species at 3 μM (on Hb basis), and incubated at 30 °C.

reduced Hb forms, free hemin was more pro-oxidant than horse mackerel and seabass oxyHb (data not shown).

Spontaneous Hemin Loss from Pollock, Horse Mackerel, and Seabass MetHb. The affinity of the metHb forms by hemin was evaluated in 50 mM phosphate buffer, pH 6.8, and the net liberation of hemin during the incubation period at 4 °C is represented in **Figure 8**. Pollock metHb lost spontaneously a significant amount of hemin after 1 day of incubation, whereas hemin loss was not detected from horse mackerel and seabass metHb at the same period. The release of hemin continued to be more severe for pollack metHb during the initial 4 days but was similar to that found for horse mackerel metHb after 6−8 days of incubation (**Figure 8**). Seabass metHb was demonstrated to be the metHb species with the strongest affinity for hemin.

Native IEF Pattern of Pollock, Horse Mackerel, and Seabass Hemoglobins. To investigate the presence of different hemoglobin isoforms differing in isoelectric point (p*I*), native IEF on gel was performed on fish hemoglobins in the range of pH 3.5−9.5. The results revealed that pollock Hb included isoforms with high anodic character, which presented p*I* as acidic as 5.2 (**Figure 9**). Horse mackerel Hb contained at least one isoform more anodic than seabass Hb given that the most acidic isoforms of horse

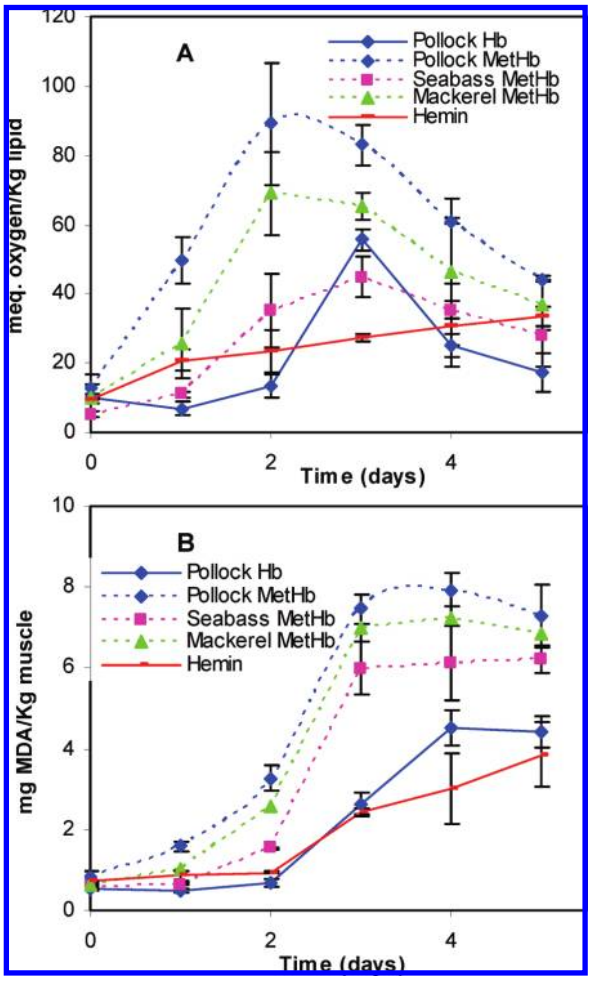

Figure 7. Pro-oxidative capacity of free hemin and metHb from Atlantic pollock, seabass, and horse mackerel in washed minced horse mackerel muscle by monitoring the formation of lipid peroxides (**A**) and TBARS (**B**). Washed minced muscle was supplemented with free hemin or metHb species at 3 μmol/kg of muscle (on Hb basis) and incubated at 4 °C.

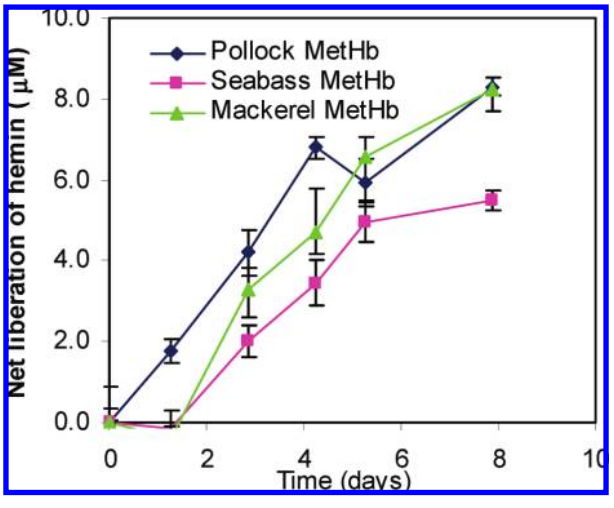

Figure 8. Spontaneous hemin loss of metHb from Atlantic pollock, seabass, and horse mackerel. MetHb was diluted to 5 μM in 50 mM phosphate buffer, pH 6.8, and incubated at 4 °C.

mackerel Hb and seabass Hb were found to possess p*I* values of 5.5 and 5.8, respectively. As general rule, the IEF bands corresponding to pollock Hb were much more poorly defined than

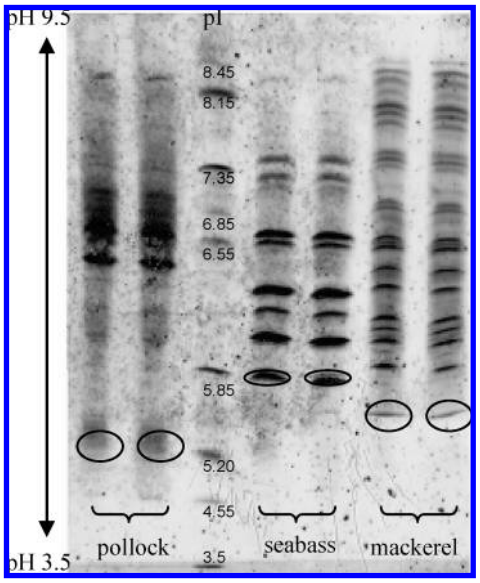

Figure 9. Native isoelectrofocusing (IEF) pattern of Atlantic pollock, seabass, and horse mackerel Hb. IEF electrophoresis was performed in the range of pH 3.5−9.5. Two lines were run with the corresponding fish Hb, and one line was run with the marker of isoelectric point (p*I*). Circles indicate Hb isoforms with the highest anodic character.

those for horse mackerel and seabass Hb. This could be attributed to a partial oxidative degradation of pollock Hb during the IEF, because pollock Hb was much less stable than other fish hemoglobins studied.

DISCUSSION

Pelagic species such as Atlantic mackerel and herring contain typically amounts of hemoglobin ranging from 3 to 7 μmol of hemoglobin/kg of light muscle (*5*, *20*), whereas cod, a representative whitefish species, possesses up to 0.2 μmol of hemoglobin/kg of muscle (*20*). Different investigations have demonstrated that fish hemoglobins catalyze extensively lipid oxidation at those hemoglobin levels found in pelagic species (*5*, *21*, *22*), and such pro-oxidative activity of hemoglobin displays a positive concentration dependence in either washed fish muscle (*5*, *23*) or fish membranes (*6*). Conversely, the incorporation of added fish triacylglycerols did not significantly enhance rates of hemoglobin-mediated lipid oxidation in washed cod muscle, the lipid substrate of which is mainly formed by phospholipids (*23*). Previous investigations by Igene et al. (*24*) reported major contributions of the phospholipid fraction in the development of rancidity in meat model systems. Therefore, the elucidation of the inherent factors of hemoglobin responsible of its pro-oxidative activity should be valuable information to develop a new generation of antioxidant strategies to inhibit lipid oxidation in meat-based foods.

Our results clearly indicate a higher pro-oxidant potential for pollock Hb than for seabass Hb in washed fish muscle, whereas hemoglobin from horse mackerel has an intermediate activity (**Figure 2**). In liposomes, there are much lower differences among the activities of fish hemoglobins, but still, pollack Hb and seabass Hb were the strongest and poorest promoters of lipid oxidation, respectively (**Figure 1**). Undeland et al. (*21*) have shown a much higher catalytic activity promoting lipid oxidation in a washed fish muscle for pollock Hb, followed in decreasing order by mackerel > menhaden > flounder. Richards et al. (*22*) have reported that mackerel and herring hemoglobins oxidized washed fish muscle more rapidly as compared to trout

hemoglobin. Trout hemoglobin has also exhibited a more elevated pro-oxidative ability than hemoglobin from tilapia (*Oreochromis niloticus*), a warm-water fish species (*25*). Most of these studies have related the pronounced pro-oxidant capacity of fish hemoglobins with high autoxidation rates and poor oxygenation, and the present investigation points to the same tendency. The relative deoxygenation and autoxidation rate followed the order pollock Hb > horse mackerel Hb > seabass Hb (**Table 1** and **Figure 3**), according to the high content in anodic isoforms for pollock Hb (**Figure 9**). It has been previously described that anodic isoforms have poor oxygen affinity at the pH values normally found in post-mortem fish muscle (*26*) and that the deoxygenation state makes the ferrous Hb molecule less compact (*27*, *28*). In addition, the present investigation has also revealed an extensive spontaneous liberation of hemin for those fish Hbs possessing greater pro-oxidative activity (pollock Hb > horse mackerel Hb > seabass Hb). Therefore, the capacity to catalyze lipid oxidation appears to be related to at least two inherent factors of fish Hb, rapid formation of metHb and unforced hemin liberation, which are directly involved in two principal pro-oxidative mechanisms: (i) the formation of highly oxidizing ferryl Hb radicals and (ii) the generation of free radicals through lipid hydroperoxide decomposition. The similar relative susceptibility of fish Hb to be oxidized to metHb and to release hemin can be explained by the poor hemin affinity observed for metHb species (**Figure 8**).

The present investigation is new evidence of a much higher pro-oxidative activity for metHb species compared to the corresponding reduced Hb forms and free hemin (**Figures 6** and **7**). It is in concordance with a previous study that showed more severe promotion of lipid oxidation for met forms of recombinant wild-type sperm whale Mb than for the ferrous forms in washed cod muscle (*29*). Additionally, the same research demonstrated that hemin alone was less effective as a lipid oxidation promoter than hemin in the presence of bovine serum albumin. Taking into consideration that metHb does not exhibit superior capacity than ferrous Hb to generate free radicals through hydroperoxide fragmentation (*9*), the stronger capacity of metHb to promote lipid oxidation is hypothesized to be due to a more significant efficiency to produce ferryl Hb radical species, which is able to initiate lipid peroxidation by direct abstraction of a hydrogen atom from a PUFA. Reeder and Wilson (*30*) demonstrated that metMb reacts with the lipid hydroperoxide hydroperoxyoctadecadienoic acid to render mainly the ferryl Mb form, whereas changes for the oxyMb are more complex, with ferrous, ferric, and ferryl species present. The stronger pro-oxidative ability observed for metHb than for free hemin appears to indicate a more decisive contribution of the metHb formation on the pro-oxidative activity of hemoglobin. However, the liberation of hemin may have a central role at the initial stages of lipid oxidation, considering the rapid and extensive promotion of free radicals by free hemin under conditions of low hydroperoxide/hemin ratios (*9*). Indeed, free hemin was significantly more effective than the most pro-oxidant Hb, pollock Hb, in catalyzing the formation of lipid peroxides during the initial phase of lipid oxidation in washed fish muscle (**Figure 7A**).

An important achievement of the present study was to elucidate the influence of two representative primary and secondary lipid oxidation byproducts on metHb formation and hemin liberation. Lipid oxidation generates a wide range of secondary aldehyde products including *n*-alkanals, *trans*-2-alkenals, 4-hydroxy-*trans*-2-alkenals, and malonaldehyde (*31*). The volatile *trans*-2-pentenal has been detected as a lipid oxidation product either in fish oil enriched emulsion (*32*) or in fish muscle (*33*). The interaction of the fish hemoglobins with either *trans*-2-pentenal or linolein hydroperoxides significantly accelerates the formation of metHb without modifying the relative redox stability of hemoglobins (**Figure 4**). Hemin liberation is stimulated by the presence of *trans*-2-pentenal (**Figure 5**), whereas linolein hydroperoxides induce lower levels of free hemin, probably due to the facility of the free radicals generated during the hemin/hydroperoxide interaction to decompose free hemin (*9*). The generation of hydroperoxide-derived free radicals can also explain the superior destabilizing effects on Hb redox state by incubation with linolein hydroperoxides instead of *trans*-2-pentenal (**Figure 4**). With regard to the reaction between aldehyde products and heme proteins, Faustman et al. (*34*) revealed that monounsaturated aldehydes more actively accelerate metMb formation than saturated aldehydes. By using 4-hydroxynonenal (HNE) as a model, Faustman and co-workers (*34*, *35*) have demonstrated that α,β-unsaturated aldehydes are able to modify covalently bovine and equine oxyMb through Michael addition in multiple histidine residues.

To conclude, the capacity of fish hemoglobins to catalyze lipid oxidation was found to be directly related with their intrinsic redox instability in terms of metHb formation and hemin liberation: pollock Hb > horse mackerel Hb > seabass Hb. Representative primary and secondary lipid oxidation byproducts, linolein hydroperoxides and *trans*-2-pentenal, are proposed as activators of the hemoglobin-catalyzed lipid oxidation, considering their ability to accelerate metHb formation and hemin liberation from hemoglobin. The present investigation puts emphasis on the importance of controlling metHb formation and hemin loss to design more successful treatments against hemoglobin-promoted lipid oxidation.

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LITERATURE CITED

(1) Frankel, E. N. *Lipid Oxidation*; The Oily Press: Dundee, Scotland, 1998.
(2) Erickson, M. C. Lipid oxidation of muscle foods. In *Food Lipids. Chemistry, Nutrition, and Biotechnology*; Akoh, C. C., Min, D. B., Eds.; Dekker: New York, 2002; pp 365−411.
(3) Richards, M. P.; Dettman, M. A. Comparative analysis of different hemoglobins: Autoxidation, reaction with peroxide, and lipid oxidation. *J. Agric. Food Chem.* **2003**, *51*, 3886–3891.
(4) Richards, M. P.; Modra, A. M.; Li, R. Role of deoxyhemoglobin in lipid oxidation of washed cod muscle mediated by trout, poultry and beef hemoglobins. *Meat Sci.* **2002**, *62*, 157–163.
(5) Richards, M. P.; Hultin, H. O. Contribution of blood and blood components to lipid oxidation in fish muscle. *J. Agric. Food Chem.* **2002**, *50*, 555–564.
(6) Pazos, M.; Medina, I.; Hultin, H. O. Effect of ph on hemoglobin-catalized lipid oxidation in cod muscle membranes in vitro and in situ. *J. Agric. Food Chem.* **2005**, *53*, 3605–3612.
(7) O'brien, P. J. Intracellular mechanisms for the decomposition of a lipid peroxide. I. Decomposition of a lipid peroxide by metal ions, heme compounds and nucleophiles. *Can. J. Biochem.* **1969**, *47*, 485–492.
(8) Pazos, M.; Lois, S.; Torres, J. L.; Medina, I. Inhibition of hemoglobin- and iron-promoted oxidation in fish microsomes by natural phenolics. *J. Agric. Food Chem.* **2006**, *54*, 4417–4423.
(9) Pazos, M.; Andersen, M. L.; Skibsted, L. H. Heme-mediated production of free radicals via preformed lipid hydroperoxide fragmentation. *J. Agric. Food Chem.* **2008**, *56*, 11478–11484.
(10) Kanner, J. Oxidative processes in meat and meat products quality implications. *Meat Sci.* **1994**, *36*, 169–189.
(11) Richards, M. P.; Hultin, H. O. Effect of pH on lipid oxidation using trout hemolysate as a catalyst: a possible role for deoxyhemoglobin. *J. Agric. Food Chem.* **2000**, *48*, 3141–3147.

(12) Svistunenko, D. A.; Dunne, J.; Fryer, M.; Nicholls, P.; Reeder, B. J.; Wilson, M. T.; Bigotti, M. G.; Cutruzzola, F.; Cooper, C. E. Comparative study of tyrosine radicals in hemoglobin and myoglobins treated with hydrogen peroxide. *Biophys. J.* **2002**, *83*, 2845–2855.
(13) Brown, W. D. Chromatography of myoglobin on diethylaminoethyl cellulose columns. *J. Biol. Chem.* **1961**, *236*, 2238–2240.
(14) Winterbourn, C. C. Oxidative reaction of hemoglobin. In *Methods in Enzymology. Oxygen Radicals in Biological Systems*; Packer, L., Glazer, A. N., Eds.; Academic Press: London, U.K., 1990; Vol. *186*, pp 265−272.
(15) Huang, S. W.; Frankel, E. N. Antioxidant activity of tea catechins in different lipid systems. *J. Agric. Food Chem.* **1997**, *45*, 3033–3038.
(16) Frankel, E. N.; Huang, S. W.; Kanner, J.; German, J. B. Interfacial phenomena in the evaluation of antioxidants: bulk oils vs emulsions. *J. Agric. Food Chem.* **1994**, *42*, 1054–1059.
(17) Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. In *Methods in Enzymology: Biomembranes. Specialiced Techniques*; Fleischer, S., Packer, L., Eds.; Academic Press: New York, 1978; Vol. *52*, pp 302−310.
(18) Chapman, R. A.; Mackay, K. The estimation of peroxides in fats and oils by the ferric thiocyanate method. *J. Am. Oil Chem. Soc.* **1949**, *26*, 360–363.
(19) McDonald, R. E.; Hultin, H. O. Some characteristics of the enzymic lipid peroxidation system in the microsomal fraction of flounder skeletal muscle. *J. Food Sci.* **1987**, *52*, 15–21.
(20) Larsson, K.; Almgren, A.; Undeland, I. Hemoglobin-mediated lipid oxidation and compositional characteristics of washed fish mince model systems made from cod (*Gadus morhua*), herring (*Clupea harengus*), and salmon (*Salmo salar*) muscle. *J. Agric. Food Chem.* **2007**, *55*, 9027–9035.
(21) Undeland, I.; Kristinsson, H. G.; Hultin, H. O. Hemoglobin-mediated oxidation of washed minced cod muscle phospholipids: effect of pH and hemoglobin source. *J. Agric. Food Chem.* **2004**, *52*, 4444–4451.
(22) Richards, M. P.; Hultin, H. O. Effects of added hemolysate from mackerel, herring and rainbow trout on lipid oxidation of washed cod muscle. *Fish. Sci.* **2003**, *69*, 1298–1300.
(23) Undeland, I.; Hultin, H. O.; Richards, M. P. Added triacylglycerols do not hasten hemoglobin-mediated lipid oxidation in washed minced cod muscle. *J. Agric. Food Chem.* **2002**, *50*, 6847–6853.
(24) Igene, J. O.; Pearson, A. M. Role of phospholipids and triglycerides in warmed-over flavor development in meat model systems. *J. Food Sci.* **1979**, *44*, 1285–1290.
(25) Richards, M. P.; Nelson, N. M.; Kristinsson, H. G.; Mony, S. S. J.; Petty, H. T.; Oliveira, A. C. M. Effects of fish heme protein structure and lipid substrate composition on hemoglobin-mediated lipid oxidation. *J. Agric. Food Chem.* **2007**, *55*, 3643–3654.
(26) Harrington, J. P. Structural and functional studies of the king salmon, *Oncorhynchus tshawytscha*, hemoglobins. *Comp. Biochem. Physiol. Part B* **1986**, *84*, 111–116.
(27) Stryer, L. Oxygen-transporting proteins: myoglobin and hemoglobin. In *Biochemistry*; Freeman, W. H., Ed.; New York, 1988; pp 143−176.
(28) Richards, M. P.; Ostdal, H.; Andersen, H. J. Deoxyhemoglobin-mediated lipid oxidation in washed fish muscle. *J. Agric. Food Chem.* **2002**, *50*, 1278–1283.
(29) Grunwald, E. W.; Richards, M. P. Mechanisms of heme protein-mediated lipid oxidation using hemoglobin and myoglobin variants in raw and heated washed muscle. *J. Agric. Food Chem.* **2006**, *54*, 8271–8280.
(30) Reeder, B. J.; Wilson, M. T. Mechanism of reaction of myoglobin with the lipid hydroperoxide hydroperoxyoctadecadienoic acid. *Biochem. J.* **1998**, *330*, 1317–1323.
(31) Esterbauer, H.; Schaur, R. J.; Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biol. Med.* **1991**, *11*, 81–128.
(32) Iglesias, J.; Lois, S.; Medina, I. Development of a solid-phase microextraction method for determination of volatile oxidation compounds in fish oil emulsions. *J. Chromatogr., A* **2007**, *1163*, 277–287.
(33) Iglesias, J.; Medina, I. Solid-phase microextraction method for the determination of volatile compounds associated to oxidation of fish muscle. *J. Chromatogr., A* **2008**, *1192*, 9–16.
(34) Faustman, C.; Liebler, D. C.; McClure, T. D.; Sun, Q. α,β-unsaturated aldehydes accelerate oxymyoglobin oxidation. *J. Agric. Food Chem.* **1999**, *47*, 3140–3144.
(35) Suman, S. P.; Faustman, C.; Stamer, S. L.; Liebler, D. C. Proteomics of lipid oxidation-induced oxidation of porcine and bovine oxymyoglobins. *Proteomics* **2007**, *7*, 628–640.

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