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ARTICLE

Capacity of Reductants and Chelators To Prevent Lipid Oxidation Catalyzed by Fish Hemoglobin

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The efficiency of different reductants (reduced glutathione, ascorbic acid, and catalase) and metal chelators [ethylenediaminetetraacetic acid (EDTA), citric acid, sodium tripolyphosphate (STPP), and adenosine-5′-triphosphate (ATP)] to inhibit lipid oxidation promoted by fish hemoglobin was investigated. The inhibitory activity on hemoglobin-catalyzed lipid oxidation was also evaluated for grape oligomeric catechins (proanthocyanidins), which have both reducing and chelating properties. The antioxidant activity was studied in two different lipid oxidation models, liposomes and washed minced fish muscle. Grape proanthocyanidins were found to be significantly more effective than other reductants to prevent hemoglobin-mediated lipid oxidation in both liposomes and washed fish muscle. Reduced glutathione was also efficient to retard lipid oxidation at the same molarity in washed fish muscle, whereas catalase and ascorbic acid showed a lower antioxidant activity. Metal chelators were less active than reductants, and consequently, the former were necessarily evaluated at much higher concentration than grape proanthocyanidins and reducing compounds. STPP was found to be the iron chelator with the strongest efficiency to delay hemoglobin-mediated lipid oxidation followed by EDTA. Citric acid and ATP were ineffective in retarding lipid oxidation in both systems. Grape proanthocyanidins provided the most extensive protection to preserve hemoglobin at ferrous state in washed fish muscle. Our results draw attention to the greater capacity of reducing compounds to prevent fish hemoglobin-mediated lipid oxidation in comparison with iron chelators, suggesting that the free radical scavenging and/or reduction of ferrylHb species are crucial actions to avoid the pro-oxidant capacity of fish hemoglobin.

KEYWORDS: Lipid oxidation; fish hemoglobin; antioxidant; reductant; iron chelator; grape proanthocyanidins

INTRODUCTION

Lipid oxidation is a principal cause of quality deterioration and shelf life shortening in seafood products, having a negative effect on flavor, color, texture, nutritive value, and safety during storage and processing (*1*). The vulnerability of fish muscle to develop lipid oxidation is partially attributed to the coexistence of high proportions of easily oxidiziable polyunsaturated fatty acids (PUFAs) with catalytic amounts of heme proteins and redox-active metals, which are able to initiate and propagate oxidative degradations on PUFAs (*2*, *3*).

It is described that dark-muscle fish species have higher hemoglobin levels than lean fish species (*4*). Hemoglobin has been found to be the predominant heme protein in mackerel light muscle (6 μmol of hemoglobin/kg) and trout whole muscle (11 μmol of hemoglobin/kg), while myoglobin and hemoglobin are abundantly present in mackerel dark muscle (*5*). Fish hemoglobin has demonstrated the capacity to trigger lipid oxidation in fish membranes (*6*, *7*), liposomes (*8*), and also in a matrix similar to fish muscle but devoid of hemoglobin such as washed fish muscle (*5*, *8*, *9*).

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Several pathways have been proposed to contribute to the pro-oxidant activity of hemoglobin: (i) generation of chain-propagating free radicals (peroxyl, alkoxyl, etc.) through fragmentation of lipid hydroperoxides (*10*); (ii) abstraction of hydrogen atoms from PUFAs by hypervalent ferrylHb species, which can be formed by reaction of ferric metHb with hydrogen peroxide (H_2O_2) or lipid hydroperoxides (*3*); and (iii) release of inorganic iron ions and hemin (oxidized form of heme group) from hemoglobin (*11*). Ferrous ions are well-recognized lipid oxidation promoters through the Fenton reaction to produce the highly oxidizing hydroxyl radical in the presence of H_2O_2 (*3*). Hemin, ferrous ions, and, to a lesser extent, ferric ions can also activate lipid oxidation by decomposition of preformed lipid hydroperoxides to generate free radicals (*10*, *12*). A previous study revealed that different lipid oxidation byproducts (hydroperoxides and aldehydes) accelerate hemoglobin oxidation to metHb and hemin release and that fish hemoglobins with stronger pro-oxidant capacities are those with weaker resistance to undergo oxidation to metHb and hemin loss (*8*). The coexistence of multiple pro-oxidant mechanisms that are influenced by the dynamic formation of lipid oxidation byproduct adds complexity to the pro-oxidant scheme of hemoglobin.

The supplementation of bioactive compounds with antioxidant properties is an emerging strategy to inhibit undesirable lipid

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oxidative reactions catalyzed by hemoglobin in meat-based food. An important activity to retard fish hemoglobin-catalyzed lipid oxidation has recently been recognized for compounds of diverse sources, such as press juice from different skeletal muscle (*13*, *14*), cranberry concentrate juice powder (*15*), white grape dietary fiber (*16*), and white grape pomace (*7*). However, more research is required to get comprehensible information about which of the pro-oxidative mechanisms attributed to hemoglobin have major contributions in muscle-based foods and about which are the essential physicochemical properties to formulate effective antioxidant additives to prevent hemoglobin-promoted lipid oxidation.

The aim of the present paper was to get more insight on efficient procedures based on antioxidant additives to prevent hemoglobin-mediated lipid oxidation. Several compounds bearing reducing capacity (reduced glutathione (GSH), ascorbic acid, and catalase) or iron-chelating ability [ethylenediaminetetraacetic acid (EDTA), citric acid, sodium tripolyphosphate (STPP), and adenosine-5′-triphosphate (ATP)] and both reducing and chelating properties (grape proanthocyanidins) were evaluated as antioxidants against fish hemoglobin-catalyzed lipid oxidation. The antioxidant activity was investigated in two different models, liposomes and washed minced fish muscle, which are considered adequate to investigate the pro-oxidant capacity of hemoglobin since both are highly concentrated in membrane lipids, primary substrates of lipid oxidation, and are devoid of hemoglobin. Atlantic Pollock Hb was selected to trigger oxidation due to its extensive pro-oxidative activity in both systems (*8*), with the purpose of evaluating antioxidant abilities under drastic oxidative conditions. The effect of reductants and iron chelators on hemoglobin redox stability was also studied by measuring the loss of redness in washed fish muscle. Finally, the capacity of grape proanthocyanidins to avoid hemoglobin autoxidation was evaluated in vitro.

MATERIALS AND METHODS

Materials. Atlantic pollock (*Pollachius pollachius*) and horse mackerel (*Trachurus trachurus*) were obtained fresh in the rigor state from a local market. Bovine hemoglobin, sodium heparin, sodium chloride, 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 a 30% L-α-phosphatidylcholine, thiobarbituric acid, trichloroacetic acid, 1,1,3,3-tetraethoxypropane (TEP), butylated hydroxytolune (BHT), $FeCl_2 \cdot 4H_2O$, $FeCl_3 \cdot 6H_2O$, streptomycin sulfate, ascorbic acid, catalase, GSH, citric acid, EDTA, ATP, STPP, L-histidine, and potassium chloride (KCl) were purchased from Sigma (Steinheim, Germany). Carbon monoxide (99.97% purity) was provided by Air Liquide (Porriño, Spain). Grape proanthocyanidins were kindly supplied by Dr. M. J. Nuñez (Universidad de Santiago de Compostela). All chemicals and solvents used were either analytical or high-performance liquid chromatography grade (Merck, Darmstadt, Germany).

Grape Proanthocyanidins. A grape fraction rich in oligomeric catechins (proanthocyanidins) was prepared by fractionation of a commercial grape seed extract (Le Grandonnenque, Cruviers-Lascours, France) that contained 40% proanthocyanidins. Fractionation was performed by size exclusion chromatography based on Toyopearl resin as previously described Torres et al. (*17*). Grape proanthocyanidins were characterized in terms of mean molecular weight, mean polymerization degree, and percentage of galloylation (presence of esters with galloyl moieties) by depolymerization with cysteamine according with Torres and Lozano (*18*). Mean molecular weight, polymerization degree, and galloylation porcentage were found to be 760 g/mol, 2.4 units, and 15%, respectively.

Fish Hemoglobin Extraction. Blood was obtained from the caudal vein with a transfer glass pipet rinsed with 150 mM NaCl and sodium heparin solution (30 units/mL) after cutting off the tail of Atlantic pollock in the rigor state. In the experiments conducted to evaluate in vitro a potential effect of grape proanthocyanidins on redox stability of fish hemoglobin, blood was isolated from horse mackerel since more stable fish hemoglobin was required. Blood was immediately mixed with approximately 1 volume of the saline sodium heparin solution, and hemolysate was then prepared according to Richards and Hultin (*19*). Either pollock or horse mackerel hemoglobin was predominantly oxygenated as indicated the appearance of two well-defined absorption peaks at approximately 540 and 570 mn, which are attributed to oxygenated Hb (OxyHb). Hemoglobin was stored at −80 °C and thawed just before used.

Determination of Hemoglobin Concentration. Hemoglobin was quantified according to Brown (*20*). Briefly, hemoglobin was diluted in 50 mM Tris buffer, pH 8.6, 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. A standard curve was built by relating the absorbance at the peak (≈416 nm) minus the absorbance at the valley (465 nm) with known concentrations of commercial hemoglobin from bovine blood.

Effect of Grape Proanthocyanidins on in Vitro Redox Stability of Fish Hemoglobin. The experiments were conducted by incubating 10 μM horse mackerel Hb with 21 μM grape proanthocyanidins in a 5 mM L-histidine buffer with 0.12 M KCl, pH 6.8, at 4 °C. These concentrations were selected to investigate the effect of grape polyphenols in a hemoglobin/polyphenolic molar ratio similar to that found when pelagic fish muscle (5−18 μmol Hb/kg muscle) is supplemented with an effective concentration of grape proanthocyanidins (50 ppm or 57 μmol/kg muscle) (*21*). The redox stability of hemoglobin was evaluated by monitoring the spectral changes suffered by hemoglobin in the absence or the presence of grape proanthocyanidins. The formation of metHb was calculated by adaptation of the Winterbourn's equation (*22*) that estimates the concentration of metHb in μM (on hemoglobin basis) considering the absorbance at 576 and 630 nm:

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

Inhibition of Hemoglobin-Catalyzed Lipid Oxidation in Liposomes. Liposomes were prepared by adaptation of a procedure previously described by Huang and Frankel (*23*). Briefly, lecithin from soybean was dissolved in a concentration of 0.8% (w/w) by magnetic stirring in the buffer solution and subsequently subjected to sonication for 10 min in a water bath ultrasonicator (P-Selecta, Barcelona, Spain). The liposomes were prepared in 50 mM phosphate buffer, pH 6.8, when reductants and grape proanthocyanidins were evaluated. In the case of the experiments conducted to test iron chelators, a buffer solution containing 5 mM L-histidine and 0.12 M KCl, pH 6.8, was employed to avoid the chelating ability of the phosphate buffer. GSH, ascorbic acid, and grape proanthocyanidins were added at 30 μM. Catalase was employed at a concentration of 200 units/L (1 unit decomposes 1 μmol H_2O_2/min). The chelating compounds (EDTA, citric acid, STPP, and ATP) were evaluated at a much higher concentration (1 mM) because of their lower inhibitory activity. Liposomes were incubated at 30 °C in a thermostatic water bath, and lipid oxidation was initiated by adding 3 μM pollock Hb. The formation of conjugated dienes and thiobarbituric acid reactive substances (TBARS) was used to monitor the development of lipid oxidation.

Inhibition of Hemoglobin-Catalyzed Lipid Oxidation in Washed Minced Fish Muscle. Washed minced muscle was prepared from light muscle of horse mackerel by the procedure described by Richards and Hultin (*5*). 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. In those experiments conducted to test chelating compounds, the phosphate buffer was substituted by a buffer solution containing 0.12 M KCl and 5 mM L-histidine, pH 6.8, to avoid the chelating ability of the phosphate buffer. Washed fish muscle was supplemented with ascorbic acid, GSH, and grape proanthocyanidins at 60 μmol/kg muscle. Catalase was employed at a concentration of 40000 units/kg muscle (1 unit decomposes 1 μmol H_2O_2/min). The chelating agents (EDTA, citric acid, STPP, and ATP) were added at 2 mmol/kg muscle. Lipid oxidation was initiated by adding 3 μmol pollock Hb per kg muscle and monitored by peroxide value (PV) and TBARS during storage at 4 °C. The loss of redness was also investigated as an index for hemoglobin redox stability. The final moisture of the washed fish muscle ranged from 80 to 84%, and the lipid content was 0.7−0.8%.

Loss of Redness. Changes in the redness (a^* value) of washed fish muscle supplemented with Hb were measured using a colorimeter Chroma

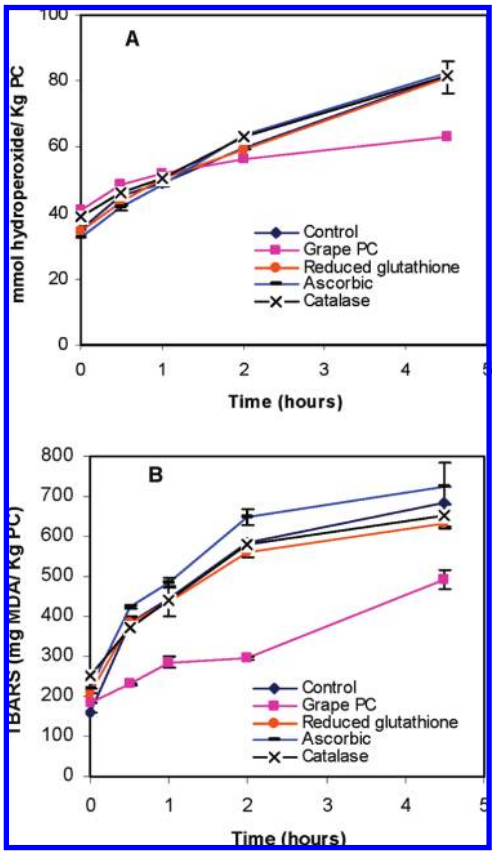

Figure 1. Antioxidant effect of reductants (GSH, ascorbic acid, and catalase) and grape proanthocyanidins on hemoglobin-mediated lipid oxidation in liposomes. GSH, ascorbic acid, and the grape proanthocyanidins were added at 30 μM, while catalase was supplemented at 200 units/L. Lipid oxidation was triggered by adding 3 μM pollock Hb and was monitored by following the formation of conjugated dienes (**A**) and TBARS (**B**) during incubation at 30 °C.

Meter CR-200 (Minolta Corp., Nieuwegein, Netherlands). The instrument was first calibrated using a white Minolta calibration plate. Redness (a^* value) was measured with approximately 1 g of washed muscle placed on a glass microslide and covered up with a cover glass.

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). Conjugated dienes were expressed as mmol hydroperoxides/kg phosphatidylcholine (PC) as previously described by Frankel and co-workers (*24*).

PV. PV was determined in washed minced muscle using an adaptation of the ferric thiocyanate method (*25*) by Buege and Aust (*26*). PV was expressed in milliequivalents (mequiv) of oxygen per kg of lipid.

TBARS Analysis. TBARS analysis was performed in liposomes and washed minced muscle according to the method by McDonald and Hultin (*27*). A standard curve was built by using TEP as standard. The results are expressed in terms of mg malonaldehyde (MDA) per kg of phosphatidylcholine (PC) or muscle, respectively, in liposomes and washed fish muscle.

Statistical Analysis. The experiments were performed twice, and data are reported as means ± standard deviations of three replicates (n = 3). The data were analyzed by one-way analysis of variance (ANOVA) and the least-squares difference method. Differences were considered as statistically significant for a confidential interval of 95% ($p < 0.05$). Statistical analyses were performed with the software Statistica 6.0.

RESULTS

Antioxidant Activity of Reductants and Grape Proanthocyanidins on Hemoglobin-Mediated Lipid Oxidation. The results revealed a stronger efficiency of grape proanthocyanidins to inhibit the pollock hemoglobin-mediated formation of conjugated dienes in liposomes at the propagation stages of lipid oxidation, while GSH, catalase, and ascorbic acid were not active at the same molar concentration (30 μM) (**Figure 1A**). Grape proanthocyanidins also exhibited a significant reduction of TBARS formation during the entire monitoring time (**Figure 1B**). However, the supplementation of liposomes with GSH and catalase was ineffective to retard the generation of TBARS, while ascorbic acid resulted to some extent pro-oxidant by promoting the formation of TBARS.

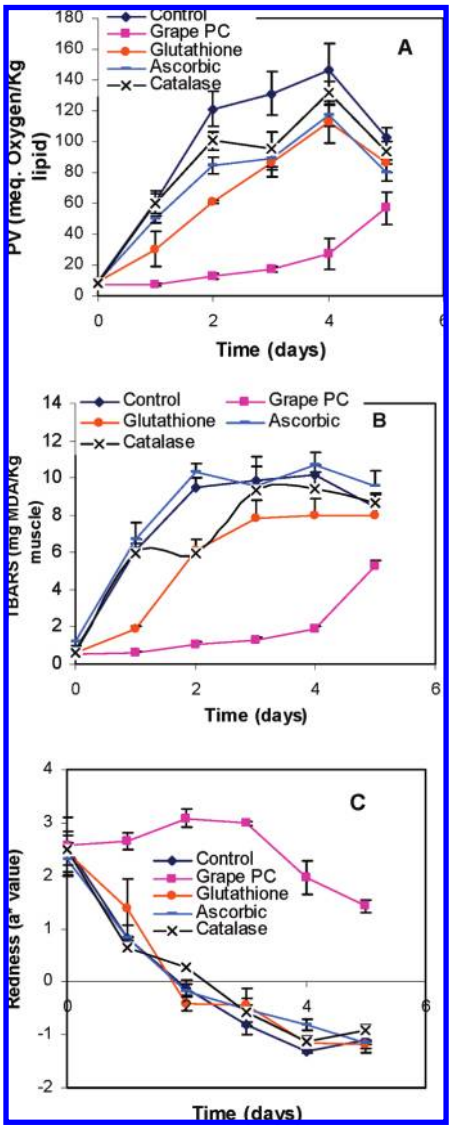

Figure 2. Antioxidant effect of reductants (GSH, ascorbic acid, and catalase) and grape proanthocyanidins on hemoglobin-mediated lipid oxidation in washed minced horse mackerel muscle. GSH, ascorbic acid, and grape polyphenols were added at 60 μmol/kg muscle, while catalase was supplemented at 40000 units/kg muscle. Lipid oxidation was triggered by adding 3 μmol of pollock Hb/kg muscle and was monitored by following the PV, TBARS, and the loss of redness (a^* value) during incubation at 4 °C.

Grape proanthocyanidins also exhibited the strongest inhibitory capacity on hemoglobin-promoted lipid oxidation in washed fish muscle (**Figure 2**). Washed fish muscle supplemented with grape proanthocyanidins (60 μmol/kg muscle) showed induction periods for the formation of lipid peroxides and TBARS of approximately 4 days, while control muscle without the addition of exogenous reducing compounds apparently did not exhibit induction periods for the formation of those lipid oxidation

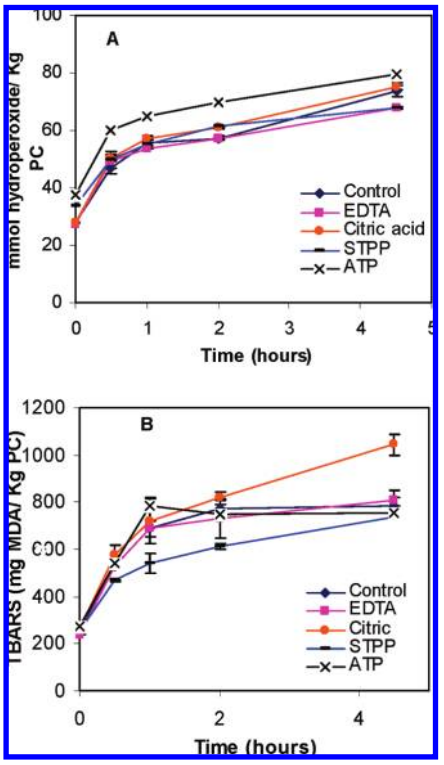

Figure 3. Antioxidant effect of iron chelators (EDTA, citric acid, STPP, and ATP) on hemoglobin-mediated lipid oxidation in liposomes. All chelating compounds were employed at 1 mM. Lipid oxidation was triggered by adding 3 μM pollock Hb and was monitored by following the formation of conjugated dienes and TBARS during incubation at 30 °C.

products. The other reductants were not able to increase induction periods, with the exception of GSH that increased the induction period for the formation of TBARS up to 1 day (**Figure 2B**). GSH was also active to decrease the formation of peroxides and TBARS during the propagation of lipid oxidation. Ascorbic acid and catalase reduced to some extent the formation of peroxides, but they were not able to diminish the generation of TBARS. The effect of reductants on the oxidation rate of hemoglobin was monitored in washed fish muscle by the loss of redness. The results indicated a drastic attenuation of the redness at day 1 for control samples, whereas fish muscle supplemented with grape proanthocyanidins was able to maintain redness at initial values after 3 days of storage (**Figure 2C**). However, GSH, ascorbic acid, and catalase were not active to retard the loss of redness and, therefore, hemoglobin oxidation. In summary, the relative capacity of reductants to inhibit hemoglobin-promoted lipid oxidation in washed fish muscle followed the order grape proanthocyanidins $\gg$ GSH $>$ ascorbic acid $\approx$ catalase.

Antioxidant Activity of Iron Chelators on Hemoglobin-Mediated Lipid Oxidation. Different iron chelators (EDTA, citric acid, STPP, and ATP) were evaluated as antioxidants to control the pro-oxidative action of the inorganic iron released from hemoglobin. The chelating compounds were tested in a wide concentration range in liposomes, and even levels of 1 mM scarcely delayed lipid oxidation in terms of formation of conjugated dienes and TBARS (**Figure 3**). STPP has shown a significant ability to diminish the formation of TBARS during the propagation period, but this chelator was found ineffective to prevent the generation of conjugated dienes. The other chelating compounds were not active to inhibit the formation of neither conjugated dienes nor TBARS.

In washed fish muscle, STPP was also found to be significantly more effective to delay the formation of lipid peroxides, followed

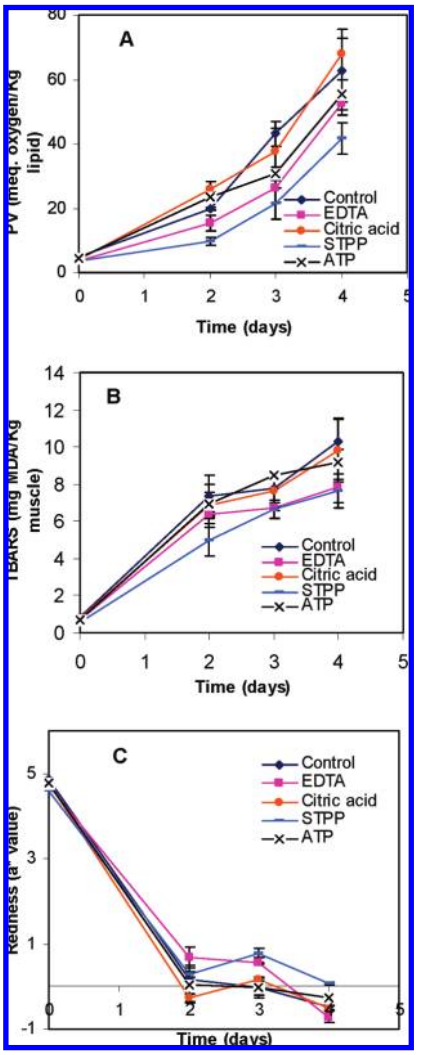

Figure 4. Antioxidant effect of iron chelators (EDTA, citric acid, STPP, and ATP) on hemoglobin-mediated lipid oxidation in washed minced horse mackerel muscle. Chelating compounds were added at 2 mmol/kg muscle. Lipid oxidation was triggered by adding 3 μmol of pollock Hb/kg muscle and was monitored by following the PV, TBARS, and the loss of redness (a^* value) during incubation at 4 °C.

in decreasing order of efficiency by EDTA (**Figure 4A**). The formation rates of TBARS have also indicated lower propagation of lipid oxidation in fish muscle supplemented with STPP and EDTA. Citric acid and ATP were ineffective to prevent the formation of both lipid oxidation products, lipid peroxides, and TBARS. Regarding a potential influence of iron chelators on redox stability of hemoglobin, chelators used were not able to retard the rapid loss of redness observed between days 0 and 2. However, fish muscle supplemented with STPP and EDTA showed a significantly higher redness value than control samples at days 2 and 3 (**Figure 4C**).

Effect of Grape Proanthocyanidins on in Vitro Redox Stability of Fish Hemoglobin. The influence of proanthocyanidins on hemoglobin autoxidation rate was evaluated in vitro under hemoglobin/polyphenolic molar ratios similar to those found when pelagic fish muscle is supplemented with an effective concentration of grape proanthocyanidins (50 ppm or 57 μmol/kg muscle). Horse mackerel was used as the hemoglobin source because it has a higher redox stability than pollock Hb (*8*); therefore, horse mackerel Hb was considered more adequate to distinguish potential protective/harmful effects of grape proanthocyanidins

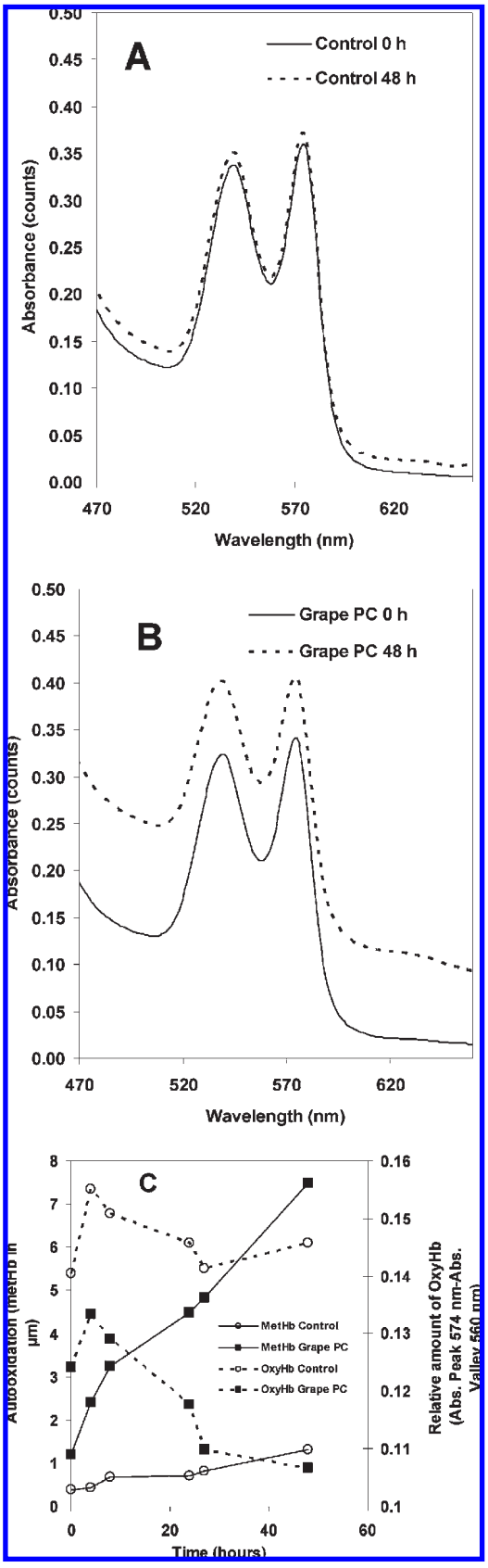

Figure 5. Spectral changes of horse mackerel Hb incubated in the absence (**A**) and the presence of grape proanthocyanidins (**B**) and effect of grape proanthocyanidins on metHb formation (**C**).

on hemoglobin redox stability. The results showed that control hemoglobin without polyphenolic supplementation suffered lower spectral changes during 48 h of incubation than hemoglobin supplemented with grape proanthocyanidins (**Figure 5A**,**B**). The attenuation of the difference of absorbance between the peak at 570 nm and the valley at 540 nm observed in hemoglobin supplemented with proanthocyanidins is attributed to a reduction of oxygenated hemoglobin (OxyHb) levels, whereas the increment of absorbance at 630 nm denoted an important hemoglobin oxidation to metHb. In fact, grape proanthocyanidins were found to extensively accelerate the formation of metHb from approximately 1.0 to 7.5 μM after 48 h of incubation (**Figure 5C**). However, control hemoglobin without proanthocyanidins reached maximum values of metHb lower than 2.0 μM after the same incubation period.

DISCUSSION

There are several pathways by which the reductants here evaluated, not including catalase, may retard the propagation of hemoglobin-mediated lipid oxidation, such as free radical scavenging and deactivation of hypervalent ferryl Hb. Catalase could inhibit the pro-oxidant activity of hemoglobin through its recognized ability to decompose hydrogen peroxide, which may produce oxidizing ferryl Hb species by reaction with metHb (*28*). Regarding the free radical scavenging capacity through electron transfer to reactive free radical species, the FRAP (ferric ion reducing antioxidant parameter) estimates a donation of 3.2−6.0 electrons per molecule for grape proanthocyanidins with polymerization of 1.7−2.7 catechin units and galloylation percentages of 15−25% (*7*), which are similar to those used in our experiments. The FRAP value for ascorbic acid is two electrons per molecule (*29*). Our results certainly revealed a stronger efficiency of grape proanthocyanidins to inhibit lipid oxidation triggered by fish hemoglobin in both liposomes and washed fish muscle, in comparison with other reductants. Therefore, this higher antioxidant ability of grape proanthocyanidins is in accordance with their superior competence to scavenge free radicals via electron donation. In washed fish muscle, the three amino acid peptide GSH (γ-glutamyl-cysteinyl-glycine) was also active in retarding the pro-oxidant activity of hemoglobin, followed in decreasing order of efficiency by ascorbic acid and catalase, which exhibited similar antioxidant activity. GSH is provided of lower reducing capacity since it can donate only a single electron per molecule to reactive free radicals with the resultant formation of oxidized glutathione (GSSG), and its reduction potential [$E°$ = 0.92 V (*30*)] is notably higher than those of ascorbic acid [$E°$ = 0.28 V (*30*)] and galloylated/nongalloylated catechins [$E°$ (epicatechin) = 0.56 V; $E°$ (epigallocatechin gallate or EGCG) = 0.43 V (*31*)]. Consequently, the reducing hierarchy in terms of FRAP value or reduction potential suggests a superior inhibition of lipid oxidation by ascorbic acid than for GSH. However, it should be considered that ascorbic acid can exert pro-oxidant effects by decomposing lipid hydroperoxides to reactive aldehyde compounds (*32*). In fact, the presence of ascorbic acid caused a significant diminution of PV in washed fish muscle, but it was not effective to reduce TBARS formation.

Catalase exhibited low efficiency to prevent the development of hemoglobin-promoted lipid oxidation in liposomes and washed fish muscle. Gorelik and Kanner (*33*) have also reported the ineffectiveness of catalase, alone or combined with superoxide dismutase (SOD), to inhibit TBARS formation during the incubation of muscle membranes with oxyMb and ferrous ions. A relevant conclusion inferred of the reduced inhibitory activity by catalase should be the low contribution of hydrogen peroxide in the pro-oxidant mechanism of fish hemoglobin in both liposomes and washed fish muscle. This fact does not denote that the highly oxidizing ferryl Hb species are not implicated in

the pro-oxidative action of hemoglobin given that ferryl species can also be generated by the interaction of metHb with preformed lipid hydroperoxides (*34*). Additionally, the reaction of metHb with lipid hydroperoxides may have a greater pro-oxidant effect than the corresponding reaction with hydrogen peroxide considering the oxidizing power of additional reaction byproduct. The production of ferryl species via the hydrogen peroxide mediation generates water molecules as byproduct (*3*). In contrast, the interaction of metHb with lipid hydroperoxides induces hydroperoxide decomposition to form either free radical species (peroxyl, alkoxyl, alkyl, and hydroxyl) implicated in the radical chain reactions of lipid oxidation (*10*, *35*) or reactive aldehyde products proficient to establish adducts with biomolecules (DNA and proteins) (*36*).

As a general tendency, iron chelators showed minor antioxidant activity on hemoglobin-mediated lipid oxidation than reductants or grape proanthocyanidins; therefore, chelating compounds were necessarily evaluated at much higher concentration. Concerning the chelating property of grape proanthocyanidins, a previous study has shown that 10 μM analogous grape proanthocyanidins (mean polymerization, 1.7−2.7 units; polymerization percentage, 15−25%) are able to complex 7.0−12.3 μM ferrous iron (*7*). EDTA holds major capacity to establish complexes with ferrous ions since 10 μM EDTA is competent to chelate up to 12.9 μM ferrous iron (result obtained in our laboratory). Taking into consideration that grape proanthocyanidins are notably more efficient to prevent hemoglobin-catalyzed lipid oxidation than EDTA, these data indicate a minor role of the chelating ability of grape polyphenols on their extensive antioxidant ability against hemoglobin-mediated lipid oxidation. Consequently, the reducing ability alone and/or combined with iron-chelating property is proposed to be a determinant to effectively prevent lipid oxidation promoted by fish hemoglobin. It is in accordance with previous studies that reported a significant correlation between the electron-donating capacity of different phenolic compounds and their ability to inhibit lipid oxidation in fillets and mince from horse mackerel, a dark-muscle fish species endowed with catalytic amounts of hemoglobin (*37*, *38*). Moreover, the elevated antioxidant effect of wine phenolics in a lipid model system activated with myoglobin has been related with their capacity to reduce the catalytic ferrylMb species (*39*). More recent studies have demonstrated that EGCG and green tea extracts are highly effective in recycling ferryl back to metHb (*40*, *41*).

The redness in washed fish muscle supplemented with fresh hemoglobin is essentially provided by the bright red color of oxyHb species, and therefore, hemoglobin oxidation to the brownish ferric metHb provokes loss of redness. Grape proanthocyanidins were effective to retard the loss of redness in washed fish muscle, and consequently, proanthocyanidins prevent hemoglobin against oxidation in fish muscle. These results contrast with those obtained in our in vitro experiments that revealed an increment of metHb formation in the presence of proanthocyanidins. Jia and Alayash (*41*) have also reported a faster oxidation of hemoglobin to metHb in the presence of EGCG, suggesting the implication of hydrogen peroxide (H_2O_2) as metHb formation was almost completely stopped by the addition of catalase and SOD. EGCG has the ability to produce superoxide radicals from molecular oxygen (*42*), and superoxide radicals can easily dismutated to hydrogen peroxide. Although similar Hb/proanthocyanidin ratios were employed in vitro and in washed fish muscle, the interaction between Hb and proanthocyanidins (or ROS generated from them) is probably stronger in vitro since Hb and grape polyphenols are the unique reactive components in the in vitro systems. However, the washed fish muscle is a more complex matrix, and consequently, polyphenols (or ROS generated from them) can interact with majority components such as proteins and phospholipids.

To summarize, compounds bearing reducing abilities demonstrated higher antioxidant ability on hemoglobin-promoted lipid oxidation than iron chelators, suggesting that the free radical scavenging and/or reduction of ferrylHb species play a more important role to prevent the pro-oxidative action of hemoglobin than the iron chelation released from hemoglobin. The present paper also stresses the strong ability of grape proanthocyanidins to inhibit hemoglobin-catalyzed lipid oxidation, according with previous studies that indicated their effectiveness to prevent lipid oxidation in dark-muscle fish species (*38*, *43*). Grape proanthocyanidins are proposed as an effective ingredient to stabilize health-promoting PUFAs in functional foods containing fish hemoglobin in catalytic amounts.

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