Effect of Heating Oxyhemoglobin and Methemoglobin on Microsomes Oxidation

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ABSTRACT

Different heme proteins such as hemoglobin (Hb) have been proposed to be major prooxidants in raw and cooked meats. Despite the fact that the content of Hb in meat is considerable, little attention has been devoted to Hb in comparison to myoglobin. To understand the mechanisms and differentiate between the prooxidant and antioxidant potential of oxyhemoglobin (OxyHb) and methemoglobin (MetHb), their prooxidant activity, protein solubility, radical scavenging capacity, iron content and the relative weight of non-chelatable iron on lipid oxidation were determined as a function of thermal treatments. The ability of native OxyHb and MetHb to promote lipid oxidation was similar and higher than those Hb heated at 68 and 90 C but not different from that at 45 C. However, the prooxidant activity of MetHb heated at 68 and 90 C were similar whereas OxyHb heated at 68 C was higher than that heated at 90 C. The decreased prooxidant activity of heat denatured Hb was associated with a decrease in the solubility of heme iron while free iron showed little impact on the lipid oxidation onset.

KEYWORDS: Oxyhemoglobin, methemoglobin, lipid oxidation, heme iron

INTRODUCTION

Iron, copper and heme species such as hemoglobin (Hb) and myoglobin (Mb) contribute to lipid oxidation in different muscle-based foods (Rhee, Ziprin et al. 1987, Monahan, Crackel et al. 1993, Kanner, German et al. 1987). However, the pathways and relative importance by which all these compounds are able to initiate and propagate lipid oxidation in raw and cooked meats is still not completely understood (Baron, Andersen 2002, Johns, Birkinshaw et al. 1989, Min, Ahn 2005, Decker, Hultin 1992, Kanner 1994, Carlsen, Moller et al. 2005).

Hb is the major heme compound found in muscle foods from several fish species (Richards, Hultin 2002). In chicken breast, Hb has been reported to be the major (Kranen, Van Kuppevelt et al. 1999) or almost the unique heme pigment present (Hazell 1982) whereas in dark meat and in other different poultry species the ratio between Hb and Mb varies from 20% to 40% (Niewiarowicz, Pikul et al. 1986). In mammal species, this ratio has been reported to range from 7% to 35% (Hazell 1982, Oellingrath, Iversen et al. 1990, Han, Mcmillin et al. 1994). Therefore, even though the Hb content can vary a lot among animal species and muscle types, it is clear that the Hb levels present in muscle-based foods have the potential to substantially contribute to lipid oxidation (Alvarado, Richards et al. 2007).

Hb consists of four globular protein subunits and each contains one heme group. Heme groups consist of an iron atom contained in the center of a large heterocyclic organic ring called porphyrin which can be bound tightly to heme proteins like Hb. Hb can be in the ferrous form with (OxyHb) or without (DeoxyHb) the presence of oxygen and can autooxidize to the ferric form (MetHb). The presence of oxygen and other ligands in the Hb cause conformational changes that have been reported to promote oxidation differently (Richards, Dettmann 2003).

The formation of ferryl and/or perferryl species upon reaction with hydrogen peroxide or lipid hydropexides has been reported to be either truly initiators or important catalysts of lipid oxidation in raw muscle meat products (Kanner, German et al. 1987, Baron, Andersen 2002, Kanner, Harel 1985). Various factors such as the ability of the Hb to autoxidize and release of hematin, the heme-iron moiety non-bound to the protein, have also been reported to be crucial in promoting lipid oxidation (Richards, Dettmann 2003, Grunwald, Richards 2006a, Richards, Dettmann et al. 2005).

Upon cooking, there is an increased susceptibility to oxidation, however; the explanation for that phenomenon has not been completely elucidated. Several factors such as the release of hematin from Hb and iron from the porphyrin moiety and from other proteins such as ferritin and transferrin have been reported in meats (Monahan, Crackel et al. 1993, Grunwald, Richards 2006a, Han, Mcmillin et al. 1995). However, heating treatments provoke protein precipitation an this loss of solubility has been reported to be determinant for Mb in inhibiting oxidation (Bou, Guardiola et al. 2008). In addition, denaturation may cause conformational changes increasing or decreasing the exposure of different amino acids. This different exposure can influence the ability of proteins to chelate prooxidants and scavenge free radicals thus affecting the antioxidant-prooxidant balance of food products (Elias, Kellerby et al. 2008).

Therefore, in proteins like Hb, which has been reported to promote oxidation, it is important to study the possible mechanisms by which native and denatured Hb are able to promote oxidation in order to develop strategies to efficiently inhibit oxidative rancidity. In this frame, the aim of this work was to determine how denaturation of Hb at different temperatures changes its prooxidative activity and antioxidative properties in a muscle microsome membrane model system.

MATERIAL AND METHODS

Reagents and standards. Hb from bovine blood, fluorescein sodium salt, ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate, 2-thiobarbituric acid (TBA), 1,1,3,3-Tetraethoxypropane (TEP), L-ascorbic acid, ferrous sulfate heptahydrate, 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), nitrilotriacetate, hydroxylamine hydrochloride, butylated hydroxytoluene (BHT) and 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) were from Sigma-Aldrich Co (St. Louis, MO). Potassium chloride, sodium nitrite, sodium phosphate dibasic and monobasic, hydrochloric acid, ammonium acetate and sodium hydrosulfite were obtained form Fisher-Scientific (Pittsburgh, PA). Trichloroacetic (TCA) anhydride, ferric chloride and acetone were from Acros Organics (Fair Lawn, NJ). The chemicals used for the Lowry method (Lowry, Rosebrough et al. 1951) were of ACS grade.

Preparation of microsomes. Pork tenderloin muscle microsomes were isolated according to the method of Brannan and Decker (Brannan, Decker 2001). Frozen pork was diced into approximately 0.5 mm cubes and then chopped in a stainless steel blender for 1 min, and the resulting paste (25 g) was homogenized in 90 mL of 0.12 M KCl / 25 mM phosphate buffer, pH 7.2, in a tissuemizer (20000 rpm; Tekmar, Cincinnati, OH) for 2 min, followed by centrifugation for 30 min at 10000 *g* at 4 C (Sorvall Ultra 80, DuPont, Wilmington, DE). The resulting supernatant was ultracentrifuged for 60 min at 100000 *g* to pellet insoluble muscle components including the microsomes. Myofibrillar proteins were then solubilized from the pellet in 0.6 M KCl / 25 mM phosphate buffer, pH 7.2, and a microsome-containing pellet was isolated by centrifugation for 60 min at 100000 *g*. Isolated microsomes were standardized to 30 mg of protein / mL of 0.12 M / 25 mM phosphate buffer, pH 7.2, and stored at -80 C until use. Protein in the microsomal fraction was determined by using the method of Lowry et al. (Lowry, Rosebrough et al. 1951).

Preparation of Oxy and Methemoglobin. A commercial Hb stock solution was prepared by weighing 0.16 g of Hb standard dissolved in 3 mL cold phosphate buffer (50 mM, pH = 7.3) and kept at 4 C.

The stock solution of Hb was chemically reduced to OxyHb by mixing with sodium hydrosulfite (ratio 1:0.9 w/w) and allowed to react for 10 min at 4 C. Another Hb stock solution was fully oxidized to MetHb by mixing potassium ferricyanide (2.3 %) and allowed to react for 5 min at 4 C. Then, the excess of sodium hydrosulfite or potassium ferricyanide were removed by passing each solution through an Econo-Pac 10 DG disposable desalting column (Bio-Rad Laboratories, Hercules, CA; exclusion limit 6000 Da) as follows. Each Hb solution (3 mL) was layered onto the column and allowed to pass into the column bed. Then, 4 mL of phosphate buffer was passed into the stationary phase to elute the respectively Hb out of the column. In a 1:50 dilution kept at 4 C the DeoxyHb, OxyHb and MetHb ratios were calculated as reported elsewhere (Benesch, Benesch et al. 1973) by measuring the absorbance at 560, 576 and 630 nm using a Shimadzu UV-visible scanning spectrophotometer model UV-2101PC (Shimadzu Scientific Instruments, Columbia, MD). Only those solutions containing a minimum yield of 90% of OxyHb or MetHb conversion were used.

Then, the freshly prepared OxyHb and MetHb desalted solutions were adjusted to pH = 5.6 and diluted to the final Hb required concentration according to the Snell-Marini equation (Snell, Marini 1988) by measuring the absorbance at 523 nm (isobestic point). Those fresh solutions were capped and placed in a water bath set at 25 C, and water bath was set to 90 C. When the temperature of the water bath reached 45, 68 and 90 C tubes were immediately taken out and kept on ice. Samples were used the same day as preparation except for samples used for ORAC assay and heme and nonheme iron analyses which were immediately stored at -80 C until use.

Measurement of lipid oxidation. A solution containing Hb and microsomes (final concentrations 0.08 g/dL and 9 mg protein/mL, respectively) dissolved in 50 mM phosphate buffer, pH = 5.6 were mixed and incubated for different periods at 37 C in a water bath under gentle agitation.

Lipid oxidation was monitored by measuring thiobarbituric acid reactive substances (TBARS) by means of a modified procedure described elsewhere (Bou, Guardiola et al. 2008). Briefly, at each incubation time, 1 mL of the mixture of microsomes plus Hb was mixed with 1 mL of a TBA solution containing 20% TCA, 0.5% TBA, 0.2% EDTA and 30 mM HCl in screw capped tubes. Immediately after, 30 µL of 3% BHT in ethanol was added and tubes were then closed and vortexed. Subsequently, samples were heated in a boiling water bath for 15 min, cooled at room temperature, and centrifuged at 1750 *g* for 20 min. The absorbance of the supernatant was measured at 532 nm, and the results were reported as micromoles of malondialdehyde (MDA) per kg of microsomal protein. Concentrations were determined from a MDA standard curve produced from TEP.

UV-Visible spectrophotometry of heated myoglobin. Samples of appropriately diluted Hb solutions (50 mM phosphate buffer, pH 5.6) were placed in the thermoblock of an Ultrospec 3000 Pro model spectrophotometer (Biochrom Ltd., Cambridge, UK). The thermoblock was equilibrated at 25 C and programmed at a heating rate of 0.8 C / min up to 97 C. The absorbance was recorded at 290 nm, to monitor changes in tryptophan (Trp) absorbance.

Protein solubility. Solubility of native Hb and heated Hb was determined after centrifugation of samples at 1750 *g* for 10 min and then determining the protein content of the supernatant fraction by the Lowry method (Lowry, Rosebrough et al. 1951).

Iron content. The content of heme and non-heme iron was determined in the whole Hb solutions and in the Hb supernatant fractions which were obtained after centrifugation at 1750 *g* for 10 min.

Heme concentrations were determined using the acidified acetone extraction method of Hornsey (Hornsey 1956) with slight modifications. Five hundred µL of the samples were added to 2.5 mL of acetone and 125 µL of 3 N HCl. After 1 hr at room temperature samples were centrifuged at 1750 *g* for 20 min. The absorbance of this supernantant was measured and heme content was calculated using a molar extinction coefficient of 4800 $M^{-1}cm^{-1}$ at 640 nm for chlorohemin.

A slightly modified method described by Rhee and Ziprin (Rhee, Ziprin 1987) was used to measure non-heme iron. One hundred µL of sodium nitrite (0.16% w/v) and 1.5 mL of extraction solution (6N HCl plus 40% TCA in equal volumes) were added to screw-cap tubes containing 500 µL of Mb samples. The tubes were closed, mixed and incubated in a water bath at 65 C for 20 hr. After cooling, the mixtures were centrifuged at 1750 *g* for 10 min and the supernatants were passed through 0.45 µm filters. To one mL of each filtrate, 1 mL of 0.8% ascorbic acid was added and the samples were allowed to stand for 15 min. Then, 1 mL of 16% ammonium acetate and 1 mL of 0.8 mM ferrozine were added and the absorbance at 562 nm was measured after 10 minutes. Concentrations were obtained using a standard curve from 0 to 2 mg of iron / L produced from ferric chloride.

Oxidative capacity of non-chelatable iron. Native or heated Hb solutions (0.16 g/dL) with or without added EDTA (1.8 mM) dissolved in 50 mM phosphate buffer (pH = 5.6) were mixed with equal volumes of microsomes (10 mg protein/mL, pH = 5.6) and incubated for 8 hr at 37 C in a water bath under gentle agitation. Subsequently, the lipid oxidation was monitored by measuring TBARS as described previously and results were expressed as follows:

$$\frac{(x_i - \bar{x}_{EDTA})}{\bar{x}_{no\ EDTA}} \times 100$$

where x_i are the different TBARS amounts found in samples without added EDTA, ξ_{EDTA} is the average of TBARS amounts from those samples in which EDTA has been added and $\xi_{no\ EDTA}$ is the average of TBARS amounts from those samples in which EDTA has not been added. All TBARS amounts were expressed as micromoles of MDA per kg of microsomal protein.

Oxygen Radical Absorbance Capacity (ORAC). A 500 mM solution of AAPH in 75 mM potassium phosphate buffer at pH 7.0 was prepared for each experiment and kept on ice. Fluorescein was dissolved to a concentration of 50 nM in phosphate buffer containing 0.1 mM EDTA before each set of experiments. For each run, fluorescein was equilibrated to 37 °C in a water bath for 15 min. Reagents were added in the order of native Hb or heated Hb in 75 mM phosphate buffer (pH 7.0), fluorescein and AAPH at a final concentration of 6 µM, 45 nM and 20 mM, respectively. Fluorescence was recorded from 0 to 50 min every 10 min by taking 4 mL aliquots in which 40 µL of 500 mM ascorbic acid was added to stop the reaction followed by centrifugation for 10 min at 1750 *g*. The fluorescence (excitation = 493 nm, emission = 515 nm; Hitachi F-2000 flourometer, Tokyo, Japan) of the supernatants were measured at 37 C. The ORAC values of the Hb or heated Hb were calculated using the area under the curve (AUC) which was calculated as follows:

$$AUC = \sum_{i=0}^{i=n} \frac{F_i}{F_0}$$

where F_0 is the initial fluorescence reading and F_i is the fluorescence reading at time i.

Statistical analyses. All samples were measured in triplicate. A 1-way analysis of variance (ANOVA) was used to determine whether the heating temperature affect protein solubility, the different iron contents, the AUC values obtained from the ORAC assay and the oxidative capacity of non-chelatable iron of each Hb.

By taking into consideration each incubation time, a 1-way ANOVA was carried out to study whether the heating temperatures affected the pro-oxidant activity of OxyHb and MetHb. When ANOVAs indicated temperature effects, means were separated by the Scheffé's test. In all cases, $P \leq 0.05$ was considered significant.

RESULTS

Changes in protein properties as a result of heating. The Tryp band absorbance (290 nm) of OxyHb and MetHb showed similar profiles with the difference that the profile of OxyHb was slightly shifted to higher temperatures (Figure 1) which is in agreement with the reported lower thermal stability of oxidized heme forms (Wittung-Stafshede 1999). In both cases, conformational changes started to occur at temperatures higher than 55 C. The Tryp absorbance increased rapidly in OxyHb and MetHb at temperatures higher than 60 C indicating that both Hb were unfolded at these temperatures.

The maximum unfold for MetHb occurred at 69 C whereas the maximum for OxyHb was reached at 72.5 C. After their respectively maximums, the absorbance rapidly decreased until both Hb were completely denatured. Several authors reported differences in the prooxidant capacity of Mb heated at several temperatures (Kristensen, Andersen 1997, Berisha, Endo et al. 2000) thus, according to these profiles, we decided to compare the prooxidant and antioxidant activity of OxyHb and MetHb at 4 temperatures according to their conformational changes (native, 45 C, 68 C and 90 C).

Changes in protein solubility provide valuable information occurred on conformational changes. OxyHb and MetHb either native or heated at 45 C showed no differences whereas those heated at 68 C showed lower solubility confirming that some denaturation had occurred at this temperature (Table 1). Heating at higher temperatures led to much lower protein solubility which

indicated extensive protein aggregation and precipitation. This explains the lowered absorbance recorded at 290 nm when Hb was heated at high temperatures.

OxyHb and MetHb on microsomes oxidation. To study the pro-oxidant activity of Hb, this was added into microsomes to an Hb / protein ratio similar to that found in meats. The ability of OxyHb in the presence of microsomes to promote oxidation showed no differences after 2 hr of incubation whereas the blank containing only OxyHb showed lower and steady TBARS values over time (Figure 2). After 4 hr of incubation, OxyHb heated at 90 C recorded lower TBARS values than native OxyHb whereas the other heating treatments showed no differences on microsomes lipid oxidation when compared with native Hb or Hb heated at 90 C (Figure 2). Native OxyHb and OxyHb heated at 45 C showed the same capacity in promoting oxidation after ≥ 8hr of incubation whereas the prooxidant activity of OxyHb heated at 68 C was significantly lower. With the exception of the blank, OxyHb heated at 90 C showed the lowest prooxidant activity.

As for MetHb, TBARS values of the blank were lower than those containing microsomes over time (Figure 3). Differences between heating treatments were observed after 6 hr of incubation. At this time, MetHb heated at 90 C showed lower TBARS values than the other MetHb heated below this temperature (Figure 3). Native MetHb and that heated at 45 C showed higher oxidation values after ≥ 8 hr of incubation whereas MetHb heated at 68 C and 90 C, which had a similar ability to oxidation, recorded lower TBARS values.

Some controversy exists about whether the heme or free iron from both Mb and Hb is the major responsible for lipid oxidation in cooked meats (Johns, Birkinshaw et al. 1989, Grunwald, Richards 2006a, Han, Mcmillin et al. 1995, Schricker, Miller 1983). In order to a better understanding we conducted a trial in which OxyHb and MetHb either native or heated were incubated for 8 hr at 37 C

in the presence of microsomes with or without added EDTA. The relative percentage of the microsomes oxidation caused by non-chelatable iron is shown in Table 1. Results indicated that the addition of EDTA provoked a relative decrease in TBARS values (10-12 %) when OxyHb was heated at temperatures ≥ 68 C. However, the addition of the chelator almost had no effect when heated at lower temperatures.

The ability to promote oxidation of native MetHb was almost unaffected by the addition of EDTA which indicated that the chelatable iron almost had no effect on microsomes oxidation (Table 1). However, when MetHb was heated at any studied temperature, TBARS values were decreased (11-17%). This reduction indicates that, in heated MetHb, chelatable but especially non-chelatable iron participated on microsomes oxidation.

Iron and heme content as a result of heating. The heme content was measured in the whole sample and in the supernatant fraction obtained after centrifugation. Therefore, results provided valuable information about the destruction of the porphyrin ring as a consequence of the thermal treatments and the distribution of the heme iron. In the whole samples, OxyHb and MetHb heme iron content showed no significant differences between treatments (Table 1). However, differences in the heme iron content were recorded in the supernatant fraction. OxyHb heated at 45 C showed no differences for the heme content in the supernatant fraction in comparison to native OxyHb but above this temperature the heme content was lowered as temperature increased (Table 1). Similar trends were observed for MetHb although MetHb heated at 68 C did not show a different heme content in the supernatant fraction compared with MetHb heated at 45 C or at 90 C (Table 1).

The non-heme iron content indicates those changes in the free iron fraction that can occur for instance because of the iron release from Hb. In the whole sample, no differences were recorded as a consequence of heating for OxyHb and MetHb

(Table 1) which was in agreement with the fact that the porphyrin ring structure had not been destroyed. In the supernatant fraction, OxyHb showed no changes in the non-heme iron content with temperature. Conversely, the non-heme iron content in the supernatant fraction of MetHb heated at 90 C and 68 C was decreased in comparison to native and that heated at 45 C (Table 1). This decrease in the water soluble nonheme iron content suggests that the pool of free iron was bound to Hb and thus removed upon precipitation of the protein.

OxyHb and MetHb antioxidant capacity. The ORAC assay showed that native OxyHb and that heated at 45 C had a higher antioxidant activity than OxyHb heated at 68 C which in turn was higher than that heated at 90 C (Table 1). Likewise, MetHb heated at 90 C showed lower antioxidant capacity than the other MetHb treatments. The overall decrease of the antioxidant capacity of the Hb as a consequence of heating was likely due to a lower exposure of amino acids with radical scavenging capacity (Elias, Kellerby et al. 2008).

DISCUSSION

Mb and Hb have been reported to promote lipid oxidation in muscle based foods (Baron, Andersen 2002, Kanner, Harel 1985, Grunwald, Richards 2006a, Richards, Dettmann et al. 2005). The ability of Mb and Hb to promote oxidation is attributed to their heme group so the oxidation mechanisms are common although subject to several conditions that may result in enhancement or decrease of their prooxidative activity (Carlsen, Moller et al. 2005).

A faster autooxidation rate and low hematin affinity, which is increased in ferric heme forms, have been related with the increase of the lipid oxidation onset (Richards, Dettmann et al. 2005, Grunwald, Richards 2006b, Richards, Nelson et al. 2007). As for autoxidation, a series of reactions can be set (Carlsen, Moller et al. 2005, Richards, Dettmann 2003, Gorelik, Kanner 2001, Yusa, Shikama 1987):

$$\text{OxyHb(II)}O_2 \rightarrow \text{DeoxyHb(II)} + O_2 \qquad [1]$$

$$\text{DeoxyHb(II)} + O_2 \rightarrow \text{MetHb(III)} + O_2^{\bullet -} \qquad [2]$$

$$O_2^{\bullet -} + O_2^{\bullet -} \rightarrow H_2O_2 + O_2 \qquad [3]$$

The reaction [1] is not favorable since the OxyHb is more stable than DeoxyHb but once it is formed it autoxidizes rapidly in the presence of oxygen (Richards, Dettmann 2003). Therefore, that limited reaction may explain the delayed pattern in microsomes oxidation when comparing native OxyHb and MetHb (Figures 2 and 3) whereas the formation of hydrogen peroxide [3] and the presence of lipid hydroperoxides led to the formation of ferryl (Hb(IV)=O) and perferryl ($Hb^{\bullet}$(IV)=O) species which are efficient promoters of lipid oxidation (Baron, Andersen 2002, Kanner, Harel 1985). This hydrogen peroxide formation might explain why the oxidation values found in microsomes were slightly higher in the presence of OxyHb. In addition, the allosterism of Hb upon deoxygenation and reduction may provoke that the catalytically active heme groups in the OxyHb were less exposed to their surroundings or more loosely in comparison to MetHb thus explaining those differences in the prooxidant activity.

Heating can also favor hematin release from Hb which is insoluble in water. Because of that and unless it remained attached to the protein while this is soluble, this fact should provoke a decrease of the heme iron content in the supernatant fraction. Heating Hb at 45 C seemed to have no effect on heme group insolubilization or destruction although a slight decrease was observed for MetHb (Table 1). This temperature is just before some changes in the Tryp band were observed (Figure 1) which was in agreement with the lack of effect on protein solubility and antioxidant capacity measured through ORAC values. All these observations were in agreement with the recorded lack of effect on the susceptibility to oxidation after heating Hb at 45 C in comparison with their respective native Hb (Figures 2 and 3).

The increased heme moiety exposure reported after protein unfolding as a consequence of heating at a moderate/high temperatures (60-70 C) (Kristensen, Andersen 1997) and the release of hematin (Grunwald, Richards 2006a) and iron (Decker, Hultin 1992) from Mb and Hb at higher temperatures have been indicated as major causes of the increased susceptibility to oxidation in cooked meats. In our conditions, heme and non-heme iron content results indicated that the iron-porphyrin moiety was quite resistant to thermal treatments even when heated at 90 C (Table 1) which was also in agreement with other works (Bou, Guardiola et al. 2008, Kristensen, Andersen 1997, Han, Mcmillin et al. 1993). However, heme iron contents were decreased in the supernatant fractions as thermal treatment increased (Table 1). Unfortunately, from our results it is not possible to know whether the heme moiety is completely released, in the form of hematin, or not since heme iron precipitation was concomitant with the protein solubility.

By looking at TBARS values and heme iron content in the supernatant fraction it was observed that they were related since the addition of OxyHb heated at 68 C into microsomes led to intermediate values for both susceptibility to oxidation and heme content (Table 1 and Figure 2). Similarly, there were no differences

between MetHb heated at 68 and that heated at 90 C for both parameters (Table 1 and Figure 3). This fact supports the hypothesis that Hb, which precipitated and aggregated upon heating, cocooned the catalitically active heme iron since both the heme moiety and heme crevice are hydrophobic. Therefore, in case that the heme moiety was located inside the aggregate, it could only interact with the denatured Hb and would explain the poor prooxidant effect when different heated Hb were added into the microsomes mixture. This relationship between heme iron loss of solubility and prooxidant activity has been reported previously in Mb (Bou, Guardiola et al. 2008, Berisha, Endo et al. 2000).

Because of the low affinity to water, hematin easily associates to different components present in the media such as bovine serum albumin and membrane components (Ledward 1971, Everse, Hsia 1997, Avissar, Shaklai et al. 1984). Therefore, either the direct interaction with membranes rich in polyunsatured lipids or through other proteins present in media will likely serve to shuttle hematin to membranes and, in consequence, favor the increased susceptibility to oxidation in cooked meats. Grunwald et al. (Grunwald, Richards 2006a) also suggested this hypothesis after they observed that lipid oxidation was promoted in washed cod muscle when hematin was added together with bovine serum albumin. However, this mechanism was impeded in our model system since Hb was precipitated before adding to microsomes thus explaining the differences in the prooxidative activity of heated heme proteins among studies.

Amino acids, peptides and proteins have been reported to act as antioxidants due to its ability in scavenging free radicals and chelating prooxidative metals (Elias, Kellerby et al. 2008, Chan, Decker 1994). Changes in the exposure of some amino acids could be related with the more maintained antioxidant capacity of MetHb in comparison to OxyHb when they were heated (Table 1). This effect was correlated with the overall poor prooxidant activity of heated Hb. Protein denaturation could also have increased the exposure of amino acid residues such as histidine, glutamic acid and aspartic acid known to bind metals and/or

hinder iron from the surface (Elias, Kellerby et al. 2008). The likely decreased exposure of some amino acid residues of the MetHb might explain the lowered non-heme iron content in the supernatant found in MetHb heated at 68 C and 90 C (Table 1). This explanation about iron encapsulation is in agreement with a previous work that reported a lowered iron release after dialysis when Mb was at heated at 100 C in comparison to that heated at 74 C (Berisha, Yasushi et al. 2003).

Regardless of the heating temperature, the latter authors also found that the released iron was responsible for only 20% of the prooxidant activity compared with the Mb itself when after heating this was added to a media containing linoleic acid (Berisha, Yasushi et al. 2003). In order to evaluate the impact of free iron versus heme on lipid oxidation, we added to the microsome solution native and heated Hb either with or without added EDTA. Results showed that chelatable iron had a low impact in the promotion of lipid oxidation in native Hb proteins thus indicating that oxidation was only due to its catalytic activity rather than free iron (Table 1). In heated OxyHb, the non-heme content in the supernatant was constant with temperature but only about 10-12% of the oxidation could be attributed to the effect of the free iron. This indicated that heme iron, in the native form or as in other heat-denatured heme forms, was the main responsible of the oxidation. MetHb heated at 90 C showed the maximum percent (17%) of lipid oxidation reduction which could be due to the low net prooxidant activity and low heme content in the supernatant of this heated MetHb rather than the free iron release since this was decreased in comparison to native MetHb.

Collectively, these results indicated that the content in heme iron in the supernatant largely influenced the susceptibility to oxidation whereas the free iron content had little effect. Because of its hydrophobicity, the porphyrin group seemed to be hidden inside the Hb denatured aggregate thus provoking the reduced susceptibility to oxidation in our system. Future works should approach

those factors that can affect the delivery of heme groups to different targets susceptible to oxidize. Nevertheless, other factors such the ability of the different denatured Hb to chelate iron and the free radical scavenging capacity also determine the overall antioxidant pro-oxidant balance.

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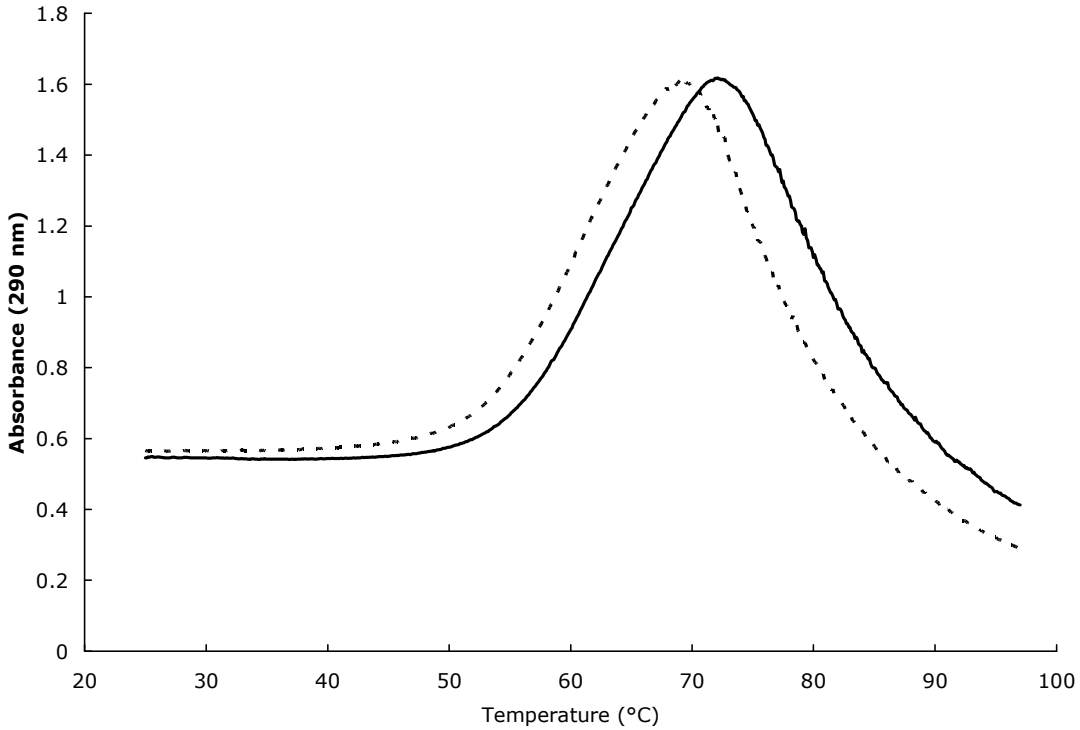

Figure 1. Absorbance changes of oxyhemoglobin (solid line) and methemoglobin (dashed line) solutions (0.04 g/dL; pH = 5.6) at 290 nm with a heating rate of 0.8 C/min.

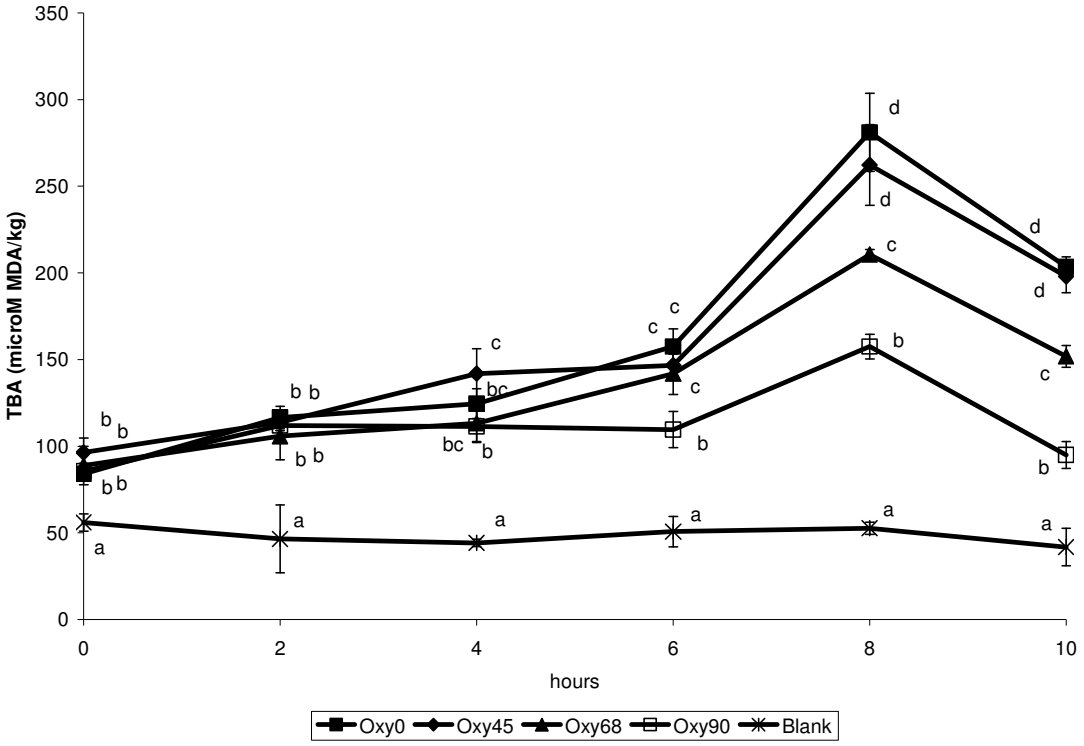

Figure 2. Effect of oxyhemoglobin (final concentration 0.08 g/dL) heated at different temperatures on the formation of thiobarbituric acid reactive substances (TBA; µmols MDA/kg protein) in the presence of muscle microsomes (final concentration 9 mg/mL) at 37 C for different incubation times. Blank correspond to samples made with native oxyhemoglobin. For each incubation time, values corresponding to a certain variable with different letter differ significantly ($P \leq 0.05$).

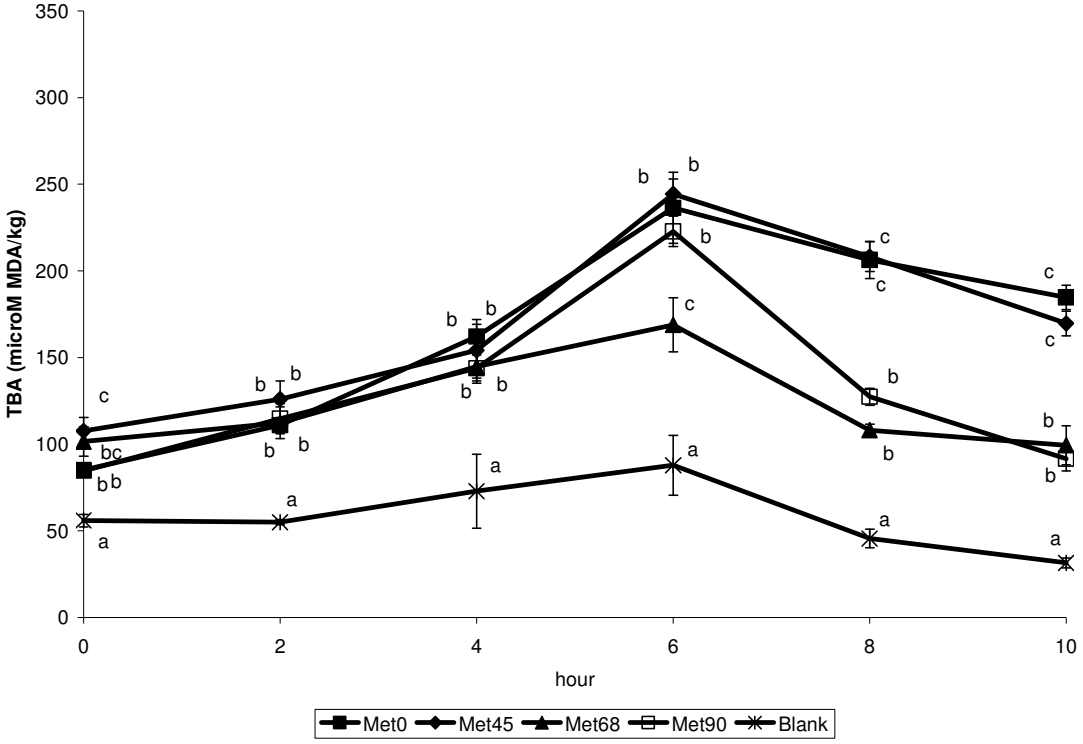

Figure 3. Effect of methemoglobin (final concentration 0.08 g/dL) heated at different temperatures on the formation of thiobarbituric acid reactive substances (TBA; µmols MDA/kg protein) in the presence of muscle microsomes (final concentration 9 mg/mL) at 37 C for different incubation times. Blank correspond to samples made with native methemoglobin. For each incubation time, values corresponding to a certain variable with different letter differ significantly ($P \leq 0.05$).

Table 1. Effect of heating oxyhemoglobin and methemoglobin on protein solubility, radical scavenging capacity, oxidative capacity of non-chelatable iron, heme and non-heme content in either the whole sample or in the supernatant after centrifugation[1].

Heating temperature (C)	Protein solubility (mg/L)	Radical scavenging capacity (AUC)[2]	Heme in whole sample (microM)	Heme in the supernatant (microM)	Non-heme in whole sample (microM)	Non-heme in the supernatant (microM)	Oxidative capacity of non-chelatable iron (%)
	OxyHemoglobin						
Native	971 a	3.61 a	35 a	35 a	105 a	64 a	0.3 a
45	956 a	3.67 a	31 a	34 a	106 a	78 a	3.9 a
68	515 b	2.59 b	32 a	20 b	86 a	63 a	10.5 b
90	12 c	1.82 c	33 a	6 c	93 a	50 a	11.9 b
SE[3]	9.9	0.089	1.7	2.4	9.5	9.4	0.74
	MetHemoglobin						
Native	868 a	3.49 a	30 a	31 a	112 a	94 ab	3.3 a
45	864 a	3.81 a	30 a	26 ab	89 a	124 a	11.7 b
68	468 b	3.38 a	26 a	13 bc	108 a	69 b	14.5 b
90	10 c	2.35 b	26 a	0.4 c	92 a	58 b	17.1 b
SE	10.0	0.15	3.1	2.7	6.4	7.2	1.4

[1] Values correspond to means obtained from an ANOVA (n = 12). Means corresponding to a certain variable and for each level of certain hemoglobin bearing no common letters are statistically different ($P \leq 0.05$).

[2] The radical scavenging capacity was calculated using the area under the curve of the oxygen radical absorbance capacity (ORAC) assay.

[3] SE means the standard error which is formed by dividing the pooled standard deviation by the square root of the number of observations at each level.

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