The Effect of Heating Temperature on the Prooxidant and Hydroperoxide Decomposition Activity of Myoglobin

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Solutions of myoglobin from the heart of a horse were heated at various temperatures to assess the effect of heating temperature on the prooxidant and hydroperoxide decomposition activity of myoglobin. The solutions of the heated myoglobin were incubated with linoleic acid in various systems and the prooxidant activity was evaluated by measuring the absorbance at 234 nm and thiobarbituric acid (TBA) value. H_2O_2 was added to the reaction mixture to activate the myoglobin. To observe the lipid hydroperoxide decomposition activity of the heated myoglobin, the heat-denatured myoglobin was incubated with oxidized linoleic acid and the residual hydroperoxides were assessed fluorometrically. Both the absorbance at 234 nm and TBA values gave similar results and the incubation of heated myoglobin with linoleic acid resulted in a rather weak prooxidant activity regardless of the heating temperature. The supplementation of H_2O_2 activated myoglobin, whose activity was greatest when heated at 74°C, decreased when heated at 100°C. As a model of pre-cooked frozen foods, a part of the heated myoglobin solution was frozen and reheated. The prooxidant activity of the reheated myoglobin was similar and greatest by heating at 74°C, but when heated at 100°C it was less prominent than that of the control (unheated). The lipid hydroperoxide-decomposition activity of the heat-denatured myoglobin (heated or reheated under the same conditions) showed a slight alteration up to 74°C, however, heating at 100°C significantly lowered this activity.

Keywords : myoglobin, heating, prooxidant activity, linoleic acid

Flavor is one of the major factors defining the quality of meat and meat products. The degeneration of meat flavor is connected to lipid peroxidation which is initiated by myoglobin (Apte & Morrissey, 1987a, b; Tichivangana & Morrissey, 1985), and the accumulation of oxidized denatured myoglobin results in loss of the fresh appearance and a dark color (Faustman *et al.*, 1992). The oxidation of polyunsaturated fatty acids in meats and meat products has attracted great deal of attention because of the concern about the rancid odor, especially the “warmed-over flavor” in cooked meats. Cooked meats developed a rancid flavor more rapidly than uncooked meat during refrigerated storage (Han *et al.*, 1995). The lipid oxidation is mainly due to the reactions between microsomal lipids and myoglobin (Eriksson *et al.*, 1971) affected by cooking temperature (Apte & Morrissey, 1987a; Ingene *et al.*, 1979).

Generation of the warmed-over flavor as a specific off-flavor phenomenon occurring during the heating of pre-cooked foods containing meat and meat products (Asghar *et al.*, 1988; Ingene *et al.*, 1979; Love & Pearson, 1974) has been attributed to heme catalysts such as myoglobin. Cooking destroys the heme pigments and releases a significant amount of non-heme iron which accelerates the lipid oxidation (Tichivangana & Morrissey, 1985).

Apart from the non-heme iron, the catalytic activity of myoglobin itself is modulated by cooking due to the conformational changes where the catalytically active heme group becomes more favorably exposed to the surrounding lipid molecules

(Eriksson *et al.*, 1971).

To observe the prooxidant activity of the heated myoglobin different methods of assessing hydroperoxide formation and decomposition have been employed.

1. Absorbance at 234 nm to follow the formation of the hydroperoxide as a transition step in secondary product formation (Frankel, 1980; 1984) responsible for the generation of warmed over-flavor in meat and meat products. Some studies have suggested that the myoglobin-catalyzed lipid peroxidation is activated by H_2O_2 (Fox *et al.*, 1974) which is generated endogenously in the muscle systems (Fygle & Melo, 1996; Giulivi & Davies, 1990). In some cases analogous to this situation, H_2O_2 was added to the reaction mixtures.

2. Thiobarbituric acid (TBA) test which reflects the overall degree of the lipid oxidation by measuring both hydroperoxides and secondary oxidation products such as alkenals (Kosugi & Kikugawa, 1989).

3. Isomeric composition of the linoleic acid peroxidation products aimed at observing alterations in the specificity of the prooxidant activity of the heat-denatured myoglobin during the catalysis of formation of the isomers of linoleic acid.

4. Fluorometrical determination (Akasaka *et al.*, 1992) of the hydroperoxide decomposition activity of the heat-denatured myoglobin against the linoleic acid hydroperoxides.

Materials and Methods

Linoleic acid purification Linoleic acid (Sigma, St. Louis, MO) was purified by a silicic acid column to delete the oxidation products. The peroxide value (PV) was determined iodometri-

cally (Japan Oil Chem. Soc., 2. 5. 2) and stored at -30°C in hexane under nitrogen.

Preparation of linoleic acid hydroperoxides Linoleic acid (Sigma) was irradiated by a fluorescence lamp (15 W) at 5°C for 4 days in the presence of methylene blue (1 mg methylene blue for 1 g linoleic acid). Hydroperoxides were purified by a silicic acid column (Fujino, 1969), run with hexane:diethyl ether (50:50, v/v) and stored in hexane under nitrogen at -30°C . The hydroperoxide preparation gave a single pink spot on TLC plates (Kieselgel 60 Merck) developed with hexane:diethyl ether:acetic acid (80:20:1, v/v/v) and sprayed with a 1% diphenyl-*p*-phenylenediamine ethanol solution (Sherma, 1972). PV determined iodometrically was 4900.

Heat-treatment of myoglobin solutions Three milliliters of 0.24 mM myoglobin (horse heart, Sigma) in 0.2 M phosphate buffer (pH 7.0) were heated at different temperatures (from 64°C to 100°C) for different periods of time (from 5 to 30 min) in a test tube. In an attempt to simulate the cooking and storage of pre-cooked frozen meat products, some heated samples were frozen overnight at -30°C and then reheated under the same conditions.

Absorbance at 234 nm (Ben-Aziz *et al.*, 1970) Linoleic acid (1 ml of 0.6 mM linoleic acid in the solution of 0.2 M phosphate buffer at pH 7.0 containing 20% ethanol, PV=0) was incubated with 1 ml of 0.6 μM myoglobin in 0.2 M phosphate buffer (pH 7) and 1 ml of 0.2 M phosphate buffer (pH 7). In some cases, myoglobin was activated by 1 ml of 29.1 or 58.2 μM H_2O_2 which was introduced in the reaction systems 30 s after the incubation began by substituting 1 ml of the phosphate buffer. The reaction mixtures were incubated in a glass cell at 20°C in the dark and the absorbance at 234 nm was recorded every min on a JASCO Uvidec-320 spectrophotometer (JASCO, Tokyo). The results of incubation in all experiments are the average of 4 determinations $\pm$ S.D.

Assay of TBA reactive substances Linoleic acid (9 ml of 3.36 mM in 0.2 M phosphate buffer pH 7 containing 20% ethanol, PV=0) was incubated with heat-denatured myoglobin (1 ml of 0.24 mM in 0.2 M phosphate buffer) for 48 h in the dark at 5°C . In another set of experiments, myoglobin was activated by 30 μM H_2O_2 and incubated under the same conditions.

TBA reactive substances were assayed principally according to the procedure of Ohkawa *et al.* (1978). An aliquot of 1 ml of the reaction mixture was incubated with 1.5 ml of 0.8% of 2-thiobarbituric acid, 1.5 ml 20% acetic acid buffer at pH 3.5 adjusted with saturated NaOH solution, 0.2 ml 8.1% sodium dodecyl sulfate and 1 ml of EDTA solution (10%) which was added to the reaction mixture to avoid the interference of myoglobin during the TBA measurement. The TBA values were expressed in μmol equivalent of malonaldehyde based on the standard solution of 1,1,3,3-tetraethoxypropane (Tokyo Kasei, Tokyo) as an equivalent to the malonaldehyde.

Isomeric composition of hydroperoxides The hydroperoxide composition was determined after the incubation of myoglobin and pure linoleic acid in two different experimental sets and conditions.

Incubation at 5°C and HPLC (Iwase *et al.*, 1997) Nine milliliters of 3.36 mM linoleic acid in 0.2 M phosphate buffer at pH 7 containing 20% ethanol was incubated with 1 ml of 0.24 mM myoglobin in 0.2 M phosphate buffer in the dark at 5°C for

12 h. The incubation was stopped with EDTA (10%) and hydroperoxides were reduced to alcohols with NaBH_4 (Chan *et al.*, 1976a), acidified to pH 3 with 0.1 N HCl, extracted with diethyl ether, and dissolved in an HPLC-grade hexane. The sample (free hydroxy linoleic acid) was subjected to the HPLC system equipped with a JASCO UV-970 detector at 234 nm. The column was Finepak Sil-5 (4.6 $\times$ 250 mm, JASCO, 35°C) run with 1 ml/min hexane : 2-propanol : acetic acid (97.5 : 2.5 : 0.2, v/v/v).

Incubation in the presence of hydrogen peroxide and HPLC (Chan *et al.*, 1976, Chan & Levett, 1976) Linoleic acid (1 ml of 6 mM in 0.2 M phosphate buffer at pH 7 containing 20% ethanol, PV=0) was incubated with 1 ml of 6 μM myoglobin and 1 ml of 582 μM H_2O_2 for 20 min at 20°C in a glass cell. The following steps for HPLC were similar to those mentioned above. A sample of methyl hydroxy linoleic acid isomers esterified with diazomethane was injected into the same HPLC system equipped with a Shodex SIL-5B column (4.6 $\times$ 200 mm, Showa Denko, Tokyo, 35°C) run with 1 ml/min hexane : 2-propanol (99 : 1, v/v).

Each isomer of linoleic acid hydroperoxides was identified according to the HPLC charts of known compounds: soybean lipoygenase L-1 (Gardner, 1975), 13-hydroperoxy-9Z,11E-octadecadienoate (9Z,11E-13-OOH); autoxidation (Chan *et al.*, 1976, Chan & Levett, 1976), 9-hydroperoxy-10E,12Z-octadecadienoate (10E,12Z-9-OOH), 9-hydroperoxy-10E,12E-octadecadienoate (10E,12E-9-OOH), 13-hydroperoxy-9Z,11E-octadecadienoate (9Z,11E-13-OOH), 13-hydroperoxy-9E,11E-octadecadienoate (9E,11E-13-OOH).

Fluorometrical determination of hydroperoxides The fluorometry of the hydroperoxides was performed by employing diphenyl-1-pyrenylphosphine (DPPP, TOSO, Tokyo; Akasaka *et al.*, 1992). The correlation between the fluorescence intensity and the hydroperoxide concentration was checked prior to the experimental work.

Linoleic acid hydroperoxides (3 ml of 0.428 μM in 0.2 M phosphate buffer at pH 7.0 containing 20% ethanol, PV=4900) and heat-denatured myoglobin (3 ml of 5 nM in 0.2 M phosphate buffer at pH 7) were incubated at 25°C for 5 and 10 min. One milliliter of the reaction mixture was mixed with 1 ml of 10% EDTA to inactivate the myoglobin. Hydroperoxides were extracted with 4 ml of chloroform and dried over Na_2SO_4 . DPPP (100 μl of 1 mg/10 ml chloroform) and the lipid samples (0.1 μl) were mixed and incubated at 63°C for 1 h. The fluorescence intensity was recorded on a JASCO FP-750 spectrofluorometer with Ex at 352 nm and Em at 380 nm. The quantity of hydroperoxides was plotted based on a standard curve of fish oil with known PV.

Results

During the incubation of the pure linoleic acid with myoglobin, the absorbance at 234 nm increased progressively. Because a longer incubation time than 10 min did not result in further absorbance increase (data not shown), the absorbance during the first 10 min was monitored. However, no impact on the prooxidant activity effect was observed by the heating temperature of myoglobin (Fig. 1).

The supplementation of H_2O_2 aided in the increase of the prooxidant activity of myoglobin as shown in Fig. 1 and the effects were enhanced at a higher concentration. Moreover, the

Fig. 1. The absorbance at 234 nm for the reactions between linoleic acid and heat-denatured myoglobin. "a" (—) control (myoglobin or linoleic acid alone), "b" (■) unheated myoglobin, "c" (●) myoglobin heated at 74°C and "d" (▲) myoglobin heated at 100°C. The results and standard deviations are the mean of 4 parallels.

myoglobin heated at 74°C exhibited the highest activity in all the cases.

Reheating of myoglobin under the same conditions did not give any effect on the prooxidant activity compared with the myoglobin heated once. In the presence of H_2O_2 , similar to the myoglobin heated only once, the prooxidant activity of the heat-denatured myoglobin was increased along with H_2O_2 concentration increase. However, the myoglobin denatured at 100°C had an even lower prooxidant activity compared with that of the unheated myoglobin, as very clearly observed during the activation with 19.4 μM H_2O_2 (Fig. 1).

The differences between absorption spectra obtained from the H_2O_2 activated myoglobin with respect to the inactivated myoglobin resulted in horizontal straight lines (results not shown). This evidenced the important activation role of H_2O_2 in the very first stage of the reactions of myoglobin with lipids.

Increase in the denaturation temperature of myoglobin resulted in an increase of the TBA values up to 74°C; denaturation above this temperature resulted in a decrease of these values. Prolongation of the heating time of myoglobin from 5 to 30 min more clearly showed the effect of the heating temperature on its prooxidant activity (Fig. 2).

Reheating of the myoglobin under the same conditions re-

Fig. 2. TBA values for the reaction between linoleic acid and heat-denatured myoglobin. Myoglobin was heated for different periods of time and incubated with linoleic acid for 48 h. The results and standard deviations are the mean of 4 parallels.

Fig. 3. TBA values for the reaction between linoleic acid and reheated myoglobin. Myoglobin was heat-denatured for 10 and 30 min, deep frozen at -30°C and reheated under the same conditions. The results and standard deviations are the mean of 4 parallels.

sulted in much higher TBA values than in the myoglobin heated once (Fig. 3), and the TBA values for that heated twice for 10 min were even higher than those of the myoglobin heated once for 30 min (Fig. 3a versus Fig. 2d). However, the effect of the heating temperature was less distinct.

Activation of the myoglobin with H_2O_2 (Fig. 4) resulted in higher TBA values compared with the non-activated systems for the myoglobin heated for 10 and 30 min (Fig. 4 versus Fig. 2). The differences between the highest TBA values at 74°C and the lowest at 100°C, however, were less sharp than their respective counterparts of non-activated myoglobin.

The incubation of linoleic acid with heated myoglobin at 20°C in the presence of H₂O₂ resulted in an equilibrated balance between 9 and 13- isomer groups of linoleic acid hydroperoxides (9-OOH/13-OOH=50/50) suggesting non-specific addition of oxygen to alkyl radicals (Table 1). The reheating of myoglobin resulted in the same finding without any significant alteration in the hydroperoxide composition as a function of temperature (data not shown).

In an effort to reduce the effect of the isomerization of hydroperoxides or their decomposition during incubation (Chan *et al.*, 1976, Chan & Levett, 1976), linoleic acid was incubated at 5°C without H₂O₂ as an activator. During the incubation time of 12 h among the 4 isomers, 13-OOH isomers formation was dominant

compared with 9-OOH isomers (about 70% versus 30%, respectively, the column could not separate 2 isomers of 9-OOH). However, the imbalance between these two (13-OOH and 9-OOH) isomers was less apparent for the myoglobin reheated under the same conditions (Table 2).

The hydroperoxide decomposition activity of the heated myoglobin assessed by the fluorometrical measurement of hydroperoxides showed no activation by heating at 74°C, however, reduction in this activity was observed for the myoglobin heated at 100°C (Fig. 5).

The hydroperoxide alone did not show any decomposition tendency unless the myoglobin was added (results not shown).

Discussion

The diene formation observed by the absorbance at 234 nm showed that the prooxidant activity of myoglobin remained relatively low even after heating. The activity was enhanced by the addition of H₂O₂ and was almost proportional to the additive concentration. The formation of H₂O₂ in biological systems, typically the disproportionation and reduction of superoxide anion and 2 e⁻ reduction of molecular oxygen, is well known. The latter reaction is catalyzed by various oxidases (Bostick & Hercules,

Fig. 4. TBA values for the reaction between linoleic acid and heat-denatured myoglobin. Myoglobin was heated for different periods of time, activated with H₂O₂ and incubated with linoleic acid for 48 h. The results and standard deviations are the mean of 4 parallels.

Table 1. The isomeric composition of linoleic acid hydroperoxides formed by the incubation at 20°C of linoleic acid with H₂O₂-activated heat-denatured myoglobin.

Isomers	Control	Unheated	74°C	100°C
9Z, 11E-13-OOH	38±3	40±8	34±2	35±3
9E, 11E-13-OOH	11±4	15±5	12±2	18±1
10Z, 12Z-9-OOH	27±2	31±3	32±5	31±3
10E, 12E-9-OOH	24±8	14±9	16±4	16±4

Data are means of four independent samples. Control is the linoleic acid incubated alone. Unheated represents the native myoglobin and 74 or 100°C are temperatures at which myoglobin was heated.

Fig. 5. Decomposition of linoleic acid hydroperoxides during the reaction with heat-denatured myoglobin. Myoglobin was heated for 30 min at 74°C and 100°C. Some heated myoglobin samples were deep frozen at -30°C and reheated under the same conditions. The results and standard deviations are the mean of 4 parallels. a, unheated; b, 74°C; c, 100°C.

Table 2. The isomeric composition of linoleic acid hydroperoxides formed by the incubation at 5°C of linoleic acid with heat-denatured myoglobin.

Isomers	Myoglobin heated once			Myoglobin heated twice		
	Unheated	74°C	100°C	Unheated	74°C	100°C
9Z, 11E-13-OOH	30±6	23±3	40±2	27±4	34±9	30±2
9E, 11E-13-OOH	42±4	45±7	30±9	32±8	27±3	32±2
10E, 12(E & Z)-9-OOH	28±3	32±1	30±2	41±1	38±7	38±2

Data are means of four independent samples. Control is the linoleic acid incubated alone. Unheated represents the native myoglobin and 74 or 100°C are temperatures at which myoglobin was heated.

1975). The occurrence of low levels of H_2O_2 naturally formed was reported in various types of foods: fish products (Umemoto, 1985), vegetable oils (Coxon *et al.*, 1987) and instant coffee (Fujita *et al.*, 1985). The effects of heating on the prooxidant activity of the H_2O_2 -activated myoglobin were similar to that of myoglobin and the activity reached maximum when heated to 74°C.

The important feature of the TBA value as an index of lipid peroxidation is that TBA can react most favorably with the secondary oxidation products alk-2-enals and alk-2,4-dienals but can also react with hydroperoxides (Kosugi & Kikugawa, 1989). This means that TBA values can reflect the overall progress of lipid peroxidation. In this study, the temperature aided the prooxidant activity of the heat-denatured myoglobin up to 74°C, however, further heating to 100°C resulted in the opposite effect. This was in accordance with the results of the formation and the decomposition of conjugated diene, mostly derived from hydroperoxides. However, when myoglobin was heated twice or supplemented with H_2O_2 , the effect of the heating temperature was less significant. The exception was 100°C where heating always decreased the prooxidant activity as compared with the unheated myoglobin.

Incubation at low temperatures permitted the approach of the linoleic acid from the 13 carbon site chain. However, reheating of myoglobin caused structural changes which promoted random contacts of the linoleic acid with heme.

The activation factors of H_2O_2 or reheating of the myoglobin solutions under the same conditions enhanced the prooxidant activity of the heat-denatured myoglobin. However, the difference in the prooxidant activities in myoglobin heated at different temperatures was small. We can thus propose that all these activation factors, regardless of their simulation role in the prooxidant activity, do not overcome the reduction effects of the structure coiling at very high temperatures. This resulted in lower prooxidant activity of heat-denatured myoglobin, as compared with that heated at middle range temperatures.

The acceleration of lipid oxidation by cooked myoglobin was reported by Tichivangana and Morrissey (1985) who suggested the possible involvement of heat-destroyed heme proteins on the prooxidant activity. However, our results in the model systems suggest that the oxidative deterioration in the cooked meats is aided by myoglobin up to certain temperatures, but then further heat-denaturation of myoglobin at least partially reduces the catalytic effect of myoglobin as a prooxidant. The activation of the denatured myoglobin with H_2O_2 may be due to the oxidation of Fe(III) in metmyoglobin to the ferryl species ($MbFe[IV]=O$) with a single oxygen atom bound to heme iron (Davies, 1991). This reaction proceeded very rapidly during incubation by comparing the prooxidant activity of myoglobin with and without H_2O_2 (Fig. 2 versus Fig. 4, Rao *et al.*, 1994).

From the observations in this paper, cooking of meat and the generation of an "off-flavor" may be aided by myoglobin up to certain middle range heating temperatures. At higher temperatures, factors other than prooxidant activity of myoglobin, such as the thermal decomposition of lipid hydroperoxides and the formation of secondary products of lipid oxidation processes, might be more important in advancing the peroxidation of the lipids.

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