Lipid Peroxidation of Muscle Food: The Role of the Cytosolic Fraction†

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The cytosolic extract from turkey muscle tissues contained compounds that partially inhibited membrane lipid peroxidation initiated by an iron-redox cycle (ascorbic acid–ferric ions and iron–NADPH enzymatic reaction). The cytosolic extract, however, totally inhibited lipid peroxidation catalyzed by H_2O_2-activated metmyoglobin. The cytosolic extract was separated into low and high molecular weight fractions, and both fractions exhibited inhibitory effects on H_2O_2-activated myoglobin. Membrane lipid peroxidation initiated by the iron-redox cycle was inhibited partially by the high molecular weight fraction but accelerated by the low molecular weight fraction. Turkey muscle cytosol contains reducing compounds at a level equivalent to ~3 mg of ascorbic acid/100 g of fresh weight or at a concentration of approximately 150 μM. These reducing compounds, 80% of which contain ascorbic acid, seem to be responsible for the activation of the iron-redox cycle and for the inhibition of H_2O_2-activated myoglobin toward membranal lipid peroxidation.

INTRODUCTION

Lipid peroxidation of muscle foods is a well-known phenomenon that affects product quality (Asghar et al., 1988; St. Angelo and Bailey, 1987). The role of metal ions and metaloproteins in the catalysis of this reaction was studied by many researchers (Sato and Hegarty, 1971; Igene et al., 1979; Tichivangana and Morrisey, 1985; Rhee et al., 1987; Rhee, 1988; Asghar et al., 1988) including the authors (Kanner et al., 1988a–c).

In spite of many years of research, the involvement of heme proteins and non-heme iron compounds in the process of lipid peroxidation in muscle foods has not been fully resolved. Researchers utilized water-extracted muscle residue as a model system to study the mechanism by which lipid peroxidation occurs in muscle foods (Sato and Hegarty, 1971; Igene et al., 1979; Tichivangana and Morrisey, 1985; Rhee et al., 1987; Asghar et al., 1988; Rhee, 1988). However, it is apparent that aqueous extraction of muscle tissues might also have extracted several essential compounds, such as enzymes, H_2O_2 (Harel and Kanner, 1985), and reducing compounds or chelating agents which may affect the overall catalysis of muscle lipid peroxidation in situ.

This study was conducted to evaluate the effect of the cytosolic extract (soluble fraction) on lipid peroxidation of the water-extracted muscle residue (insoluble fraction) stimulated by H_2O_2-activated myoglobin and iron ion dependent enzymatic and nonenzymatic reactions.

MATERIALS AND METHODS

Materials. Ascorbic acid, trichloroacetic acid, and hydrogen peroxide (30% for synthesis) were purchased from Merck (Darmstadt, FRG). Myoglobin type I from equine skeletal muscle, β-nicotinamide adenine dinucleotide disodium salt reduced from type X (NADPH), ascorbic acid oxidase, ammonium acetate, sodium acetate, Ferrozine, thiobarbituric acid (TBA), and Neocupreine were all obtained from Sigma Chemical Co. (St. Louis, MO). $FeCl_3 \cdot 6H_2O$ was from Riedel-de Haen (Seelze, Holland), and adenosine 5′-diphosphate salt (ADP) was purchased from Boehringer-Mannheim GmbH (Mannheim, FRG).

Methods. Isolation of the water-extracted muscle residue fraction from fresh turkey muscle was carried out by mincing the tissues with a Moulinex food processor for 30 s. The minced tissues (10 g) were further homogenized in 40 mL of 0.05 M (pH 6.5) acetate with a Polytron homogenizer. The homogenate was centrifuged for 30 min at 100000*g* at 4 °C. The resultant supernatant fraction was used as the soluble cytosolic extract. The pellet was washed and centrifuged three more times with acetate.

Separation of the cytosolic fraction to low (LMW) and high (HMW) molecular weight compounds was done by an Amicon Model 202 (RC8050) instrument, using a cutoff membrane of 10 000 MW.

Lipid peroxidation was determined in a model system by thiobarbituric acid according to the procedure of Bidlack et al. (1973). Thiobarbituric acid reactive substances (TBA-RS) were calculated as malondialdehyde (MDA) with an extinction coefficient of $E_{532} = 1.55 \times 10^5$ M^{-1} cm^{-1} (Buege and Aust, 1978). Lipid peroxidation in the model system containing washed muscle residue was initiated by H_2O_2-activated myoglobin (Kanner et al., 1985a), ascorbic acid–$FeCl_3$ (iron-redox cycle; Kanner et al., 1985), and enzymic reaction of the cytochrome P450 reductase in the presence of NADPH (400 μM), ADP (400 μM), and $FeCl_3$ (10 μM) at pH 6.5 and 37 °C (Kanner et al., 1988c).

Catalase activity was determined by using oxygen evolution from the breakdown of H_2O_2 by the enzyme. The rate of oxygen accumulation was determined by an oxygen monitor (Yellow Spring Instrument Co., Model 53) with a Clark electrode and recorded (Shapira, 1986). The test system contained 10 mM acetate at pH 6.5, 1 mM H_2O_2, and various amounts of soluble cytosolic extracts before and after heating at 98 °C for 5 min.

Chelatable iron ions were assayed by using ferric chloride for standard calibration. The determination of iron ions was done by a method developed by Stookey (1970) using Ferrozine reagent. Ferrozine forms with ferrous ions a complex of high extinction coefficient of $E_{562} \times 2.79 \times 10^4$ M^{-1} cm^{-1}.

Reducing compounds were assayed by using ascorbic acid for standard calibration. The determination of reducing compounds was made by a modified method of Sullivan and Clarke (1955). This method was adapted by Sikic et al. (1977); however, we used Ferrozine reagent instead of α,α-dipyridyl (Stookey, 1970). Minced muscle tissues (1 g) were homogenized with double-distilled water treated with Chelex 100 (3 mL); 1 mL of this mixture was reacted with fresh ferric chloride solution (1 mL, 1 mM) and stirred for 2 min at room temperature, after which time trichloroacetic acid was added (1 mL, 11.3%). The mixture was centrifuged at 10000*g* for 10 min. The supernatant (2 mL) was reacted with ammonium acetate (0.8 mL, 0.34 M) and Ferrozine reagent (0.2 mL, 1 mM) for 10 min; the color developed was determined at 562 nm. Ascorbic acid was calculated from total

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Table I. Total Reducing Compounds (RC) and Ascorbic Acid in Turkey Muscle Tissues

treatment	mg/100 mg of wet weight ± SE	
	total RC[a]	ascorbic acid
dark muscles	3.10 ± 0.2	2.40 ± 0.2
light muscles	2.70 ± 0.3	2.10 ± 0.2

[a] Total reducing compounds are expressed as ascorbic acid. Ascorbic acid was calculated from total reducing compounds, after decomposition of ascorbic acid by ascorbic acid oxidase (Kanner and Shapira, 1989).

Table II. Catalase Activity in Dark and White Turkey Muscle Tissues

treatment	catalase activity,[a] units/g of wet weight ± SE
dark tissue	117.0 ± 6
white tissue	28.8 ± 4

[a] Catalase units = $^1/_2$ μmol of O_2 generated min^{-1} (g of wet weight)$^{-1}$ [or 1 μmol of H_2O_2 decomposed min^{-1} (g of wet weight)$^{-1}$]. The cytosolic extract was determined before and after heating at 98 °C for 5 min. Catalase activity was totally inhibited in heated samples.

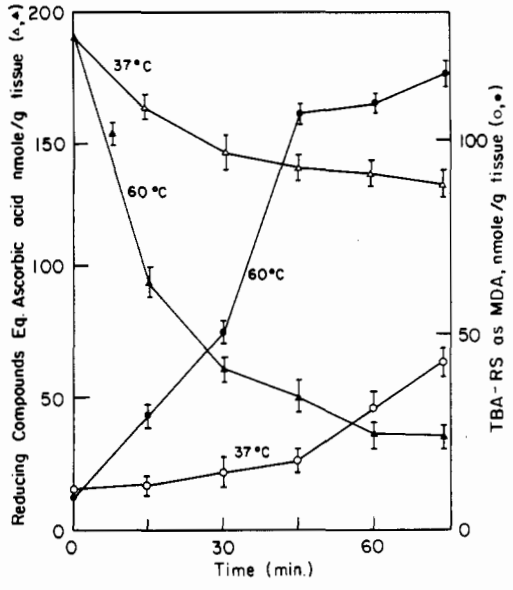

Figure 1. Lipid peroxidation and reducing compounds decrease during incubation of dark muscle homogenate (1:3 w/v) at 37 and 60 °C.

reducing compounds after its oxidation by ascorbic acid oxidase (Kanner and Shapira, 1989). The results were expressed as means of triplicates, and in the figures (Figures 1–5) each bar (I) denotes the standard deviation.

RESULTS

The cytosol from dark and light muscles contains reducing compounds (RC) at a concentration of ~3 mg equiv of ascorbic acid/100 g of wet tissue (Table I). The results showed that about 80% of the RC was ascorbic acid. The concentration of the RC was in the range 140–180 μM equiv of ascorbic acid. The cytosol also contains catalase (Table II). Catalase activity was completely inhibited after heating of the cytosol at 98 °C for 5 min. These results, together with our previous work (Harel and Kanner, 1985), show that the activity derives from true catalase and not from a pseudocatalatic activity of heme proteins which are not affected by heat. Incubation of muscle homogenate at 37 °C, especially at 60 °C, significantly reduced the RC, while the TBA-RS was elevated (Figure 1). Similar results were obtained by using minced muscle tissues (results not shown).

Table III. Lipid Peroxidation Initiated by H_2O_2-Activated Metmyoglobin As Affected by the Cytosolic Fraction before or after Heating[a]

treatment	lipid peroxidation, %
washed muscle residue + buffer	100
+ cytosolic fraction	2
+ heated cytosolic fraction (95 °C, 5 min)	15
+ heated cytosolic fraction (95 °C, 60 min)	98

[a] H_2O_2-activated metmyoglobin was generated by incubating H_2O_2 with metmyoglobin for 3 min before it was added to the washed muscle residue system (Kanner and Harel, 1985a). Washed muscle residue was diluted with cytosolic fraction 1:4 (w/v) or 50 mM acetate, pH 6.5, and incubated at 37 °C for 30 min. Lipid peroxidation was determined by the accumulation of TBA-RS. The cytosolic fraction was heated to 95 °C for 5–60 min and cooled before it was added to the muscle system.

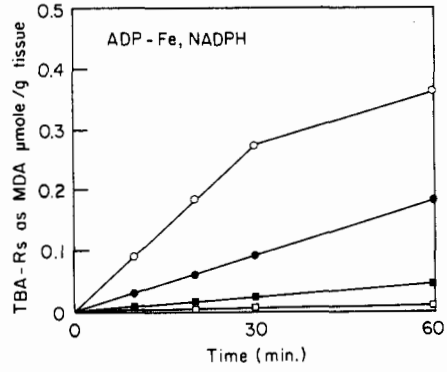

Figure 2. Enzyme-dependent lipid peroxidation of dark turkey washed muscle residue as affected by cytosolic extract (at pH 6.5 and 37 °C). (□) Controls, water-extracted muscle residue (equivalent to 1 g of muscle wet eight) in the presence of 50 mM acetate (3 mL), pH 6.5. (■) Controls, water muscle residue (equivalent to 1 g of muscle wet weight) in the presence of first cytosolic extract (3 mL), pH 6.5. (○) Washed muscle residue in acetate plus ADP (400 μM), $FeCl_3$ (10 μM), and NADPH (400 μM). (●) washed muscle residue in the presence of cytosolic extract (1:3 w/v) plus ADP (400 μM), $FeCl_3$ (10 μM), and NADPH (400 μM).

Water-extracted muscle residue was incubated in the presence of H_2O_2-activated metmyoglobin (30 μM each), which catalyzed membrane lipid peroxidation. This catalysis was inhibited completely by the cytosolic extract, from both dark (Table III) and white (results not shown) muscles. It was possible to eliminate the inhibitor effect by heating the extract before it was added to the model system. This treatment almost completely oxidized the cytosolic RC (Figure 1).

Water-extracted dark muscle residue incubated in the presence of NADPH (400 μM), ADP (400 μM), and $FeCl_3$ (10 μM) (enzymic system) (Lin and Hultin, 1976; Rhee, 1988) accelerated membrane lipid peroxidation. However, when the system contained the cytosolic fraction, this catalysis was significantly suppressed (Figure 2).

A third system was included, which could initiate membrane lipid peroxidation (Kanner et al., 1986). This system contained water-extracted muscle residue incubated in the presence of ascorbic acid (200 μM) and $FeCl_3$ (10 μM), the iron-redox cycle system. The system containing ascorbic acid and iron ions accelerated membrane lipid peroxidation, but when the system contained the cytosolic fraction, this catalysis was significantly suppressed, especially during the first stage of peroxidation

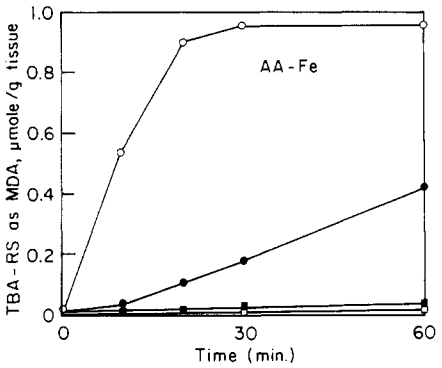

Figure 3. Iron redox cycle dependent lipid peroxidation of dark turkey washed muscle residue, as affected by cytosolic extract (at pH 6.5 and 37 °C). (□) Washed muscle residue in acetate. (■) Washed muscle residue in the presence of cytosolic extract (1:3 w/v). (○) Washed muscle residue in acetate plus ascorbic acid (200 μM) and $FeCl_3$ (10 μM). (●) Washed muscle residue in the presence of cytosolic extract (1:3 w/v) plus ascorbic acid (200 μM) and $FeCl_3$ (10 μM).

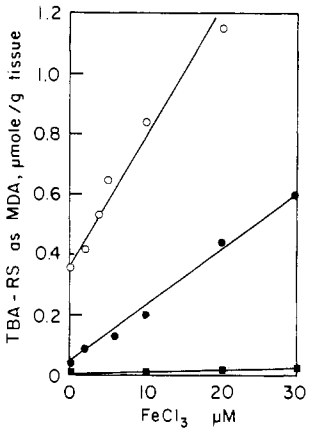

Figure 4. Catalysis of muscle lipid peroxidation by ferric chloride ions in the presence of reducing compounds or cytosolic muscle extract at 37 °C. (■) Water-extracted muscle residue (equivalent to 1 g of muscle wet weight) in the presence of 50 mM acetate (3 mL), pH 6.5. (●) Water-extracted muscle residue (equivalent to 1 g of muscle wet weight) in the presence of first cytosolic extract (3 mL) pH 6.5. (○) Water-extracted muscle residue (equivalent to 1 g of muscle wet weight) in the presence of ascorbic acid (200 μM) in 50 mM acetate, pH 6.5.

(Figure 3). Almost the same effect was observed with the cytosol extracted from light muscles (results not shown).

Figure 4 illustrates the effect of iron ions on the catalysis of membrane lipid peroxidation. The addition of iron ions to water-extracted muscle residue did not accelerate lipid peroxidation. The addition of the cytosolic fraction to the model system, containing water-extracted muscle residue and iron ions, stimulated lipid peroxidation. However, ascorbic acid added at a concentration similar to that of the RC in the cytosol accelerated lipid peroxidation at a rate that was 4-fold higher than that produced by the cytosol. These results indicated that the cytosol contained not only compounds that may activate iron toward lipid peroxidation but also compounds that inhibit the same reaction.

The cytosolic extract was separated into low and high molecular weight compounds by membrane filtration. H_2O_2-activated metmyoglobin-dependent lipid peroxidation was inhibited by both low and high molecular weight compounds; however, iron redox cycle dependent lipid peroxidation was inhibited only partially by the high molecular weight fraction and was accelerated by the low molecular weight fraction (Figure 5). The amount of chelatable iron ions in the eluent from muscle extracted with distilled water was 0.8 ± 0.09 μg/g of fresh weight.

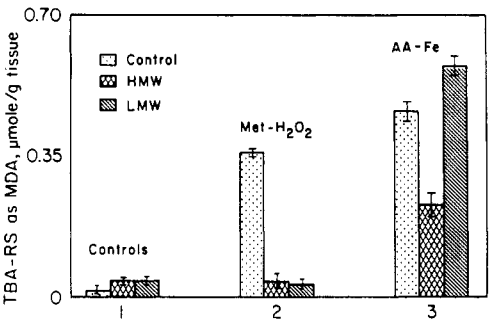

Figure 5. Effect of cytosolic fractions on lipid peroxidation of washed muscle residue by several systems. (1) Washed muscle residue in the presence of cytosolic fractions without catalyzers. (2) Washed muscle residue in the presence of LMW and HMW reconstituted to the cytosolic volume (1:3 w/v) plus activated H_2O_2–metmyoglobin (30 μM each). (3) Washed muscle residue in the presence of LMW and HMW reconstituted to the cytosolic volume (1:3 w/v) in the presence of ascorbic acid (200 μM) and $FeCl_3$ (10 μM).

DISCUSSION

Many studies have been directed toward the identification of the catalysts that promote muscle lipid peroxidation. In the beginning, lipid peroxidation was attributed to heme catalysts (Watts and Peng, 1947; Tappel, 1953, 1962). The involvement of heme proteins as catalyzers of lipid peroxidation was first described by Robinson (1924), who found that hemoglobin accelerates the peroxidation of unsaturated fatty acids. Lipid peroxidation by heme compounds exhibits an induction period which was postulated to be dependent on preformed hydroperoxides (Tappel, 1962). Heme proteins such as hemoglobin or myoglobin accelerate the decomposition of hydroperoxides to free radicals which, in the presence of oxygen, propagate lipid peroxidation (O'Brien, 1969; Kendrick and Watts, 1969; Kanner, 1974).

In studies employing model systems of linoleate emulsions, Liu and Watts (1970) suggested that both heme and non-heme iron are important catalysts of lipid peroxidation in muscle products. Sato and Hegarty (1971) directed a study of muscle lipid peroxidation using a water-washed muscle residue. They indicated that the substances responsible for initiating the reaction were water-soluble. Sato and Hegarty (1971) and others (Love and Pearson, 1974; Igene et al., 1979; Tichivangana and Morrisey, 1985) proposed that non-heme iron plays a major role in the catalysis of muscle lipid peroxidation and that myoglobin is not directly responsible for off-flavors developed during storage of cooked muscle foods.

However, all these model systems suffer from many errors. Model systems using linoleate emulsion contain preformed hydroperoxides at different levels (Kanner, 1974), and they could not simulate lipid peroxidation in muscle cells, which are mostly of a membranal nature, containing unoxidized phospholipids. Model systems containing washed muscle tissues better simulate the situation in muscle tissues. However, most of the researchers using this model system had omitted from it several important compounds which, during the exhaustive washing or dialysis of the muscle samples, were removed (Love and Pearson, 1970; Sato and Hegarty, 1971; Igene et al., 1979; Tichivangana and Morrisey, 1985; Rhee et al., 1987; Asghar et al., 1988).

During incubation of minced muscle tissue hydrogen peroxide is generated (Harel and Kanner, 1985), and this

compound can activate oxy- or metmyoglobin to a ferryl compound (Kanner and Harel, 1985a). Ferrylmyoglobin, which contains tetravalent iron, could oxidize many molecules such as membrane fatty acids (sarcosomes) (Kanner et al., 1988b), free fatty acids (Grisham, 1985), β-carotene and reducing compounds (Kanner and Harel, 1985b), and proteins (Rice et al., 1983). However, hydrogen peroxide is only one of the cytosolic compounds that could affect lipid peroxidation in muscle foods.

Reducing compounds are among the other important compounds that may affect the process of lipid peroxidation (Kanner and Harel, 1985a). The model system in this study was in operation at a temperature/time condition that left the controls (washed muscle tissue residue, with and without the cytosolic extract) at a low and insignificant lipid peroxidation.

The present study demonstrated that the cytosolic extract contained compounds which totally inhibited membrane lipid peroxidation by H_2O_2-activated myoglobin or partially inhibited peroxidation by iron–NADPH enzymic reaction and iron–ascorbic acid reaction. Several other researchers have shown that cytosolic compounds may affect lipid peroxidation (Slabyj and Hultin, 1983; Han and Liston, 1989).

The addition of the muscle cytosolic fraction to a model system containing only water-extracted muscle residue and ferric ions accelerated lipid peroxidation. Ascorbic acid added to the same model system, at a concentration similar to that of the cytosol, accelerated lipid peroxidation at a significantly higher rate. The lower activity of the cytosolic fraction toward lipid peroxidation seems to derive from compounds that inhibit the pro-oxidative reaction.

The inhibitory effect of the cytosolic extract was also determined after separation in low and high molecular compounds. It is assumed that the inhibitory effect of the high molecular weight compounds toward lipid peroxidation by the iron-redox cycle is derived from the capability of this fraction to chelate iron ion and prevent its penetrating the membranes; this effect is similar to that of EDTA (Kanner et al., 1986). Our results show that most of the chelatable iron ions in muscle tissues are connected with the high molecular weight fraction (Kanner et al., unpublished results). This fraction contains proteins, enzymes, and especially catalase; however, catalase cannot inhibit membrane lipid peroxidation initiated by the iron redox cycle (Kanner et al., 1986).

It seems that H_2O_2-activated myoglobin was inhibited especially by the reductive activity of both fractions. The low molecular weight fraction seems to contain most of the reducing compounds which, in the model system of the iron redox cycle, even accelerated the peroxidative process.

In one of our earlier studies (Harel and Kanner, 1985) we showed that H_2O_2-activated myoglobin could be one of the main initiators of lipid peroxidation in muscle foods. However, this study was conducted in a muscle system of sarcosomes without the cytosolic fraction.

Our work demonstrated that H_2O_2-activated metmyoglobin, in the presence of the cytosolic fraction, cannot initiate muscle lipid peroxidation. The present results fit our previous works which showed that myoglobin and hemoglobin are inhibitors of hydroxyl radicals generated in a model system of iron redox cycle (Harel and Kanner, 1989). We assume that myoglobin and hemoglobin in the presence of hydrogen peroxide are activated to ferryl compounds. Ferryl compounds could initiate lipid peroxidation (Kanner and Harel, 1985a). However, in a system containing reducing compounds, such as muscle cells, peroxidation is prevented by competitive oxidation of the reducing compounds, as

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{4+}{=}O + HO^-$$

$$Fe^{4+}{=}O + AH_2 \rightarrow Fe^{3+} + A + H_2O + H^+$$

where Fe^{3+} is metmyoglobin, Fe^{4+} is ferrylmyoglobin, and AH_2 is a reducing agent.

In addition, catalase found in muscle cells will prevent part of the reaction of H_2O_2 with heme proteins.

Heme proteins seem to affect lipid peroxidation in muscle foods only after heating, which inhibits catalase and decreases the amount of the reducing compounds to a low level. At this stage, heme proteins affect especially the propagation process by breaking down hydroperoxides to free radicals.

Recent data indicated that lipid peroxidation in biological tissues (Halliwell and Gutteridge, 1986; Reif et al., 1989) and muscle foods (Kanner et al., 1988a–c) is initiated by "free" iron ions and hydrogen peroxide. This reaction is cycled by superoxide (Haber–Weiss) or by reducing compounds, e.g., ascorbic acid (redox cycle) (Kanner et al., 1987). Furthermore, ferrous ions work especially to propagate lipid peroxidation by breaking down hydroperoxides to free radicals.

In conclusion, the cytosolic fraction contains reducing compounds that stimulate the iron redox cycle dependent lipid peroxidation but totally inhibit the catalysis by activated heme proteins. It seems that the cytosolic fraction also contains compounds which generally suppress muscle lipid peroxidation, acting as antioxidants.

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