# Catalysis of Linoleate Oxidation by Soluble Chicken Muscle Proteins

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**A soluble fraction of a chicken *Musculus gastrocnemius* muscle was used to characterize the catalyst of linoleate oxidation. Separation of the chicken muscle extract into low (free metal) and high (protein) molecular weight fractions revealed that the molecular weight of the major catalyst of linoleate oxidation in chicken muscle extract was greater than 700 daltons. Catalysis of linoleate oxidation by the protein fraction exhibited a pH optimum of 5.9. Subjecting the protein fraction to heat treatments at increasing temperatures (30-90 C) decreased the catalysis of linoleate oxidation. Addition of two mM EDTA had no effect on the catalysis of linoleate oxidation. Cyanide (2 mM), glutathione (1 mM) and cysteine (1 mM) decreased the oxidation of linoleate by the protein fraction 21.0%, 22.9% and 29.0% respectively. Characterization of the oxidative catalyst in chicken muscle extract indicated that free metals and hemoproteins contribute to overall catalysis of linoleate oxidation but are not the sole catalysts. Heat inactivation of the oxidative catalyst and the observed pH optimum suggests that the unidentified catalyst is proteinacious and may be an enzyme.**

Meats and meat products are susceptible to lipid oxidation. Oxidative deterioration of lipids in meats can directly affect many quality parameters such as color, texture, flavor and nutritive value (1). Lipid oxidation occurs in both phospholipids and triglycerides, but Igene et al. (2) reported that phospholipids were oxidized initially, followed by oxidation of triglycerides after a prolonged induction period. Igene et al. (2) also reported that highly unsaturated phospholipids are major contributors to the development of off-flavors. Beef and chicken phospholipids contain equal amounts of unsaturated fatty acids, but the fatty acids of chicken triglycerides are over 69% unsaturated compared to 55% unsaturation in beef triglycerides (3). The large percentage of unsaturated fatty acids in the lipids of poultry muscle results in products susceptible to oxidative deterioration.

The use of mechanically deboned chicken in emulsified and other processed meat products has increased in recent years (4). The crushing and sieving nature of the deboning process results in loss of cell integrity and introduction of lipid oxidation catalysts into the membrane lipids. Mechanical deboning also increases the surface area of the meat and incorporates high levels of oxygen into the meat matrix. Instability of mechanically deboned chicken toward lipid oxidation results in rapid loss of color and functional properties, as well as the rapid development of off-flavors (4).

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Hemoproteins (5) and free transition metals (6) are both powerful oxidation catalysts of unsaturated fatty acids in muscle and model systems. Hemoproteins have been reported to be the major catalyst of lipid oxidation in beef, chicken, turkey, fish and mechanically deboned chicken (1, 7, 8). Free transition metals are important catalysts of lipid oxidation in myoglobin-free liver and kidney homogenates, but play only minor roles in the catalysis of linoleate oxidation in tissues containing myoglobin (9). Igene et al. (10), Sato and Hegarty (11) and Love and Pearson (12) concluded that non-heme iron was the principle catalyst of lipid oxidation in cooked meat. Lui and Watts (13) found non-heme iron to be a major catalyst of oxidative rancidity in raw beef and pork muscle. Hultin (14) has shown that lipid oxidation can be catalyzed by muscle microsomal fractions of poultry and fish in the presence of NADH or NADPH, ADP and $Fe^{+3}$. The combined effect of the reported catalysts (free transition metals, heme, hemoproteins and muscle microsomes) on lipid oxidation in meat has not been elucidated.

## MATERIALS AND METHODS

All chemicals used were purchased from Sigma Chemical Co. (St. Louis, Missouri) unless otherwise indicated. Linoleic acid (99% pure) was dissolved in 95% ethanol, (41.2 mg linoleate/ml ethanol), vacuum sealed in glass ampules (2 ml/ampule) and stored at −20 C prior to use.

Linoleate emulsions were prepared as described by Wills (6). Two ml of the linoleate/ethanol mixture, diluted to five ml with an additional 95% ethanol, were added to 45 ml 0.1 M sodium phosphate buffer, pH 6.2, to form a 0.006 M linoleate emulsion. Oxidation of the linoleate emulsion was measured by oxygen uptake using an Orion 97-08-00 oxygen probe with a 970801 membrane/electrolyte module and an Orion microprocessor ionalyzer/901. Fifty-ml graduated cylinders with ground glass fittings were used as reaction vessels. All glassware was soaked in 2% EDTA for 24 hr and rinsed thoroughly in deionized glass distilled water. Oxygen consumption data was reported as the log of oxygen concentration versus time to obtain linearity.

Six-week-old male Hubbard chickens were obtained from the Washington State University Poultry Center, Pullman, Washington. The chickens were slaughtered, eviscerated and immediately placed in an ice bath for 24 hr. The *Musculus gastrocnemius* muscle was removed, cut into two-g samples, vacuum sealed in nylon-poly pouches and stored at −20 C. Muscle extract was prepared by grinding two g of the *Musculus gastrocnemius* muscle in 10 ml of 0.1 M sodium phosphate buffer, pH 6.2, with a teflon tissue grinder in an ice bath. The homogenate was stored for

24 hr at 4 C for complete extraction of the soluble muscle proteins, vacuum filtered through Whatman #4 filter paper and centrifuged at 5000 rpm for 10 min on a Sorvall GLC-2 laboratory centrifuge equipped with a HL-4 head.

Free metals and other low molecular weight compounds were separated from the proteins of the chicken muscle extract by Sephadex G10 gel filtration chromatography (1 × 45 cm columns with 0.1 M sodium phosphate buffer, pH 6.2, at a flow rate of 20 ml/hr). The protein fractions of the chicken muscle extract were collected, pooled and divided into aliquots.

The effects of heat treatment on the ability of the protein fraction to catalyze linoleate oxidation were determined by subjecting the protein fraction to a two-min heat treatment (30-90 C) in a water bath with constant stirring, followed by immediate cooling to 4 C. The combined supernatant and precipitate of the heated protein fraction was added to the linoleate emulsion for evaluation of oxidation rates. Determination of the effect of emulsion pH on the oxidation of linoleate by the protein fraction was performed in 0.1 M phosphate buffer over a pH range of 4.75-7.75.

The effect of lipid oxidation inhibitors was determined by adding inhibitors directly to phosphate buffer used in emulsion preparation. Final concentrations of two mM were used for ethylene diaminetetraacetic acid (EDTA) and Drabkin reagent (potassium cyanide/potassium ferricyanide). Glutathione and cysteine were added to a final concentration of one mM.

## RESULTS AND DISCUSSION

Chicken muscle extract was separated into low molecular weight free metal and high molecular weight protein fractions by Sephadex G10 chromatography. The rates of linoleate oxidation catalyzed by the low molecular weight and protein fractions, along with a reconstituted crude extract, are presented in Figure 1. Reconstitution of the crude extract decreased oxidation of linoleate when compared to catalysis by the protein fraction. The decrease in oxidation rates observed in the reconstituted extract can be explained by the presence of low molecular weight inhibitors of linoleate oxidation. Ascorbic acid, glutathione, cysteine, histidine (9), manganese and cobalt salts (15), which are present in muscle, inhibit hemoprotein catalyzed oxidation of linoleate. The protein fraction of the chicken muscle extract is responsible for more than 98% of the total oxidation observed in the crude extract.

Proteinacious catalysts of lipid oxidation in muscle were established by Wills (9) and Tappel (1), and oxidation observed in animal muscle was attributed to hemoproteins. Sephadex G10 separation of low molecular weight compounds from proteins confirms that the major catalyst of linoleate oxidation is proteinacious in nature. Determination of the contribution of protein compared to free metal catalysis of linoleate oxidation by chicken muscle extract was not possible because of the presence of oxidation inhibitors in the low molecular weight fraction.

The effect of heat treatment on catalysis of linoleate by the protein fraction is presented in Figure 2. The

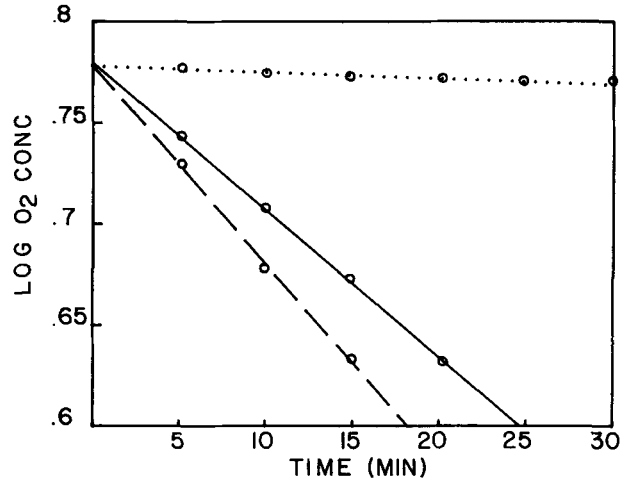


**Fig. 1. Catalysis of linoleate oxidation by the low molecular weight fraction (. . . . . ), the protein fraction ( - - - - ) and the reconstituted crude extract (———) of the chicken muscle extract.**

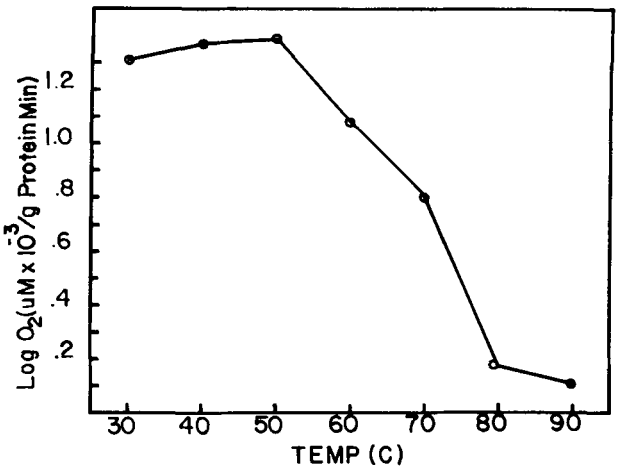


**Fig. 2. Effect of heat treatments (30-90 C) of the protein fraction of chicken muscle extract (1 min) on catalysis of linoleate oxidation.**

oxidative rate catalyzed by the protein fraction decreased sharply as the protein fraction was heated to 60-90 C for two min. Total inhibition of linoleate oxidation was not observed. Protein precipitation was observed at 60 C and above. Heating the protein fraction from 30-50 C had no effect on the rate of linoleate oxidation. Eriksson et al. (16) reported that nonenzymatic catalase and peroxidase was characterized by increasing oxidation rates as hemoproteins were subjected to increasing heat treatments from 25 to 100 C. Increases in the oxidation of linoleate by hemoproteins as a result of heat treatments have been attributed to conformational changes of the hemoproteins, which resulted in greater exposure of the catalytic hematin group to fatty acids, or to the release of non-heme iron (10, 16). The decrease in linoleate oxidation by heat treatment at increasing temperatures (Fig. 2) suggests that hemoproteins are not solely responsible for the observed oxidation rates.

Player and Hultin (17) have reported a 62% decrease

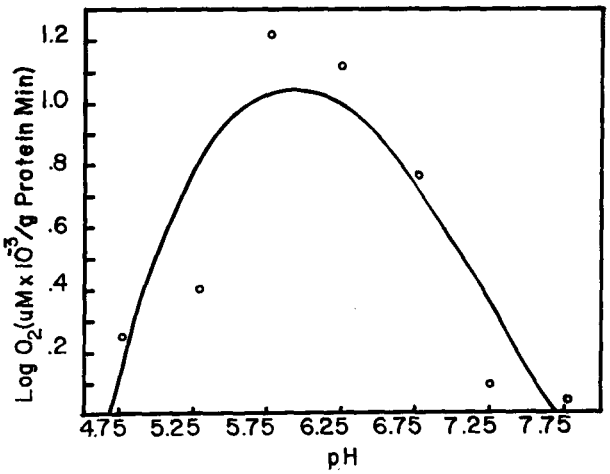


**Fig. 3. Catalysis of linoleate oxidation at different pH's by the protein fraction.**

**TABLE 1**

**Effect of Various Compounds on Linoleate Oxidation by the Protein Fraction of Chicken Muscle Extract**

| Incubation condition | % Oxidation uptake |
|---|---|
| Control[a] | 100.0 |
| Linoleate emulsion alone (6 mM) | <0.1 |
| EDTA (2.0 mM) | 94.1 |
| $KCN/K_3Fe(CN)_6$ (2.0 mM) | 79.0 |
| Glutathione (1.0 mM) | 77.1 |
| Cysteine (1.0 mM) | 71.0 |

[a]The control system consisted of the protein fraction separated by Sephadex G10 in a 6-mM linoleate emulsion. Aliquots for the control and subsequent inhibitor studies were equivalent to 1 ml of chicken muscle extract.

in the formation of thiobarbituric acid reacting materials by heating chicken breast microsomes to 80 C for five min in the presence of NADH, $Fe^{+3}$ and ADP. No data were reported on heating at temperatures less than 80 C. The observed decrease in linoleate oxidation by 60-90 C heat treated protein fraction and the formation of protein precipitate at 60 C demonstrated that denaturation of proteins inhibited linoleate oxidation. The observed decrease in oxidation rates suggests that the proteins responsible for the catalysis of linoleate may be enzymatic in nature, or that the catalytic sites of the proteins become inaccessible to the linoleate molecules as the proteins aggregate.

The effect of emulsion pH on the oxidation of linoleate by the protein fraction of chicken muscle extract is presented in Figure 3. The optimal pH of catalysis was approximately 5.9. Figure 3 cannot be explained by catalysis of linoleate oxidation by free metals or hemoproteins alone. Transition metal catalysis of unsaturated fatty acid oxidation increases as the pH decreases below 7.0. Wills (9) reported an increase in non-heme iron catalyzed oxidation of linoleate with decreasing pH, and Marcuse and Fredricsson (18) reported similar results with ferrous and cupric ion catalyzed oxidation. Lui and Watts (13) observed an increase in hemoprotein catalyzed oxidation of unsaturated fatty acids at alkaline pH. Player and Hultin (17) reported a pH optimum of 5.5 for oxygen uptake of microsomal fractions of chicken breast muscle in the presence of $Fe^{+3}$, ADP and NADPH. The pH optimum (Fig. 3) is similar to that of chicken breast microsomal oxidation. A pH optimum of 5.9 suggests that the oxidation of linoleate by the protein fraction of chicken muscle may be at least partially enzymatic in nature.

To test the hypothesis that neither free metals nor hemoproteins were the major catalysts, an inhibitor study was conducted. EDTA, Drabkin reagent, glutathione and cysteine were added separately to aliquots of the protein fraction of chicken muscle extract (Table 1). The addition of two mM EDTA did not significantly decrease the oxidation of linoleate by the protein fraction of the chicken muscle extract. The chelation of free metals by EDTA inhibits metal catalyzed oxidation of unsaturated fatty acids (19). The absence of a significant decrease in the catalytic rate reemphasizes that free transition metals do not play a significant role in the catalysis of linoleate oxidation by the protein fraction. Lack of inhibition upon addition of EDTA also indicates that microsomes are not major catalysts of linoleate oxidation. Player and Hultin (17) reported strong inhibition of chicken muscle microsomal lipid oxidation in the presence of EDTA.

Catalysis of linoleate oxidation by a cyanide-treated protein fraction of the chicken muscle extract was decreased by 21.0%. O'Brien (20) reported complete inhibition of linoleate hydroperoxide decomposition by hemoproteins in the presence of cyanide. Cyanide inhibits hemoprotein catalysis of hydroperoxide decomposition by strongly binding to the sixth coordination site of the iron in the heme moiety. Formation of stable cyanohemichrome inhibits lipid peroxides from entering the sixth coordination site of the iron, thus inhibiting hydroperoxide decomposition and significantly decreasing the rate of lipid oxidation (21). Addition of two mM potassium cyanide/potassium ferricyanide to the protein fraction caused a 10-15 nm red shift in Soret absorbance as expected with the formation of cyanmetmyoglobin and cyanmethemoglobin (22). Hultin (14) also reported decreases in unsaturated fatty acid oxidation by chicken muscle microsomes upon addition of potassium cyanide. The decrease in oxidation rates of 21.0% observed upon the addition of cyanide to the protein fraction (Table 1) suggests that hemoproteins contribute to the oxidation of linoleate by the protein fraction of the chicken muscle extract but are not solely responsible for the overall catalytic rates observed. The results of the inhibitor study indicate that chicken muscle microsomes and free metals are not significant catalysts of linoleate oxidation in chicken muscle extract.

Addition of one mM glutathione (GSH) to the protein fraction of chicken muscle extract decreased linoleate oxidation 22.9% (Table 1). Wills (9) found complete inhibition of linoleate oxidation by tissue homogenates in the presence of one mM GSH, but increases in oxidation rates were observed at greater GSH concentration (2-5 mM). GSH (1 mM) also

inhibited hemoprotein catalysis of linoleate oxidation, but accelerated linoleate oxidation by iron (6). The observed decrease in oxidation of linoleate by the protein fraction of chicken muscle extract upon the addition of one mM GSH suggests that free metals are not significant catalysts of linoleate oxidation. The 22.9% decrease also indicates that hemoproteins are not the sole catalyst of linoleate oxidation by the protein fraction of chicken muscle extract.

The presence of one mM cysteine decreased catalysis of linoleate oxidation by the protein fraction of chicken muscle extract 29% (Table 1). Wills (6) found that 3.3 mM cysteine initiated the rapid oxidation of linoleate by $Fe^{+3}$, but one mM cysteine decreased linoleate oxidation by hemoproteins. The observed decrease in oxidation of linoleate by the one mM cysteine-treated protein fraction reemphasized that free metals are not important catalysts, and that hemoproteins are not the sole catalyst of linoleate oxidation in chicken muscle extract.

Lipid oxidation observed in meat has been attributed to the catalytic action of free metals, microsomes, heme and hemoproteins (23). The contribution of each of these catalysts to the overall oxidation observed in muscle has not been elucidated previously. We determined that heme and hemoproteins contributed to the overall rate of lipid oxidation but were not the major catalysts. Free metals were found to be minor catalysts of lipid oxidation in raw poultry meat. The major catalysts of lipid oxidation in chicken muscle found in this study have not been reported previously. The major cataysts have molecular weights greater than 700 daltons and have temperature and pH profiles indicative of enzymes. Inhibitor studies indicate that the major catalyst of lipid oxidation does not contain a heme moiety and is not microsomal in nature. These findings suggest that the unidentified catalyst of linoleate oxidation in chicken muscle is proteinacious and may be an enzyme.

Further studies are needed to verify the proteinacious and possibly enzymic nature of the catalysts of lipid oxidation found in this study.

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