Catalysis of Linoleate Oxidation by Nonheme- and Heme-Soluble Chicken Muscle Proteins

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Catalysis of lipid oxidation in chicken muscle was studied on an extract of the chicken Musculus gastrocnemius muscle. Chicken muscle extract was separated with Sephadex G200 gel filtration into three major protein fractions. The intermediate molecular weight fraction (42000–250000 Da) of chicken muscle extract catalyzed 92.5% of the total linoleate oxidation by chicken muscle extract. The intermediate molecular weight fraction was further separated into four protein fractions by Sephadex CM25 cation-exchange chromatography. Native polyacrylamide gel electrophoresis and a staining technique specific for linoleate oxidizing proteins revealed prooxidants in three of the CM25 fractions. Hemoglobin, a prooxidant of the intermediate molecular weight fraction, was responsible for 30.4% of the total linoleate oxidation. The other two prooxidants were nonheme as evidenced by the absence of Soret absorbance. The major nonheme prooxidant of the intermediate weight fraction had a molecular weight of 51000 and was responsible for 52% of the total linoleate oxidation.

INTRODUCTION

Lipid oxidation is a major cause of quality deterioration in mechanically deboned meat (Dawson and Gartner, 1983). Oxidative deterioration in muscle foods is characterized by undesirable changes in flavor, color, texture, and nutritional quality (Tappel, 1952). Mechanically deboned muscle foods are especially susceptible to lipid oxidation because of increased surface area and aeration that occurs during the mechanical deboning process (Dawson and Gartner, 1983).

Tappel (1952) reported hemoproteins to be the major catalyst of lipid oxidation in beef, chicken, turkey, and fish muscle. The role hemoproteins play in catalysis of lipid oxidation in chicken muscle is not well understood. Lee et al. (1975) characterized catalysts of linoleate oxidation in mechanically deboned chicken by determining the effects of pH, ascorbic acid, chelating agents, thiol compounds, cyanide, and the destruction of hemoproteins on oxidation rates. Lee concluded that hemoproteins were the predominant and possibly sole catalyst of lipid oxidation in mechanically deboned chicken.

Additional work by Mast and MacNeil (1976), Chen and Waimaleongora-ek (1981), and Igene et al. (1979, 1985) did not show hemoproteins to be the sole catalyst of lipid oxidation. Mast and MacNeil (1976) observed that pasteurized mechanically deboned chicken (59–60 °C for 2–6 min) had decreased thiobarbituric acid values compared to unpasteurized controls. Chen and Waimaleongora-ek (1981) reported increased lipid oxidation as the pH of ground chicken meat was increased (3.08–9.5). The reported increase in stability of chicken lipids by heating and decreasing pH does not support the theory of hemoprotein catalysis of lipid oxidation in mechanically deboned chicken. Igene et al. (1979, 1985) demonstrated that nonheme iron was a significant factor in lipid oxidation in cooked chicken. Ericksson et al. (1971) reported increased catalysis of unsaturated fatty acid oxidation as hemoproteins were heated (25–100 °C). Liu and Watts (1970) observed increasing hemoprotein catalysis of lipid oxidation with increasing pH (5.6–7.8).

The objectives of this study were to resolve and identify soluble proteins of chicken muscle responsible for catalysis of linoleate oxidation.

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MATERIALS AND METHODS

All chemicals used were purchased from Sigma Chemical Co. unless otherwise indicated. Linoleic acid (99% pure) was dissolved in 95% ethanol, (41.2 mg of linoleate/mL of 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 (1965). Two milliliters of the linoleate/ethanol mixture, diluted to 5 mL with additional 95% ethanol, was added to 45 mL of 0.1 M sodium phosphate buffer, pH 6.2, to form a 0.006 M linoleate emulsion. Atmospheric oxygen was incorporated into the emulsion by shaking. Oxidation of the linoleate emulsion was measured by oxygen uptake by an Orion 97-08-00 oxygen probe with a 970801 membrane/electrolyte module and an Orion microprocessor ionalyzer/901. Fifty-milliliter graduated cylinders with ground-glass fittings were used as reaction vessels. The diameter of the ground-glass fitting was identical with the diameter of the oxygen probe, resulting in an air-tight seal when the probe was immersed in the linoleate emulsion. The reaction vessel was painted black to decrease the effect of photoxidation. All glassware was soaked in 2% EDTA for 24 h and rinsed thoroughly in deionized glass-distilled water. Oxygen consumption data were reported as the log of oxygen concentration (μmol of O_2) vs. time to obtain linearity, and all oxidation experiments were performed at 25 °C.

Six-week-old male Hubbard chickens were obtained from the Washington State University Poultry Center, Pullman, WA. The chickens were slaughtered, eviscerated, and immediately placed in an ice bath for 24 h. The Musculus gastrocnemius muscle was removed, cut into 2-g samples, vacuum sealed in nylon-poly pouches, and stored at −20 °C. Muscle extract was prepared by grinding 2 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 h at 4 °C for complete extraction of the soluble muscle proteins, vacuum filtered through Watman #4 filter paper, and centrifuged at 4600g for 10 min on a Sorvall GLC-2 laboratory centrifuge equipped with a HL-4 head.

Soret absorbance was determined spectrophotometrically according to the method of Henry et al. (1974). Protein fractions were scanned from 600 to 400 nm to determine the absorbance of the α, β, and γ spectra characteristic to hemoproteins. Protein concentrations were determined with the dye-binding method described

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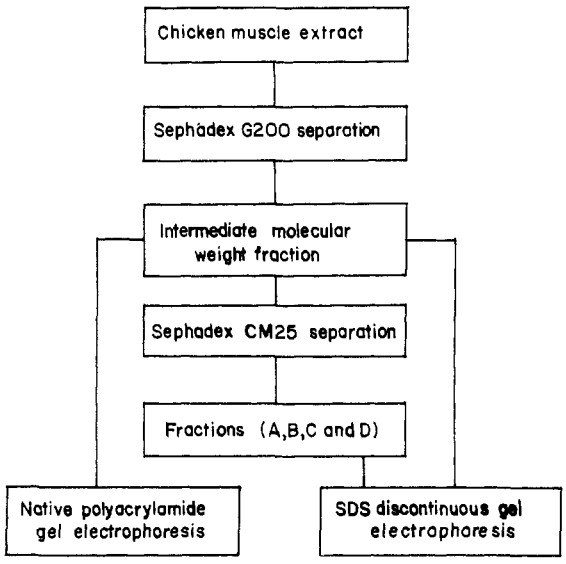

Figure 1. Experimental scheme of prooxidant isolation and identification.

by Bradford (1976) using bovine serum albumin as the standard.

Chicken muscle extract proteins were resolved with Sephadex G200 gel exclusion chromatography followed by CM25 cation-exchange chromatography. G200 separation was performed with a 1 × 45 cm column, 0.1 M phosphate buffer, pH 6.2, at 3.3 mL/h. Molecular weight calibrations were made on catalase, aldolase, bovine hemoglobin, and bovine myoglobin standards (250000, 160000, 64000, and 16000 Da, respectively). Eluate was continuously monitored with an Isco Type B monitor with a 280–310-nm filter.

Cation-exchange CM25 Sephadex chromatography was performed with a 1 × 45 cm column at pH 5.6, 14 mL/h. Three milliliters of the protein extract was loaded onto the column and eluted with a 150 mL of 1–1000 mM phosphate buffer gradient. All column chromatography was performed at 4 °C.

The effect of cyanide on catalysis of linoleate oxidation was determined by adding Drabkin reagent (Henry et al., 1974) to a final cyanide concentration of 2.0 mM. Chymotrypsin was added to the G200 intermediate molecular weight fraction to a concentration of 10^{-6} g/mL and incubated at room temperature for 1 h. Protein fractions were prepared for electrophoresis by concentration on an Amicon Ultrafilter with PM10 membranes and by dialyzing against 0.1 M phosphate buffer, pH 6.2.

Native and SDS electrophoresis was performed according to the method of Laemmli (1970). SDS and native gels were stained with Coomassie Brilliant Blue. Molecular weight estimations were related to phosphorylase *b*, bovine serum albumin, carbonic anhydrase, trypsin inhibitor, and lysozyme standards (97400, 66000, 29000, 20100, and 14300 Da, respectively). Proteins that catalyze linoleate oxidation were determined by electrophoresis and staining as described by Guss (1967). A 7.5% polyacrylamide gel with 0.5% soluble potato starch was used to resolve the proteins. After electrophoresis the gels were incubated in a linoleate/ethanol/phosphate buffer for 20 min, followed by incubation in acidic potassium iodide. Linoleate oxidizing proteins were revealed as blue–brown bands.

RESULTS AND DISCUSSION

In Figure 1 the analytical steps of our study of the catalysts of linoleate oxidation in chicken muscle are schematically outlined.

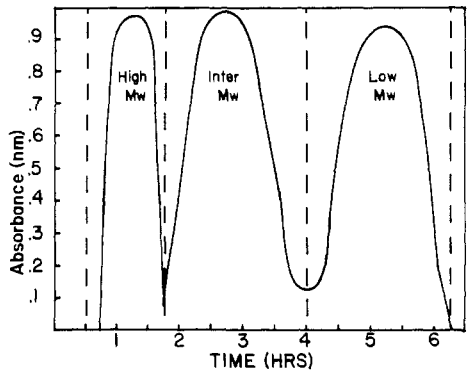

Figure 2. Elution profile of G200 gel filtration of crude chicken muscle extract.

Table I. Catalysis of Linoleate Oxidation as Monitored by Oxygen Consumption by G200 and CM25 Chromatography Fractions of Chicken Muscle Extract

column	fraction	log O_2 consump, μmol O_2/extr from 1 g muscle per h	% total O_2 consump	protein, mg/extr from 1 g muscle
lipid control[a]		0.0011		0.00
protein control[b]		0.0006		ND[c]
G200[d]	low MW	0.0071	6.1	ND
	inter MW	0.1082	92.5	ND
	high MW	0.0016	1.4	ND
CM25[d]	A	0.0081	8.7	3.38
	B	0.0083	9.0	0.16
	C	0.0482	52.0	1.30
	D	0.0282	30.4	3.41

[a] Linoleic acid. [b] Crude extract. No linoleic acid. [c] Not determined. [d] All data corrected for controls.

Chicken extract was resolved into three protein fractions, low, intermediate, and high molecular weight, by Sephadex G200 chromatography. The elution profile for the separation is shown in Figure 2. Catalysis of linoleate oxidation by each fraction is shown in Table I. The intermediate molecular weight fraction (42000–250000 Da) was responsible for 92.5% of the total catalysis of linoleate oxidation by chicken muscle extract. High (>250000 Da) and low (<42000 Da) molecular weight fractions catalyzed 1.4% and 6.1% of the total oxidation, respectively.

The high molecular weight fraction, which was the least active catalyst of lipid oxidation, contains cellular material over 250000 Da such as proteins, lipid vesicles, and muscle microsomes. The low molecular weight fraction catalyzed 6.1% of the total linoleate oxidation by chicken muscle extract. The low molecular weight fraction contains cellular material such as metal salts and the hemoproteins myoglobin and cytochrome *c*, which have been reported to catalyze lipid oxidation in muscle foods (Gardner, 1975) and probably account for the observed oxidation.

The intermediate molecular weight fraction catalyzed 92.5% of linoleate oxidation by the chicken muscle extract. Soret absorbance indicated that the intermediate molecular weight fraction contained proteins with heme moieties. Iodine staining of native polyacrylamide electrophoresis gels revealed three proteins in the intermediate molecular weight fraction capable of oxidizing linoleate (Figure 3). The fourth peak corresponded to insoluble proteins that remained on the gel front.

Addition of 2 mM Drabkin reagent (ferricyanide/cyanide) to the intermediate molecular weight fraction decreased oxidation 33.3% (Table II). O'Brien (1969) reported complete inhibition of hemoprotein-catalyzed hydroperoxide decomposition by hemoproteins in the pres-

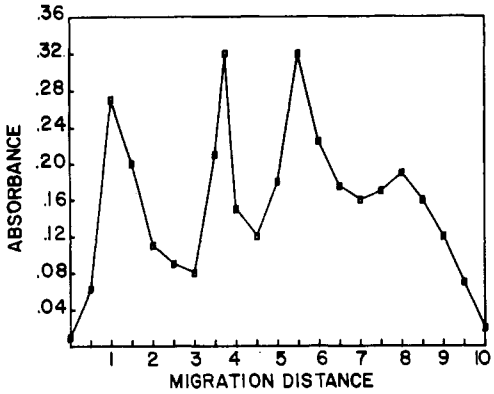

Figure 3. Densitometer scan of a polyacrylamide gel stained for linoleate oxidizing proteins in the G200 intermediate molecular weight fraction.

Table II. Effect of Drabkins Reagent (Cyanide) and Chymotrypsin on the Catalysis of Linoleate Oxidation as Monitored by Oxygen Consumption, by the Intermediate Molecular Weight G200 Fraction of Chicken Muscle Extract

treatment	log O_2 consump, μmol O_2/extr from 1 g muscle per h	% redn in O_2 consump
control	0.0809	
drabkins	0.0540	33.3
chymotrypsin	0.0773	4.4

ence of cyanide. The absence of strong inhibition of linoleate oxidation by Drabkins reagent indicates that hemoproteins may not be solely responsible for the observed oxidation.

Addition of 1×10^{-6} g of chymotrypsin/aliquot of intermediate molecular weight fraction decreased linoleate oxidation 4.4% (Table II). Addition of chymotrypsin to the intermediate molecular weight fraction of chicken muscle extract will hydrolyze accessible peptide bonds between the aromatic and other amino acids. Therefore, the added chymotrypsin would be expected to cleave some of the proteins in the intermediate molecular weight fraction. Absence of a significant decrease in linoleate oxidation upon addition of chymotrypsin suggests that the catalyst responsible for the observed linoleate oxidation may not be enzymatic.

Proteins of the intermediate weight fraction were resolved with Sephadex CM25 cation-exchange resin. Four protein fractions, A–D, were collected. The elution profile is shown in Figure 4. Fraction D was the only fraction that contained hemoproteins as evidenced by Soret absorbance. Catalysis of linoleate oxidation by the four CM25 fractions is shown in Table I. Fraction C was the most active prooxidant of the four fractions catalyzing 52% of the total linoleate oxidation by the intermediate molecular weight fraction. Fractions A, B, and D catalyzed 8.7%, 9.0%, and 30.4% of the observed linoleate oxidation, respectively.

CM25 fractions were subjected to native polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. Protein concentration within CM25 fractions was insufficient to give significant iodide oxidation and subsequent staining of the starch gels. Therefore, the relative mobilities of the proteins in the CM25 Coomassie-stained gels were compared to the relative mobilities of the proteins in G200 iodide-stained gels to determine which CM25 fractions contained the proxidant proteins. Relative mobilities (R_f) of proteins in the four CM25 fractions and the G200 intermediate molecular weight fraction are presented in Table III. Complete separation of fraction A and B and

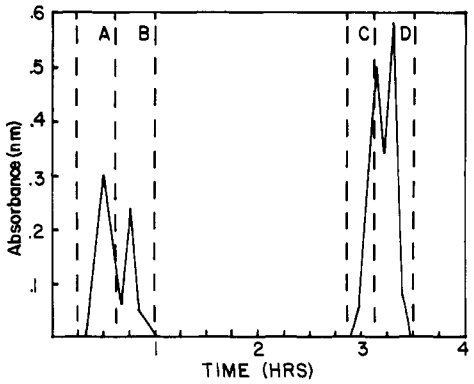

Figure 4. Elution profile of CM25 gradient cation-exchange chromatography.

Table III. Relative Mobilities of the G200 Intermediate Molecular Weight and CM25 Fractions Obtained from Native Discontinuous Polyacrylamide Gel Electrophoresis

fraction	protein no.	rel mobility	fraction	protein no.	rel mobility
G200	1[a]	0.064	CM25 fraction A	6	0.208
	2[a]	0.088		7	0.372
	3	0.112		8	0.424
	4[a]	0.160		9	0.440
	5[b]	0.192	CM25 fraction B	1[c]	0.056
	6	0.212		3	0.116
	7	0.376	CM25 fraction C	2[c]	0.088
	8	0.424	CM25 fraction D	4[c]	0.152
	9	0.440			

[a] Proteins showing positive iodide oxidation staining. [b] Observed in the intermediate molecular weight fraction of G200 separation but not in the CM25 fractions. [c] Proteins with relative mobilities corresponding to the relative mobilities of the G200 proteins that showed positive iodide oxidation staining.

C from D was not possible with CM25 cation exchange. Table III indicates the fraction in which the majority of protein (staining intensity) was observed.

Table III indicates that proteins capable of catalyzing linoleate oxidation in chicken muscle extract are found in the CM25 fractions B–D. Observed oxidation of linoleate by fraction A could not be explained. Linoleate oxidation by fraction A may be the result of residual prooxidant from fraction B. Protein 5 was observed in the intermediate molecular weight fraction but not in CM25 fractions. Absence of protein 5 in CM25 fractions could be the result of inability to detect the protein during elution or the inability to elute the protein from CM25 resin with 1 M phosphate buffer.

The molecular weight of the proteins in the G200 intermediate molecular weight and CM25 fractions was determined by polyacrylamide–SDS electrophoresis (Table IV). Table IV indicated the fraction in which the majority of the proteins was observed. SDS discontinuous electrophoresis revealed 14 proteins within the intermediate molecular weight fraction of chicken muscle extract compared to 9 for native PAGE of the intermediate molecular weight fraction. Proteins H and N were observed within the intermediate molecular weight fraction, but not in CM25 fractions. Absence of proteins in CM25 fractions could be the result of inability to detect or elute the proteins from the cation exchange resin.

Identification and molecular weight determination of proteins responsible for linoleate oxidation within CM25 fraction B could not be made because of the presence of four proteins within the fraction and the inability to conduct iodide staining on SDS denatured proteins. Fraction C contained the protein responsible for the majority of linoleate oxidation catalyzed by the intermediate

Table IV. Relative Mobilities of the Intermediate Molecular Weight Fraction of G200 and CM25 Fractions Obtained from SDS Discontinuous Gel Electrophoresis

fraction	protein	rel mobility	mol wt
G200	A	0.203	91 000
	B	0.216	88 000
	C	0.305	71 000
	D	0.322	66 000
	E	0.330	64 000
	F	0.381	57 000
	G	0.411	51 000
	H[b]	0.443	46 000
	I	0.470	44 000
	J	0.500	42 000
	K	0.530	38 000
	L	0.661	26 000
	M	0.686	25 000
	N[b]	0.720	23 000
CM25 fraction A	D	0.318	66 000
	E	0.330	64 000
	L	0.652	26 000
	M	0.686	25 000
CM25 fraction B	A	0.208	91 000
	B	0.216	88 000
	C	0.301	71 000
	J	0.500	42 000
CM25 fraction C	G	0.411	51 000
CM25 fraction D	F	0.386	55 000
	I	0.470	44 000
	K	0.530	38 000

[a] Molecular weight expressed as daltons. [b] Observed in G200 intermediate molecular weight fraction but not in CM25 fractions.

molecular weight fraction of chicken muscle extract. The protein of fraction C had a molecular weight of 51 000 Da. Fraction D contained three proteins or protein subunits. The prooxidant of fraction D had a molecular weight of 38 000 Da. A molecular weight of 38 000 Da corresponds to the molecular weight of hemoglobin dimers, as determined by SDS discontinuous electrophoresis of a chicken hemoglobin standard.

Catalysis of linoleate oxidation by chicken muscle extract is not solely due to metal salts and hemoproteins. Catalysis of linoleate oxidation by the intermediate molecular weight fraction of chicken muscle extract is largely due to three proteins. Hemoglobin was responsible for 30.4% of the linoleate oxidation, and two nonheme proteins were responsible for over 61.0% of the oxidation by the intermediate molecular weight fraction. The nonheme proteins may contain transition metals (copper, cobalt, nonheme iron). Further studies are needed to purify and characterize the two nonheme proteins.

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Registry No. Linoleic acid, 60-33-3.

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