Lipoxygenase in Chicken Muscle

S. Grossman, M. Bergman, and D. Sklan*

The presence of lipoxygenase-type enzymes was demonstrated in chick muscles. Examination of the oxidation products of [^{14}C]arachidonic acid revealed the presence of 15-lipoxygenase. The enzyme was partially purified by affinity chromatography on linoleoyl-aminoethyl-Sepharose. The enzyme was stable on frozen storage, and activity was almost completely preserved after 12-month storage at −20 °C. During this period the content of *cis,cis*-1,4-pentadiene fatty acids decreased slightly. It is suggested that lipoxygenase may be responsible for some of the oxidative changes occurring in fatty acids on frozen storage of chicken meat.

Poultry meat, in particular turkey meat, is susceptible to the development of rancidity and off-flavors on frozen storage. This has been attributed, at least in part, to oxidation of the polyunsaturated fatty acids (PUFA) (Wilson et al., 1976). The sensitivity of the tissue to oxidative deterioration is a function of the PUFA content (Sklan et al., 1983a); high tocopherol retards oxidation (Sklan et al., 1983b). This oxidation has been assumed to occur mainly through catalysis by heme iron; however, evidence for non-heme-soluble protein catalysis has recently been published (Decker and Schanus, 1986; Rhee et al., 1987).

Lipoxygenase (linoleate:oxygen oxidoreductase, E.C. 1.13.11.12) catalyzes the hydroperoxidation of fatty acids interrupted by *cis*-methylene (Tappel, 1963). The existence of mammalian lipoxygenase was first recognized in human platelets by Nutgeren (1975). Since then, the presence of lipoxygenase has been reported in lung, kidney, testes, and white blood cells (Goetzl et al., 1980; Borgeat and Samuelsson, 1979; Shahin et al., 1978; Grossman et al., 1986) and in gill tissue of fish (German et al., 1986).

In this study, the presence of a lipoxygenase enzyme in chicken muscle is reported and its partial purification and characterization are described.

MATERIALS AND METHODS

Pectoralis and gastrocnemius muscles were excised from 8–9-week-old male broilers immediately after slaughtering and stored at −20 °C for differing periods of time.

A microsomal fraction was prepared by sequential centrifugation (Wlodawe and Samuelsson, 1973) and in some cases stored at −20 °C for several days. Particles were suspended in 0.2 M phosphate buffer, pH 6.5, for assay of lipoxygenase.

Lipoxygenase was determined polarographically or spectrophotometrically at pH 6.5 according to Grossman and Zakut (1979) using linoleic acid as substrate. Oxidation products of lipoxygenase from whole homogenate tissues were followed with [^{14}C]arachidonic acid (Radiochemical Center, Amersham). The reaction mixture contained 0.1 μCi of labeled substrate and 2–3 mg of muscle homogenate in a final volume of 0.5 mL by adding 50 mM Tris-HCl, pH 7.4. Inhibitors added to the assay were nordihydroguaiaretic acid (NDGA) or indomethacin at final concentrations of 20 or 200 μM, respectively. For both inhibitors a stock alkaline solution was prepared. The reaction mixtures were incubated for 15 min at 42 °C with gentle shaking. The reaction was stopped by acidifying to pH 3–4 with 10% formic acid. The tubes were extracted with 5 mL of diethyl ether, which was evaporated to dryness, taken up in diethyl ether, and separated by thin-layer chromatography on 0.2-mm plastic plates of silica gel F (Merck Inc, Darmstadt). Elution was with diethyl ether–petroleum ether–acetic acid, 100:50:1. Separated components were visualized with ultraviolet light or with I_2 vapor. Labeled substrates were determined by autoradiography with Curix RP-2 films (Alfa) with exposure times of 10–14 days.

Products were identified by high-performance liquid chromatography (HPLC) on a reversed phase Ultrasphere ODS column by the method of Funk et al. (1976). 5-HETE, 12-HETE, and 15-HETE served as standards. Eluent was monitored at 254 nm and ^{14}C with a radioactive flow detector.

The content of polyunsaturated free fatty acids in muscle lipids was determined with heptadecanoic acid as internal standard following extraction with chloroform–methanol (Folch et al., 1957), separation by TLC, and gas chromatography (Sklan et al., 1983b).

The microsomal lipoxygenase was purified by affinity chromatography on linoleoyl-aminoethyl-Sepharose as described by Grossman et al. (1972) using Sepharose 4B rather than agarose. The microsomal fraction (2 g wet weight) was homogenized with 5 mL of 5 mM sodium acetate buffer, pH 5.0, 1% Triton X-100 using a glass tissue homogenizer. All steps were carried out at 0–4 °C. The homogenate was centrifuged at 12000*g* for 10 min.

The solubilized lipoxygenase supernatant was applied to the linoleyl-aminoethyl-Sepharose column (10 × 2 cm) equilibrated with 5 mM sodium acetate buffer, pH 5.0. The column was eluted until absorbance at 280 nm was zero and then with 200 mM acetate, pH 5.0; an additional elution with 100 mM sodium borate, pH 9.0, was carried out. Fractions of 2 mL were collected from the column and protein content and lipoxygenase activity monitored.

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Table I. Linoleate Oxidation by Microsomal Fraction from Pectoralis and Gastrocnemius of Chicken with Storage at −20 °C

	activity,[a] L O_2/min per mg protein		
	fresh	6-month storage	12-month storage
pectoralis	0.30	0.28	0.24
gastrocnemius	0.30	0.38	0.31

[a] Activity was followed polarographically as described in Materials and Methods.

RESULTS

Preliminary experiments showed that muscle homogenates in 0.2 M sodium phosphate buffer, pH 6.5, contained peroxidative activity when measured polarographically with linoleic acid as substrate. However, the activity obtained with microsomal fractions from these homogen-

Department of Life Sciences, University of Bar Ilan, Ramat-Gan, Israel (S.G., M.B.), and Faculty of Agriculture, Hebrew University, Rehovot 76-100, Israel (D.S.).

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Table II. Contents of Polyunsaturated Free Fatty Acids in Breast and Leg Muscles Lipids with Storage at -18 °C

	% PUFA[a]/total lipids		
	fresh	6-month storage	12-month storage
pectoralis	30.7 ± 2.9 a	27.3 ± 2.6 ab	26.3 ± 2.6 b
gastrocnemius	34.2 ± 3.5 a	28.9 ± 2.7 b	26.1 ● 3.4 b

[a] 18:2 + 18:3 + 20:4 as percent of total fatty acids. Values are means ± SD, n = 8. Numbers in rows not followed by the same letter differ significantly ($P < 0.05$).

ates was 6–7-fold higher; some typical values are shown in Table I. A microsomal fraction from either pectoralis or gastrocnemius showed activity of a similar order of magnitude on a protein basis. This activity remained relatively stable during long periods of frozen storage. Examination of the content of polyunsaturated free fatty acids during this period revealed a slight decrease with time of storage (Table II). This decrease was greater in the gastrocnemius then in the pectoralis.

Gastrocnemius homogenate was active in oxidizing both linoleate and arachidonate. A typical TLC pattern of products obtained after incubation of the homogenate with [^{14}C]arachidonic acid is shown in Figure 1. Major products had R_f 0.88, 0.84, 0.69, and 0.47. NDGA, an inhibitor of the enzyme lipoxygenase, significantly inhibited production of these products, while indomethacin, a characteristic inhibitor of the cyclooxygenase pathway, did not appear to have any significant effect. Denaturing of the enzyme by boiling did not inhbit the production of the compound with R_f 0.47.

With known standards, the fraction with R_f 0.69 was identified as 15-hydroxyeicosatetraenoic acid (HETE), a well-documented product of the enzyme 15-lipoxygenase. The final identification of this product was achieved by HPLC (Figure 2).

Solubilization of the Oxidation Activity from the Microsomal Fraction. Various techniques including sonication, using solutions of different ionic strengths, and using detergents were tried to solubilize linoleate oxidizing activity. Optimal results, approximately 70% solubilization, were obtained when either 1% Triton X-100 or Tween 20 was used in an acetate buffer at pH 5.0–7.0.

Purification of Lipoxygenase. A microsomal fraction from gastrocnemius solubilized with 1% Tween 20 in 5 mM sodium acetate buffer, pH 5.0, was applied to a column of linoleoyl-aminoethyl-Sepharose. A typical profile of the enzyme elution is shown in Figure 3. The inactive proteins are eluted with 5 mM sodium acetate. Increasing the ionic strength of the buffer resulted in the elution of a fraction with high specific activity for linoleate oxidization. A second active fraction was eluted by raising the pH with 100 mM sodium borate buffer, pH 9.0.

A typical purification procedure is shown in Table III. The overall enrichment obtained was 6–7-fold for each of the affinity-purified fractions, with a total yield of 109% for the first eluted fraction and 77% for the second fraction.

Kinetic Properties. The pH–activity profile for the second active fraction from the affinity chromatography

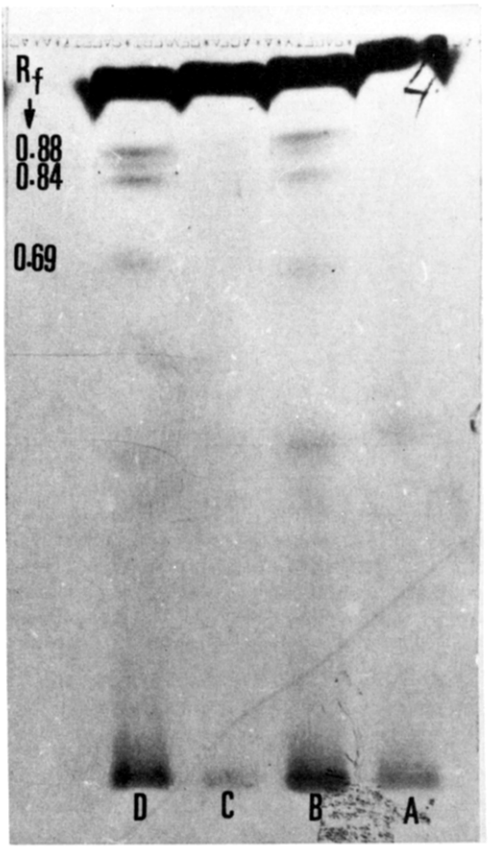

Figure 1. Thin-layer chromatograms of products obtained by incubation of gastrocnemius homogenate with [^{14}C]arachidonic acid: A, boiled enzyme and substrate (control); B, enzyme and substrate; C, enzyme, substrate, and NDGA (10^{-4} M); D, enzyme, substrate, and indomethacin (10^{-6} M).

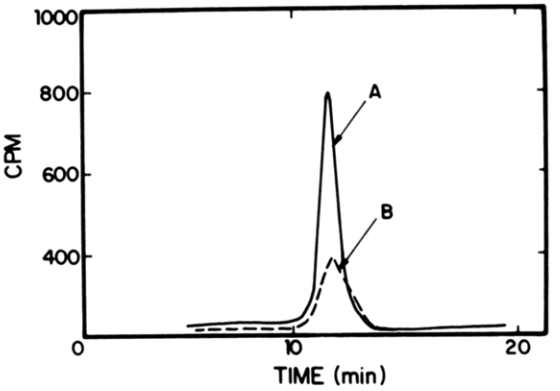

Figure 2. Reversed-phase HPLC separation of arachidonic acid metabolites (see Materials and Methods for details): (A) 15-HETE standard; (B) arachidonic acid oxidized product with R_f 0.69 on TLC.

was determined. The optimum pH for linoleate oxidation was pH 7.0, with very little activity observed below pH 5.0 and above 8.5.

A K_m value of 1.0×10^{-4} M was found for this fraction with linoleate as the substrate under optimal conditions.

Table III. Purification of Lipoxygenase from Gastrocnemius[a]

step	total protein, mg	total act., OD/min	sp act., OD/min per mg protein	yield, %	enrichment
solubilized microsomal fraction	7.8	1.40	0.10	100	1.0
affinity chromatography					
200 mM, pH 5.0	2.4	1.53	0.64	109	6.4
100 mM, pH 9.0	1.5	1.08	0.71	77	7.1

[a] Lipoxygenase activity was determined spectrophotometrically following conjugated diene formation at 234 nm.

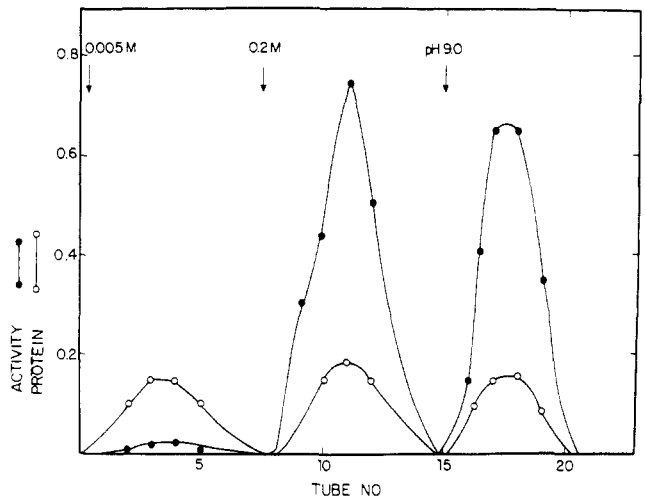

Figure 3. Affinity chromatography profile of lipoxygenase activity in solubilized gastrocnemius microsomal fraction on linoleoyl-aminoethyl-Sepharose. Protein (15 mg) in 5 mM acetate buffer was applied to the column (15 × 1.5 cm). Lipoxygenase activity was determined spectrophotometrically, and the change in absorbance at 234 nm/mL per min is shown (filled circles) together with protein (mg/mL).

DISCUSSION

The demonstration of the presence of lipoxygenase-type enzymes in chicken muscle appears to be of importance in understanding biochemical changes occurring on storage.

In the present study it was demonstrated that 15-lipoxygenase is present in chick gastrocnemius. The enzyme was purified by affinity chromatography, and its product, 15-HETE, was identified by TLC and HPLC. However, as is apparent from Figure 1, 15-lipoxygenase does not appear to be the only lipoxygenase present in the tissue. The products with R_f 0.88 and 0.84 (Figure 1) are probably derived from a lipoxygenase-like enzyme as they were inhibited by NDGA but not by indomethacin, which inhibits the cyclooxygenase pathway. These two products were also found in pectoralis (not shown) and do not appear to belong to the 5- or 12-lipoxygenase pathways. Preliminary data obtained in our laboratory suggested that these may be epoxy and keto compounds derived from the 15-lipoxygenase; however, until more complete identification is made, this remains speculative.

In this study it was again elucidated that the linoleoyl-aminoethyl-Sepharose column developed for the purification of plant lipoxygenases (Grossman et al., 1972) can be used for the purification of animal lipoxygenases. On the basis of this technique some animal lipoxygenases have been successfully purified (Shahin et al., 1978; Sofer, 1983; Grossman et al., 1986). The high yield of lipoxygenase activity obtained in the affinity chromatography procedure can be attributed to the removal of detergent or other inhibitors.

The existence of a catalyst, stable on freezing, such as the lipoxygenase enzyme in muscle tissue is likely to stimulate the development of rancidity in the stored tissues through the formation of free radicals and peroxides. The data presented here support this explanation, in that polyunsaturated free fatty acid concentrations decreased with storage time while the enzyme activity decreased only slightly with storage. The decrease in PUFA in gastrocnemius was greater than that in pectoralis, and this may be due to the higher activity of the lipoxygenase in this tissue. Previous studies have indicated that lipolysis and oxidation occur concurrently in muscles of both turkey (Sklan et al., 1983a) and chickens (Sklan and Tenne, 1984) on cold storage. It was assumed that the lipolysis occurring on storage provided the substrate for oxidation. However, the mechanism of the latter reaction was not described. It is possible that the presence of stable lipoxygenase in the tissue may explain the level and mechanism of this oxidation.

Registry No. 15-Lipoxygenase, 82249-77-2; lipoxygenase, 9029-60-1; linoleic acid, 60-33-3; arachidonic acid, 506-32-1.

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