Identification and Characterization of a 15-Lipoxygenase from Fish Gills

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An arachidonic acid 15-lipoxygenase activity was discovered in the gill tissue of teleost fishes during purification of the previously recognized and more preponderant 12-lipoxygenase enzyme. The total activity of this enzyme following purification using hydroxylapatite was significantly greater than in the crude tissue preparation, suggesting that an inhibition was removed during purification. The enzyme was active toward polyunsaturated fatty acids present in the tissue producing hydroxylated metabolites from fatty acids with 18-, 20-, and 22-carbon chain lengths at carbons 13, 15, and 17, respectively. The enzyme was further purified by using a Superose gel filtration column and eluted with an apparent molecular weight of 70 000.

INTRODUCTION

Lipoxygenases present in animal tissues, which catalyze the production of a variety of physiologically active metabolites (Borgeat, 1985), have also been shown to initiate lipid peroxidation (German et al., 1985; German and Kinsella, 1985, 1986a,b) and recently to promote the generation of volatile flavors (Josephson and Lindsay, 1986). In contrast to autoxidation, in which hydroperoxides of unsaturated fatty acids are formed without considerable specificity, lipoxygenases are highly substrate and position specific and stereospecific (Kuhn et al., 1986). These enzymatic initiators could therefore provide an important level of control of the quantity and characteristics of the spectrum of volatile compounds produced from fresh animal tissues. Thus, the unique flavor properties of certain foods such as fresh fish may be dictated not simply by the composition of flavor precursors such as polyunsaturated fatty acids but also by the quantities of specific enzyme catalysts. We have been studying the freshwater and marine fish gill as models for lipoxygenases and the role of these enzymatic reactions in lipid oxidation. Previous work has demonstrated relatively high activities of the arachidonic acid 12-lipoxygenase from fish gill which generates the same 12-(*S*) chiral product of arachidonic acid formed by the 12-lipoxygenase in mammalian lungs and platelets (German et al., 1985; Winkler et al., 1990). In recent studies designed to purify this 12-lipoxygenase activity we have discovered a second lipoxygenase in these tissues. In this paper we report the substrate specificity and molecular properties of this enzyme.

MATERIALS AND METHODS

Gill Tissue Preparation. In these studies freshwater trout *Salmo gairdneri* (1 kg) and marine rockfish *Sebastes flavidus* (2–4 kg) were used as sources for the fish gill lipoxygenases. Results were essentially the same for both species of fish, and the results reported in Figures 1–4 are those for the trout samples. Gill tissue (3–20 g) was removed from the animals and homogenized in 5 volumes of 0.05 M potassium phosphate buffer, pH 7.8, containing 5 mM reduced glutathione. The homogenate was then centrifuged for 90 min at 100000*g* to spin down debris, organelles, and membrane fractions. This high-speed supernatant was then brought to 45% saturation with solid ammonium sulfate and stirred on ice for 30 min. The insoluble protein was sedimented by a 10-min spin at 12000*g*. This ammonium sulfate precipitate was resuspended in 5 mL of phosphate buffer and frozen as single-drop beads in liquid nitrogen.

Hydroxylapatite (fast flow, Calbiochem) chromatography was accomplished by passing 0.1 mL of the resuspended ammonium sulfate fraction through a 2-mL column (0.4 g of dry hydroxylapatite). Elution was initially performed by using a linear gradient of potassium phosphate from 0.05 to 0.5 M, pH 7.8, with reduced glutathione (5 mM). For routine preparations of the 15-lipoxygenase enzyme, 0.05 M potassium phosphate was used to elute essentially all the activity directly, followed by a step to 0.2 M potassium phosphate, which eluted the 12-lipoxygenase.

Enzyme Assays. Fish enzyme preparations were added to 0.05 M potassium phosphate buffer, pH 7.8, with 1 mM glutathione, and subsequently purified fatty acids (NuChek Prep, Elysian, MN) in ethanol were added to appropriate final concentrations. Lipid products were extracted with 2 volumes of ethyl acetate from buffer acidified to pH 3.5 with dilute acetic acid (German et al., 1987).

Product Analyses. High-pressure liquid chromatographic analyses of chromophore-containing products were performed on a Hewlett-Packard 1090 HPLC equipped with a diode array detector. Reverse-phase analyses were performed on a 25 × 4.6 mm C-18 column (Supelco, Bellefonte, PA). The compounds were eluted isocratically by using a solvent system of methanol/water (70:30) buffered with 5 mM ammonium acetate to an apparent pH of 5.7 and containing 0.5 mM EDTA. Chiral analyses were performed on a Bakerbond ionically bonded DNTB chiral phase on methyl esters formed by using ethereal diazonmethane as described (Kuhn et al., 1987). UV spectra of eluting compounds were recorded automatically by using the diode array detector. Retention times, extinction coefficients, and spectra were compared and standardized with authentic compounds [hydroxy isomers: 5(*S*)-, 15(*S*)-, and 12(*S*)- and 12(*R*)-HETE (hydroxyeicosatetraenoic acid)] kindly provided by Merck-Frosst or purchased from Cayman. Gas chromatography/mass spectrometry analyses were performed on products after methyl esters were formed with ethereal diazomethane and trimethylsilyl ethers of free hydroxyl groups with BSTFA (Supelco, State College, PA). Gas chromatography was performed on a 25-m DB 23 column (J&W Scientific, Folsom, CA), and EI mass spectra were obtained on a VG ZAB-HS-2F spectrometer (VG Analytical, Wythenshawe, England).

RESULTS AND DISCUSSION

Crude homogenates of freshwater and marine teleost fishes and the lungs of various mammals exhibit an active 12-lipoxygenase. A more precise designation for these enzymes' specificity is that they are $n-9$ specific in that they add oxygen from the n or methylene terminus, generating in this case an $n-9$ hydroperoxide product. Thus, the enzymes metabolize both endogenous and exogenous long-chain polyunsaturated fatty acids, i.e., 20:4 $n6$, 20:5 $n3$, and 22:6 $n3$, to the corresponding $n-9$ hy-

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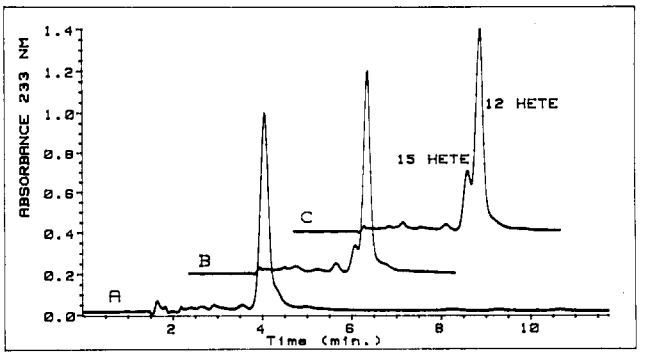

Figure 1. RP-HPLC elution profiles of hydroxy fatty acids formed by trout gill homogenates. Arachidonic acid was added to a final concentration of 20 μM and incubated for 20 min with enzyme preparations of trout gill enzymes at stages of purification: (A) crude homogenate; (B) high-speed supernatant; (C) 30–45% ammonium sulfate fraction. Absorption spectra and retention times of peaks were identical with those of standard 15- and 12-HETEs.

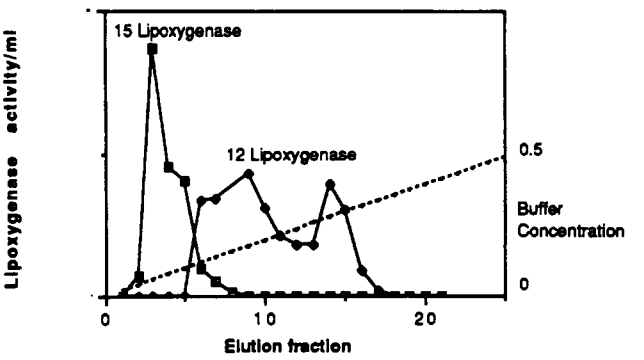

Figure 2. Elution of trout gill lipoxygenase activities from hydroxylapatite. Fractions (1 mL) from the hydroxyapatite column were assayed with 20 μM arachidonic acid. Products were extracted, separated, and identified on RP-HPLC as described. Activity is reported as nanomoles of arachidonic acid product formed per fraction.

droperoxide products. The specificity of this $n-9$ enzyme activity in these tissues is apparently quite high as >95% of the detectable monohydroxy derivatives of polyunsaturated fatty acids (PUFA) in these tissues reflect this specificity for the $n-9$ hydroperoxide product.

We have been purifying the $n-9$ lipoxygenase activity from teleost fishes and discovered during purification experiments an increasing quantity of additional products of the substrate fatty acids as apparent, for example, in HPLC analyses of products of the substrate arachidonic acid metabolism (Figure 1). In this case the difference was an increase in products with hydroxyl function not at the $n-9$ but rather at the $n-6$ position. This suggested an $n-6$ lipoxygenase metabolic activity. These could have arisen via an alteration in the absolute specificity of the single $n-9$ lipoxygenase enzyme or the appearance of a previously undetected $n-6$ lipoxygenase enzyme. Further purification of these enzyme activities was pursued to answer this question.

The stability of lipoxygenase enzymes from animal tissues has been widely recognized to be unstable to purification attempts, and most column steps yield extremely poor yields. We did find success, however, using short hydroxylapatite columns. The 30–45% ammonium sulfate precipitate containing both apparent enzyme activities was applied to a hydroxylapatite column in buffer containing 5 mM glutathione. Fractions eluting from the hydroxylapatite column were assayed and two lipoxygenase peaks eluted separately (Figure 2). Reverse-phase HPLC analyses capable of separating positional isomers of the products of arachidonic acid were then performed. The first peak, eluting at low ionic strength, was apparently not the 12-lipoxygenase but rather a previously unrecognized lipoxygenase activity. That is, the products of incubation of this enzyme with arachidonic acid chromatographed on the HPLC column precisely with standard 15-HETE. Further analyses were performed to identify this product. The ultraviolet absorption spectrum was consistent with a conjugated diene structure identical with that of standard 15-HETE. Spectra of the products of incubation with arachidonic acid 20:4 $n6$ and 18:2 $n6$, 18:3 $n3$, 20:3 $n6$, 20:3 $n3$, 20:5 $n3$, and 22:6 $n3$ were identical. The product of incubation of this enzyme fraction with arachidonic acid was then methylated and converted to trimethylsilyl ethers and analyzed by mass spectrometry. These results confirmed that the hydroxyl addition site on arachidonic acid was at carbon 15 (Figure 3).

A final structural characterization was performed to determine the stereochemistry of the oxygen addition site of the enzyme. Analyses of the methyl ester of this 15-HETE on a chiral stationary phase HPLC column revealed that this metabolite had the S configuration (data not shown). Thus, the enzyme is similar in the position and chirality of its catalytic reaction to the 15-lipoxygenase

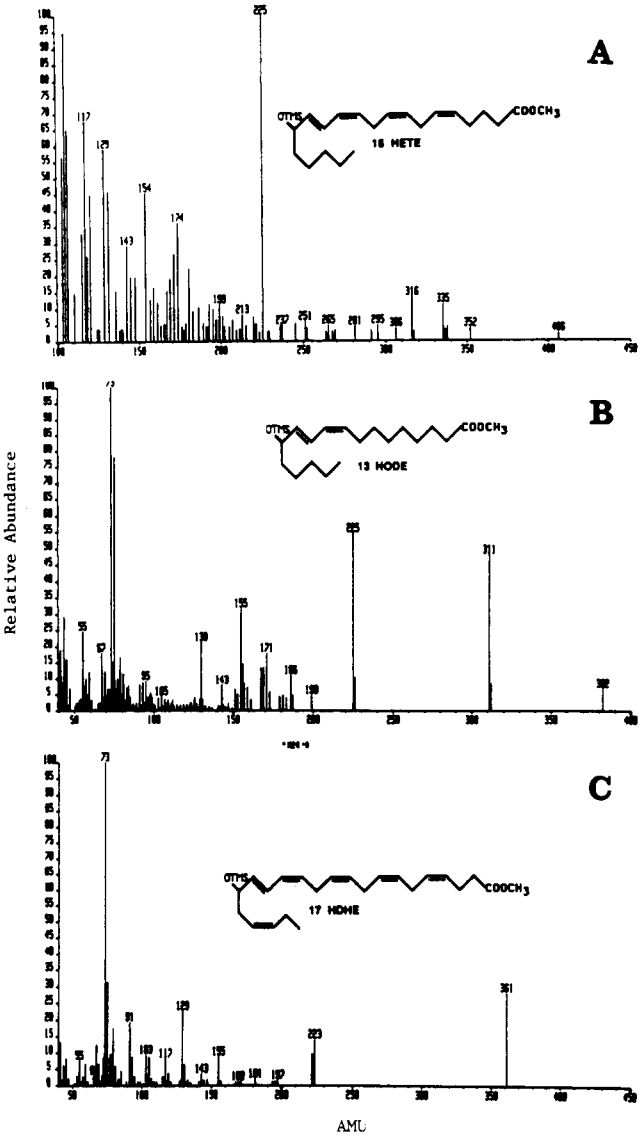

Figure 3. Mass spectra of products of arachidonic acid (A), linoleic acid (B), and docosahexaenoic acid (C) formed by the partially purified $n-6$ lipoxygenase from trout gill.

Table I. Substrate Specificity of the 15-Lipoxygenase from Fish Gills[a]

substrate fatty acid	product formed	substrate fatty acid	product formed
18:2 *n6*	13(*S*)-HODE	20:4 *n6*	15(*S*)-HETE
18:3 *n3*	13-HOTE	20:5 *n3*	15-HEPE
20:3 *n6*	15-HETE	22:6 *n3*	17-HDHE
20:3 *n3*	15-HETE		

[a] Substrate fatty acids were incubated with partially purified enzyme in phosphate buffer for 10 min as described under Materials and Methods. Products were extracted, analyzed by HPLC, methylated, and analyzed by chiral HPLC where chirality is indicated and silylated and analyzed by GC/MS as described.

characterized in leukocytes, reticulocytes, and soybeans (Shewe et al., 1986).

The substrate specificity of this enzyme was determined by incubating a variety of fatty acids with the hydroxylapatite purified enzyme preparation. Fatty acids with double bonds at both the $n - 6$ and $n - 9$ positions were substrates for activity in fatty acids with chain lengths from 18 to 22 carbons. The respective substrates tested and products formed as confirmed by HPLC and GC/MS are summarized in Table I.

This enzyme activity was analyzed for its apparent molecular weight by using fast protein liquid chromatography (FPLC) over a Superose size exclusion column (Pharmacia). Although the enzyme was not sufficiently pure to characterize the molecular weight as eluting protein peak, it was possible to analyze each 0.5-mL fraction for enzyme activity. The intent was to ensure with these analyses that we were measuring precisely the $n - 6$ lipoxygenase rather than the potentially contaminating $n - 9$ enzyme or nonspecific lipid peroxidation initiators possibly present. Therefore, each fraction eluting from the column was assayed as described for its positional lipoxygenase activity. Products arising from incubating arachidonic acid with the various molecular weight fractions eluting from this column are shown as the respective RP-HPLC chromatograms (Figure 4). Just one lipoxygenase activity was detected, and the activity was confined to one fraction. When compared with molecular weight standards run under similar conditions, the results indicated that the enzyme eluted from this column with an apparent molecular weight of 70 000 ± 5000. This is consistent with that found for mammalian lipoxygenases but significantly smaller than the soybean enzyme (Shewe et al., 1986).

The identification of this activity in purified enzyme preparations raised the question as to why it was so difficult to detect in crude homogenates. Subsequent experiments were undertaken to determine the basis of this change in relative activity. The initial objective was to determine if the 15-lipoxygenase was simply kinetically uncompetitive with the 12-lipoxygenase for available substrate. That is, if the apparent concentration required for half-maximal velocity of the 12-lipoxygenase was much lower than for the 15-lipoxygenase, substrate would be preferentially consumed by it as a result of that competitive advantage.

Kinetic analyses of the oxygen consumption rates by the enzyme preparations using arachidonic and eicosatrienoic acids found an apparent K_m of 25 μM at 25 °C in the assay buffer described for these fatty acid substrates. This is essentially similar to that previously recognized for the 12-lipoxygenase (German et al., 1985). Thus, on the basis of substrate concentration required for maximal activity there was no strong competitive advantage for the 12-lipoxygenase. This tended to argue that an actual

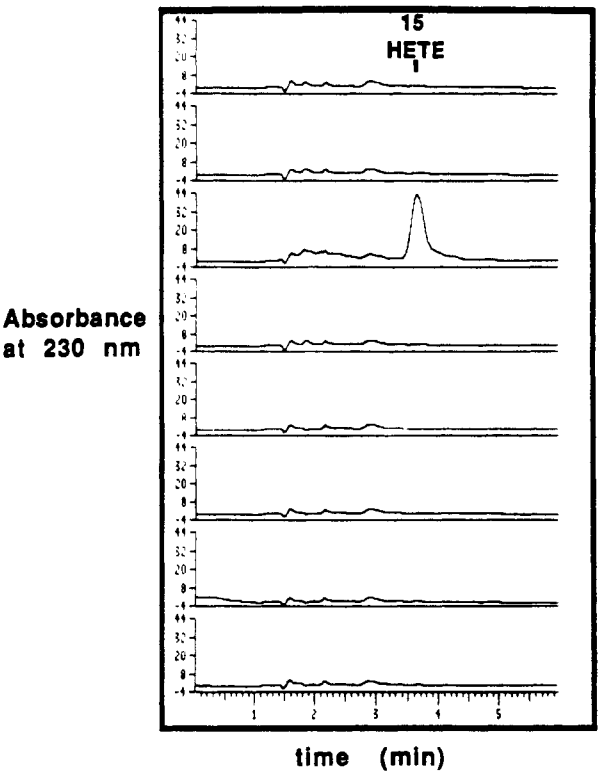

Figure 4. RP-HPLC of products of lipoxygenase activity associated with the molecular weight fractions eluting from Superose FPLC column. Fractions (1 mL) from sizing column were assayed for lipoxygenase activity as described.

inhibition was operating in the tissue preparations, preventing full expression of the existing 15-lipoxygenase enzyme activity.

Purification experiments also suggested that there was a net increase in 15-lipoxygenase activity during column separation. That is, when 20:4 *n6* arachidonic acid was used as substrate and the ammonium sulfate precipitate at 1 mg protein concentration assayed for net lipoxygenase activity, the entire apparent activity of the 15-lipoxygenase was 0.2 μmol/min. The sum of activity eluting from the hydroxylapatite column as the more purified 15-lipoxygenase was 1.9 μmol/min for the same milligram of protein. This dramatic enhancement in total activity, however, could have resulted from competition for the same substrate by the two lipoxygenases. That is, in the ammonium sulfate precipitate both the 12- and 15-lipoxygenase activities are present and thus both products are generated, while in the purified fractions only the single enzyme is present and need not compete for substrate. Therefore, experiments were performed by using a substrate with a double-bond configuration that would be metabolized solely by the 15-lipoxygenase.

Eicosatrienoic acid 20:3 *n3* contains double bonds at the $n - 3$, $n - 6$, and $n - 9$ positions. Previous experiments have demonstrated that the 12-lipoxygenase requires double bonds at carbons $n - 9$ and $n - 12$ (German et al., 1985), while the 15-lipoxygenase requires double bonds at carbons $n - 6$ and $n - 9$. Thus, the 20:3 *n3* fatty acid satisfies only the structural requirements of the 15-lipoxygenase and was metabolized to the 15-hydroxyeicosatrienoic acid derivative. This fatty acid was incubated with crude enzyme preparations containing both enzymes and with separate preparations of each enzyme purified over hydroxylapatite. Total synthetic activity yielding 15-hydroxy 20:3 was again significantly greater in the pooled samples containing 15-lipoxygenase purified over hydroxylapatite than in the entire sample applied to the column.

Apparent activity applied to the column using 20:3 *n*3 as the substrate was 0.1 μmol of product min^{-1} (mg of protein)$^{-1}$, and the total activity assayed eluting from the column was 0.5 μmol/min. This suggests that there is an actual inhibitory activity specific for the 15-lipoxygenase, associated with gill tissue, which is removed on subsequent purification. The nature of this activity is the subject of ongoing investigations.

The presence of this enzyme and its natural inhibition have several implications for fish flavor/odor and stability. The role of lipoxygenases as a class in the initiation of lipid peroxidation and the production of specific flavor compounds is increasingly apparent in plant and in animal tissues. An important property of this pathway for volatile production from polyunsaturated fatty acids is the specificity of volatiles produced due to the positional specificity of the oxygen addition and double-bond rearrangement. This is in contrast to nonenzymatic autoxidation which yields a relatively nonspecific spectrum of volatiles. In fact, this distinction frequently represents the difference between desirable flavors and objectionable off-flavors. Different lipoxygenases present would then result in distinct volatiles from the same precursors. The possibility that separate lipoxygenase enzymes in animal tissues could generate different volatile patterns depending on the condition of the tissues is an obvious implication of the discovery of this second lipoxygenase displaying alternate specificity and activity. It is not yet known what tissues in addition to gills express these enzymes. The probability of distinct tissue and cell type differences in their distribution also argues for variation in flavor production. As a separate but related question, the possibility that endogenous activators and inhibitors of these enzymes could be manipulated to control lipid peroxidation and volatile production is intriguing.

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