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Lipoxygenase Generation of Specific Volatile Flavor Carbonyl Compounds in Fish Tissues

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Lipoxygenase present in fish tissues can initiate the oxidation of polyunsaturated fatty acids to produce acyl hydroperoxides. With a gill homogenate as a model system, the capacity of gill lipoxygenase to generate short-chain volatile carbonyl compounds depended on the concentrations of enzyme, substrate fatty acids, and duration of incubation period. The major volatile compounds generated from the oxidation of arachidonic and eicosapentaenoic acids by gill 12-lipoxygenase were 1-octen-3-ol, 2-octenal, 2-nonenal, 2-nonadienal, 1,5-octadien-3-ol, and 2,5-octadien-1-ol. The generation of oxidative flavors was related to the activity of the lipoxygenase and was inhibited by lipoxygenase inhibitors, esculetin, and BHA.

Fish lipids are very susceptible to oxidation because of their high degree of acyl unsaturation. Lipid oxidation causes changes in the flavor, color, and texture of fish and seafood products (Khayat and Schwall, 1983). Thus, oxidative off-flavored short-chain carbonyl compounds, such as 4-heptenal, 2,4-heptadienal, 2-pentenal, 3-hexenal, and 2,4-decadienal, increased during cold storage of cod (McGill et al., 1977). These apparently result from the oxidation of $n-3$ polyunsaturated fatty acids released from phospholipids (Ross and Love, 1979). Because the problems of flavor and quality deterioration in fish are closely associated with the oxidative instability of lipids, more information is needed concerning the mechanism of initiation of lipid oxidation (Kanner et al., 1987). Understanding this may provide the means for improving the flavor stability and quality of fish.

Several enzymatic and nonenzymatic processes, such as autoxidation, photosensitized oxidation, lipoxygenase, peroxidase, and microsomal enzymes, have been suggested as initiators of lipid oxidation in fish tissues (Kanner and Kinsella, 1983; Frankel, 1985; Slabyj and Hultin, 1984; German and Kinsella, 1986; Josephson et al., 1987). Lipoxygenase in trout gill and skin tissues can oxidize polyunsaturated fatty acids into position-specific hydroperoxides (German and Kinsella, 1985, 1986; German et al., 1986). These hydroperoxides then may be decomposed by homolytic cleavage and β-scission to produce various fragmentation products, such as aldehydes, alcohols, ketones, and hydrocarbons. These compounds influence the flavor quality and palatability of fish products (Hsieh and Kinsella, 1988).

However, the relative importance of fish lipoxygenase in the initiation of lipid oxidation and its actual contribution to the development of oxidative flavor in fish has not been quantified. Because of its potential in initiating lipid oxidation, the influence of lipoxygenase on the biogeneration of fish flavor and off-flavor is of practical interest. In this report, the contribution of lipoxygenase-initiated oxidation of polyunsaturated fatty acids to the generation of specific oxidative volatile carbonyl compounds was determined.

MATERIALS AND METHODS

Materials. Arachidonic acid (20:4n6) and eicosapentaenoic acid (20:5n3) were obtained from NuChek Prep (Elysian, MN). [U-^{14}C]- and [1-^{14}C]arachidonic acid were bought from New England Nuclear (Boston, MA). *tert*-Butyl hydroxyanisole (BHA), *tert*-butylhydroxytoluene (BHT), esculetin, and esculin were purchased from Sigma Chemical Co. (St. Louis, MO). Flavor standards such as 1-octen-3-ol and 2-octenal were purchased from Aldrich Chemical Co. (Milwaukee, WI). 2-Nonenal and 2,6-nonadienal were obtained from Alfa Products (Danvers, MA). Tenax GC (mesh size 80/100) was bought from Alltech Associates, Inc. (Deerfield, IL), and thoroughly washed with hexane to condition it for the purge-and-trap collection procedure. Ethyl acetate and pesticide-grade hexane were obtained from Fisher Scientific Co. (Rochester, NY). Methanol was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Chloroform and glacial

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acetic acid were purchased from Mallinckrodt Inc. (St. Louis, MO).

Lipoxygenase Preparation. Samples of gill tissue (1 g) were excised from young freshly killed rainbow trout (15 g) (Tunison Fish Laboratory, Cortland, NY) and homogenized in 40 mL of 0.05 M (pH 7.4) phosphate buffer on a Polytron homogenizer. The homogenate was centifuged for 15 min at 15000*g* at 4 °C. The resultant supernatant fraction was used as the crude enzyme source without further purification (German and Kinsella, 1985; German et al., 1986). Protein concentration was estimated by the Lowry method with bovine serum albumin as standard (Lowry et al., 1951).

The activity of lipoxygenase was determined by incubating the gill extract with arachidonic acid (50 μM) using labeled [1-^{14}C]arachidonic acid (specific activity 1000 cpm/nmol) as tracer in the presence of added glutathione (1 mM). The primary products of lipoxygenase activity on polyunsaturated fatty acids are the corresponding hydroperoxides, which are unstable and are readily reduced to their hydroxy analogues by the glutathione peroxidase present in the enzyme preparation (Bell et al., 1980; German and Kinsella, 1986). Hence, lipoxygenase activity was estimated from the conversion of arachidonic acid into its 12-hydroxy analogue during a 10-min incubation period at 25 °C. The reaction was quenched by acidification with formic acid to pH 3.0, and the products were extracted twice with ethyl acetate as previously described (German et al., 1986). The extracted lipids were separated by thin-layer chromatography (TLC) using a solvent system consisting of chloroform/methanol/acetic acid/water (90:8:1:0.8, v/v/v/v) (Powell, 1982). The predominant radiolabeled product, 12-hydroxy-5,8,10,14-eicosatetraenoic acid generated from arachidonic acid by gill lipoxygenase was detected by autoradiography as reported (German and Kinsella, 1986). The extent of product formation during the reaction period was quantified by measuring the radioactivity by liquid scintillation counting (German and Kinsella, 1985).

Purge-and-Trap Technique for Isolation of Volatiles. Because of its high lipoxygenase (LO) activity, fish gill homogenate was used as a model system to study the capacity of LO to generate short-chain volatile carbonyl compounds. The volatile compounds generated from polyunsaturated fatty acids (PUFA) by the gill enzyme system were collected by a purge-and-trap technique as described in Figure 1. The efficiency of recovery of flavor volatile compounds by the purge-and-trap procedures was calibrated against an internal standard, 2-nonanone, that is absent from fish tissue. The internal standard, 2-nonanone, was initially dispersed in 20 mL of gill LO preparation in a 50-mL serum bottle to give a final concentration of 20 ppb. The preparation containing 2-nonanone was then purged by prepurified oxygen-free nitrogen, and the effluent 2-nonanone was collected on Tenax GC (mesh size 80/100). The 2-nonanone was eluted from the Tenax GC powder with 1 mL of hexane, twice, concentrated, and injected into GC for quantification as described below. The influence of purging rate and purging time on the recovery of volatile compounds was determined (Hsieh, 1988). Maximum recovery was obtained with a nitrogen flow rate of 40 mL/min for a 90-min trapping period.

With this purge system, the effects of concentration of enyzmes, substrate, and fatty acid and time of incubation on the capacity of fish lipoxygenase system to generate oxidative volatile compounds were determined by quantifying recovery of radiolabeled volatile carbonyl compounds generated from [U-^{14}C]arachidonic acid. The fish

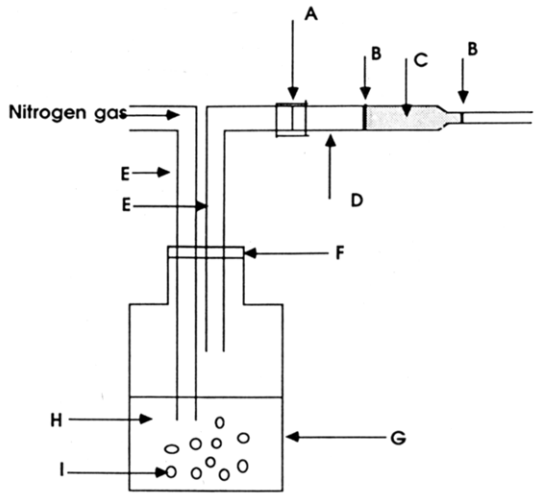

Figure 1. Purge-and-trap apparatus used for the collection of volatile flavor compounds generated by the gill lipoxygenase system. The gill enzyme preparation (20 mL) (A) was placed in a 50-mL serum bottle (B) capped with a Teflon-faced silicon seal (C). Nitrogen gas (D) was continuously introduced through a $^1/_4$-in. glass tubing (E) into the gill preparation held at 25 °C. The volatile compounds generated in this system were transformed by the effluent gas (E) and trapped on Tenax GC powder (F) in a Pasteur pipet (G) retained at both ends with a plug of glass wool (H).

enzyme preparation (20 mL, 1 mg of protein/mL), in 0.05 M phosphate buffer (pH 7.4) without added glutathione, was preincubated at 25 °C with arachidonic acid (50 μM) using [U-^{14}C]arachidonic acid (specific activity 1000 cpm/nmol). In the absence of added glutathione, the acyl hydroperoxides formed by LO were degraded to yield volatile carbonyl compounds. The effect of duration of incubation period on the capacity of gill enzyme system to generate volatile compounds was determined. After specified periods, the volatile compounds generated by the above system at 25 °C were recovered by purging with nitrogen gas at 40 mL/min for 90 min and trapped on Tenax GC. The total trapped radiolabeled volatile compounds were then eluted by hexane from the trapping powder, and their radioactivity was measured by liquid scintillation counting.

A similar system was used to quantify the amounts of specific volatile carbonyl compounds (as identified by gas chromatography–mass spectrometry) generated from endogenous PUFA by the lipoxygenase (LO) in the presence and absence of LO inhibitors. Nonanone was used as an internal standard.

The effects of concentration of enyzme on the generation of volatile compounds were determined. Solutions containing increasing enzyme concentrations (0.4–1 mg of protein/mL) were prepared by diluting the original gill supernatant (5 mg of protein/mL) with 0.05 M phosphate buffer (pH 7.4). The enzyme preparation was then incubated with arachidonic acid (50 μM, 1000 cpm/nmol) for 2 h. The radiolabeled volatile compounds generated in the gill enzyme system were then collected by purge-and-trap

technique. The radioactivity of the trapped volatile compounds was measured by liquid scintillation counting.

The effects of substrate concentration (arachidonic acid) on the production of oxidative volatile compounds were determined. The gill enzyme system (1 mg of protein/mL) was incubated with different concentrations (1–10 μM) of radioactive arachidonic acid for 2 h (optimum period). The radiolabeled volatiles generated in the gill enzyme system were then collected by the purge-and-trap technique and eluted by hexane, and the radioactivity was measured by liquid scintillation counting. All assays were done in triplicate.

In order to determine whether lipoxygenase was responsible for the generation of some volatile compounds by the gill system, the inhibitory effects of phenolic antioxidants, *tert*-butylhydroxyanisole (BHA), *tert*-butylhydroxytoluene (BHT), esculetin (6,7-dihydroxycoumarin), and esculin (esculetin 6-glucoside), on lipoxygenase and flavor biogeneration was studied. Gill lipoxygenase (1 mg of protein/mL) was prepared without the addition of exogenous glutathione. The gill lipoxygenase preparation was preincubated with specific antioxidants at 25 °C for 10 min. Then, radiolabeled [U-^{14}C]arachidonic acid (50 μM, specific activity 100 cpm/nmol) was added to initiate enzymatic reaction. After incubation the radiolabeled volatile compounds generated in the gill enzyme system were collected and eluted, and their radioactivity was measured by liquid scintillation counting as stated above. At least three concentrations of each antioxidant were used to evaluate the inhibitory effects.

The inhibitory effect of each antioxidant on the lipoxygenase-generated hydroxy fatty acids was also estimated by autoradiography and liquid scintillation counting (German and Kinsella, 1986). At least five concentrations of each inhibitor were used to determine its inhibitory potency. The IC_{50} value, the concentration of an inhibitor causing 50% reduction of enzyme activity, was calculated by linear regression analyses (Steel and Torrie, 1980).

Identification of Volatile Compounds. In order to identify the volatile compounds generated by lipoxygenase, the volatile compounds generated by gill lipoxygenase incubated with arachidonic acid (or eicosapentaenoic acid) were collected after the internal standard (2-nonanone) was added. After incubation, the volatile compounds were purged from the incubation system, trapped on Tenax GC, eluted by hexane, and concentrated. The samples were injected into a gas chromatograph with a Supelcowax 10 capillary bonded-phase column (30-m length, 0.32-mm inner diameter, 0.25-μm film thickness) for quantitative analysis. The GC analyses were carried out by direct on-column injection using a Hewlett-Packard 5830A gas chromatograph (Hewlett-Packard, Avondale, PA) with a flame ionization detector. Running conditions were as follows: initial temperature 70 °C for 5 min; programmed at 5 °C/min up to 200 °C; injector temperature 220 °C; detector temperature 220 °C. Nitrogen was used as both carrier and makeup gas with flow rates at 2 and 60 mL/min, respectively. The septum purge rate was 5 mL/min. The flow rates of hydrogen and air were 30 and 240 mL/min, respectively.

A Hewlett-Packard 5995A GC–MS system with a HP 59970A workstation for acquisition and analyses of GC–MS data was used for identification of the principal volatile compounds separated by GC. The same GC column and chromatographic conditions as stated above were used for GC–MS analysis. The conditions for analyses of mass spectra were as follows: ionization voltage 70 eV; ion acceleration voltage 2000 V; transfer line temperature 280 °C; mass analyzer temperature 180 °C; ion source temperature 180 °C. Identification of short-chain flavor compounds from the gill enzyme system was based on computer matching of full or partial mass spectra of compounds published in EPA/NIH Mass Spectral Data Base (Heller and Milne, 1976, 1980) and the coincidence of mass spectral patterns from isolated compounds with those of the authentic standard compounds.

Table I. Effect of Concentrations of Protein and Arachidonic Acid on the Generation of Short-Chain Carbonyl Compounds by Gill Lipoxygenase System[a]

protein concn, mg/mL	arachidonic acid, μM	rec of trapped flavor compds, % (mean ± SEM)
0.4	50	0.635 ± 0.025
0.5	50	0.685 ± 0.027
0.7	50	0.744 ± 0.020
1.0	50	0.787 ± 0.020
1.0	1	0.362 ± 0.016
1.0	5	0.549 ± 0.018
1.0	20	0.704 ± 0.024
1.0	50	0.786 ± 0.032
1.0	100	0.795 ± 0.034

[a] Reactions were performed in the gill enzyme assay mixture with specific quantities of partially purified enzyme. The enzyme was preincubated with [U-^{14}C]arachidonic acid at 25 °C for 2 h. Then, volatile compounds from the system were purged, collected, and analyzed as stated in Materials and Methods. The recovery of 2-nonanone was about 95% among samples. Triplicate data were obtained for each treatment.

RESULTS

The role of lipoxygenase in generating oxidative short-chain carbonyl compounds (volatile compounds) was evaluated by collection of radiolabeled volatile compounds generated from [U-^{14}C]arachidonic acid following incubation with gill extract. The formation of volatile compounds by gill enzyme system was dependent on the duration of preincubation, concentrations of protein (enzyme), and concentration of substrate arachidonic acid. The amounts of total volatile compounds collected from gill system increased (i.e., 0.695, 0.765, 0.774, and 0.738% of radioactive AA after 1, 2, 6, and 8 h, respectively) with the increased incubation time up to 2 h. There was no further increase in the recovered volatile compounds following incubation beyond 2 h. The amounts of volatile compounds recovered following incubation also increased with both substrate and enzyme concentrations (Table I).

On the basis of these observations, all subsequent analyses were conducted at 25 °C in a reaction mixture at pH 7.5 containing 1 mg of protein/mL and 50 μM fatty acid and an incubation period of 2 h. The recovery of volatile compounds from the model system was 90–95% based on the recovery of added nonanone.

The involvement of lipoxygenase in the generation of volatile carbonyl compounds in the gill model system was evaluated by using antioxidants and lipoxygenase inhibitors such as BHA, BHT, esculetin, and esculin (esculetin 6-glucoside) and analyzing products. The inhibition of gill lipoxygenase, as determined by the formation of hydroxy fatty acids, was dependent on the concentration of inhibitors. Esculetin was a potent inhibitor of gill lipoxygenase (IC_{50} = 0.02 μM), whereas esculin was much less potent with an IC_{50} = 500 μM. BHA inhibited gill lipoxygenase with IC_{50} = 5 μM, whereas BHT had a negligible inhibitory effect (IC_{50} > 1 mM).

In concordance with inhibition of LO-generated hydroxy fatty acids, the generation of volatile compounds by the gill enzyme was also inhibited by these antioxidants.

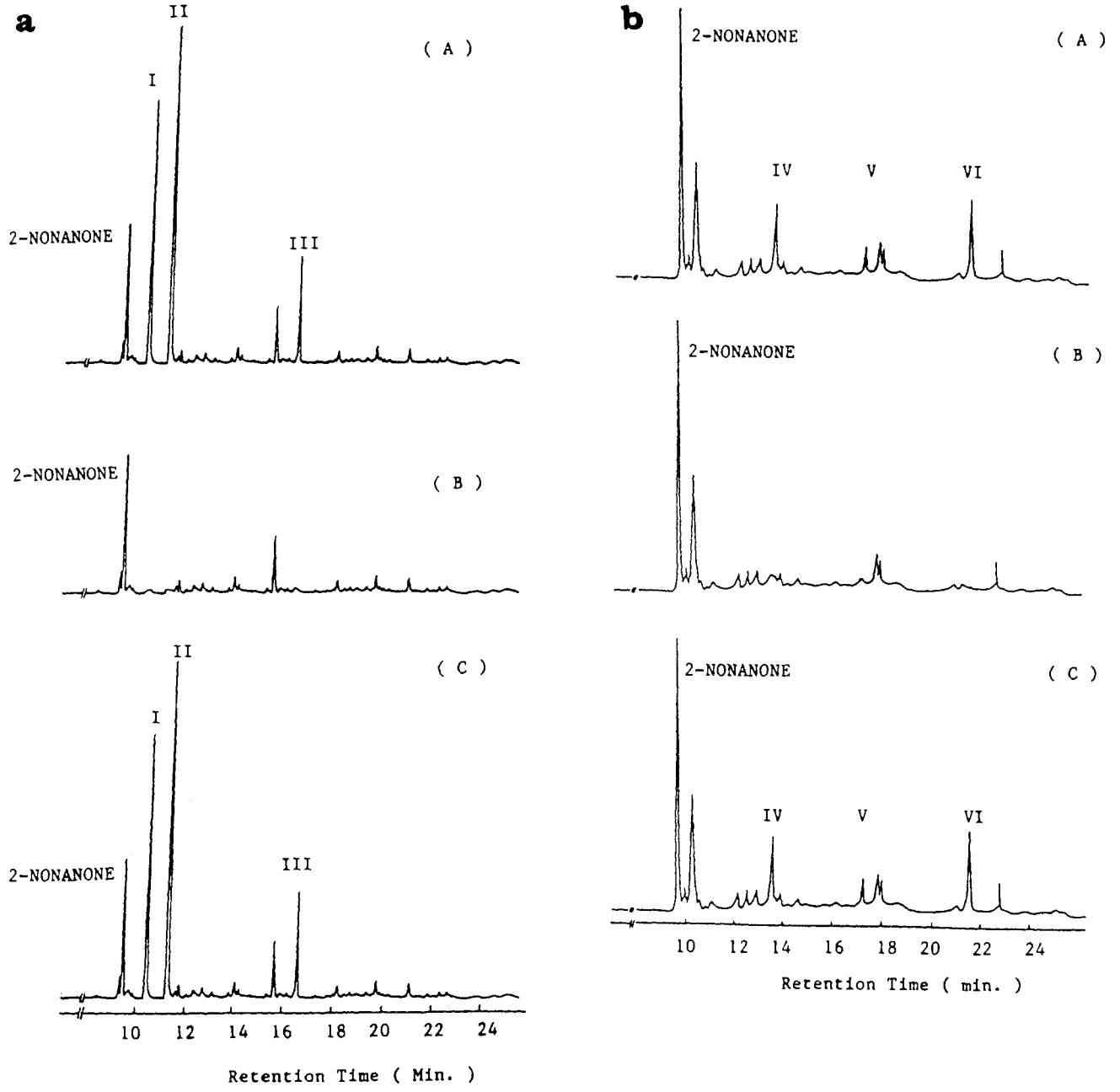

Figure 2. Gas chromatograms of volatile compounds collected from the trout gill homogenate incubated with (a) arachidonic acid and (b) eicosapentaenoic acid. Legend: (A) no additive; (B) 1 μM esculetin; (C) 1 μM esculin. Gill homogenate (1 mg of protein/mL) was incubated with 50 μM arachidonic or eicosapentaenoic acid for 2 h. Volatile compounds were then collected and analyzed as stated in Materials and Methods. Peaks: I, 2-octenal; II, 1-octen-3-ol; III, 2-nonenal; IV, 1,5-octadien-3-ol; V, 2,6-nonadienal; VI, 2,5-octadien-1-ol.

Esculetin at 0.01 μM inhibited the generation of volatile compounds by 40%, while at 1 μM esculetin the inhibition was almost complete (Table II). Esculin at 1 mM caused 50% inhibition in the generation of volatile compounds. BHA was much more potent as an inhibitor of volatile generation than BHT. The reduced formation of volatile compounds by BHA at 10 and 500 μM was 45 and 90%, respectively, while BHT at 500 μM caused 50% inhibition.

In order to identify the flavor compounds formed by gill lipoxygenase, the volatile compounds from gill enzyme preparation incubated with polyunsaturated fatty acids in the presence of 1 μM esculetin or esculin were separated by gas chromatography. The three major volatile compounds produced by gill lipoxygenase following the addition of arachidonic and eicosapentaenoic acids (Figure 2) were analyzed. The structure and identity of these three compounds were determined by comparing their retention times by GC analyses and their mass spectra with authentic standards. The retention time and mass spectra of compound I from samples incubated with arachidonic acid (Figure 2) were consistent with those of 2-octenol (MW = 126). Mass fragments at m/e 97 (M - 29, loss of CHO^+) and 108 (M - 18, loss of H_2O) were characteristic patterns of an aldehyde. The mass fragment at m/e 55 (CH=CHCHO) was that of 2-alkenal (Heller and Milne, 1980).

The characteristic mass fragments of compound II (Table III) were at m/e 57 ($CHOHCH{=}CH_2$), 97 (M - 31, loss of CH_2OH), 110 (M - 18, loss of H_2O). Fragments at m/e M - 18 and M - 31 were typical for alkyl alcohol. The fragment at m/e 57 corresponds to the position of hydroxyl group and the presence of a double bond. These fragmentation patterns were consistent with that of 1-octen-3-ol (MW = 128).

The characteristic fragments of compounds III (Figure 3), at m/e 55 (CH=CHCHO), 97 (M - 43), 111 (M - 29,

Table II. Effects of Esculetin, Esculin, BHA, and BHT on the Generation of Short-Chain Carbonyl Compounds by Gill Lipoxygenase System[a]

treatment	concn	rec of trapped flavor compd, g (mean ± SEM)
control		0.715 ± 0.027
esculetin	1 nM	0.673 ± 0.032
	10 nM	0.450 ± 0.037
	100 nM	0.172 ± 0.016
	1000 nM	0.080 ± 0.014
esculin	1 nM	0.723 ± 0.021
	10 nM	0.685 ± 0.018
	100 nM	0.460 ± 0.024
	1000 nM	0.307 ± 0.024
BHA	1 μM	0.440 ± 0.033
	10 μM	0.325 ± 0.027
	100 μM	0.173 ± 0.020
	500 μM	0.105 ± 0.021
BHT	1 μM	0.525 ± 0.039
	500 μM	0.346 ± 0.039
	1000 μM	0.320 ± 0.024

[a] Reactions were performed in the standard assay mixture containing 1 mg of partially purified enzyme. The enzyme was preincubated with inhibitor for 10 min. Then, [U-^{14}C]arachidonic acid (50 μM, 100 cpm/nmol of arachidonic acid) was introduced and incubated at 25 °C for an additional 2 h. Then, volatile compounds from the system were purged, collected, and analyzed as stated in Materials and Methods. The recovery of 2-nonanone was 90–95% among samples.

loss of CHO^+), and 122 (M – 18, loss of H_2O), were consistent with those 2-nonenal (MW = 140).

The retention data and mass spectra of volatile compounds IV–VI generated in samples incubated with eicosapentaenoic acid were consistent with those of 1,5-octadien-3-ol, 2,5-octadien-1-ol, and 2,6-nonadienal. The diagnostic fragments of compound IV (Table III) at *m/e* 57 (CH_2=$CHCHOH$), 95 (M – 18 – 15, loss of H_2O and CH_3), and 108 (M – 18, loss of H_2O) were consistent with 1,5-octadien-3-ol (MW = 126).

The characteristic mass fragments of compound V, at *m/e* 69 (CH_3CH_2CH=$CHCH_2$) and 70 (CH_2CH=$CHCH$-OH), were those of 2,6-nonadienal (MW = 138).

The characteristic mass fragments of compound VI (Figure 4) at *m/e* 79 (M – 18 and 29, loss of H_2O and C_2H_5), 95 (M – 31, loss of CH_2OH), 95 (M – 18 – 15, loss of H_2O and CH_3), and 108 (M – 18 loss of H_2O) were consistent with the structure of 2,5-octadien-1-ol (MW = 126).

These fragmentation patterns of the six compounds were consistent with those of the commerical standards and published mass spectral data (Table III) (Heller and Milne, 1976, 1980; Tressl et al., 1982; Wurzenberger and Grosch, 1986).

When gill lipoxygenase preparations were incubated with esculetin (1 mM), 2-octenal, 1-octen-3-ol, and 2-nonenal were not detected when arachidonic acid was used as substrate, and 2,6-nonadienal, 1,5-octadien-3-ol, and 2,6-nonadienal were not detected when eicosapentaenoic acid

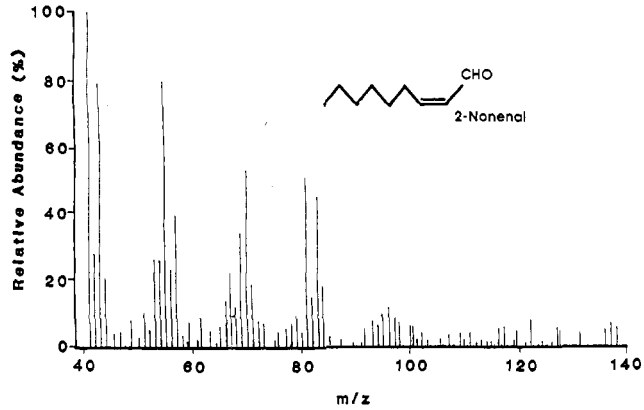

Figure 3. Partial mass spectrum of compound III, 2-nonenal, generated by lipoxygenase in trout gill homogenate incubated with arachidonic acid. Gill homogenate (1 mg of protein/mL) was incubated with 50 μM arachidonic acid for 2 h. Volatile compounds were then collected, separated, and identified as stated in Materials and Methods.

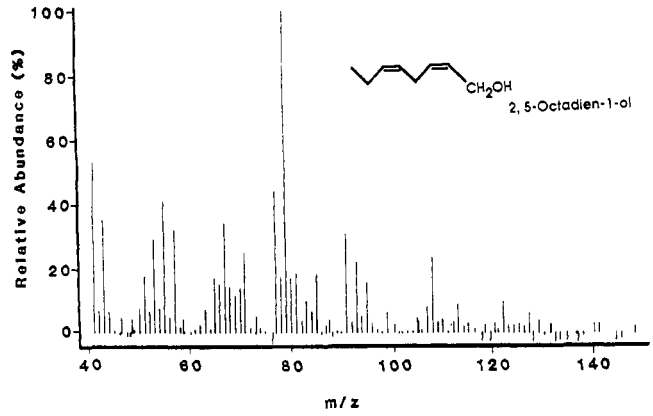

Figure 4. Partial mass spectrum of compound IV, 2,5-octadien-1-ol, generated by trout gill homogenate incubated with eicosapentaenoic acid. Gill homogenate (1 mg of protein/mL) was incubated with 50 μM eicosapentaenoic acid for 2 h. Volatile compounds were then collected.

used as substrate (Figure 2). However, in the presence of esculin (1 mM), which did not inhibit gill lipoxygenase, these specific compounds were generated and there were negligible differences between the volatile compounds generated by control and esculin-treated lipoxygenase. These volatiles were not formed following heating of the gill preparation at 80 °C for 10 min.

DISCUSSION

The lipoxygenase present in gill and skin tissues of several species of fishes (Hsieh et al., 1988) catalyzes the oxidation of polyunsaturated fatty acids to produce specific hydroperoxides (German and Kinsella, 1985; Hsieh and Kinsella, 1988). Because of its ability to initiate lipid oxidation, the influence of lipoxygenase on the biogeneration of specific flavor and off-flavor compounds in fish

Table III. Characteristic Mass Fragments and Their Relative Abundances of Some Eight- and Nine-Carbon Carbonyl Compounds Formed by 12-Lipoxygenase in Gill Enzyme System

compound	MW	characteristic mass fragments (relative abundance, %)
2-octenal[a]	126	41 (100), 55 (95), 70 (70), 83 (44), 97 (10), 108 (4)
1-octen-3-ol[a]	128	41 (47), 55 (52), 57 (100), 70 (33), 85 (16), 95 (7), 97 (16), 110 (10)
2-nonenal[a]	140	41 (100), 55 (71), 70 (52), 83 (50), 95 (13), 97 (10), 122 (10)
1,5-octadien-3-ol[b]	126	41 (54), 55 (100), 57 (55), 70 (26), 95 (21), 108 (15)
2,6-nonadienal[b]	138	41 (100), 69 (50), 70 (55), 95 (10)
2,5-octadien-1-ol[b]	126	41 (54), 55 (41), 67 (33), 79 (100), 93 (22), 95 (15), 108 (19)

[a] The volatile compounds produced after the addition of arachidonic acid into gill lipoxygenase system. [b] The volatile compounds produced after the addition of eicosapentaenoic acid into gill lipoxygenase system.

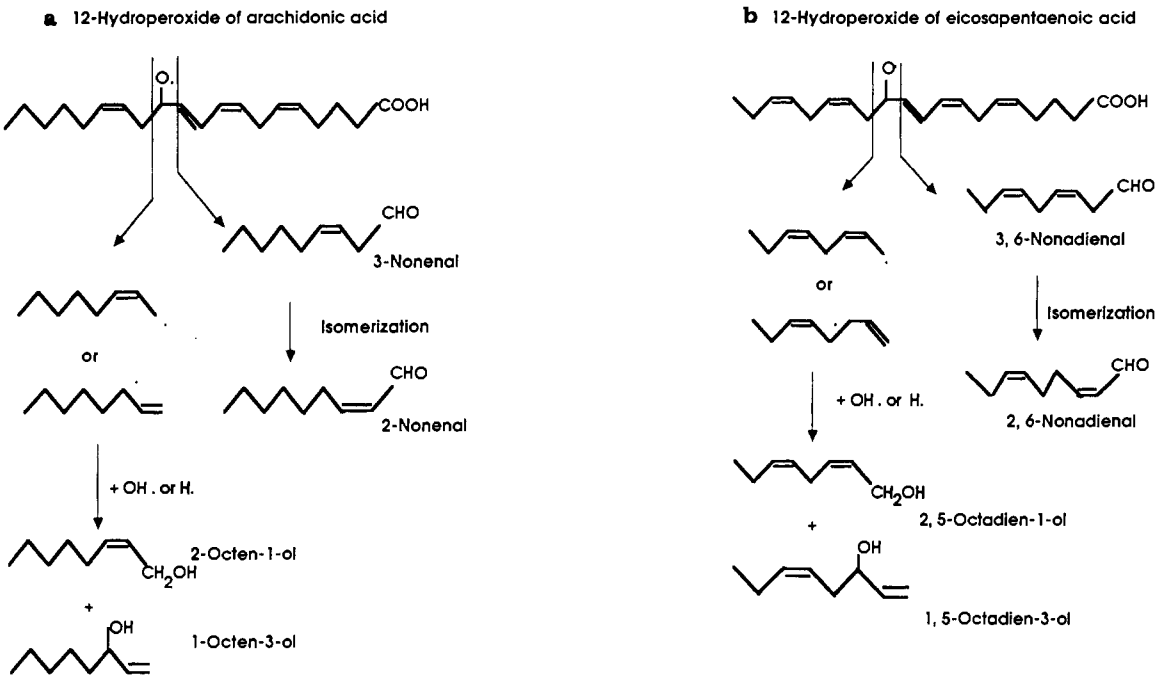

Figure 5. Proposed reaction schemes showing lipoxygenase-catalyzed oxidation of arachidonic acids (a) and eicosapentaenoic acid (b) with the subsequent generation of oxidative flavor compounds.

and seafoods is of practical interest. Hence, we studied the capacity of gill LO to generate flavorful carbonyl compounds, which at low concentrations can impart fresh-fish aroma (Josephson et al., 1983) but at higher concentrations (ppm) may convey off-flavors to fish.

The gill lipoxygenase preparation provided an excellent enzyme model system for studying the impact of lipoxygenase activity on the generation of specific oxidative flavor compounds in fish tissue. Factors such as enzyme and substrate fatty acid concentration and incubation time affected the formation of specific hydroperoxides from added unsaturated fatty acids. Significantly, these parameters had comparable effects on the generation of specific oxidative flavor compounds in this system. The capacity and potential of fish gill model system to produce specific flavor compounds was directly related to the activity of lipoxygenase. The higher the activity of lipoxygenase in this model system, the greater the amount of acyl hydroperoxides synthesized and of the resultant oxidative flavor compounds formed.

The involvement of lipoxygenase in the generation of volatile flavor compounds in gill system was demonstrated by the effects of specific lipoxygenase inhibitors. Phenolic antioxidants such as BHA and BHT, with oxidation potentials of 0.65 and 0.68 V, respectively, are traditionally considered as antioxidants that scavenge alkoxy and alkperoxy radicals (Scott, 1965). Esculetin and esculin, with oxidation potentials of 0.76 V, are also potential radical scavengers. However, despite the similarities in their oxidation potentials, our data indicated that BHA and esculetin were more potent as inhibitors of lipoxygenase than BHT and esculin, respectively. Comparable disparities in inhibitory potencies between BHA and BHT have been observed with lipoxygenase of skin reticulocytes (Nakadate et al., 1984; Schewe et al., 1986). A dihydroxy structure in the coumarin skeleton of esculetin was required for the inhibition of platelet 12-lipoxygenase (Sekiya et al., 1982) and 5-lipoxygenase in polymorphonuclear leukocytes (Kimura et al., 1985). Glycosylation of esculetin to form esculin caused loss of its inhibitory potency on lipoxygenase. These data indicated that esculetin and BHA could be used as specific inhibitors of lipoxygenase and hence were useful in studying the generation of oxidative flavor compounds in the trout gill system. The generation of short-chain volatile compounds was associated with the activity of gill lipoxygenase. Thus, whereas BHA and esculin did not inhibit lipoxygenase-initiated oxidative reactions and the subsequent generation of oxidative volatile compounds, the inhibition of lipoxygenase activity by BHA and esculetin inhibited the formation of specific hydroperoxides and also the volatile flavor compounds.

By the GC chromatograms of flavor compounds generated by trout gill incubated with specific lipoxygenase inhibitors such as BHA and esculetin are compared, we observed that the formation of specific aldehydes and alcohols in trout gill enzyme system paralleled the activity of 12-lipoxygenases. These results suggested that lipoxygenase activity not only is important as an initiator of lipid oxidation but also can generate oxidative volatile compounds in this gill enzyme model system (Figure 5).

The initial product of LO-initiated lipid oxidation is specific. Thus, arachidonic and eicosapentaenoic acids are oxidized by gill lipoxygenase to produce their respective 12-hydroperoxides (German and Kinsella, 1985). In vivo, these hydroperoxides are reduced to their hydroxy analogues in the presence of glutathione peroxidase (German and Kinsella, 1986). As lipid oxidation proceeds, especially postmortem, the accumulation of hydroperoxides may be further oxidized to epoxide, hydroxy, epoxide, trihydroxy fatty acids, etc. (Frankel, 1985). In the presence of metal ions, these reactive oxygenated derivatives may be cleaved by homolytic cleavage and β-scission to generate alkyl, alkoxy, or alkperoxy radicals, etc. (Frankel, 1985).

The decomposition products obtained from enzyme-generated hydroperoxides of PUFA are specific. The 12-hydroperoxy arachidonic acid, the initial oxidation product by gill 12-lipxoygenase, decomposes to produce 3-nonenal, 1-octene, or 2-octene radical. The 3-nonenal usually isomerizes into 2-nonenal, and the 1-octene radical may accept a hydrogen or hydroxyl radical to form 1-octene or 1-octen-3-ol. On the other hand, the 2-octene radical may receive a hydrogen or hydroxyl radical to produce 2-octene or 2-octen-1-ol (Figure 5). By a similar mechanism, the 12-hydroperoxyeicosapentaenoic acid can be decomposed

to generate 2,6-nonadienal, 1,5-octadien-3-ol, and 2,5-octadien-1-ol, respectively (Figure 5).

The compounds 2-nonenal and 2,6-nonadienal occur in freshwater fish (Josephson et al., 1984) and 1-octen-3-ol, 1,5-octadien-3-ol, and 2,5-octadien-1-ol contribute to the fresh aroma of various species of freshwater and saltwater fish (Josephson et al., 1983, 1984).

The key point in controlling lipid oxidation is at the initiation stage of the process. Both enzymatic and nonenzymatic reactions may be responsible for initiation reactions in fish and seafood tissues (Kanner and Kinsella, 1983; Slabyj and Hultin, 1984; German and Kinsella, 1986; Josephson et al., 1987; Hsieh et al., 1988). Lipoxygenase and peroxidase are plausible sources of the initial peroxides, i.e. acyl hydroperoxides generated from polyunsaturated fatty acids. These are also sources of short-chain oxidative flavor compounds and free radicals, which may lead to extensive chain reactions and autoxidation. 4-Heptenal, 2,4-heptadienal, 2-pentenal, 3-hexenal, 2,4-decadienal, and 2,4,7-decatrienal are the important compounds derived via oxidation and have been associated with oxidized fish flavors (McGill et al., 1977; Khayat and Schwall, 1983).

Research in this laboratory has focused on the nature of enzyme-initiated lipid oxidation in tissues (Kanner and Kinsella, 1983; German and Kinsella, 1985; Kanner et al., 1987; Hsieh et al., 1988). We initially demonstrated that lipoxygenase from trout tissue is capable of catalyzing the oxidation of arachidonic, eicosapentaenoic, and docosahexaenoic acids to produce acyl hydroperoxides (German and Kinsella, 1985; German et al., 1986). In this study, we demonstrated lipoxygenase-initiated oxidation of PUFA results in the generation of specific oxidative flavor compounds in fish tissues. These may be important in imparting fresh aroma to fish and also in causing off-flavors. The 12-lipoxygenase activity is also present in fish skin tissue (Hsieh et al., 1988), which may play a role in generating off-flavors in fish.

In conclusion, our results demonstrate that lipoxygenase in the gill tissue has the potential to be an important initiator of lipid oxidation and that the gill enzyme system may be a valid model to study the effect of enzymes on the initiation of lipid oxidation and the generation of volatile compounds. Lipoxygenase activity can cause the hydroperoxidation of unsaturated fatty acids and the generation of oxidative volatile compounds. Proper postharvest methods such as low-temperature storage on ice, avoidance of bruising or injury, and mild heat treatments, i.e. blanching (Hsieh et al., 1988) and application of specific antioxidants (such as BHA and esculetin) may be employed to control or retard lipoxygenase-initiated lipid oxidation and retard quality deterioration of fish postmortem.

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Registry No. 20:6*n*4, 506-32-1; 20:5*n*3, 10417-94-4; LO, 63551-74-6; BHA, 25013-16-5; BHT, 128-37-0; $H_3C(CH_2)_5CH{=}CHCHO$, 2463-53-8; $H_3CCH_2(CH{=}CHCH_2)_2OH$, 83861-75-0; $H_3C(CH_2)_4CH(OH)CH{=}CH_2$, 3391-86-4; $H_3CCH_2CH{=}CH(CH_2)_2CH{=}CHCHO$, 26370-28-5; $H_3CCH_2CH{=}CHCH_2CH(OH)CH{=}CH_2$, 83861-74-9; 2-octenal, 2363-89-5; esculetin, 305-01-1; esculin, 531-75-9.

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Antioxidant Effect of Riboflavin in Aqueous Solution

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The antioxidant effects of riboflavin in aqueous solution under irradiation and in the dark were studied. When measurements were made soon after treatment, the linoleic acid hydroperoxide produced increased as the concentration of riboflavin increased. The linoleic acid hydroperoxide produced during light irradiation might arise because of active oxygen produced by the light-sensitizing effect of riboflavin. When the solution was left in the dark after light irradiation and assayed later, the decomposition of hydroperoxide and the recovery at 444 nm tended to increase with higher concentrations of riboflavin. The recovery at 444 nm in the dark might result from a reversible reaction if the reduced was produced by light irradiation to the oxidized form, in the dark. These results suggested that hydroperoxide acted as an electron acceptor in the reversible reaction and that this radical might participate in the reversible reaction from the reduced to the oxidized form, thereby becoming decomposed.

Very fatty foods, which contain much unsaturated fatty acid, in particular, are readily peroxidized by transition-metal ions, active oxygen, and visible or UV light (Agarwal et al., 1984; Kellogg and Fridovich, 1975; Maier and Tappel, 1959; Yamashoji et al., 1979; Thomas et al., 1985; Truby et al., 1987). Consequently, various antioxidants are used to prevent peroxidization of fat in such foods. The most commonly used antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and vitamin E. However, tests with experimental animals suggest that BHA and BHT have adverse effects on the body under some circumstances (Ito et al., 1985; Ponder and Green, 1985; Witschi and Morse, 1985). Vitamin E has no undesirable effects on the body but is unstable to heat (Kajimoto et al., 1987a,b). The antioxidant effect of vitamin E is not of use in very fatty foods that are treated with heat.

In Japan, riboflavin tetrabutyrate is used as a food additive to enrich the vitamin B_2 level. Riboflavin tetrabutyrate has an antioxidant effect on emulsions in the dark (Toyosaki et al., 1987a); the effect is due to the riboflavin itself (Toyosaki et al., 1987b). However, contrary to expectation, riboflavin tetrabutyrate stimulates oxidation under light irradiation. The cause might be the active oxygen produced, which acts in fat peroxidation, or the cause might be the photolysis of riboflavin itself, which did away with its antioxidant effect. Contradictory findings have been reported. Ohama and Yagi (1969) showed that hydroperoxide production is suppressed when a mixture of riboflavin tetrabutyrate and linoleic acid is irradiated with light. Terada (1975) reported that the suppression is due to the riboflavin moiety. The reason for the discrepancy is unknown but may have arisen from differences in the experimental conditions. The purpose of this report is to confirm whether the antioxidant effect of riboflavin occurs even during light irradiation; we assumed, based on the report of Terada (1975) and results of our own group (Toyosaki et al., 1987a,b), that riboflavin has an antioxidant effect. The antioxidant effect of riboflavin being again suggested by our results here, the next step was to identify the mechanism of this effect.

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MATERIALS AND METHODS

Materials. Materials were purchased from the following sources: riboflavin (more than 99% pure), linoleic acid (more than 99% pure), lipoxygenase (from soybean, Type I, lyophilized), and peroxidase (from horseradish, Type X, crystallized) were from Sigma Chemical Co. (St. Louis, MO); Kieselgel 60 G and 60 PF_{254} were from Merck (Darmstadt, FRG); the 0.25-mm thin-layer chromatography (TLC) was done with use of a TLC apparatus from Yazawa Scientific Apparatus Mfg. Co., Ltd. (Tokyo, Japan); nitro blue tetrazolium (NBT), *N',N'*-dimethylformamide, and *o*-dianisidine were from Tokyo Kasei Co., Ltd. (Tokyo, Japan); all other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Preparation of Linoleic Acid Hydroperoxide. Linoleic acid hydroperoxide was prepared by the procedure of Matsuda et al. (1978) by enzymatic peroxidation with lipoxygenase. The standard reaction mixture containing 32 mM linoleic acid, 0.1% Tween 80, 50 mM Na_2HPO_4/NaOH buffer (pH 9.0), and 50 units of the lipoxygenase in a total volume of 20 mL was incubated in a conical flask to facilitate flushing with pure oxygen. The reaction mixture was stirred mechanically for 40 min under a stream of pure oxygen. After incubation, hydroperoxide was extracted with diethyl ether. The formation of linoleic acid hydroperoxide was monitored by measurement of the increase in the absorbance at 234 nm. The hydroperoxide was purified by TLC with *n*-hexane/diethyl ether/acetic acid (60:40:1, v/v/v) as the solvent system and monitored under UV light.

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