Shaw, P.; Berry, R. "Hexose–Amino Acid Degradation Studies Involving Formation of Pyrroles, Furans, and Other Low Molecular Weight Products". *J. Agric. Food Chem.* **1977**, *25*, 641–644.

Stadtman, F. H.; Chichester, C. O.; Mackinney, G. "Carbon Dioxide Production in the Browning Reaction". *J. Am. Chem. Soc.* **1952**, *74*, 3194–3196.

Wolfrom, M. L.; Schlicht, R. C.; Langer, A. W.; Rooney, C. S. "Chemical Interactions of Amino Compounds and Sugars. 4. The Repeating Unit in Browning Polymers". *J. Am. Chem. Soc.* **1953**, *75*, 1031.

Lipoxygenase in Fish Tissue: Some Properties of the 12-Lipoxygenase from Trout Gill

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The gill and skin tissues from several fish species contain active lipoxygenase, which is capable of oxidizing polyunsaturated fatty acids into hydroperoxides. Gill lipoxygenase of rainbow trout exhibited similar reactivities toward arachidonic, eicosapentaenoic, and docosahexaenoic acids, but low reactivity toward linoleic acid. The lipoxygenase exhibited activity from pH 7 to 9 with optimum pH at 7.5. The enzyme was rapidly inactivated at temperatures above 40 °C. Stability of gill lipoxygenase was enhanced in the presence of glutathione. Inactivation of lipoxygenase by sulfhydryl-specific reagents suggested that thiol groups were involved in its activity.

The demand for high-quality fish and seafood products should increase because of their apparent beneficial effects on health (Herold and Kinsella, 1986). However, the high degree of unsaturation of fish lipids makes them susceptible to oxidation and quality deterioration especially if fish is not handled properly. The problem of quality deterioration in fish is closely related to oxidative instability of lipids (McDonald et al., 1979). To control or minimize lipid oxidation, more basic information concerning the initiation mechanism of lipid oxidation is needed.

When fish is killed and tissue damaged, certain enzymes such as lipoxygenase of fish gill and skin (German and Kinsella, 1985, 1986a), peroxidase of fish blood (Kanner and Kinsella, 1983), and microsomal NADH peroxidase of fish muscle (Slabyj and Hultin, 1984) may become uncontrolled and initiate lipid peroxidaton. Lipoxygenase is present in gill and skin tissues of fish and capable of initiating oxidation of polyunsaturated fatty acids to produce unstable hydroperoxides (German and Kinsella, 1985, 1986a). These hydroperoxides, following carbon–carbon cleavage at the hydroperoxide group, are potential precursors of many compounds, such as hexanal, 4-heptenal, and 2,4-heptadienal. These carbonyls are sources of oxidative off-flavors that can adversely affect taste and smell of fish (Josephson et al., 1984).

Because of its potential role in generating oxidative off-flavors in fish, the properties of lipoxygenase as they affect fish qualities are of practical interest. Recently we observed lipoxygenase in trout gill and skin tissues (German and Kinsella, 1985, 1986a). The present study was undertaken to determine the presence and relative activities of lipoxygenase in gill and skin tissues of several species of fresh water fish. Furthermore, because its activity and concentration is high in trout gill tissue, we used gill lipoxygenase to determine properties (the optimum pH, substrate specificity, heat stability, enzyme self-inactiva-

tion, and the effects of thiol reagents on lipoxygenase activity) of this lipoxygenase.

MATERIALS AND METHODS

Materials. Linoleic (18:2, $n - 6$), arachidonic (20:4, $n - 6$), eicosapentaenoic (20:5, $n - 3$), and docosahexaenoic (22:6, $n - 3$) acids were obtained from Nu-Chek Prep (Elysian, NY). Radioactive [1-^{14}C]arachidonic, [1-^{14}C]eicosapentaenoic, [1-^{14}C]docosahexaenoic, and [1-^{14}C]linoleic acids were purchased from New England Nuclear (Boston, MA). Glutathione, iodoacetamide, and p-chloromercuribenzoate (pCMB) were purchased from Sigma Chemical Co. (St. Louis, MO). Ethyl acetate was obtained from Fisher Scientific Co. (Rochester, NY). Methanol was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Chloroform and glacial acetic acid were from Mallinckrodt Inc. (St. Louis, MO).

Lipoxygenase Preparation. Samples of gill tissue (1 g) were carefully excised from young freshly killed rainbow trout (15 g) (Tunison Fish Laboratory, Cortland, NY). The gill tissue was homogenized in 40 mL of 0.05 M pH 7.4 phosphate buffer with 1 mM glutathione (r-L-glutamyl-L-cysteinylglycine) on a Polytron homogenizer. The homogenate was centrifuged for 15 min at 15000g at 4 °C. The resultant supernatant fraction was used as the crude enzyme source without further purification. The enzyme preparation was either used immediately or frozen in liquid nitrogen as droplets and stored at −70 °C until use. Protein concentration was estimated by using phenol reagents with bovine serum albumin as standard (Lowry et al., 1951).

Skin tissues (1 g) were carefully excised from young freshly killed rainbow trout, and residues of muscle on the skin were completely removed. They were then cut into small pieces to facilitate homogenization. The skin homogenate was then centrifuged, and protein concentration was determined as stated above.

Lipoxygenase Assay. To assay lipoxygenase activity, the enzyme preparation (1 mg of protein/mL) was incubated at 25 °C with a polyunsaturated fatty acid such as arachidonic acid (50 μM) using labeled [1-^{14}C]eicosatet-

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raenoic acid as tracer. The primary products of lipoxygenase activity on polyunsaturated fatty acids are their hydroperoxides. Hydroperoxides are unstable and readily reduced to their hydroxy analogues by glutathione and glutathione peroxidase present in the enzyme preparation. Glutathione peroxidase has been demonstrated to be present in the fish gill tissue (Bell et al., 1980). Hence, lipoxygenase activity was calculated from the conversion of arachidonic acid into its 12-hydroxy analogue during a 10-min incubation period. The reaction was quenched by acidification with formic acid to pH 3.0. The resultant products were twice extracted with 1 mL of ethyl acetate. Extracted lipids were then separated by thin-layer chromatography using a solvent system consisting of chloroform/methanol/acetic acid/water (90:8:1:0.8, v/v/v/v) (Powell, 1982). The radiolabeled product, 12-hydroxy-5,8,10,14-eicosatetraenoic acid, from labeled arachidonic acid, was generated due to the activity of gill lipoxygenase and was qualitatively detected by autoradiography as reported (German and Kinsella, 1985). The extent of product formation during the reaction period was quantified by locating and scraping the corresponding bands and measuring the radioactivity in each band by liquid scintillation counting (German and Kinsella, 1985, 1986b).

The effect of pH on gill lipoxygenase activity was determined by carrying out the assay at different pHs (from 4 to 9). The enzyme assay proceeded at 25 °C, and analysis of product formation was performed as stated above.

The effect of temperature on gill lipoxygenase activity was examined by heating the enzyme preparation to a specific temperature between 0 and 90 °C within 1 min. Arachidonic acid was then added, and the assay mixture was incubated at that temperature for an additional 10 min. The products were quantified as stated before. The stability of gill lipoxygenase at various temperatures (40, 50, 60 °C) was evaluated by incubating the enzyme preparation at specific temperatures for different times and then quickly cooling to 25 °C. Enzyme activity was then assayed at 25 °C, and analysis of product formation was performed as stated above.

The influence of thiol reagents on the gill lipoxygenase activity was performed on the standard assay mixture containing one of the following compounds: glutathione, 2-mercaptoethanol, iodoacetamide, or *p*-chloromercuribenzoate. The enzyme was preincubated with one of the compounds at 25 °C for 10 min. The enzyme assay and analysis of product formation were then performed as stated above.

Enzyme Velocity Calculations. To accurately determine the maximal rate of lipoxygenase-catalyzed reaction and its lag time (time required to reach maximal rate following the addition of substrate), lipoxygenase activity was monitored by oxygen consumption by a polarographic oxygen electrode cell (Hansatech Ltd., Norwich, England).

The enzyme preparation (1 mg of protein/mL) was equilibrated in phosphate buffer (0.05 M, pH 7.4) in a water-jacketed oxygen electrode chamber at 25 °C for 1 min. The enzyme activity was initiated by the addition of substrate fatty acid (50 M) in ethanol (1 μL). Oxygen consumption was continuously monitored by an Apple IIe computer. The experimental data were smoothed to eliminate random electronic noise. The first differential of the oxygen concentration vs time (dO_2/dt) was calculated. The maximal rate and lag time required to reach this rate were obtained from these first differential plots. The appropriate program for performing these analyses was written in Forth for the Apple IIe computer. The K_m of gill lipoxygenase was determined with the data obtained

Table I. Relative Activity of Lipoxygenase in Gill and Skin Tissues of Fish

	rel. act. compared to trout tissue, %	
fish species	gill	skin
freshwater drum (*Aplodinotus grunniens*)	160	17
white perch (*Morone americanus*)	156	8
emerald shiner (*Notropis atherinoides*)	154	40
white bass (*Morone chrysops*)	151	nd
gizzard shad (*Dorosoma cepedianum*)	140	50
rock bass (*Ambloplites rupestris*)	136	nd
brown bullhead (*Ictalurus nebulosus*)	133	36
sheephead	124	nd
rainbow trout (*Salmo gairdneri*)	100[a]	100[b]
channel catfish (*Ictalurus punctatus*)	88	84
salmon (*Salmo salar*)	79	44
smallmouth bass (*Micropterus dolomieui*)	43	13
yellow perch (*Perca flavescens*)	40	5
blue gill (*Lepomis macrohirus*)	31	nd
pumpkin seed (*Lepomis gibbosus*)	26	20

[a] 100% activity corresponded to the formation of 30 nmol of 12-hydroxy-5,8,10,14-eicosatetraenoic acid by 1 mg of partially purified gill lipoxygenase from rainbow trout following 10-min incubation (details in Methods). [b] 100% activity corresponded to the formation of 20 nmol of 12-hydroxy-5,8,10,14-eicosatetraenoic acid by 1 mg of partially purified gill lipoxygenase from rainbow trout following 10-min incubation (details in Methods).

from the maximal oxygen consumption rates at different substrate fatty acid concentrations.

RESULTS AND DISCUSSION

The gill and skin tissues from many freshwater fish species contained active lipoxygenase activity (Table I). However, there was no detectable lipoxygenase activity in muscle tissues. In general the activity of lipoxygenase was higher in fish gill than in skin tissues. In both tissues, the lipoxygenase activity varied among different fish species, as there was 8 times more lipoxygenase activity in gill of freshwater drum than of pumpkin seed and 20 times more lipoxygenase activity in skin of rainbow trout than of yellow perch. Also there was little correlation in lipoxygenase activity between gill and skin tissues of the same fish species.

Because of the high lipoxygenase activity in trout gill, the plentiful supply of fresh trout, and its similarities to the skin lipoxygenase in substrate specificity, all subsequent studies were conducted using trout gill lipoxygenase. Furthermore, a significant and practical advantage of using gill lipoxygenase is its freedom from other oxidation reactions (German and Kinsella, 1986b). In the presence of glutathione peroxidase, the major reaction product generated by rainbow trout lipoxygenase preparation from arachidonic acid (peak I) was 12-hydroxy-5,8,10,14-eicosatetraenoic acid (peak II) (Figure 1) as identified by German and Kinsella (1985, 1986a). About 70% of the substrate, arachidonic acid, was converted into its 12-hydroxy fatty acid. The rate of oxidation of arachidonic acid increased with the enzyme concentration up to 4 mg/mL. Boiled enzyme preparation showed no activity.

Effect of pH on Enzyme. Trout gill lipoxygenase showed optimum activity around pH 7.5 (Figure 2). The enzyme was less sensitive to pH change in the alkaline range than in the acidic range. A decrease in pH from 7.5 to 6.0 resulted in the loss of 90% of the activity, while raising pH from 7.5 to 9.0 reduced the activity by 40%. A similar optimum pH was observed among lipoxygenases from human platelet (Wallach and Brown, 1971, 1981), rat lung tissue (Yokoyama et al., 1983), and skin tissue of
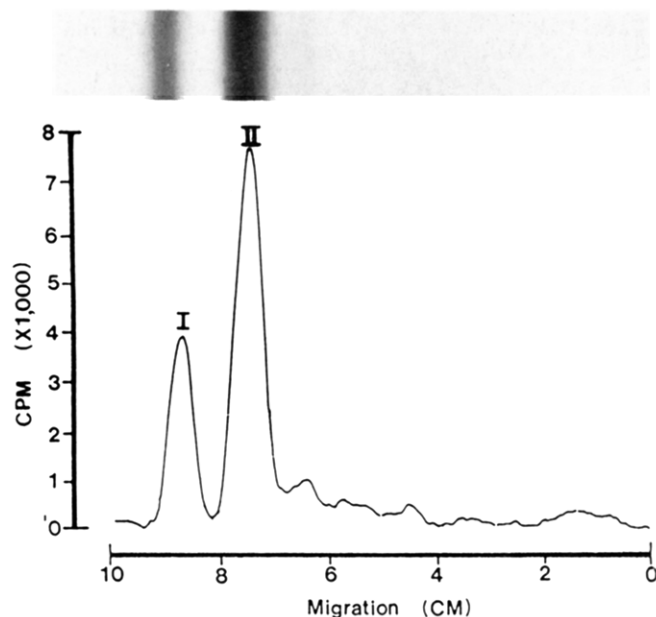
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Figure 1. Autoradiogram and scan of products generated following incubation of lipoxygenase of trout gill tissue with arachidonic acid. Gill 12-lipoxygenase (1 mg of protein) was incubated with 50 μM [1-^{14}C]arachidonic acid in 1 mL of phosphate buffer containing 1 mM glutathione for 10 min. Extraction, thin-layer chromatography, and detection of labeled products were performed as described in Materials and Methods. Peak I is arachidonic acid, 30% of the total area, whereas peak II is its 12-hydroxy fatty acid, 70% of the total area.
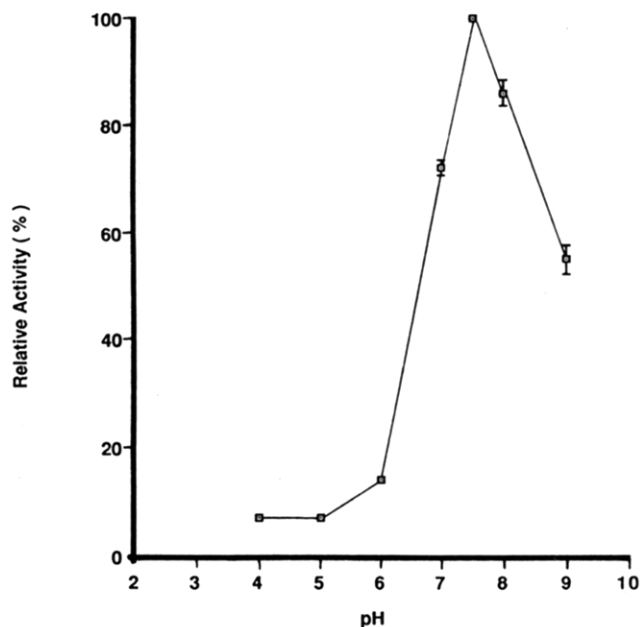

Figure 2. Effect of pH on the activity of gill lipoxygenase. Reactions were carried in the assay mixture at various pH. Extraction, thin-layer chromatography, and detection of labeled products were performed as described in Materials and Methods. The bars represent standard error of mean.

guinea pig (Ruzicka et al., 1983).

Substrate Specificity. Eicosapentaenoic and docosahexaenoic acids are abundant in fish lipids; the lipoxygenase of trout gill exhibited no selectivity for these over arachidonic acid (20:4, $n - 6$) (Figure 3). However, the enzyme showed much less reactivity toward linoleic acid (18:2, $n - 6$).

The 12-lipoxygenase from human platelet showed similar affinities for substrate fatty acids such as dihomo-r-
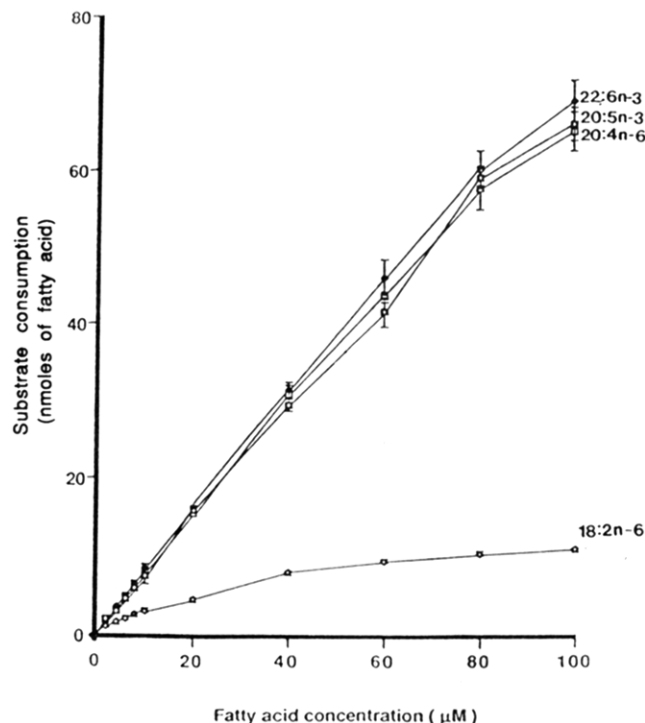

Figure 3. Substrate specificity of trout gill lipoxygenase. Reactions were performed in the presence of a fatty acid such as arachidonic, eicosapentaenoic, docosahexaenoic, or linolenic. The substrate consumption rate is expressed as nanomoles of fatty acids used/milliliter per minute. Extraction, thin-layer chromatography, and detection of labeled products were performed as described in Materials and Methods. The bars represent standard error of mean.

linolenic acid, arachidonic acid, and 5,8,11,14,17-eicosapentaenoic acid (K_m in the range of 10^{-5} M) (Wallach and Brown, 1981). However, in the case of 12-lipoxygenase from rat lung, eicosapentaenoic acid was used preferentially to arachidonic acid, while little reactivity was shown toward linoleic and linolenic acids (Yokoyama et al., 1983).

The K_m of rainbow trout 12-lipoxygenase for arachidonic acid was 20 μM as determined by oxygen consumption and polarography. This value is similar to that of 12-lipoxygenase from human platelet (Wallach and Brown, 1981) and skin tissue of guinea pig (Ruzicka et al., 1983).

Effect of Temperature. Because of the potential role of lipoxygenase in causing off-flavors, practical methods to minimize lipoxygenase activity are of interest. Therefore, the effects of temperatures on gill lipoxygenase were studied. Gill lipoxygenase retained over 90% of its activity between 10 and 30 °C (Figure 4a). There was still 60% of lipoxygenase activity present at 0 °C. The high activity of fish lipoxygenase at temperatures near freezing suggests that it may be important in the initiation of fish lipid oxidation at refrigeration conditions. At 40 °C, 80% of activity was retained. However an abrupt decline in activity occurred at 50 °C, and only minimal activity was observed at temperatures above 60 °C.

The effects of temperatures on gill lipoxygenase were studied further by incubating at 40, 50, and 60 °C for different times. Of enzyme activity, 80% was retained after incubation at 40 °C for 10 min (Figure 4b). However heating at 50 °C for 2 and 10 min decreased lipoxygenase activity by 50 and 95%, respectively. Over 90% of the lipoxygenase activity was lost by heating at 60 °C for 20 s. Lipoxygenases of human platelets (Wallach and Brown, 1981) and rat lung (Yokoyama et al., 1983) were also heat-labile. The instability of gill lipoxygenase above 50 °C may provide an approach for controlling enzyme ac-
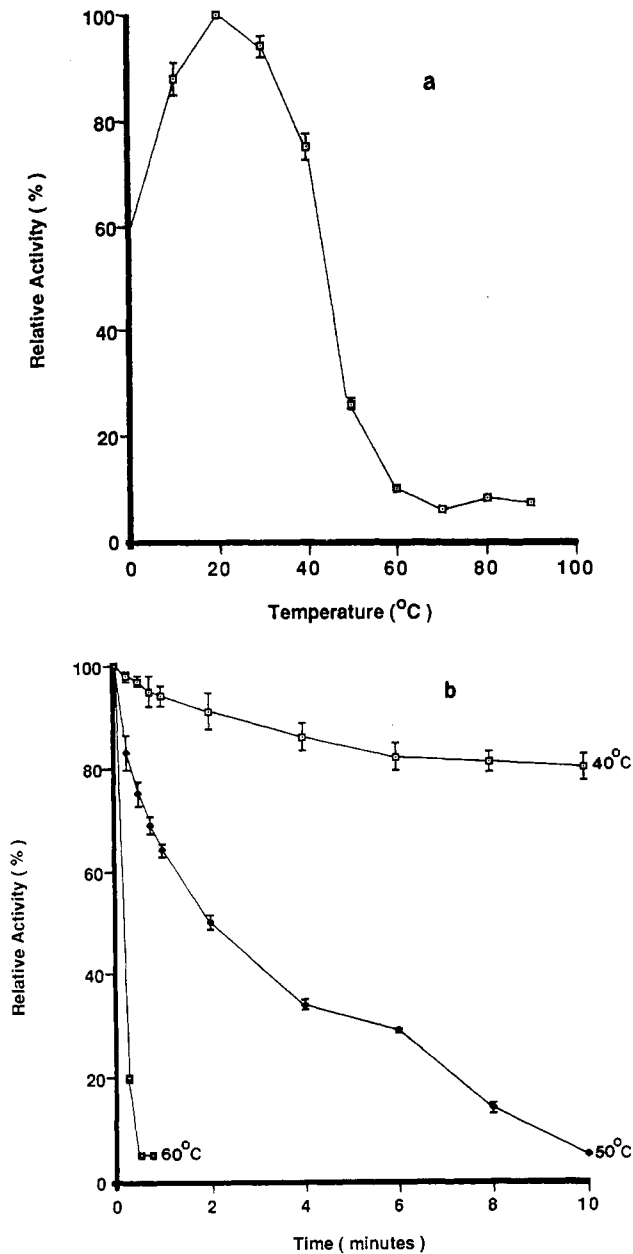
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Figure 5. Autoinactivation of gill lipoxygenase during storage at 4 °C. Reactions were performed in the assay mixture with (a) no added glutathione or (b) 1 mM glutathione. Extraction, thin-layer chromatography, and detection of labeled products were performed as described in Materials and Methods. The bars represent standard error of mean.

Figure 4. Effect of temperature on the activities of trout gill lipoxygenase. Reactions were carried in the standard assay mixture (a) at various temperatures for 10 min and (b) heated at different temperatures for different times. Extraction, thin-layer chromatography, and detection of labeled products were performed as described in Materials and Methods. The bars represent standard error of mean.

Figure 6. Effect of thiol reagents on the activities of gill lipoxygenase. Reactions were performed in the standard assay mixture containing one of the following reagents: (a) glutathione; (b) 2-mercaptoethanol; (c) iodoacetamide; (d) *p*-chloromercuribenzoate. Extraction, thin-layer chromatography, and detection of labeled products were performed as described in Materials and Methods. The bars represent standard error of mean.

tivity and help improve flavor stability and the quality of fish and seafood products.

Hydroperoxides and Stability of Lipoxygenase. The time-dependent diminution in activity of gill lipoxygenase stored at 4 °C was shown in Figure 5. The half-life of the enzyme (1.5 h) was shortened by the addition of substrate fatty acid, such as arachidonic acid, or the elevation of temperature to 25 °C. This loss of activity was not due to the exhaustion of substrate fatty acids as addition of new substrate failed to restore the activity. The inactivation of enzyme by oxygenated substrate also occurred with lipoxygenases of reticulocyte (Rapport et al., 1979) and blood platelet (Lapetina and Cuatrecasas, 1979). The instability of lipoxygenase may be caused by its oxygenation activity and the accumulation of excess amounts of enzyme-generated hydroperoxides (>1 μM) (Egan et al., 1979).
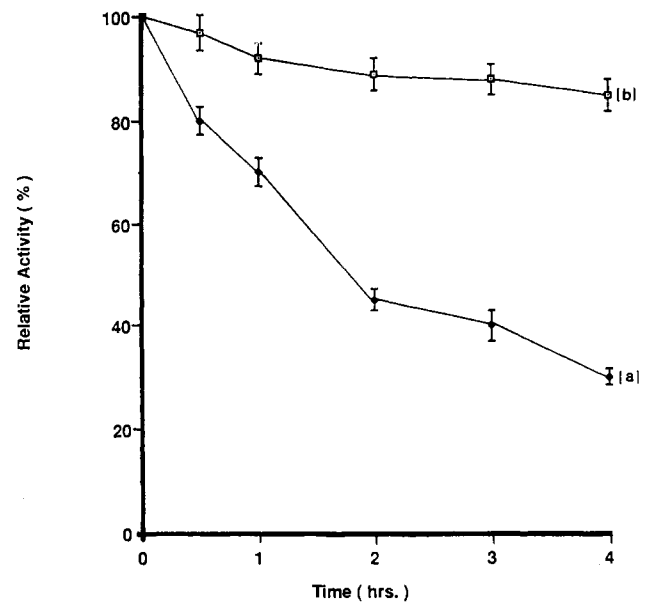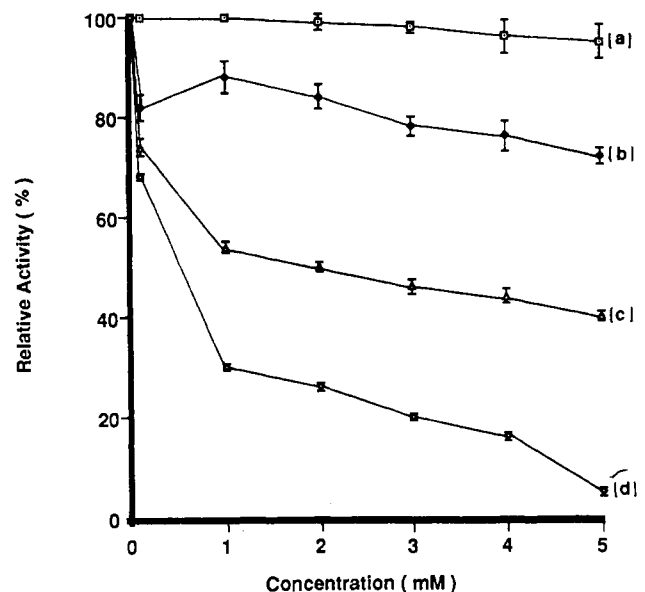
The stability of gill lipoxygenase was significantly improved by addition of 1 mM glutathione to the preparation (Figure 5). Glutathione may be used by glutathione peroxidase in trout tissue (Bell et al., 1980) to reduce hydroperoxides to its stable hydroxy analogues and remove excess hydroperoxides to keep the lipoxygenase active. 2-Mercaptoethanol had a lower potency on stabilizing lipoxygenase activity than glutathione (Figure 6). The effect of 2-mercaptoethanol may be ascribed to the maintenance of essential sulfhydryl groups in gill lipoxygenase in the reduced state or the liberation of glutathione to facilitate glutathione peroxidase reaction to keep lipoxygenase from self-inactivation (Flohe, 1982).

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The addition of high concentrations (5 mM) of reduced glutathione or 2-mercaptoethanol to the gill lipoxygenase preparation might reduce the available concentrations of hydroperoxides to such an extent that the lipoxygenase activity was limited (Figure 6). The requirement of low levels of hydroperoxides (<10 nM) for activation of lipoxygenases has also been reported for many animal (Yokoyama et al., 1983) and plant tissues (Haining and Axelrod, 1958; Smith and Lands, 1972; Egan et al., 1983). The actions of critical amounts of hydroperoxides in the catalytic cycle of lipoxygenase reaction are not only relevant to activate the enzyme for initiating lipid oxidation but also required for the continuation of oxidation (Papatheofanis and Lands, 1985).

Thiol Groups and Lipoxygenase Activity. The lipoxygenase apparently requires thiol groups for its activity. Sulfhydryl groups are present and important for the activity of lipoxygenases from various sources such as platelet (Ruzicka et al., 1983), soybean (Siegel et al., 1980), and guinea pig skin (Spaapeen et al., 1980). In this study, p-chloromercuribenzoate (pCMB) and iodoacetamide were used to ascertain whether thiol groups were needed for activities of gill lipoxygenase. pCMB at 1 mM concentration reduced gill lipoxygenase activity by 50% (Figure 6) while 5 mM pCMB blocked all essential sulfhydryl groups in the enzyme and inactivated it completely. Iodoacetamide, at corresponding concentrations, was only half as effective as pCMB as an inactivator of gill lipoxygenase (Figure 6). pCMB inhibited lipoxygenases in platelets (Siegel et al., 1980) and skin tissue of guinea pig (Ruzicka et al., 1983). However, pCMB did not inhibit lipoxygenase of rat lung (Yokoyama et al., 1983). Modification of sulfhydryl groups of soybean lipoxygenase by alkylmercuric halides has also been assumed to block sulfhydryl groups in the active site of the enzyme (Spaapeen et al., 1980). The modification of the active site apparently induced conformational changes in the active site of the enzyme, which reduced activity and adversely affected its affinity toward fatty acids, its substrate specificity, reaction mechanism, and kinetics (Grossman et al., 1984).

These results demonstrate the potential of gill lipoxygenase in initiating fatty acid oxidation and generating hydroperoxides in fish tissue. Under postharvest conditions, after fish are caught, the release of tissue lipoxygenases from endogenous constraints could generate significant quantities of reactive lipid hydroperoxides. In the presence of metal ions (such as ferric and ferrous ions) hydroperoxides could serve as potential sources of free-radical species (Kanner et al., 1987), which in turn further catalyze autoxidation and generate off-flavors and products that may react with other components and result in flavor, color, and quality deterioration in fish and seafood products (Simic and Karel, 1980). Proper postharvest methods, such as low-temperature storage on ice or at 0 °C, avoidance of bruising or injury, mild heating (blanching), and control of pH may be employed to control or retard lipoxygenase-initiated lipid oxidations and quality deterioration.

ACKNOWLEDGMENT

LITERATURE CITED

Bell, J. G.; Cowey, C. B.; Youngson, A. "Rainbow Trout Liver Microsomal Lipid Peroxidation. The Effect of Purified Glutathione Peroxidase, Glutathione S-Transferase and Other Factors". *Biochim. Biophys. Acta* **1980**, *795*, 91–99.

Egan, R. W.; Gale, P. H.; Kuehl, F. A., Jr. "Reduction of Hydroperoxides in the Prostaglandin Pathway by a Microsomal Peroxidase". *J. Biol. Chem.* **1979**, *254*, 3295–3302.

Egan, R. W.; Tischler, A. N.; Baptista, E. M.; Ham, E. A.; Soderman, D. D.; Gale, P. H. "Specific Inhibition and Oxidative Regulation of 5-Lipoxygenase". In *Advances in Prostaglandin, Thromboxane, and Leukotriene Research*; Samuelsson, B., Paoletti, R. Ramwell, P., Eds.; Raven: New York, 1983; Vol. 2, pp 151–157.

Flohe, L. "Glutathione Peroxidase Brought into Focus". In *Free Radicals in Biology*; Pryor, W. E. M., Ed.; Academic: New York, 1982; Vol. 5, pp 223–249.

German, J. B.; Kinsella, J. E. "Lipid Oxidation in Fish Tissue. Enzymatic Initiation by Lipoxygenase". *J. Agric. Food Chem.* **1985**, *33*, 680–683.

German, J. B.; Kinsella, J. E. "Lipoxygenase in Trout Gill Tissue Acting on Arachidonic, Eicosapentaenoic, and Docosahexaenoic Acids". *Biochim. Biophys. Acta* **1986a**, *875*, 12–20.

German, J. B.; Kinsella, J. E. "Hydroperoxide Metabolism in Trout Gill Tissue: Effect of Glutathione on Lipoxygenase Products Generated from Arachidonic Acid and Docosahexaenoic Acid". *Biochim. Biophys. Acta* **1986b**, *879*, 378–387.

Grossman, S.; Klein, B. P.; Cohen, B.; Pinsky, A. "Methylmercuric Iodide Modification of Lipoxygenase-1: Effect of the Anaerobic Reaction and Pigment Bleaching". *Biochim. Biophys. Acta* **1984**, *793*, 455–462.

Haining, J. L.; Axelrod, B. "Induction Period in the Lipoxidase-Catalyzed Oxidation of Linoleic Acid and Its Abolition by Substrate Peroxide". *J. Biol. Chem.* **1958**, *232*, 193–202.

Herold, P.; Kinsella, J. E. "Fish Oil Consumption and Decreased Risk of Cardiovascular Disease: A Comparison of Findings from Animal and Human Feeding Trials". *Am. J. Clin. Nutr.* **1986**, *43*, 566–598.

Josephson, D. B.; Lindsay, R. C.; Stuiber, D. A. "Biogenesis of Lipid Derived Volatile Aroma Compounds in Emerald Shiner (*Notropis atherinoides*)". *J. Agric. Food Chem.* **1984**, *32*, 1347–1352.

Kanner, J.; Kinsella, J. "Lipid Deterioration Initiated by Phagocytic Cells in Muscle Foods: β-Carotene Destruction by a Myeloperoxidase–Hydrogen Peroxide–Halide System". *J. Agric. Food Chem.* **1983**, *31*, 370–376.

Kanner, J.; German, J. B.; Kinsella, J. E. "Initiation of Lipid Peroxidation in Biological System: A review". In *Critical Review Food Science and Nutrition*; Furia, T. E., Ed.; CRC: Boca Raton, FL, 1987; pp 317–364.

Lapetina, E. G.; Cuatrecasas, P. "Rapid Inactivation of Cyclooxygenase after Stimulation of Intact Platelets". *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 121–125.

Lowry, O. H.; Farr, A. L.; Rosenbrough, N. J.; Randall, R. "Protein Measurement with Folin Phenol Reagent". *J. Biol. Chem.* **1951**, *193*, 265–275.

McDonald, R. E.; Kelleher, S. D.; Hultin, H. O. "Membrane Lipid Oxidation in a Microsomal Fraction of Red Hake Muscle". *J. Food Biochem.* **1979**, *3*, 125–134.

Papatheofanis, F. J.; Lands, W. E. M. "Lipoxygenase Metabolisms". In *Biochemistry of Arachidonic Acid Metabolites*; Lands, W. E. M., Ed.; Martinus Nihoff: Boston, 1985; pp 9–39.

Powell, W. S. "Prostaglandins and Arachidonic Acid Metabolites". In *Methods Enzymol.* **1982**, *85*, 467–477.

Rapport, S. M.; Schewe, T.; Wiesner, R.; Halang, K. W.; Ludwig, P.; Janicke-Hohne, M.; Tannert, C.; Hiebsch, C.; Klatt, D. "The Lipoxygenase of Reticulocytes: Purification, Characterization and Biological Dynamics of the Lipoxygenase; Its Identity with the Respiratory Inhibition of the Reticulocyte". *Eur. J. Biochem.* **1979**, *96*, 545–561.

Ruzicka, T.; Vitto, A.; Printz, M. P. "Epidermal Arachidonate Lipoxygenase". *Biochim. Biophys. Acta* **1983**, *751*, 369–374.

Siegel, M. I.; McConnell, R. T.; Porter, N. A.; Cuatrecasas, P. "Arachidonic Acid Metabolism via Lipoxygenase and 12-Hydroxy-5,8,10,14-eicosatetraenoic Aciod Peroxidase Sensitive to Anti-inflammatory Drugs". *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 308–312.

Simic, M. G., Karel, M., Eds. *Autoxidation in Food and Biological Systems*; Plenum: New York, 1980.

Slabyj, B. M.; Hultin, H. O. "Oxidation of a Lipid Emulsion by a Peroxidizing Microsomal Fraction from Herring Muscle". *J. Food Sci.* 1984, *49,* 1392–1393.

Smith, W. L.; Lands, W. E. M. "Oxygenation of Unsaturated Fatty Acids by Soybean Lipoxygenase". *J. Biol. Chem.* 1972, *247,* 1038–1047.

Spaapeen, L. J. M.; Verhagen, J.; Veldink, G. A.; Vliegenthart, J. F. G. "The Effect of Modification of Sulfhydryl Groups in Soybean Lipoxygenase-1". *Biochim. Biophys. Acta* 1980, *618,* 153–162.

Wallach, D. P.; Daniels, E. G. "Properties of a Novel Preparation of Prostaglandin Synthetase from Sheep Seminal Vesicles".

Biochim. Biophys. Acta 1971, *231,* 445–457.

Wallach, D. P.; Brown, V. R. "A Novel Preparation of Human Platelet Lipoxygenase". *Biochim. Biophys. Acta* 1981, *663,* 361–372.

Yokoyama, C.; Mizuno, K.; Mitachi, H.; Yoshimoto, T.; Yamamoto, S.; Pace-Asciak, C. R. "Partial Purification and Characterization of Arachidonate 12-Lipoxygenase from Rat Lung". *Biochim. Biophys. Acta* 1983, *750,* 237–243.

Cooxidation of β-Carotene by Soybean Lipoxygenase

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The cooxidation behavior of a natural mixture of soybean lipoxygenase isoenzymes has been studied at pH 7.4. The ratio of oxidation rates of linoleic acid and β-carotene was found to be 23.3:1. However, BHT and α-tocopherol reduce the rate of oxidation of β-carotene to a greater extent than that of linoleic acid. BHT is a more effective antioxidant than α-tocopherol, while ascorbic acid has no significant antioxidant properties. α-Tocopherol (10^{-6} M) retards the initial cooxidation of β-carotene and retinyl acetate, but cooxidation proceeds rapidly after this period. The rate of carotene bleaching increases with carotene concentration, but the ratio of the rate of linoleic acid oxidation to β-carotene oxidation is independent of temperature since the two reactions have an identical activation energy within experimental error. These observations are consistent with the accepted mechanism for lipoxygenase-catalyzed cooxidation.

Lipoxygenase, which catalyzes the oxidation of polyunsaturated fatty acids, occurs in a wide variety of plants (Eskin et al., 1977). There are two distinct groups of enzymes described as types 1 and 2. Type 1 lipoxygenase has been reported in relatively few plants and has optimum activity at pH 9 with little tendency to cause cooxidation of other lipids during the reaction. Type 2 lipoxygenase occurs widely with optimum activity at pH 6.5–7.0 and a strong tendency to catalyze the cooxidation of other compounds. Chlorophyll, carotenoids, cholesterol, cytochrome c, and thiols in dough are among the substances reported to suffer cooxidation (Eskin et al., 1977). The cooxidation of molecules during the lipoxygenase-catalyzed oxidation of linoleic acid has been ascribed to the fact that a large proportion of the peroxyl radicals is not directly converted to hydroperoxides by the enzyme (Weber and Grosch, 1976). The cooxidation of lipids during lipoxygenase-catalyzed oxidation can lead to the formation of off-flavors (Rackis et al., 1972) and a loss of nutrients (e.g., β-carotene) in foodstuffs, and therefore this study was concerned with increasing the understanding of the factors affecting the rate of the cooxidation reaction catalyzed by a natural mixture of soybean lipoxygenase isoenzymes at neutral pH. Neutral pH was selected, since this is close to the pH of soybean flour dispersed in water.

MATERIALS AND METHODS

Soybean lipoxygenase (Sigma Type 1, lyophilized, a natural mixture of isoenzymes, 125 000–175 000 units/mg

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protein), linoleic acid (99%), β-carotene (synthetic), retinyl acetate, L-α-tocopherol, and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co.

Lipoxygenase-catalyzed oxidation of linoleic acid was monitored by spectrophotometric determination of the increase in absorbance at 234 nm, according to the method of Ben Aziz et al. (1970). The hydroperoxide (LOOH) concentration was calculated with the molar absorptivity of 26 000 L mol⁻¹ cm⁻¹ (Matthew et al., 1977).

β-Carotene and retinyl acetate were determined spectrophotometrically from the absorbances at 460 and 325 nm, respectively. The molar absorptivities in the assay medium were found to be 88 000 and 37 313 L mol⁻¹ cm⁻¹, respectively.

A typical cooxidation reaction required the preparation of aqueous linoleate and aqueous β-carotene solutions. The linoleate solution was prepared from a solution of linoleic acid in ethanol (1 mL, 7.5%) mixed with Tween 80 in ethanol (0.3 mL, 10%), to which aqueous ethylenediaminetetraacetic acid (EDTA) (5 mL, 0.5%) was added before being adjusted to pH 9 with sodium hydroxide solution (1 M). The β-carotene solution was prepared by dissolving β-carotene (25 mg) and Tween 80 (0.9 mL) in chloroform (25 mL). A sample of the chloroform solution was evaporated to dryness, and EDTA solution (10 mL, 0.25%) was added. The linoleate solution (0.5 mL) was mixed with the β-carotene solution (0.5 mL), and citric acid (0.1 M)–disodium hydrogen phosphate (0.2 M) buffer (9 mL, pH 7.4) was added. A sample of the buffered linoleate/β-carotene solution (1.5 mL) was transferred to a spectrophotometer cuvette, and distilled water (0.4 mL) and lipoxygenase solution (0.1 mL containing 12 μg of enzyme) were added.