Linoleate 13(S)-Lipoxygenase in Sardine Skin

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A novel lipoxygenase was found in sardine skin. It oxidized linoleic acid more efficiently than arachidonic acid or eicosapentaenoic acid. Esterified fatty acids such as methyl linoleate and trilinolein were also oxidized. 13(S)-Hydroperoxyoctadeca-9(Z),11(E)-dienoic acid was produced as the primary oxidation product from linoleic acid by both the crude extract of skin tissue and the active fraction after gel filtration. The enzyme had an optimum pH at 7.0 and was stable over the pH range 6–9. It may participate in the initiation of lipid oxidation in fish.

INTRODUCTION

Lipoxygenase (LOX, linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a dioxygenase enzyme. It stereoselectively absorbs a hydrogen atom from an active methylene group in 1(Z),4(Z)-pentadiene structures in polyunsaturated fatty acids and forms a hydroperoxide with molecular oxygen (Hamberg and Samuelsson, 1967). It is widely distributed in both plant and animal tissues. In plant food, LOXs not only participate in the initiation of lipid peroxidation but also are responsible for the generation of beany or green leaf-like odors, in cooperation with the coexistent hydroperoxide lyase (Galliard and Chan, 1980). On the other hand, animal lipoxygenases, which have become known relatively recently, are attracting much attention due to their physiological importance in the metabolism of eicosanoids (Yoshimoto, 1989). Such being the case, very limited information is available on animal lipoxygenases in food.

Hsieh and Kinsella (1989) suggested that LOX activity was present in the gill and skin tissues of several species of fish, and trout LOXs are specific arachidonate 12-LOXs. Although Cho et al. (1989) reported the occurrence of heat labile prooxidants in sardine skin, and their activity was much higher than those in dark meats having an abundance of hemoprotein, it has not been established enzymologically whether they are true LOXs. In a previous paper (Mohri et al., 1990), we suggested the occurrence of a lipoxygenase in sardine skin. In this paper, unique characterization of sardine skin lipoxygenase by examination of substrate specificity to various fatty acids and lipids, stereospecificity of oxidation, and effect of pH on its activity and stability is described.

MATERIALS AND METHODS

Reagents. Linoleic acid (>99%) was prepared from safflower oil methyl esters by successive urea adduction, silicic acid column chromatography, vacuum distillation, and alkaline hydrolysis. Polyunsaturated fatty acids other than linoleic acid were provided by Idemitsu Petrochemical Industries Co. Ltd., Tokyo. Soybean lipoxygenase was purchased from Sigma Chemical Co., St. Louis, MO.

Preparation of Enzyme Extract. Samples of skin tissues (30 g) were carefully removed from fresh sardines (*Sardinops melanosticus*, 500 g) landed on the same day as the removal occurred. The skin tissue was homogenized in deionized water at a 1:3 ratio (tissue weight/water volume) and centrifuged at 10000*g* for 15 min. The supernatant was filtered and the residue further extracted with 90 mL of 50 mM sodium phosphate buffer at pH 7.0 and then treated similarly. Both of the supernatants were combined and used as the crude extract. All procedures were performed at 0–4 °C.

Partial purification of the crude extract was performed by Sephadex G-100 gel filtration. After concentration to a $^1/_{10}$ volume by ultrafiltration on a Millipore 10,000 NMWL polysulfone membrane, the concentrated enzyme solution was then applied to a gel filtration column (2.5 × 90 cm) preequilibrated with 50 mM sodium phosphate buffer at pH 7.0. The elution of protein was monitored by the absorbance at 280 nm.

Lipoxygenase Assay. A typical lipoxygenase assay mixture contained 0.2 M potassium phosphate, pH 7.0, 5 mM linoleic acid, 0.12% (w/v) Tween 20, and 0.3 mL of enzyme solution in a final volume of 3 mL. The activity was generally assayed spectrophotometrically according to the method of Surrey (1964). The activity was expressed as the increase of the absorbance at 234 nm, of the reaction mixture, in a 1-mL cell per milliliter of the enzyme solution at 25 °C.

The activities on various fatty acids and lipids were compared by the oxygen absorption method (Satoh et al., 1976) using a YSI Model 5300 biological oxygen monitor (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) at 25 °C. In the case of the assay of trilinolein and L-α-lecithin dilinoleoyl, 1.67 or 2.5 mM of the lipids was used, respectively. The concentration of other components was the same as that of the spectrophotometric method. The reaction velocity was calculated on the basis of oxygen consumption during the initial 10 min.

Effect of Metal Ions for Stabilization of Enzyme. Various metal ions (1 mM) were added to the partially purified enzyme obtained by gel filtration, and the mixtures were allowed to stand at 40 °C for 30 min at pH 7.0. Then, linoleic acid, of which final concentration was 5 mM, was added to the preincubated mixture, and the residual activities were assayed spectrophotometrically as described above. The activity of the enzyme solution kept at 4 °C (control) was designated as 100. Similarly, the mixtures of metal ions and linoleic acid without enzyme were also incubated. The enzyme activity after incubation with metal ions were shown after subtraction of the value of this balnk test.

Isolation and Derivatization Hydroperoxides. The reaction mixture (5 mL) was incubated for 10 min at 5 °C and immediately reduced with sodium borohydride (10 mg) to stop the reaction and stabilize hydroperoxides. After acidification to pH 3, lipids were extracted three times with equal volumes of ethyl ether, washed with distilled water until the washing became neutral, and dried over anhydrous sodium sulfate overnight.

After methylation with diazomethane, the reaction products were applied to HPLC analysis.

Isomeric Analyses Using Straight Phase and Chiral Phase HPLC. Positional and geometrical isomers of methyl hydroxyoctadecadienoates (MHODEs) were analyzed by HPLC on a Radial-Pak cartridge silica 10-μm column (100 × 8 mm, Waters Associates, Milford, MA). The column was eluted with *n*-hexane/2-propanol (99.3:0.7 v/v) at a flow rate of 1.0 mL/min. The eluent was monitored with a JASCO 875 UV detector set at

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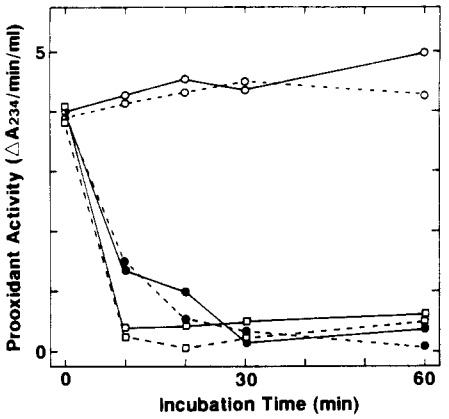

Figure 1. Thermal stability of sardine skin lipoxygenase. Enzyme solutions were incubated at various temperatures and times, described in the figure, at pH 7. After the solutions cooled to room temperature, residual activity was measured by the spectrophotometric method. (○) 30 °C; (●) 60 °C; (□) 100 °C; (—) crude extract; (- - -) crude extract after dialysis against distilled water.

Table I. Effect of Metal Ions on the Stability of Sardine Skin Lipoxygenase

metal[a]	residual act., %	metal[a]	residual act., %
control[b]	100	$FeCl_3 \cdot H_2O$	116
none	57	$CaCl_2 \cdot H_2O$	84
$CoCl_2$	52	$CuSO_4 \cdot H_2O$	45
$MgCl_2 \cdot 6H_2O$	84	$ZnCl_2$	65
$CdCl_2 \cdot H_2O$	95	$MnCl_2 \cdot 4H_2O$	30

[a] 1 mM of each metal ion was used. [b] Enzyme solution was kept at 4 °C.

234 nm. Isomeric compositions of four peaks corresponding to 9-OH,10*E*,12*Z* / 9-OH,10*E*,12*E* / 13-OH,9*Z*,11*E* / 13-OH,9*E*,11*E* were established according to the method of Hamberg and Samuelsson (1967). Optical isomers were analyzed by chiral phase HPLC. Methyl 13-hydroxy-9(*Z*),11(*E*)-octadecadienoate previously isolated by HPLC as described above was resolved by HPLC on a Chiralcel OJ (250 × 4.6 mm, Daicel Chemical Industries, Tokyo) using *n*-hexane/2-propanol (99:1 v/v) as a solvent. Authentic 13(S)-HODE was prepared according to the method of Hamberg and Samuelsson (1967) using soybean LOX.

RESULTS AND DISCUSSION

Thermal Stability. The prooxidant activity of sardine skin extract was found to be inhibited almost completely by heating at 100 °C for 10 min (Figure 1). However, the activity was relatively resistant to heating; i.e., approximately 40% of the activity remained after heating at 60 °C for 10 min, and heating at 30 °C for 60 min hardly impaired the activity. In comparison, rainbow trout gill LOX was very sensitive to heating (Hsieh et al., 1988). However, some plant LOXs, such as soybean LOX 1 (Christopher et al., 1970), immature English peas (Chen and Whitaker, 1986), and *Fusarium oxysporum* (Satoh et al., 1976), are fairly stable against heating.

Effect of Dialysis and Metal Ions on the Enzyme Activity. Dialysis against distilled water did not impair the activity (Figure 1). Porcine leukocyte 12 LOX and *F. oxysporum* LOX need a metal ion, Fe^{2+} and Co^{2+}, respectively, for the stabilization of the enzyme (Yokoyama et al., 1986). Rabbit leukocyte 5 LOX is activated by Ca^{2+} (Jakschik and Lee, 1980). The effects of several metal ions on sardine skin LOX were tested (Table I). The activity incubated without metal ions was decreased by 57%. However, preincubation with Fe^{3+} maintained the activity perfectly. This result suggests Fe^{3+} stabilized the enzyme structure from the inactivation by heating. Mg^{2+}, Cd^{2+}, and Ca^{2+} also stabilized the enzyme, but they are less effective than Fe^{3+} for stabilization.

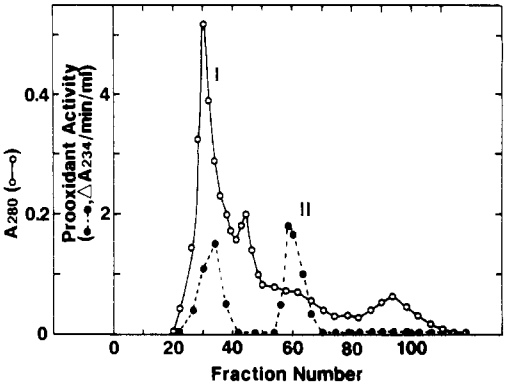

Figure 2. Partial purification of sardine skin prooxidant by Sephadex G-100 gel filtration. Crude extract was concentrated and applied to the top of the column (2.5 × 90 cm) preequilibrated with 50 mM sodium phosphate buffer, pH 7.0. The elution rate was kept at 25 mL/h, and 5-mL fractions were collected. (○) Absorbance at 280 nm; (●) prooxidant activity.

Table II. Substrate Specificity of Partially Purified Sardine Skin Lipoxygenase

substrate	rel act.	substrate	rel act.
linoleic acid	100	methyl linoleate	130
α-linolenic acid	113	trilinolein	167
γ-linolenic acid	38	L-α-lecithin dilinoleoyl	30
arachidonic acid	45	(9*E*,12*E*)-9,12-octadecadienoic acid	0
eicosapentaenoic acid	22		
docosahexaenoic acid	44		

No inhibitory effect of dialysis shows that sardine skin LOX did not always need free metal ions for activating the enzyme. However, under a critical condition such as high temperature, additive metal ions may be profitable to maintain the activity. *Fusarium* LOX was also reported to improve its stability against heating by addition of some metal ions (Satoh et al., 1976).

Partial Purification by Gel Filtration. Figure 2 shows the elution profile from a Sephadex G-100 column. The crude enzyme extract gave two major active fractions, I and II. The protein recoveries of fractions I and II through this procedure were 62% and 8% against the crude extract, respectively. However, the recoveries of activity were almost 40% and 60%, respectively. Fraction II was applied to the following experiments, not only because of its higher specific activity but also because of the reproducibility in the higher recovery of the activity.

Substrate Specificity. Substrate specificity of sardine skin LOX was examined in the partially purified preparation by measuring the activity by the oxygen absorption method. The enzyme did not oxidize (9*E*,12*E*)-9,12-octadecadienoic acid at all and was suggested to be specific for fatty acids containing (1*Z*,4*Z*)-1,4-pentadiene structures (Table II). This property is a typical characteristic of lipoxygenases (Galliard and Chan, 1980). The higher reaction velocity with linoleic acid than with arachidonic acid or eicosapentaenoic acid was different from previously known LOXs in animal tissues such as human leukocyte (Ochi et al., 1983) and fish gill (Hsieh et al., 1988) and was also different from autoxidation. Furthermore, the sardine skin LOX favorably oxidized methyl linoleate and trilinolein, while the activity for L-α-lecithin dilinoleoyl was lower than that for free acids. Although it is reported that some LOXs, i.e., soybean isoenzymes 2 and 3 (Christopher et al., 1970) and *Fusarium* (Satoh et al., 1976), oxidize esters of lipids, LOXs which oxidize neutral ester lipids as rapidly as free acids have not been known from

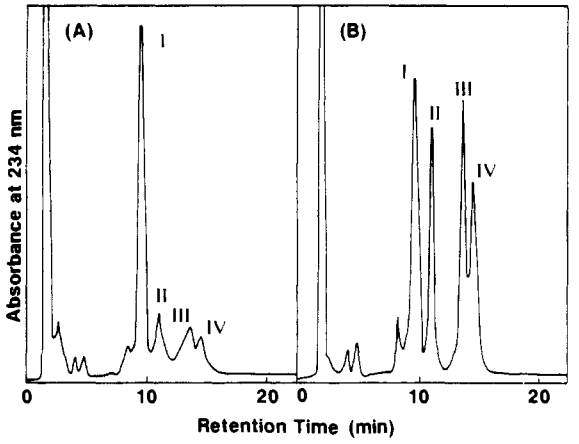

Figure 3. Analysis of positional isomers of methyl hydroxyoctadecadienoate derivatives produced by sardine skin lipoxygenase. Reaction time was 5 (A) and 30 min (B). (I) 13-OOH-9*Z*,13*E* type; (II) 13-OOH-9*E*,13*E* type; (III) 9-OOH-10*E*,12*Z* type; (IV) 9-OOH-10*E*,12*E* type.

animal tissues. Since the enzyme preparation used in this experiment had no lipase activity because neither free fatty acids nor mono- or diglycerides could be detected in the incubated reaction mixture, sardine skin LOX oxidizes esters of linoleic acid as rapidly as free fatty acids. This is an unusual property for an animal LOX, although LOXs which react with phospholipids have recently been reported from human leukocytes (Soberman et al., 1985) and reticulocytes (Schewe et al., 1986).

Steric Analysis of Products. The monohydroperoxides resulting from the oxidation of linoleic acid with the partially purified enzyme preparation were analyzed by HPLC.

Four major peaks (I–IV) were obtained from the analysis of linoleic acid oxidation products by SP-HPLC (Figure 3A). Products of the crude extract also showed a similar chromatogram (data not shown). Peak I was the most abundant and was identified as methyl 13-hydroxy octadeca-9(*Z*),11(*E*)-dienoate (13(*Z*,*E*)-MHODE) by comparing its retention time with a sample prepared from soybean LOX (Hamberg and Samuelsson, 1967). This result indicates that the sardine skin lipoxygenase predominantly catalyzes the formation of 13-hydroperoxyoctadeca-9(*Z*),11(*E*)-dienoic acid (13/9 = 95:5). It is possible that the small amount of other hydroperoxides formed was due to concomitant autoxidation during incubation and extraction procedures. Besides, the rate of hydroperoxide isomers increased rapidly when the reaction time became long and the temperature became high. When reaction time was 30 min or more, the ratio of 9 to 13 isomers changed to 1:1 (Figure 3B). This result may suggest the existence of hydroperoxide isomerases in fish skin.

13(*Z*,*E*)-MHODE isolated by SP-HPLC was applied to the steric analysis without further derivatization by chiral phase HPLC. Although 13(*Z*,*E*)-MHODE obtained from autoxidized linoleic acid separated into two peaks having equal area (Figure 4A), the product of the sardine skin prooxidant showed only one peak corresponding to the former peak in the chromatogram of autoxidized 13-(*Z*,*E*)-MHODE (Figure 4B). The chromatogram of 13-MHODE of soybean LOX, which is known to give a 13(*S*)-type hydroperoxide, showed a peak corresponding to the previous peak in the autoxidized sample (Figure 4C). These results elucidated the existence of a lipoxygenase which produces 13(*S*)-MHODE from linoleic acid in sardine skin.

Lipoxygenases which produce *S*-type hydroperoxides are known in various plants and animals (Hamberg and Samuelsson, 1967; Yoshimoto, 1989). But in fish, the optical isomerism of the hydroperoxides has not been clarified so far. In addition, while the existence of prooxidants in skin tissues has already reported (Hsieh et al., 1988; Cho et al., 1989), stereoselectivity of products has not been established. Positional selectivity products have only been studied by German and Kinsella (1985, 1986) in reporting the existence of arachidonate 12-LOX in fish tissue. This would be the first study showing the establishment of stereoselectivity of oxidation products by fish LOX.

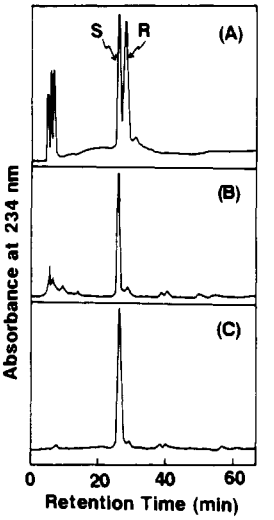

Figure 4. Steric analysis of methyl 13-hydroxy-(9*Z*,11*E*)-octadecadienoate. (A) Autoxidation; (B) sardine skin lipoxygenase; (C) soybean lipoxygenase.

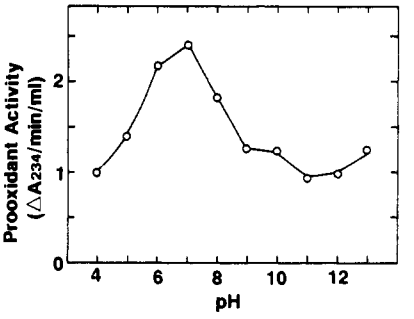

Figure 5. Effect of pH on the sardine skin lipoxygenase activity. Reactions were performed at various pHs with 0.2 M corresponding buffer: succinate buffer, pH 4–5; phosphate buffer, pH 6–8; borate buffer, pH 9–10; Ringer buffer, pH 11–12. Activity was assayed with partially purified enzyme solution by the spectrophotometric method.

Effect of pH on the Activity. As shown in Figure 5, the optimum pH for sardine skin LOX was about pH 7, quite different from that of soybean LOX 1 (Christopher et al., 1970) and *Fusarium* LOX (Satoh et al., 1976), both of which show maximum activity at alkaline pHs. However, most lipoxygenases distributed in animals and plants have optimum activities at about neutral pH (Galliard and Chen, 1980). Hsieh et al. (1988) have shown that the optimum pH of the lipoxygenase in rainbow trout gills is pH 7.0.

The effect of pH on the enzyme stability was studied by using the same buffer system as described in Figure 5. Figure 6 shows that sardine skin LOX was quite stable at 30 °C in the pH range from 6 to 9.

The physiological role of sardine skin LOX is not at all clear so far; however, LOXs in human leucocytes or skin oxidize arachidonic acid to produce leucotrienes which are mediators of inflammation (Yoshimoto, 1989). While

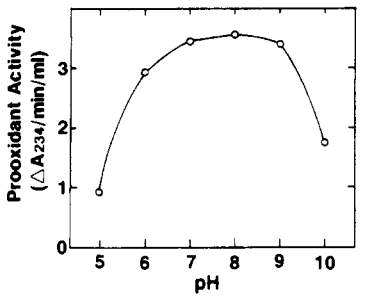

Figure 6. pH stability of sardine skin lipoxygenase. After enzyme solution was dialyzed against distilled water and concentrated, it was incubated at 30 °C for 60 min at various pHs with the corresponding buffer as shown in Figure 5.

sardine skin LOX oxidizes linoleic acid more efficiently than arachidonic acid (Table I), unlike mammalian LOXs, it may participate in a defense system in skin tissues. A similar physiological role of LOXs in plant tissues has been already suggested (Hildebrand, 1989). From a food chemical viewpoint, the enzyme will probably take part in the initiation of lipid oxidation in skin tissue. Mechanisms underlying lipid oxidation in fish have long been studied, and it is believed to be heme-catalyzed nonenzymatic oxidation (Fisher and Deng, 1977) or indirect enzymatic oxidation in which catalases and/or peroxidases etc. participate (Hsieh and Kinsella, 1989). However, German and Kinsella (1985) suggested that initial free radicals leading to the propagation phase had not been established in this process and proposed that lipoxygenase would be released posthumously and would generate significant quantities of reactive hydroperoxides. Damage to fish tissue starts in the gills and skin tissue. The existence of LOXs in these tissues strongly supports this concept. To confirm these explorations, the properties of the enzyme must be studied using a purified enzyme.

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Registry No. LOX, 9029-60-1; 13(*S*)-hydroperoxyoctadeca-9(*Z*),11(*E*)-dienoic acid, 33964-75-9; linoleic acid, 60-33-3.