Identification of 12-Lipoxygenase in the Hemolymph of Tiger Shrimp (*Penaeus japonicus* Bate)

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Lipoxygenase (LOX) activity was found in the hemolymph of live tiger shrimp (*Penaues japonicus* Bate) but not in the muscle, branchiae, midgut gland, eye, brain, and spinal cord. Oxygen consumption rate of shrimp hemolymph catalyzed oxidation of arachidonic acid was 1.32 nmol min^{-1} (mg of protein)$^{-1}$. The product was determined to be 12-hydroxyeicosatetraenoic acid (12-HETE) on the basis of retention time in HPLC analysis. The 12-HETE was found to have a conjugated structure on the basis of UV absorbance spectroscopy and to be oxygenated at the C-12 position on the basis of mass spectroscopy and HPLC analysis. The amount of 12-HETE was proportional to oxygen consumption. A 12-lipoxygenase (LOX) activity was identified in the hemolymph of shrimp. This was the first observation demonstrating LOX activity in a marine crustacean.

Keywords: *Lipoxygenase; 12-HETE; hemolymph; shrimp*

INTRODUCTION

Lipoxygenase (LOX) (EC 1.13.11.12) is widely distributed in the plant kingdom (Chen and Whitaker, 1986; Gardner, 1991). LOXs are also found in animals in the sanguineous tissue, such as platelet (Croset and Lagarde, 1985).

In aquatic animals, LOX activity was identified in the skin and gill of trout (German and Kinsella, 1986) and ayu (Zhang et al., 1992) and in eggs of starfish (Meijer et al., 1986) and sea urchin (Hawkins and Brash, 1987). LOX in egg was important in oocyte maturation (Meijer et al., 1986) or regulating membrane permeability (Perry and Epel, 1985). LOX of skin and gill was proposed to be responsible for flavor formation of fresh fish (Josephson et al., 1984, 1987; Hsieh and Kinsella, 1989).

LOX activity has been observed in a marine crustacean (Kuo and Pan, 1992). When shrimp was homogenized with $SnCl_2$, an inhibitor of lipoxygenase used in fish LOX studies (Josephson et al., 1984), and then steam distilled, the result was a decrease in the total volatiles, mainly 1-octen-3-ol and the total amount of 5,8,11-tetradecatrien-2-one isomers (Kuo and Pan, 1991). More volatile compounds were extracted from whole shrimp than the sum of volatiles extracted from shrimp head and from shrimp meat separately (Shye et al., 1987). Volatile compounds extracted by steam distillation from shrimp oil alone did not have shrimp flavor (Chen and Pan, unpublished results). Addition of shrimp hemolymph to shrimp head extract resulted in the formation of a 3-fold higher amount of 1-octen-3-ol than the control (Kuo and Pan, unpublished results). The existence of lipoxygenase in shrimp was thus proposed (Kuo and Pan, 1991, 1992). The objective of the present study is to confirm the LOX activity in shrimp hemolymph and to relate the roles of LOX to shrimp flavor formation.

MATERIALS AND METHODS

Shrimp Hemolymph. Hemolymph in 0.5–1.0 mL was drawn from a live Japanese tiger shrimp (*Penaeus japonicus* Bate) and collected into 0.5–1.0 mL of 0.9% sodium chloride containing the anticoagulant 0.02 M EDTA. Because the activity decreased rapidly in post-mortem shrimp, this mixture was used as crude LOX without further purification (Kuo and Pan, 1992).

Acidified hemolymph was prepared by adjusting the pH to 4.0 using 1 N HCl. Heat-inactivated hemolymph was obtained from incubation of hemolymph at 100 °C for 5 min.

Oxygen Consumption. The oxygen consumption of shrimp hemolymph catalyzed oxidation was measured by polarographic analysis (Hsieh et al., 1988). Crude shrimp hemolymph lipoxygenase (SH-LOX), 15–20 mg, was equilibrated in potassium phosphate buffer (0.05 M, pH 6.5) in a thermostatic incubation cell (20 mm × 68 mm) at 26 °C. Reaction was initiated by the addition of arachidonic acid (100 μM), and the oxygen concentration was measured by a biological oxygen monitor (YSI 5300, Yellow Springs, OH) equipped with a YSI Clark oxygen probe. A data acquisition system (Notebook for IBM PC computer) was used to record the change of oxygen concentration.

Assay of LOX Activity. The activity of crude SH-LOX was assayed in 0.05 M phosphate buffer (pH 6.5) containing 1 mM glutathione (reduced form, Sigma, St. Louis, MO) and was incubated with arachidonic acid (100 μM) at 26 °C for 5 min. A blank was prepared by addition of 0.5 mM esculetin into the assay mixture and incubation under the same conditions (Kuo and Pan, 1992). The reaction products were extracted with ethyl acetate and then methylated with diazomethane (Ayorinde et al., 1989). The hydroxyeicosatetraenoic acid (HETE) compounds were separated by a solid-phase extraction column (J&W Scientific, Folsom, CA) (German and Berger, 1990) and then subjected to HPLC analysis.

The protein concentration of the hemolymph was determined according to the Bradford (1976) method using bovine serum albumin (Sigma) as standard.

Identification of 12-HETE. High-pressure liquid chromatographic analyses were performed on an ODS2 column (15 cm × 4.6 mm, 5-μm particles) equipped with a Waters (Milford, MA) Model 510 pump and a Waters Model 490E UV detector and monitored at 235 nm. The HETE compounds were eluted

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Table 1. Lipoxygenase Activity Identified in Shrimp

tissue	sample size	12-HETE/mL[a]
hemolymph	1 mL	5.68 nmol
muscle	20 g	none
branchiae	20 g	none
midgut gland	20 g	none
eye	15 g	none
brain and spinal cord	15 g	none

[a] Crude extract was diluted with 0.05 M potassium phosphate buffer, pH 6.5 (1:9 v/v) and incubated with arachidonic acid (100 μM) at 26 °C for 10 min. Products were analyzed with HPLC.

Table 2. Oxygen Consumption during Oxidation of Arachidonic Acid Catalyzed by Shrimp Hemolymph

	O_2 consumption [nmol min^{-1} (mg of protein)$^{-1}$]	fold
shrimp hemolymph	1.3	1
trout gill extract[a]	20.0	15

[a] Hsieh et al. (1988). Extracted with phosphate buffer (0.05 M, pH 7.4) and determined by polarography using arachidonic acid as substrate.

isocratically by a solvent system of methanol/water (75:25 v/v) buffered with 5 mM ammonium acetate containing 0.5 mM EDTA to an apparent pH of 5.7. Six hundred nanograms of 5-HETE was used as internal standard. The LOX-catalyzed product, 12-HETE, was confirmed in comparison to an authentic standard using UV spectral and GC–MS techniques.

GC–MS Analysis. The methylated 12-HETE was derivatized with Tri-Sil reagent (Pierce, Rockford, IL) to form trimethylsilyl (TMS) 12-HETE (German and Hu, 1990) and analyzed by gas chromatography–mass spectrometry (Shimadzu QP-2000A, Kyoto, Japan) with an OV-1 column of cross-linked methyl silicone, 12.5 m × 0.22 mm (Hewlett-Packard, Palo Alto, CA), The injector was set at 250 °C. The oven temperature was programmed from 180 to 250 °C at a rate of 4 °C/min. The carrier gas was helium at a flow rate of 1.5 mL/min. The ionization voltage was 70 eV, and the ion source temperature was 200 °C.

RESULTS AND DISCUSSION

LOX activity in different tissues of shrimp is shown in Table 1. No LOX activity was found in muscle, branchiae, midgut gland, eye, brain, and spinal cord. LOX activity was only identified in the hemolymph of shrimp on the basis of the following observations.

Oxygen Consumption. Arachidonic acid was reacted with shrimp hemolymph. The oxygen consumption measured by polarographic analysis is shown in Table 2. The oxygen consumption rate of hemolymph-catalyzed oxidation of arachidonic acid was 1.32 nmol min^{-1} (mg of protein)$^{-1}$ (Table 2), as compared to that of trout gill LOX at 20 nmol min^{-1} (mg of protein)$^{-1}$ (Hsieh et al., 1988). The addition of shrimp hemolymph to arachidonic acid resulted in rapid oxygen consumption. However, the catalytic rate of shrimp hemolymph was only 6.5% of that of the crude extract of trout gill LOX.

UV Absorption Spectrum. The products of arachidonic acid treated with shrimp hemolymph were separated by a solid-phase extraction column. The UV scanning spectrum is shown in Figure 1. An absorption peak was found at 225–240 nm, indicating a conjugated diene system (Takagi et al., 1987). This spectrum was similar to those of 12-HETE or the product from arachidonic acid treated with trout gill LOX (German and Berger, 1990). A hydroperoxide with conjugated diene structure was formed from arachidonic acid treated with shrimp hemolymph.

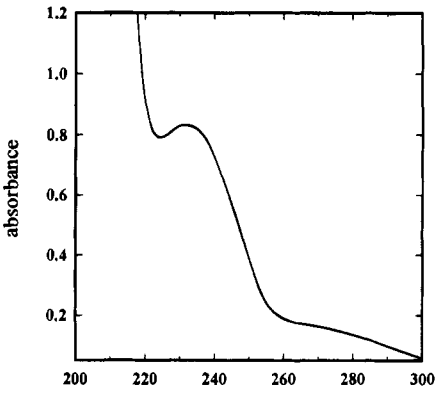


Figure 1. UV absorption spectrum of the product of arachidonic acid treated with shrimp hemolymph.

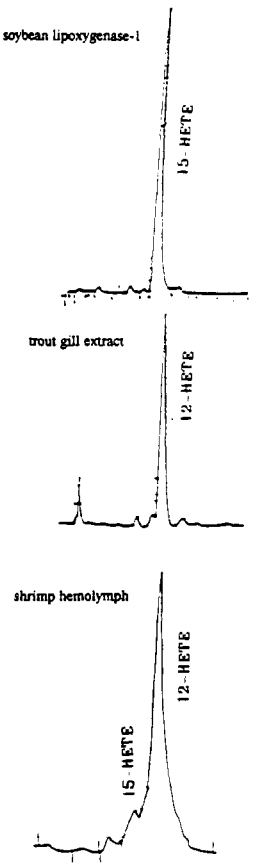


Figure 2. HPLC chromatograms of arachidonic acid treated with soybean lipoxygenase 1 (Sigma), trout gill extract (supplied by Dr. J. B. German), and shrimp hemolymph.

HPLC Chromatogram. Shrimp hemolymph was incubated with arachidonic acid. The reaction products were extracted and separated. The HPLC chromatogram is shown in Figure 2. A major peak with retention time of 13.92 min was identical to that of 12-HETE or the product of arachidonic acid treated with trout gill LOX. A minor peak was also found. Its retention time was 12.53 min, identical to that of 15-HETE. Reactions of arachidonic acid catalyzed with acidified hemolymph or heat-inactivated hemolymph or the hemolymph treated with 1 mM esculetin showed no HPLC peaks in Figure 2.

Autoxidation of arachidonic acid under bubbled oxygen resulted in a total of five peaks. They were identified as 5-, 9-, 11-, and 15-HETE and unseparated 8- and 12-HETE on the HPLC chromatograms (Van-

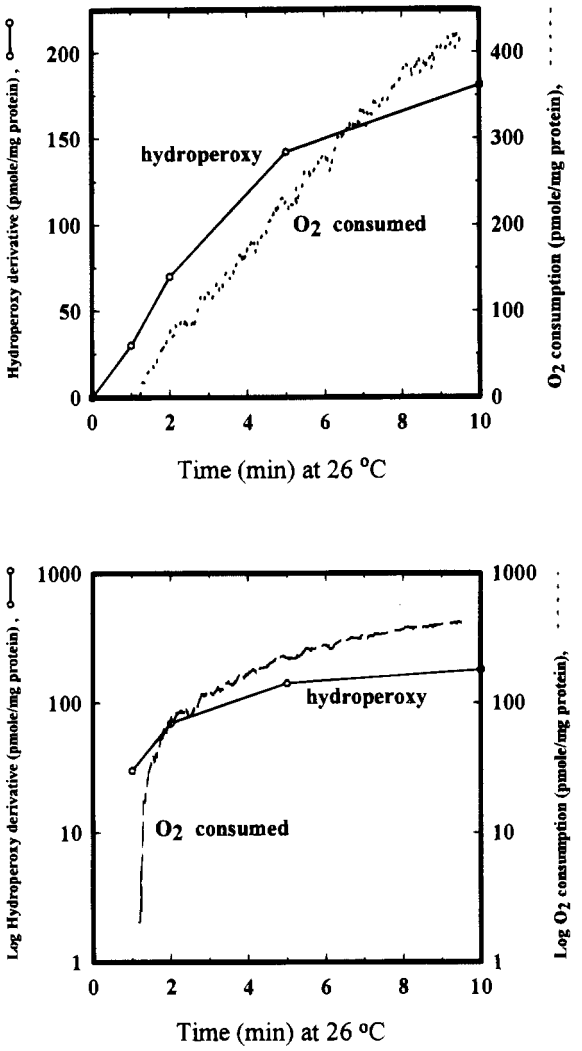


Figure 3. Oxygen consumption and hydroperoxy derivative production of arachidonic acid catalyzed by shrimp hemolymph.

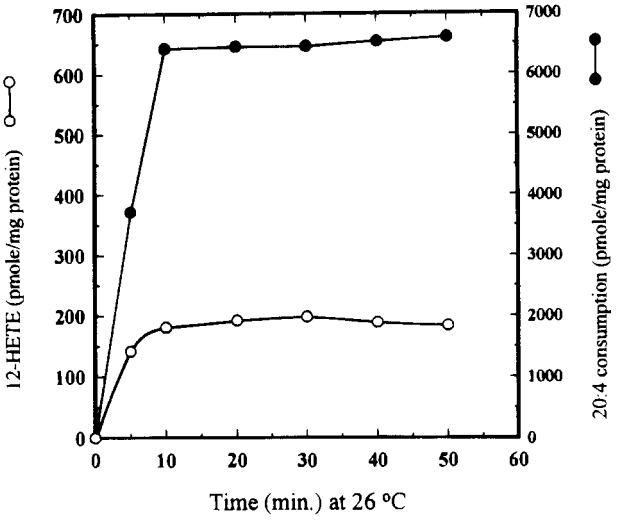
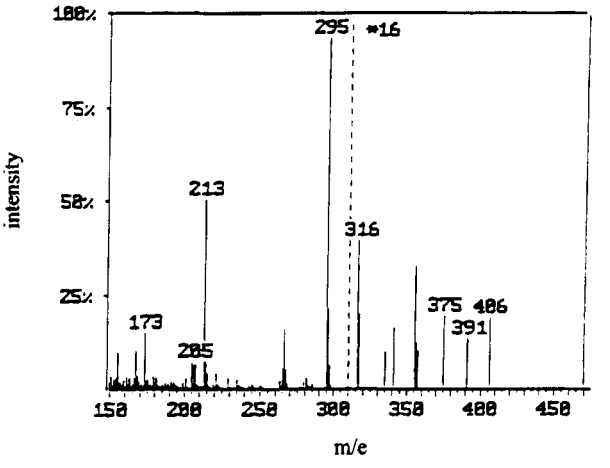
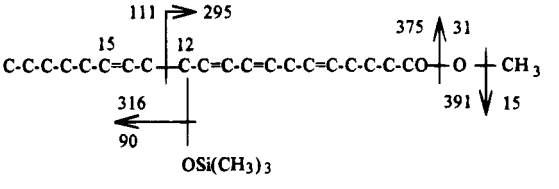


Figure 4. Time course of 12-HETE production and arachidonic acid consumption catalyzed by shrimp hemolymph at 26 °C.





trimethylsilyl 12-HETE methyl ester

Figure 5. Mass spectrum of the product of arachidonic acid treated with shrimp hemolymph.

rollins and Murphy, 1984). This was different from the HPLC pattern of a major peak of 12-HETE and two shoulders shown in Figure 2. Therefore, the 12-HETE and the two minor products were formed enzymatically but not from autoxidation. The major LOX activity in shrimp hemolymph was 12-LOX and one of the minor activities was from 15-LOX.

Oxygen consumption and 12-HETE production increased linearly with time during the first 5 min and reached a plateau after 10 min in hemolymph-catalyzed oxidation of arachidonic acid (Figure 3). The semilogarithmic plot of 12-HETE versus time indicated the 12-LOX-catalyzed reaction is not a first-order reaction. The amount of 12-HETE production was proportional to both the oxygen consumption and the arachidonic acid consumption for the first 10 min (Figure 4). Thus, the 12-HETE was derived from SH-LOX-catalyzed oxidation of arachidonic acid.

GC–MS Analysis. The mass spectrum of the major compound produced from arachidonic acid treated with shrimp hemolymph, methylated, and TMS derivatized (Figure 5) was found the same with that of the TMS derivative of 12-HETE.

According to fragmentation patterns of electron impact system, the base peak of *m/z* 295 was probably derived from cleavage of the molecular ion (M^+, 406) at carbon 12. The ion of *m/z* 391 was likely formed from scission of M^+ at CH_3 (M − 15), *m/z* 371 at OCH_3 (M − 31), *m/z* 316 at TMS (M − 90), and *m/z* 205 from cleavage of base peak at TMS (295 − 90). If the TMS was at carbon 15, i.e., hydroperoxidation of arachidonic acid at carbon 15, the base peak would have been *m/z* 225. In this case, the base peak was 295, not 225. Therefore, the TMS was at carbon 12, and the hydroperoxidation of arachidonic acid catalyzed by SH-LOX occurred at carbon 12.

Conclusion. On the basis of polarographic analysis, UV absorbance spectroscopy, HPLC separation, and GC–MS technique, 12-LOX activity was confirmed to exist in shrimp hemolymph. This is the first observation of LOX activity in marine crustacean.

Since LOXs were found in animal sanguineous tissue, such as platelet (Croset and Lagarde, 1985), leukocyte (Spector et al., 1988), neutrophil (Marcus et al., 1987), basophil (Donk et al., 1991), reticulocyte (Kunh and Brash, 1990), and gill of trout (German and Kinsella, 1986), mullet, tilapia, and crab (Kuo and Pan, unpublished results), the LOX activity in different animal tissues probably originated from blood. However, isolation and characterization of LOX from the blood of marine animals had not previously been reported in the literature. The present results confirmed the occurrence of 12-LOX activity in shrimp hemolymph. If LOX activity originated from blood, the reason no LOX activity was found in the gill of shrimp was probably the low LOX activity in the hemolymph of shrimp. Recently, a 12-LOX activity was identified in the platelet and gill of cultured gray mullet (*Mugil cephalus*), and its physiological significance is being studied (Chen and Pan, unpublished results).

The SH-LOX-catalyzed oxidation of polyunsaturated fatty acid forms hydroperoxide derivatives (Kuo and Pan, 1992), which may further undergo cleavage or other reactions to form aroma compounds of fresh seafood flavor (Josephson et al., 1984, 1987; Hsieh and Kinsella, 1989) or cooked shrimp flavor (Kuo and Pan, 1991). Thus, the enzymatic modification of seafood flavor via lipoxygenase may provide a new dimension for understanding the flavor formation mechanism and its application.

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