Preliminary Identification of Lipoxygenase in Algae (*Enteromorpha intestinalis*) for Aroma Formation

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Lipoxygenase (LOX) activity was identified in a green sea algae (*Enteromorpha intestinalis*). The oxygen consumption rate of sea algae-catalyzed oxidation of linoleic acid was 1.18 μmol/min·mg of protein. The catalytic rate was 911 times that of shrimp hemolymph LOX and 59 times that of trout gill LOX. Based on retention time in normal phase HPLC analysis and UV absorption spectrosocopy, hydroperoxidation of linoleic acid by sea algae LOX was at the C9 and C13 positions with a ratio of 1.97:1. The products of sea algae LOX treated with arachidonic acid were 12- (or 8-) and 15-HETE (hydroxyeicosatetraenoic acid) on the basis of retention time in reverse phase HPLC analysis. Thus, 12- (or 8-) and 15-LOX were the major LOX isozymes in the sea algae. The optimal pH of sea algae LOX was 7.8 and the optimal temperature was around 35 °C. Different treatments of sea algae generated volatile compounds contributing to flavor notes of clam, oyster, fresh apple, cucumber, mango, and algae identified with the GC-sniffing method. This is the first observation of LOX in marine algae with respect to its possible roles in seafood flavor formation.

Keywords: *Lipoxygenase; sea algae; seaweed; Enteromorpha intestinalis; flavor*

INTRODUCTION

Fresh fish flavor consists of volatile components similar to those of fruit or seaweed. Compounds contributing to fresh fish aroma are derived from highly unsaturated fatty acids via lipoxygenase (LOX) actions (Josephson et al., 1987). Biogensis of fresh fish volatiles via LOX was established (Josephson et al., 1984; Hsieh and Kinsella, 1989). The formation mechanism is similar to that of fruit flavor (Phillips and Galliard, 1978). The breakdown compounds of LOX-catalyzed fatty acids derivatives, i.e., C6, C8, and C9 aldehydes, ketones, and alcohols, are responsible for the characteristic odor of freshly harvested fish (Hsieh and Kinsella, 1989; Josephson and Lindsay, 1986; Pan and Kuo, 1994). Flavor notes of these compounds are green, melonlike, cucumberlike, and oysterlike, or seaweedlike (Josephson et al., 1985). 12- and 15-LOX are the two main isozymes for the formation of these compounds in aquatic animals (Hsieh and Kinsella, 1989; Zhang et al., 1992; Pan and Kuo, 1994). However, isolation and characterization of LOX from seaweed and its effect on aroma have not yet been reported in the literature.

Odors developed from polyunsaturated fatty acids pretreated with mullet gill LOX are green, grassy, and fresh fish-like (Tsai, 1994). These aromas can also be catalyzed by plant LOX (Josephson and Lindsay, 1986). We have screened a number of aquatic sources of LOX for seafood flavor formation or modification. Very high LOX activity was found in a marine green algae, *Enteromorpha intestinalis*. This algae is called "green nori" by the Japanese and is widely distributed around the world. It is used as food in Japan, China, and the Philippines. It is also used as flavorant in Hawaii (Bold and Wynne, 1985) and as fish bait in Taiwan.

The objective of the present work is to confirm LOX activity in green algae and to study its role in seafood flavor formation.

MATERIALS AND METHODS

Sea Algae. Sea algae, *Enteromorpha intestinalis*, was harvested in July 1994 from the Pacific coast of north Taiwan. The algae was maintained in cool sea water (ca. 10 °C) and transported to the laboratory immediately for analyses.

Lipid Extraction and Fatty Acid Analysis. Lipid was extracted from algae with chloroform:methanol (2:1, v/v) (Bligh and Dyer, 1959). Algal lipid, 0.1 g, was saponified and esterified with tetramethylammonium hydroxide ($TMAH/CH_3$-OH) (Fourie and Basson, 1990), and analyzed by gas chromatography (Shimadzu GC 14A, Kyoto, Japan) with a DB-23 column of 60 m × 0.252 mm (J&W Scientific, Folsom, CA). The injector was set at 250 °C. The oven temperature was programmed from 170 to 230 °C at a rate of 2 °C/min. The carrier gas was hydrogen at a flow rate of 1.0 mL/min.

Partial Purification of LOX. Fresh algae were homogenized (1:5, w/v) with 0.05 M phosphate buffer (pH 7.5) containing 1 mM glutathione (reduced form, Sigma, St. Louis, MO) at 4 °C. The homogenate was centrifuged at 20000*g* for 15 min to obtain a crude enzyme extract. Crystallized ammonium sulfate was added slowly into crude extract to 40−60% saturation. The supernatant was discarded and the precipitate was removed by centrifugation (20000*g*) for 10 min at 4 °C and dissolved in a minimum volume of phosphate buffer (0.05 M, pH 7.5). The partially purified LOX was prepared as the precipitate of the 40−60% saturation of $(NH_4)_2$-SO_4, followed by dialysis overnight against 200 volumes of 0.05 M phosphate buffer.

Polarographic Analysis. The oxygen consumption of crude algal extract catalyzed oxidation was measured by polarographic analysis (Kuo et al., 1994). Crude LOX, 0.1−0.5 mg, was equilibrated in 0.05 M potassium phosphate buffer (pH 7.5) in a thermostatic incubation cell (20 mm × 68 mm) at 26 °C. Reaction was initiated by the addition of linoleic acid (100 μM), and the oxygen concentration was measured with a biological oxygen monitor (YSI 5300, Yellow Springs, OH) equipped with a YSI Clark oxygen probe. A data

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Table 1. Total Fatty Acid Composition of Sea Algae (*Enteromorpha intestinalis*)

fatty acids	%	fatty acids	%
10:0	0.77	18:2	12.99
14:0	4.60	18:3	27.07
14:1	0.71	20:0	0.28
16:0	38.47	20:3	0.86
16:1	5.99	20:4	1.72
18:0	0.60	24:0	0.87
18:1	5.07		

acquisition system (Notebook for IBM PC computer) was used to record the oxygen concentration.

Assay of LOX Activity. The activity of algal LOX was determined by measuring the increase of absorbance at 234 nm (Surrey, 1964; Chen and Whitaker, 1986) with a Hitachi U-2000 spectrophotometer (Tokyo, Japan). Algal LOX extract, 0.1 mL, was diluted with 0.9 mL of 0.05 M phosphate buffer (pH 7.5) containing 0.01% Tween-20. The mixture was incubated with linoleic acid (100 mM) at 26 °C for 5 min. LOX activity was determined by the increase in fatty acid hydroperoxide estimated by using a molar absorptivity of 25 000 L/mol·cm at 234 nm (Vick, 1991).

HPLC Chromatography. The partially purified algal LOX was incubated with linoleic acid (100 μM) or arachidonic acid (100 μM) at 26 °C for 5 min. The reaction products were extracted with ethyl acetate and then reduced with $NaBH_4$ and methylated with diazomethane (Ayorinde et al., 1989). The resulting compounds were separated with a solid phase extraction column (J&W Scientific, Folsom, CA) and then subjected to HPLC analysis (German and Berger, 1990).

Normal phase high pressure liquid chromatographic analyses were performed on a Chirex (R) PGLY 3,5 and DNB column (25 cm × 4.6 mm, Phenomenex, Torrance, CA) equipped with a pump (Waters, Model 510, Milford, MA) and UV detector (Waters, Model 490E) monitored at 234 nm. The hydroperoxy derivatives were eluted isocratically with a solvent system of hexane/dichloromethane/ethanol (50:15:1, v/v/v). The LOX catalyzed products, 18:2-9OOH (9-HPODE, hydroperoxyoctadecadienoic acid) and 18:2-13OOH (13-HPODE) were confirmed in comparison to authentic standards (Caman, Ann Arbor, MI).

Reverse phase high pressure liquid chromatographic analyses were performed on an ODS2 column (15 cm × 4.6 mm, 5 mm particles) equipped with a Waters pump and UV detector system mentioned above. The HETE compounds were eluted 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. The LOX catalyzed products were confirmed in comparison to authentic standards of 5-, 12-, and 15-HETE (Caman).

Headspace Analysis and GC-Sniffing. The volatiles of crude algal extract (2 L) in 0.05 M phosphate buffer (pH 7.5) were purged with high purity nitrogen onto a porous polymer Tenax-TA (Chrompack, Middelburg, Netherlands) at 26 °C for 2 h at a flow rate of 60 mL/min. The total trapped volatile compounds were eluted with pentane/ether (1:1, v/v). The eluent was dried over anhydrous sodium sulfate and concentrated to minimal volume by spinning band distillation. The aroma concentrates were analyzed by using a Shimadzu GC-14A (Kyoto, Japan) equipped with a CP-WAX column, 50 m × 0.53 mm (Chrompack, Middelburg, Netherlands), in the splitless mode. On the outlet of the column, an adjustable splitter (SGE, Austin, TX) was connected to an olfactory device (SGE, Austin, TX) and FID with a ratio of flow rate of 8:1. The injector was set at 250 °C and the injection volume was 1 μL. The oven temperature was programmed from 50 to 200 °C at a rate of 1.5 °C/min. The carrier gas was hydrogen at a flow rate of 1.2 mL/min. Odor evaluation of the volatile components was performed by three panelists on the sniffing port of the olfactory device.

Treatments of Algae for Aroma Formation. Prior to the GC-sniffing test, algae were treated in four ways. (1) Algal extract in phosphate buffer containing glutathione was microwaved for 2 min in a home-use oven. (2) The algal extract was reacted with linoleic acid (400 μM) at 26 °C for 2 h. (3) Alga was dried in an oven at 150 °C for 2 h. (4) Alga was pickled in 5% NaCl (1:10, w/v) at 10 °C for 24 h. Odor developed from these four treatments was sniffed by using the olfactory device by three panelists familar with aroma characteristics of seafood flavor.

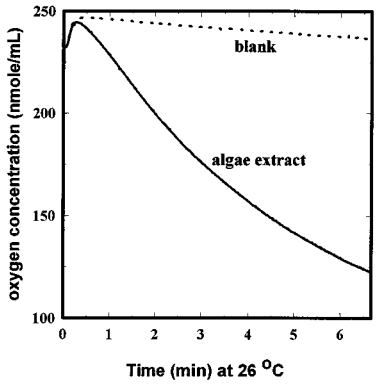

Figure 1. Progress curve of oxygen concentration of crude algal extract-catalyzed oxidation of linoleic acid (100 μM). Crude algal extract, 0.1−0.5 mg, was incubated in 0.05 M phosphate buffer (pH 7.5) at 26 °C for 5 min. Oxygen consumption was measured by polarographic analysis.

RESULT AND DISCUSSION

Lipid Content and Fatty Acid Composition of Sea Algae. The amount of algal lipid was 7.98 ± 0.72% on a dry basis. Palmitic (16:0), linolenic (18:3), and linoleic acids (18:2) were the predominant fatty acids of algal lipid, consisting of 38.47, 27.07, and 12.99% of total fatty acids, respectively, on the basis of area percent in gas chromatogram (Table 1). The amount of arachidonic acid (20:4) was 1.72%, while eicosapentaenoic (20:5) and docosahexaenoic acid (22:6) were not found in algal lipid (Table 1). The fatty acid composition of sea algae was similar to that of vegetable oil and was different from that of fish oil.

Isozymes of Sea Algal Lipoxygenase. LOX activity was identified in the sea algae on the basis of following observations.

Oxygen Consumption. Crude algal extract was assayed with linoleic acid. The oxygen consumption rate of the algal extract-catalyzed oxidation of linoleic acid was 1.18 μmol/min·mg of protein (Figure 1). This was very rapid catalytic rate as compared to that of other LOX, being half that of soybean LOX (1000 units), 911 times that of the shrimp hemolymph LOX (Kuo et al., 1994), and 59 times that of the trout gill LOX (Hsieh et al., 1988).

UV Absorption Spectrum. The products of linoleic acid treated with partially purified algal LOX separated by solid phase extraction column showed an absorption peak at 234 nm (Figure 2), indicating a conjugated diene system in the reaction product (Takagi et al., 1987). This spectrum was similar to those of the products from linoleic acid treated with soybean LOX. A hydroperoxide with conjugated diene structure was formed from linoleic acid treated with algal extract.

HPLC Chromatography. The partially purified algal LOX was incubated with linoleic acid. The reaction products were extracted and separated. The normal phase HPLC chromatogram showed two peaks with retention times of 12.29 and 13.08 min (Figure 3) being identical to those of the reduced 18:2-13OOH or 13-HODE (hydroxyoctadecadienoic acid) and the reduced 18:2-9OOH or 9-HODE. The ratio of these two isomers

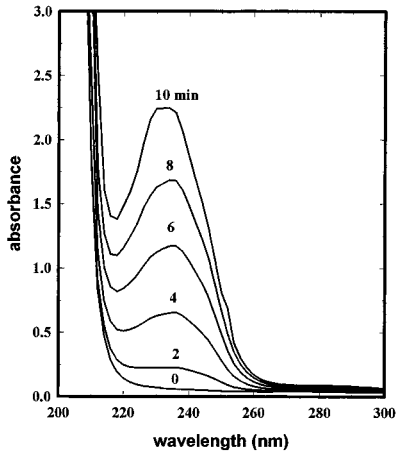

Figure 2. UV absorption spectrum of the product of linoleic acid treated with partially purified algal LOX. The partially purified algal LOX was incubated with linoleic acid (100 μM) up to 10 min at 26 °C. The products were separated with a solid phase extraction column (Si) and analyzed with UV spectrophotometer.

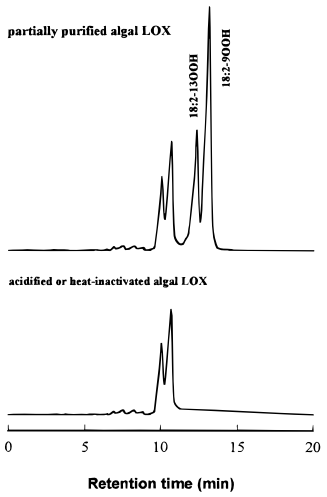

Figure 3. Normal phase HPLC chromatogram of linoleic acid treated with partially purified algal LOX.

was 1:1.97. These two peaks disappeared upon reactions of linoleic acid catalyzed with acidified or heat-inactivated algal LOX (Figure 3). Thus, algal LOX catalyzed hydroperoxidation of linoleic acid at C-9 and C-13 positions. Hydroperoxidation of 18:2 via LOXs of tea leaves (Sekiya et al., 1984), tomato (Galliard and Matthew, 1977), white clove, and watermelon (Sekiya et al., 1983) were predominant at the C-13 position, while those of rice (Yamamoto et al., 1980), corn (Gardener, 1970), cucumber (Phillips and Galliard, 1978), and potato (Galliard et al., 1974) were at the C-9 position. The catalytic properties of algal LOX seem to differ from those that exist in plants by having both LOX isozymes with an activity ratio of 1.97:1. The partially purified algal LOX was incubated with arachidonic acid. The reaction products were extracted and separated. Two peaks were found in the reverse phase HPLC chromatogram (Figure 4). The retention properties of these two peaks were identical to those of 15- and 8- or 12-HETE with a ratio of 1:2.8. Since 12-LOX seems to be the predominant isozyme in shrimp (Kuo and Pan, 1992; Kuo et al., 1994), grey mullet (Hsu and Pan, 1996), and other aquatic animals (German and Kinsella, 1986), 15- and 12-LOX were likely to be the major LOX isozymes in the sea algae.

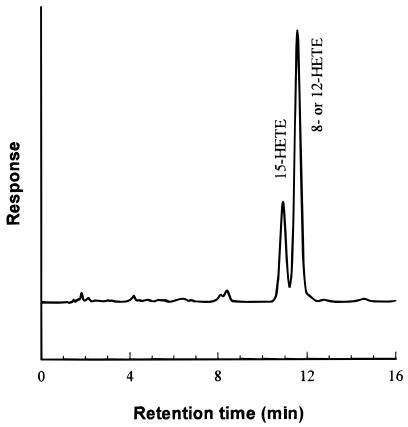

Figure 4. Reverse phase HPLC chromatogram of arachidonic acid treated with partially purified algal LOX.

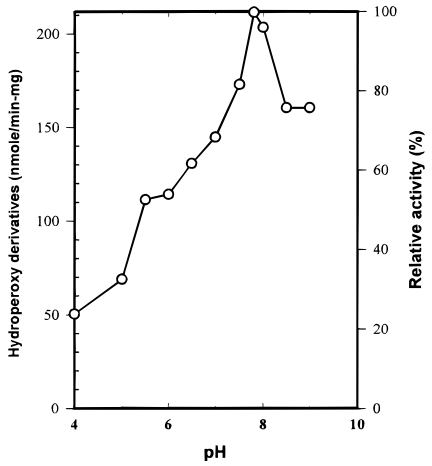

Figure 5. pH profile of algal LOX using linoleic acid as sustrate at 26 °C for 5 min. The buffer system was acetate buffer in pH 4, 5, 5.5, and 6; phosphate buffer in pH 6.5, 7, 7.5, and 7.8; tris buffer in pH 8 and 8.5, and borate buffer in pH 9.

pH Optimum of Sea Algal LOX. The optimal pH of partially purified algal LOX was 7.8 (Figure 5).The algal LOX demostrated 80% activity at pH 9.0 and over 50% activity at pH 5.5. The optimal pH of soybean LOX-1 was 9.5, soybean LOX-2, pH 6.6 (Gardner, 1991), kiwifruit LOX, pH 6.2 (Boyes et al., 1992), fresh water algal LOX, pH 8.8 (Beneytout et al., 1989), and human platelet (Wallach and Brown, 1981) and trout gill LOX (German and Kinsella, 1986), around pH 7.5−8.5. The optimal pH of algal LOX seems to be similar to those of the animals.

Temperature Optimum of Sea Algal LOX. The optimal temperature of partially purified algal LOX was around 35 °C (Figure 6). An abrupt decline in activity occurred above 50 °C, while 50% of maximal activity was retained at 26 °C. The optimal temperature of trout gill LOX was around 20 °C (Hsieh et al., 1988), and that of shrimp hemolymph LOX was at 38 °C (Kuo and Pan, 1992). The trout gill LOX exhibited over 90% of maximal activity between 10 and 30 °C and 60% activity at 0 °C (Hsieh et al., 1988), while shrimp hemolymph LOX showed 84% activity at 26 °C (Kuo and Pan, 1992). The temperature profile of algal LOX activity seems to differ from those in shrimp hemolymph and trout gill LOX.

Effects of Sea Algal LOX on Aroma. Algal extract cooked with microwaves for 2 min developed a clamlike odor. Drying algae at 150 °C for 2 h developed a typical algal aroma. Algae pickled in NaCl solution formed

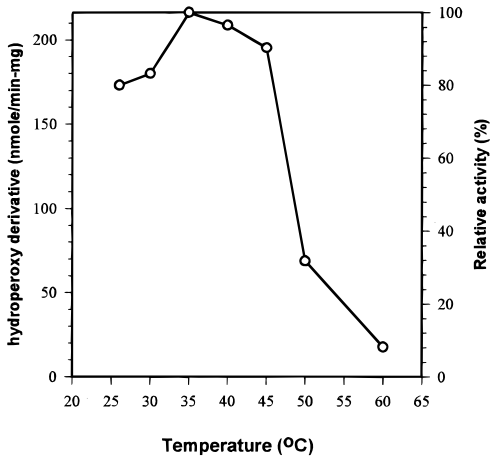

Figure 6. Temperature profile of algal LOX in 0.05 M potassium phosphate buffer (pH 7.5) using linoleic acid as substrate.

Table 2. Odors Developed from Different Treatments of Sea Algae

treatment	odor
microwave-cooking[a]	clamlike
drying[b]	algaelike
pickling[c]	oysterlike
linoleic acid[d]	sweet, melonlike

[a] Algal extract in phosphate buffer containing glutathione was microwaved in a home-use oven for 2 min. [b] Algae were dried at 150 °C for 2 h. [c] Algae were pickled in 5% NaCl at 10 °C for 24 h. [d] Algal extract was reacted with 400 μM linoleic acid at 26 °C for 2 h.

oysterlike aroma, while algae extract treated with 18:2 produced sweet and cucumber-like aroma (Table 2). The characteristic aroma compounds of oyster were mainly C-9 aldehydes or alcohols derived from LOX reaction (Josephson et al., 1985). The flavor compounds of cucumber were formed from hydroperoxide of 18:2 via LOX, followed by hydroperoxide lyase (HPLS) reactions (Hatanaka et al., 1975; Wardale et al., 1978). The HPLS activity also seemed to be present in the algal extract at pH 6.5, as hydroperoxide of 18:2 was used as substrate (data not shown). The characteristic compounds of the oysterlike or cucumber-like aroma were probably derived from reactions of algal LOX and HPLS on linoleic acid. Headspace analysis and GC-sniffing of algal extract treated with linoleic acid were shown in Figure 7. Fresh applelike, green, cucumber, mango, and algal aromas were found in the volatile concentrate of algal extract treated with linoleic acid. The overall flavor note was sweet cucumber-like. These volatile components are being identified with GC-MS.

CONCLUSION

Two lipoxygenase isozymes were found in sea algae. They catalyze peroxidation at the 9- and 13-positions on linoleic acid with a ratio of 1.97:1 and at the 12- and 15-positions on arachidonic acid with a ratio of 2.8:1. This pattern differs from those found in plants such as soybean (Gardner, 1991), pea (Chen and Whitaker, 1986), and cucumber (Gardner, 1991). The latter generally have only one predominant LOX isozyme, either the 9- or the 13-LOX on linoleic acid. Linoleic acid was more susceptible to algae LOX catalysis than arachidonic acid. The fatty acid profile of sea algal lipid resembles that of vegetable oils by having linolenic acid (27.07%) and linoleic acid (12.99% of total fatty acids)

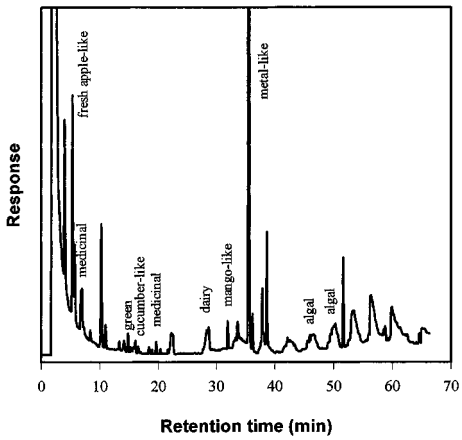

Figure 7. GC chromatogram and odor description of headspace volatile compounds of algal extract reacted with linoleic acid. The algal extract was reacted with linoleic acid (400 μM) and purged with N_2 onto a Tenax-TA column at 26 °C for 2 h at a flow rate of 60 mL/min. The trapped volatile compounds were eluted with pentane/ether 1:1 v/v and concentrated by distillation, then measured by the GC-sniffing method.

as the predominant polyunsaturated fatty acids and no EPA (20:5) or DHA (22:6) present. Flavors generated from different treatments of algae are derived from the fatty acids, C18:3, C18:2, and C20:4, present in algal lipid via one of the two LOX isozyme catalyses, possibly then followed by hydroperoxide lyase (HPLS) reactions (Hatanaka et al., 1975, Wardale et al., 1978). The HPLS activity was present in this green algae (data not shown) and was also found in many fruits (Phillips and Galliard, 1978; Wardale et al., 1978; Sekiya et al., 1983), fresh water algae (Andrianarison et al., 1989), and some fish (Josephson and Lindsay, 1986). However, the role of HPLS is not clear in the marine kingdom. Thus, the relationship between LOX and HPLS activity in this algae and their effects on lipid metabolism and flavor formation need further investigation. The volatile compounds contributing to different flavor notes resulting from different treatments of algae are being identified by GC-mass studies.

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