Purification, Substrate Specificity, and Products of a Ca^{2+}-Stimulating Lipoxygenase from Sea Algae (*Ulva lactuca)*

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A calcium-stimulated lipoxygenase (LOX) was isolated and purified 103-fold from sea algae (*Ulva lactuca)* using 40−55% saturation of ammonium sulfate fractionation, MacroPrep-Q ion exchange, and gel filtration on Sephacryl S-300. When this partially purified algal LOX was run on 4−20% polyacrylamide gel electrophoresis (PAGE), two bands with LOX activity were found using activity staining. The apparent molecular masses were estimated as 41 and 116 kDa by PAGE. The LOX activities from the sea algae were stimulated by Ca^{2+} at 0.2 mM to a maximal activity of about 8.8-fold. The optimal pH of the algal LOX was 7.5, and optimal temperature was 33 °C. At pH ranging from 5.5 to 9.0 and temperature below 40 °C, the algal LOX was stable. The algal LOX showed the highest reactivity toward 18:2 fatty acid followed by 20:4, 20:5, 22:6, and 18:3. Linoleic acid and its esters were peroxidized by algal LOX in the order linoleic acid ≫ monolinolein > dilinolein > methyl linoleate, but no activity was observed on trilinolein. On the basis of retention time in normal phase HPLC, the products of 18:2 reacting with algal LOX were 9- and 13-hydroxyoctadecadienoic acid at a ratio of 86:14; the product from 18:3 was 9-hydroxyoctadecatrienoic acid. Products derived from 20:4 were 12- and 15-hydroxyeicosatetraenoic acid at a ratio of 90:10 using reversed phase HPLC. Fatty acids of 20:5 and 22:6 yielded 12-hydroxyeicosapentaenoic acid and 14-hydroxydocosahexaenoic acid, respectively. It seems that *n*-9 LOX is the dominant LOX and *n*-6 LOX is the minor LOX in sea algae.

Keywords: *Lipoxygenase; sea algae; Ulva lactuca; purification; calcium stimulation*

INTRODUCTION

Lipoxygenase (LOX) (linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a dioxygenase that catalyzes the oxygenation of poylunsaturated fatty acids containing a *cis,cis*-1,4-pentadiene system to hydroperoxides. It is widely distributed in the plant and animal kingdoms. This enzyme plays important roles in flavor biogenesis in ripening fruits, in off-flavor production in legumes, in pigment degradation, in secretion of plant growth hormone, i.e. jasmonic acid, and in wound healing and disease resistance of leaves (Gardner, 1991).

In the aquatic plant kingdom, LOXs were identified in micro green algae (Zimmerman and Vick, 1973), fresh water blue green algae, *Oscillatoria* (Beneytout et al., 1989), and sea algae, *Enteromorpha intestinalis* (Kuo et al., 1996a,b). They were important in the production of antimicrobial substances of micro algae (Zimmerman and Vick, 1973; Beneytout et al., 1989) and flavor formation of seaweed (Kuo et al., 1996a,b).

Compounds contributing to seafood flavor were formed from polyunsaturated fatty acids via LOX action such as in shrimp. Thus, we screened a number of aquatic sources of LOX (Kuo and Pan, 1991, 1992; Pan and Kuo, 1994; Kuo et al., 1994, 1996a,b). Green sea algae have very high LOX activity. Volatile compounds contributing to flavor notes of clam, oyster, fresh apple, cucumber, melon, mango and algae were generated from different treatments of the sea algae (Kuo et al., 1996a,b). An attempt was made to purify and characterize the sea algal LOX. However, LOX activity was lost during purification (Kuo et al., 1996a,b). Therefore, the objective of the present work is to enhance its stability to purify and characterize the algal LOX.

Studies of soybean LOX-2 (Dreesen et al., 1982) and LOX from mullet gill (Hsu, 1996; Hsu and Pan, 1996), human leukocytes (Rouzer and Samuelsson, 1985), rat basophilic leukemia cell (Hogaboom et al., 1986), and human term placenta (Joseph et al., 1993) showed LOX activities were all stimulated by the presence of Ca^{2+}. In this paper, we also found that the LOX activity of sea algae was stimulated by the presence of Ca^{2+} and the LOX activity remained detectable during purification.

MATERIALS AND METHODS

Sea Algae. Sea algae, *Ulva lactuca*, were harvested in June 1995 from the Pacific coast of northern Taiwan. The algae were then kept in seawater at ca. 20 °C and transported to the laboratory immediately.

Purification of Algal LOX. All steps were performed at temperature below 4 °C. All buffers used were added with 0.2 mM calcium chloride. Fresh algae were homogenized with 25 mM phosphate buffer (pH 7.5) containing 1 mM glutathione (reduced form; Sigma, St. Louis, MO) in a ratio of 1:6, w/v. The homogenate was centrifuged at 20000*g* for 20 min to obtain a crude enzyme extract. Two liters of crude enzyme extract was fractionated by ammonium sulfate precipitation at 40−55% saturation and centrifuged at 20000*g* for 15 min. The pellet was dissolved in a minimal volume of 25 mM Tris

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Table 1. Purification of Lipoxygenase from Sea Algae (*U. lactuca*)

stage	total activity (μmol/min)	total protein (mg)	spec activity (μmol mg^{-1} min^{-1})	recovery (%)	purifn (fold)
crude extract	97.60	521.4	0.19	100	1.00
40−55% $(NH_4)SO_4$	64.46	149.2	0.43	66.04	2.31
MacroPrep-Q	37.91	8.2	4.64	38.84	24.79
Sephacryl S-300	32.25	1.7	20.41	33.04	102.66

buffer (pH 7.5) and dialyzed overnight at 4 °C against 200 volumes of the same buffer.

The dialysate was applied to a MacroPrep-Q (Bio-Rad, Hercules, CA) column (4.4 × 15 cm) equilibrated with 25 mM Tris buffer (pH 7.5) and eluted with a linear gradient of 0−0.4 M NaCl at a flow rate of 0.8 mL/min. The active fractions were collected and concentrated using ultrafiltration through a membrane with pores of nominal cutoff at 10 000 Da.

The fraction from MacroPrep-Q separation was then subjected to a Sephacryl S-300 (Pharmacia, Uppsala, Sweden) column (1.6 × 95 cm) equilibrated with 25 mM Tris buffer (pH 7.5) at a flow of 0.25 mL/min. The enzyme was eluted with the same buffer. The active fraction was collected, concentrated, and used for subsequent studies.

Polyacrylamide Gel Electrophoresis (PAGE) and Identification of LOX. PAGE of partially purified algal LOX was performed on 4−20% polyacrylamide gel using a gradient mixer (Pharmacia) and a Bio-Rad mini-PAGE instrument. The gels were stained for protein with silver nitrate (Hochstrasser et al., 1988), and a low molecular mass electrophoresis calibration kit including phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) (Pharmacia) was used as standard proteins. The LOX activity on polyacrylamide gel was identified by activity staining of either I_2-starch method (Guss et al., 1967) or a fast blue staining (Delumen and Kazeniac, 1976). LOX activity was visualized as a brown band in the I_2-starch method and as a reddish brown band in fast blue staining.

Oxygen Uptake Analysis. The oxygen consumption of crude algal extract catalyzed oxidation was measured using 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 acquisition system (Notebook for IBM PC computer) was used to record the oxygen concentration.

Assay of LOX Activity. The algal LOX was incubated with fatty acids (100 μM) at 26 °C for 5 min. 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 0.001% Tween 20 and 0.2 mM Ca^{2+}. The mixture was incubated with linoleic acid (100 μM) at 26 °C for 5 min. LOX activity was determined by the increase in fatty acid hydroperoxide using a molar absorptivity of 25 000 L mol^{-1} cm^{-1} at 234 nm for estimatation (Vick, 1991).

HPLC Chromatography. The LOX reaction products were extracted with ethyl acetate and then reduced with $NaBH_4$ and methylated with diazomethane. The resulting compounds were separated with a solid phase extraction column (J&W Scientific, Folsom, CA) and then subjected to HPLC analysis (Kuo et al., 1996a,b).

Normal phase high-pressure liquid chromatographic analyses were performed on a Bondclone silica column (30 cm × 3.9 mm, 10 μm; Phenomenex, Torrance, CA) equipped with a pump (Waters, Model 510, Milford, MA) and UV detector (Waters, Model 486) monitored at 234 nm. The reduced hydroperoxy derivatives were eluted isocratically with a solvent system of hexane/ethanol/acetic acid (98:1.9:0.1, v/v/v) at a flow rate of 0.8 mL/min. The LOX-catalyzed products, 18:2−9OOH (9-HPODE, hydroperoxyoctadecadienoic acid), 18:2−13OOH (13-HPODE), and 18:3−9OOH (9-HOTE, hydroperoxyoctadecatrienoic acid), were confirmed in comparison to authentic standards (Caman, Ann Arbor, MI).

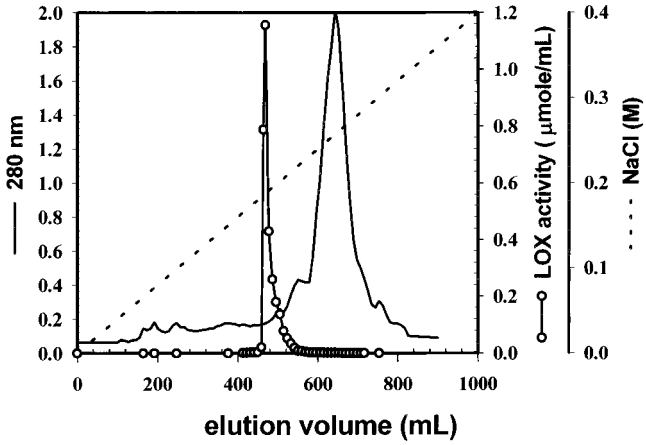

Figure 1. Elution profile of *U. lactuca* LOX on Macro Prep-Q column (4.4 × 15 cm). Column was equilibrated with 25 mM Tris buffer (pH 7.5) containing 0.2 mM Ca^{2+} and eluted with a linear gradient of 0−0.4 M NaCl at a flow rate of 0.8 mL/min. Fractions of 4.6 mL were collected and assayed for protein as absorbance at 280 nm and for LOX activity.

Reversed phase high-pressure liquid chromatographic analyses were performed on an ODS2 column (25 cm × 4.6 mm, 5 μm particles) equipped with a Waters pump and UV detector system as mentioned above. The hydroperoxy 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-, 8-, 11-, 12-, and 15-HETE (hydroxyeicosatetraenoic acid), 12- and 15-HEPE (hydroxyeicosapentaenoic acid), and 14-HDHE (hydroxydocosahexaenoic acid).

RESULTS AND DISCUSSION

Purification and Molecular Mass of LOX. The purification of algal LOX was accomplished by ammonium sulfate fractionation, MacroPrep-Q ion exchange, and gel filtration on Sephacryl S-300. The purification procedure and recovery are summarized in Table 1. LOX activity was obtained from 40 to 55% of $(NH_4)_2SO_4$ saturation, resulting in a 2.3-fold purification with 66% recovery. Seventy-one percent of total protein and chlorophyll were removed, while 34% of LOX activity was lost in this step. This phenomenon differed from that studied in fresh water algae, of which the LOX activity was in the fraction of below 30% of $(NH_4)_2SO_4$ saturation, while the recovery of LOX activity in this fraction was only 7.35% (Beneytout et al., 1989).

The dialysate of the 40−55% saturation of $(NH_4)_2SO_4$ was absorbed on a MacroPrep-Q column, and the elution profile of algal LOX with NaCl is shown in Figure 1. Fractions eluting between 240 and 280 mL appeared reddish in color with no LOX activity. Fractions eluting between 600 and 700 mL contained the highest amount of protein (Figure 1) with still no LOX activity. LOX activity was eluted between 470 and 520 mL or 0.2 M

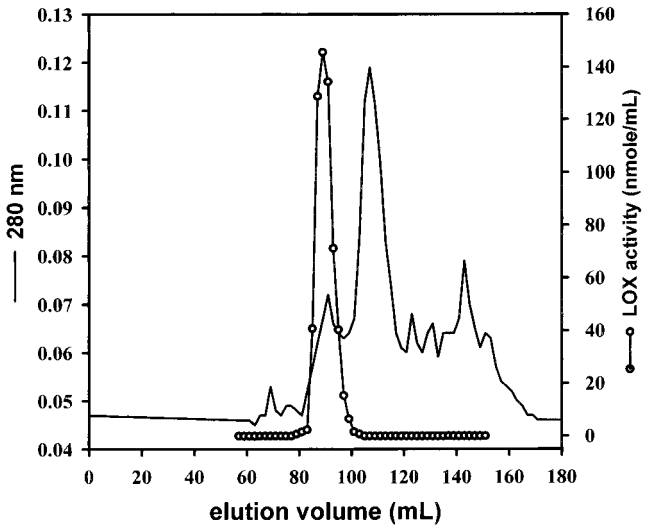

Figure 2. Elution profile of *U. lactuca* LOX on Sephacryl S-300 column (1.6 × 95 cm). Column was equilibrated with 25 mM Tris buffer (pH 7.5) containing 0.2 mM Ca^{2+} and eluted with the same buffer at a flow rate of 0.25 mL/min. Fractions of 1 mL were collected and assayed for protein as absorbance at 280 nm and for LOX activity.

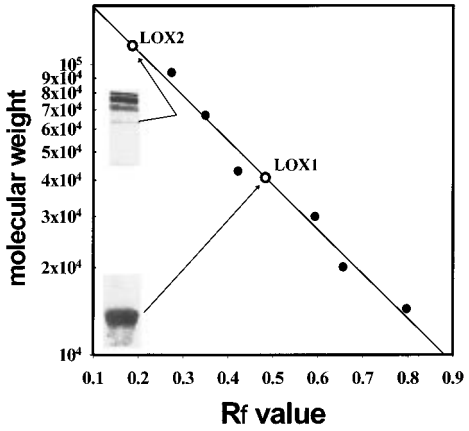

Figure 3. 4−20% polyacrylamide gel electropherogram of partially purified algal LOX from *U. lactuca*.

NaCl. The recovery after MacroPrep-Q ion exchange was 38.8%, with 24.8-fold purification (Table 1). The majority of protein was removed from MacroPrep-Q ion exchange, while 27.2% of LOX activity was also lost in this step.

The active fraction from MacroPrep-Q column separation was pooled onto a Sephacryl S-300 column. The elution profile is shown in Figure 2. LOX activity was eluted between 85 and 95 mL. The recovery of LOX activity was 33% with 102.7-fold purification (Table 1). The active fraction from Sephacryl S-300 gel filtration was collected, dialyzed, and concentrated. The purity was examined by 4−20% polyacrylamide gel electrophoresis (PAGE). The electropherogram showed six bands (Figure 3). Activity localization using I_2-starch method and fast blue staining found a band with apparent molecular mass (MM) 41 kDa as the major one and a band of 116 kDa as the minor one having LOX activity. The MM of LOX from fresh water algae was 124 kDa (Beneytout et al., 1989) and that from microalgae, 182 kDa (Vick and Zimmerman, 1987); the MM of the three isozymes from soybean, 98 kDa; the two isozymes from pea, both 95 kDa; the two isozymes from potato tuber, 35 and 85 kDa (Reddanna, et al., 1990), and LOX from trout gill, 70 kDa (German and Creveling, 1990). The MM of the major band of sea algal LOX

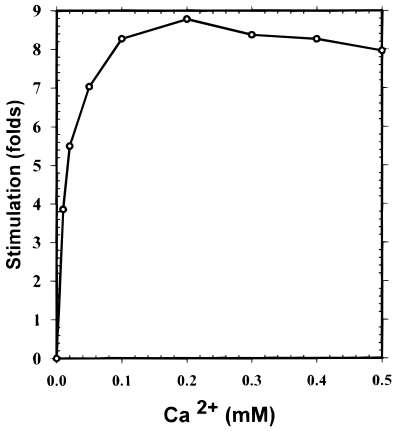

Figure 4. Stimulatory effects of Ca^{2+} on the algal LOX using linoleic acid as substrate.

was smaller than that of the fresh water algae and microalgae but larger than those found in land plants and fish gill.

When the mass of LOX was predicted on the fractions eluted from S-300 gel filtration with Tris buffer containing 0.2 mM Ca^{2+}, the MM was about 400 kDa (data not shown), larger than those of 41 and 116 kDa estimated later by PAGE. When Sephadex G-200 was used as the separation medium, the activity of sea algal LOX was eluted almost with the void volume (data not shown). It seemed that the two LOX isozymes of sea algae cross-linked in the presence of Ca^{2+} during gel filtration. To eliminate the cross-linking effect of Ca^{2+}, the enzyme preparation was dialyzed with several changes against 25 mM Tris buffer (pH 7.5) and eluted by the same buffer without Ca^{2+}. The elution profile was the same as that by the elution buffer containing Ca^{2+} (data not shown). It seemed that the intermolecular cross-link between two LOXs was not removed or that LOX of 41 kDa was bound to another protein. The fraction showing lower retention time in gel filtration was probably the cross-linked of the two LOXs or the LOX highly hydrophobic (Vick and Zimmerman, 1987).

Stimulatory Effect of Ca^{2+} on Algal LOX. The partially purified algal LOX from Sephacryl S-300 gel filtration was dialyzed against 1000 volumes of Tris buffer to remove calcium ion from LOX. Calcium ion at concentration up to 0.5 mM markedly stimulated LOX activity (Figure 4). Maximal stimulation of LOX activity to about 8.8-fold was observed at 0.2 mM Ca^{2+}. This stimulating effect of Ca^{2+} on algal LOX was similar to that on soybean LOX-2, except the latter was only 2-fold (Restrepo et al., 1973; Dreesen et al., 1982). Algal LOX was more sensitive to Ca^{2+} stimulation than the soybean LOX-2. The mechanism of Ca^{2+} activation to plant LOX has no confirmable explanation. Ca^{2+} probably unmasks the catalytic site or changes the protein conformation of LOX (Yamamoto et al., 1970). It certainly needs further investigation.

When the crude algal extract was dialyzed against 1000 volumes of Tris buffer and assayed for LOX activity with spectrophotometric method, LOX activity was not detected in the absence of calcium ion. The response of LOX activity became detectable after 0.2 mM Ca^{2+} was added to the dialysate (Table 2). A similar result was also found in the 40−55% of $(NH_4)_2SO_4$ saturation fraction of algal extract (data not shown). This is the reason the algal LOX activity disappeared during purification in the absence of Ca^{2+} (Kuo et al., 1996a,b).

Table 2. Ca^{2+} Activation of LOX after Different Treatments and Assayed with Linoleic Acid

treatment	LOX activity[a] (ΔA_{234} min^{-1} mg^{-1})
algal extract	4.05
dialyzed algal extract[b]	undetectable[c]
$CaCl_2$-treated extract[d]	3.88

[a] Algal extract, 0.1 mL, was incubated in 0.9 mL of 0.05 M potassium phosphate buffer (pH 7.5)with linoleic acid (100 μM) at 26 °C for 10 min. LOX activity was assayed with spectrophotometer at 234 nm. [b] Dialysis at 4 °C for 24 h using frequent changes of 25 mM Tris buffer (pH 7.5). [c] LOX activity of algal extract up to 0.5 mL was not detected using spectrophotometric method. Enzyme extract used at ≥0.5 mL resulted in blank overscale. [d] Dialyzed buffer was added with 0.2 mM $CaCl_2$.

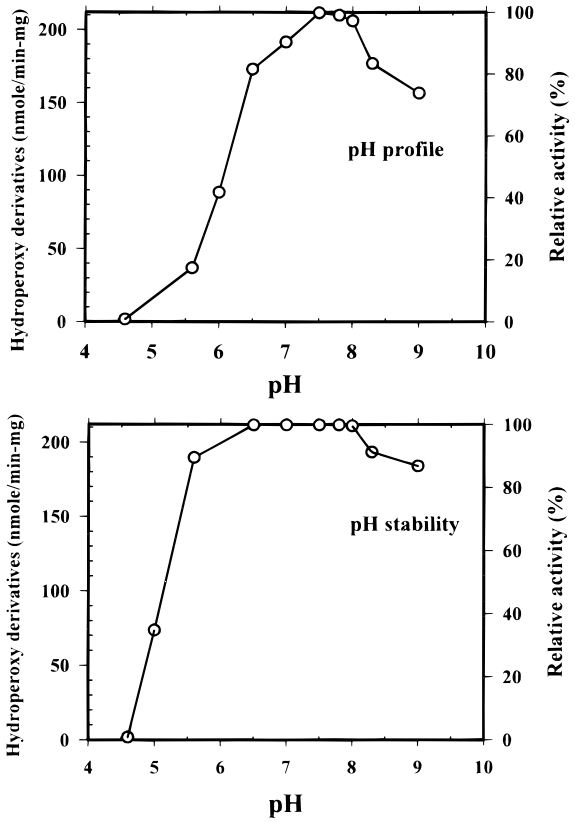

Figure 5. pH profile and pH stability of algal LOX activity in the presence of 0.2 mM Ca^{2+}. Linoleic acid was used as substrate and reacted at 26 °C for 5 min. The buffer systems for determining pH profile included acetate buffer ranging from pH 4.6 to 6, phosphate buffer ranging from pH 6.5 to 7.8, Tris buffer ranging from pH 8 to 8.3, and borate buffer at pH 9. The pH stability was performed by preincubating algal LOX at different pH values for 10 min and assayed for LOX activity.

pH Optimum and Stability. The optimal pH of algal LOX was 7.5 (Figure 5). At pH 9.0, 74% activity was observed, and at pH 5.0, only 12% activity. The partially purified algal LOX was preincubated at pH ranging from 4.5 to 9.0 for 10 min. The stability of the algal LOX was shown in Figure 5. The algal LOX is stable over a pH range from 5.5 to 9.0.

Temperature Optimum and Thermostability. The optimal temperature of partially purified algal LOX was 33 °C (Figure 6). An abrupt decline in activity occurred above 45 °C. At 26 °C 80% of the maximal activity remained. The thermostability of algal LOX is shown in Figure 6. The algal LOX was stable below 40 °C.

Substrate Specificity. Since LOX catalyzes hydroperoxidation of polyunsaturated fatty acids (PUFA) possessing a *cis,cis*-1,4-pentadiene unit, fatty acids with chain lengths of 18−22 carbons and double bonds at both the $n-6$ and $n-9$ positions, ie., 18:2, 18:3, 20:4, 20:5, and 22:6, can serve as substrates of LOX. The susceptibility of these PUFA to algal LOX was examined at 26 °C (Table 3). LOX showed the highest reactivity and hydroperoxide production toward 18:2, followed by

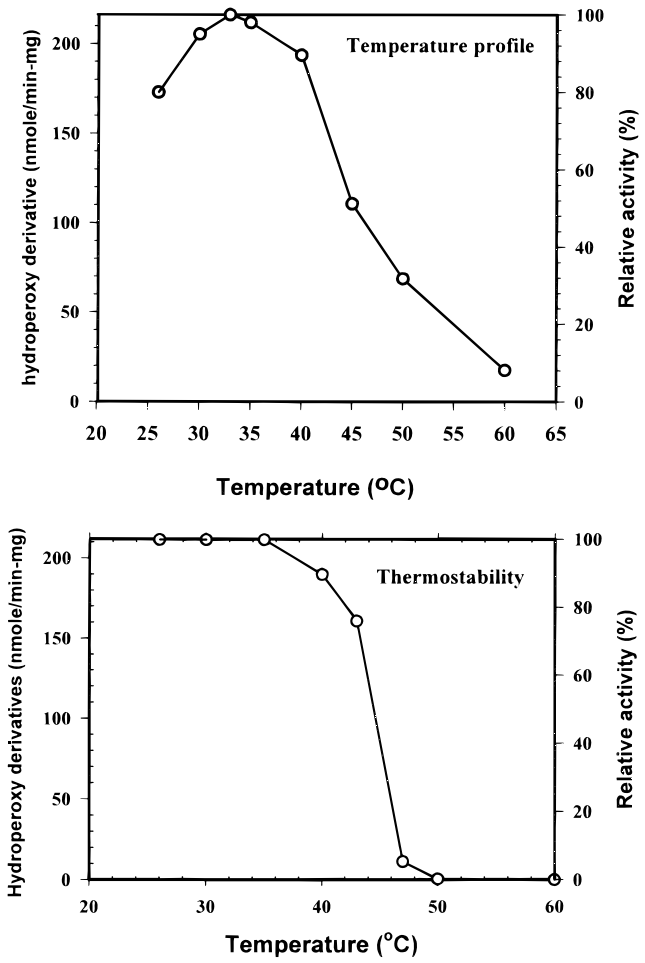

Figure 6. Temperature profile and thermostability of algal LOX. The temperature profile of LOX activity was obtained at different temperatures in 0.05 M potassium phosphate buffer (pH7.5) containing 0.2 mM Ca^{2+} using linoleic acid as substrate. The thermostability was performed by preincubating algal LOX at different temperatures for 10 min and then assayed for LOX activity.

Table 3. Formation of Hydroperoxide from Polyunsaturated Fatty Acid Reacted with Partially Purified Algal Lipoxygenase[a]

fatty acid	hydroperoxide [nmol (mg of protein)$^{-1}$ min^{-1}]	rel activity (%)
18:2 ($\omega-6$)	173.5	100
18:3 ($\omega-3$)	3.8	2.2
20:4 ($\omega-6$)	146.6	84.5
20:5 ($\omega-3$)	94.2	54.3
22:6 ($\omega-3$)	56.9	32.8
methyl linoleate[b]	7.5	4.3
monolinolein[b]	13.5	7.8
dilinolein[b]	11.6	6.7
trilinolein[b]	0	0

[a] Reacted with 50 μM substrate at 26 °C for 10 min. [b] The glycerides were dissolved in 99.8% alcohol to obtain a working solution. The algal LOX was incubated with glycerides (50 μM) in 0.05 M phosphate buffer (pH 7.5) containing 0.01% Tween 20. LOX activity was determined with spectrophotometric method or polarographic analysis as described under Materials and Methods.

Table 4. Substrate Specificity of the Partially Purified Algal LOX[a]

substrate fatty acid	product formed[b]	% of products
18:2	9-HODE	86
	13-HODE	14
18:3	9-HOTE	100
20:4	12-HETE	90
	15-HETE	10
20:5	12-HEPE	100
22:6	14-HDHE	100

[a] The partially purified algal LOX was obtained from the separation of ammonium sulfate fractionation, MacroPrep-Q ion exchange, and gel filtration on Sephacryl S-300 as described under Materials and Methods. [b] The algal LOX was incubated with 100 μM fatty acids at 26 °C for 10 min. The products were reduced, methylated, separated, and then subjected to HPLC analyses. Products from 18:2 and 18:3 were determined by normal phase HPLC and reversed phase HPLC for 20:4, 20:5, and 22:6.

20:4, 20:5, 22:6, and 18:3. The $\omega-6$ fatty acids seemed to be more susceptible to algal LOX than the $\omega-3$. The rate of oxidation catalyzed by soybean LOX on fatty acids 18:2, 22:6, 20:5, 18:3, and 20:4 in decreasing order was always above 77% compared to that on 18:2 (Holman et al., 1969). Tomato LOX showed a substrate preference of 18:2, 18:3, and 20:4 in decreasing order (Regdel et al., 1994). Reactivity of shrimp hemolymph LOX on fatty acids increased with degree of unsaturation of PUFA (Kuo et al., 1992). Grey mullet gill LOX showed the highest reactivity toward 20:4 followed by 22:6, 18:2, 18:3, and 20:5 (Hsu, 1996). The substrate specificity of LOX from algae as well as from land plants showed higher reactivity toward 18:2 than other PUFA, while LOXs from aquatic animals probably prefer 20:4.

Esters of linoleic acid (18:2) were also peroxidized by algal LOX (Table 3). The activity observed was in the order linoleic acid $\gg$ monolinolein > dilinolein > methyl linoleate. No activity was found when trilinolein was used as substrate. This result indicated that the algal LOX could catalyze linoleic acid esters at a lower rate than the free form, similar to the finding on LOX of soybean (Brash et al., 1987), canola (Khalyafa et al., 1990), shrimp hemolymph (Kuo et al., 1992, 1994), and reticulocyte (Rapoport et al., 1979), except the order of peroxidation rate was somehow different. The soybean LOX-2 was distinguished from LOX-1 by its higher affinity to fatty acid ester than that of LOX-1 (Brash et al., 1987). Therefore, the data shown in Table 3 suggested the algal LOXs resembled soybean LOX-2.

Hydroperoxidation Products. The products formed from PUFA treated with algal LOX are shown in Table 4. The products from 18:2 were reduced 18:2−9OOH (9-HODE) and 18:2−13OOH (13-HODE) with a peak area ratio of 86:14 using normal phase HPLC. Sea algae (*Enteromorpha intestinalis*) and fresh water algae also yielded 9- and 13-HODE, but at different ratios of 66.3:33.7 (Kuo et al., 1996a) and 48:52 (Beneytout et al., 1989).

The products from 20:4 treated with algal LOX were reduced 20:4−12OOH (12-HETE) and 20:4−15OOH (15-HETE) with a ratio of 90:10 using reversed phase HPLC, indicating the major LOX in the sea algae was 12-LOX and the minor was 15-LOX. These results were similar to those of sea algae, *E. intestinalis* (Kuo et al., 1996a), reticulocytes (Gardner, 1991), trout gill (German and Kinsella, 1986), and grey mullet gill (Hsu et al., 1996).

The product from algal LOX-catalyzed oxidation of 18:3 was reduced 18:3−9OOH (9-HOTE). That of 20:5 or 22:6 was reduced 20:5−12OOH (12-HEPE) or 22:6−14OOH (14-HDHE), respectively. The fact that algal LOX forms one or two major products from different PUFA is interesting. The reason for it needs further investigation.

Conclusion. A calcium-stimulated LOX was partially purified from sea algae, *U. lactuca*. This partially purified enzyme was found having two LOX activities in PAGE; the major one had a apparent MM of 41 kDa and the minor one, 116 kDa. The former was probably $n-9$ LOX and the minor one with 116 kDa was $n-6$ LOX on the basis of hydroperoxide formed from reactions with different PUFAs. The two LOXs from sea algae were probably cross-linked and stabilized in the presence of Ca^{2+} during Sephacryl S-300 gel filtration. The mechanism of stimulation and stabilization of Ca^{2+} to sea algal LOX is being studied.

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