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ORIGINAL PAPER

# Characterization and Immobilization of Marine Algal 11-Lipoxygenase from *Ulva fasciata*

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**Abstract** The lipoxygenase (LOX) of the marine green alga *Ulva fasciata* was purified and immobilized in order to improve the stability and reusability. The algal LOX was partially purified by fractionation with 35–55% saturation of ammonium sulfate and MacroPrep high Q anion exchange chromatography. The LOX was purified ten times using linoleic acid ($C_{18:2}$) or arachidonic acid ($C_{20:4}$) as substrate, the Michaelis constant ($K_m$) of LOX was 117.6, 31.3 μM, and maximum velocity ($V_{max}$) was 12.8, 23.3 μmol hydroperoxy fatty acid/min-mg protein, respectively. The algal LOX showed the highest activity towards $C_{18:4}$ followed by $C_{20:4}$, $C_{18:2}$ and methyl ester of $C_{18:4}$. LOX activity increased up to 10.5 times with increased concentration of Triton X-100 in the extraction medium reaching an optimum at 0.05%. Calcium chloride, glutathione and phenylmethylsulphonyl fluoride were found effective protectants to LOX during purification. Hydroperoxyeicosatetraenoic acid (HpETE) formed from arachidonic acid catalyzed by this purified algal LOX was reduced and identified as 11-hydroxy-5,8,12,14-eicosatetraenoic acid (11-HETE) by NP-HPLC and GC–MS. This algal 11-LOX was immobilized in alginate beads. The stability was sevenfold greater than that of the unbound lipoxygenase at 4 °C in 0.05 M Tris–HCl buffer (pH 7.5). This is the first report on immobilization of a marine algal lipoxygenase with a view to its potential role in seafood flavor formation.

**Keywords** Enzyme · Lipoxygenase · 11-HETE · Marine algae · *Ulva fasciata* · Alginate bead · Immobilization · $K_m$ · $V_{max}$ · Substrate reactivity

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## Introduction

Lipoxygenase (LOX, EC 1.13.11.12) was first isolated and crystallized from soybean [1]. Its structure was later elucidated. This enzyme is a dioxygenase that catalyzes the oxygenation of polyunsaturated fatty acids containing a *cis*,*cis*-1,4-pentadiene system to form hydroperoxides. It is widely distributed in the plant and animal kingdoms. In plants LOX plays important roles in flavor biogenesis in ripening fruits, off-flavor production and pigment degradation in legumes, in secretion of plant growth hormone, that is, jasmonic acid, and in wound healing and disease resistance of leaves [2].

Marine algal LOX has been under exploited [3–6]. However, algae are abundant in coastal waters and have long been used in Asian cuisines to enhance seafood flavor. Our previous results showed that marine algae had very high LOX activity. Treatment with algal LOX was able to improve aroma of fish oil [7] and chicken oil [8]. Volatile compounds contributing to flavor notes of clam, oyster, fresh apple, cucumber, melon, mango and algae were generated from different treatments of the marine algae [4]. The 2,4-decadienals of marine algal volatile compounds have been suggested to be produced via 11-hydroperoxyicosatetraenoic acid (HPITE) from arachidonic acid in marine green algal 11-LOX pathway [9]. The 11-HPITE

has also been named 11-hydroperoxyeicosatetraenoic (HpETE) in our studies as well as in other literatures [6, 10].

Since LOX is a labile protein, techniques for immobilization have been sought to improve its stability and to enhance its commercial applications. Several methods for immobilizing LOX have been described in literature [11–13]. In general, different sources and preparations of LOX differed in their relative stability and immobilization efficiency. Our aim was to characterize the algal LOX and to stabilize it in order to utilize it repeatedly for specific aroma generation.

## Materials and Methods

### Marine Algae

*Ulva fasciata*, was harvested in February 2006 from the Pacific coast of northern Taiwan. The algae were kept in seawater at ca. 20 °C and transported to the laboratory immediately for analyses.

### Purification of Algal LOX

All steps were performed at a temperature below 4 °C. All buffers contained 0.2 mM $CaCl_2$. Fifty grams of fresh algae were washed with distilled water, cut into small pieces and blended in a high speed homogenizer (Polytron PT-3000, Switzerland) with 250 ml of 0.05 M potassium phosphate buffer (pH 7.5) in a ratio of 1:5 (w/v) containing 1 mM glutathione, 1 mM phenylmethylsulphonyl fluoride (PMSF) and Triton X-100.

The homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000×*g* for 20 min at 4 °C to obtain a crude enzyme extract. Two liters of the crude enzyme extract was fractionated by ammonium sulfate precipitation at 35–55% saturation and centrifuged at 20,000×*g* for 15 min. The pellet was dissolved in a minimal volume of 0.05 M potassium phosphate buffer (pH 7.5) containing 1 mM glutathione and dialyzed overnight at 4 °C against 200 volumes of the same buffer.

The dialysate was applied to a MacroPrep high Q (Bio-Rad, Hercules, CA, USA) column (2.2 × 15 cm) equilibrated with 0.025 M Tris–HCl buffer (pH 7.5) and eluted with a linear gradient of 0.15–0.5 M NaCl at a flow rate of 0.8 mL/min. The eluted fraction containing LOX activity was collected, concentrated, and pooled onto the same column again for separation. The purified LOX was obtained from the elution at a flow rate of 0.3 mL/min. The homogeneity of the enzyme was determined using analytical gel electrophoresis (Bio-Rad mini-PAGE instrument, Hercules, CA, USA) [14].

### Assay of LOX Activity

The activity of algal LOX was determined by measuring the increase in absorbance at 234 nm [5] with a Hitachi U-2000 spectrophotometer (Tokyo, Japan). Algal LOX extract, 0.1 mL, was diluted with 0.9 mL of 0.05 M potassium phosphate buffer (pH 7.5) containing 0.01% Tween-20. The mixture was incubated with 100 μM linoleic acid or arachidonic acid in ethanol at 25 °C for 5 min. The reaction was terminated by acidification to pH 3 with 6 M HCl. LOX activity was determined as the increase in fatty acid hydroperoxide product estimated by using a molar absorptivity of 25,000 L $mol^{-1}$ $cm^{-1}$ at 234 nm [15].

### HPLC Chromatography

The LOX catalyzed reaction products were reduced by addition of 0.1 g of $NaBH_4$ then extracted with ethyl acetate. The solvent was removed under vacuum and then subjected to HPLC analysis [4, 14]. Normal phase high-pressure liquid chromatographic (NP-HPLC) analyses were performed on a Bondclone silica column (30 cm × 3.9 mm, 10 μm; Phenomenex, Torrance, CA, USA) equipped with a pump (Waters, Model 510, Milford, MA, USA) and a UV detector (Waters, Model 490E) monitored at 234 nm. The reduced hydroperoxy derivatives were eluted isocratically with a solvent system of hexane/isopropanol/acetic acid (100:2.0:0.1 v/v/v) at a flow rate of 1.0 mL/min. Reversed phase high-pressure liquid chromatographic (RP-HPLC) analyses were performed on an ODS18 column (25 cm × 4.6 mm, 5 μm particles) equipped with a Waters pump and a UV detector system as mentioned above. The hydroxy derivatives (HETE) were eluted isocratically by a solvent system of methanol/water/acetic acid (85:15:0.1 v/v/v) buffered with 5 mM ammonium acetate containing 0.5 mM EDTA to an apparent pH of 5.7. The LOX catalyzed products, 18:2-9OOH (9-HPODE, hydroperoxyoctadecadienoic acid), 18:2-13OOH (13-HPODE), 5-, 12-, 11-, and 15-HETE (hydroxyeicosatetraenoic acid) were confirmed by comparison with authenticated standards (Caman, Ann Arbor, MI, USA).

### GC–MS Analyses of Derivatized 11-HETE

The purified algal LOX was incubated with arachidonic acid (0.2 mM in ethanol) at 25 °C for 5 min. The reaction products were extracted with ethyl acetate and then reduced with $NaBH_4$. The solvent was removed under vacuum. The residues were reconstituted in 1 ml of methanol/ether (2:1 v/v). The HETE was esterified with 0.2 ml of 2.0 M trimethylsilyldiazomethane (TMSD, Sigma–Aldrich Chemie GmbH, Steinheim, Germany) in

hexane at 40 °C for 10 min. The resulting methyl ester was formed by silylation of the hydroxyl group with Tri-Sil reagent (Pierce, Rockford, IL, USA) at 75 °C for 30 min. Positive-ion electron impact spectra were obtained using a mass spectrometer (Hewlett-Packard 6890 N, Palo Alto, CA, USA) operated at 70 eV. The samples were run on a HP-5/MS (5% phenylmethyl-polysiloxane, Hewlett–Packard) capillary column (30 m × 0.25 μm) using helium as carrier gas. The injector was set at 240 °C. The oven temperature was programmed from 60 to 280 °C at a rate of 10 °C/min.

### Enzyme Kinetic Measurements

The enzyme steady-state kinetics Michaelis–Menten constant ($K_m$) and maximum velocity ($V_{max}$) were measured by plotting $1/V$ versus $1/S$ according to Lineweaver and Burk [16], where $V$ is velocity and $S$ is the substrate concentration being 10–160 μM of linoleic acid or arachidonic acid in 0.05 M potassium phosphate buffer, pH 7.5 reacting with 10 μL of purified algal LOX (0.5 mg protein/mL) for 5 min at 35 °C.

### Immobilization of LOX

Purified algal LOX was immobilized into an alginate sol-gel matrix using the procedure of Hsu et al. [13]. Sodium alginate (2% w/v) in 0.05 M Tris–HCl buffer (pH 7.5) was well mixed with an equal volume of algal LOX (0.5 mg/mL in 0.05 M Tris–HCl buffer, pH 7.5). Droplets of this mixture were dispensed with a peristaltic pump (Pharmacia LKB-Pump P-1, Phenomenex, Torrance, CA, USA) into a cold solution of 0.2 M $CaCl_2$ (50 ml) to form single beads, which were collected filtered and washed with 0.05 M Tris–HCl buffer (pH 7.5) then stored in the same buffer at 4 °C until use.

### Determination of Storage Stability

The free and alginate-immobilized algal LOX was stored in 10 volumes of 0.05 M Tris–HCl buffer, pH 7.5 at 4 °C for different periods of time (up to 30 days). The LOX was assayed using arachidonic acid as substrate at designated storage periods.

## Results and Discussion

### Isolation of Membrane-Bound Algal LOX with Triton X-100

Triton X-100 was required for extraction of algal LOX, of which the activity increased with the concentration of the surfactant up to 0.05%, above which the activity reached a plateau (Fig. 1). The LOX activity so recovered was 10.5 times of that extracted without the Triton X-100, indicative of 90.4% of the LOX present in algae was membrane-bound. Throughout the extraction, purification and assay of LOX, Triton X-100 was used to maintain the LOX activity. The stabilizing effect of Triton X-100 on algal LOX seems to indicate that hydrophobic interaction was required to maintain the conformation for its activity.

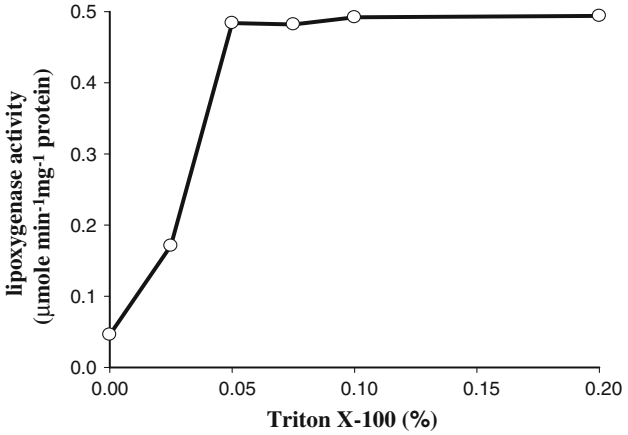


**Fig. 1** Effect of Triton X-100 concentration on extraction of lipoxygenase from *U. fasciata*

LOX activity present in algal extract was stimulated by the presence of $Ca^{2+}$ at 0.2 mM to a maximum activity of about 8.8-fold [5]. $Ca^{2+}$ probably unmasks the catalytic site or changes the protein conformation of LOX to increase its activity. Further investigation is needed [5]. Soybean leaf extracts prepared in the presence of the protease inhibitor PMSF (1 mM) and stored in an ice bath for 48 h showed an increase in LOX relative activity to about 2.2-fold when compared to the control-extract [17]. Grey mullet gill extracts prepared in the presence of reduced glutathione (1 mM) and stored at 4 °C for up to 15 h demonstrated a very pronounced stabilizing effect, being 40% higher than the control and maintained significantly higher for up to 40 h. Glutathione reduced hydroperoxide to its stable hydroxyl analogues and thus eliminated excess hydroperoxides which cause self-inactivation of LOX [18].

LOX extracts prepared in the presence of $Ca^{2+}$, PMSF, and glutathione were used in the experiments of this study (Fig. 2). The partially purified LOX added with 0.2 mM $CaCl_2$ retained 50% of activity for 48 h at 4 °C. Adding PMSF (1 mM) and glutathione (1 mM) to partial purified LOX (contained 0.2 mM $CaCl_2$) retained 50% of the activity for 96 h at 4 °C. The stability was double that of when only $CaCl_2$ was the single LOX protector. In the absence of any protector, LOX activity was lost within 24 h (Fig. 2). $CaCl_2$, glutathione and PMSF were found

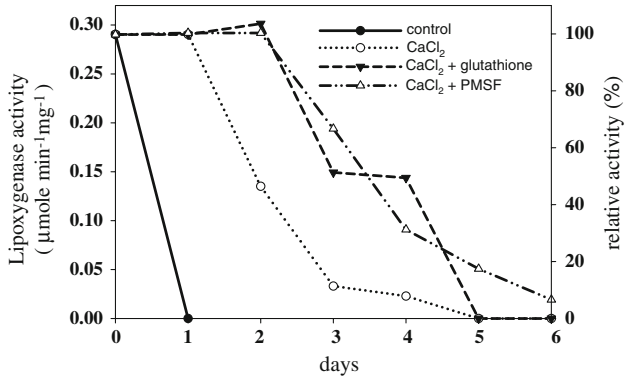


**Fig. 2** Effect of various protectants on the stability of partial-purified LOX during storage at 4 °C. (The crude enzyme extract was fractionated with 35–55% saturation of ammonium sulfate. The protectants were $CaCl_2$ 0.2 mM, glutathione 1 mM and PMSF 1 mM)

effective protectants to LOX throughout the purification steps. The crude extract of *Ulva fasciata* was fractionated with 35–55% saturation of ammonium sulfate followed by chromatography of Macroprep Q ion exchange eluted with 0.025 M Tris–HCl buffer (pH 7.5) containing 0.025% Triton X-100 and the three protectants at a linear salt gradient of 0.15–0.5 M NaCl. LOX was purified tenfold (Table 1). The purified LOX showed homogeneity as a single band in electrophoresis (data not shown).

## Confirmation of 11-LOX in Algae

The elution profile of the hydroperoxy derivatives formed from linoleic acid catalyzed by the algal LOX from *Ulva fasciata* showed a major peak from 9-LOX and a minor peak from 13-LOX (Fig. 3) similar to those found in *Enteromorpha intestinalis* [4].When the LOXs were inactivated by acidification with 6 M HCl to pH 3.0, these two peaks disappeared indicating that the two algal LOXs catalyze the hydroperoxidation of linoleic acid at C-9 or C-13 position. When arachidonic acid was used as substrate, the major algal LOX was 11-LOX and the minor isozyme was 15-LOX similar to the pattern shown in Fig. 4, but the resolution between 15-HETE, 12-HETE and 11-HETE was not as well separated in the RP-HPLC chromatogram (data not shown). The normal phase chromatogram (NP-HPLC) provided a better separation of the three HETE isomers as shown in Fig. 4. The UV spectral profiles had shown that

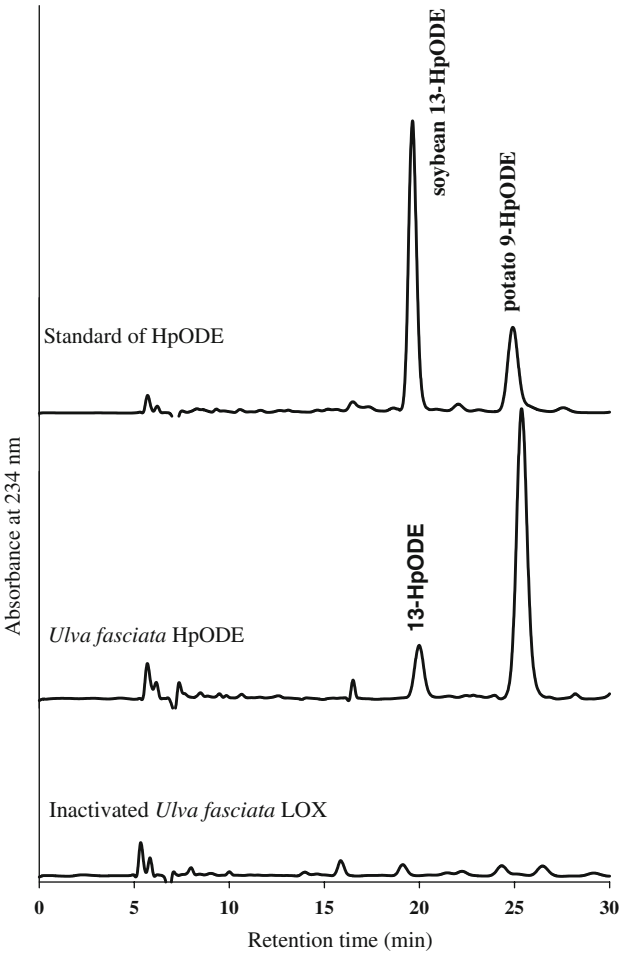


**Fig. 3** Normal phase HPLC chromatogram of hydroperoxy derivatives (HpODE) formed from linoleic acid peroxidation catalyzed by partial-purified algal LOX and acid-inactivated LOX

algal 11-HETE and 11-HETE standard have similar characteristic (Fig. 4).

The mass spectrum of the major derivative from arachidonic acid treated with algal LOX was methylated and the TMS derivatized, which was found similar to the TMS derivative of the authenticated standard of 11-HETE (Fig. 5). The fragmentation pattern of the electron impact system consisted of ions at *m/z* 406 ($M^+$); *m/z* 391 ($M^+ - 15$); *m/z* 316 ($M^+ - 90$); *m/z* 283 (C1–C11); *m/z* 225 (C11–C20) being identical to the spectrum of the 11-HETE authenticated standard [6]. The predominant ion at *m/z* 225 (C11–C20)

**Table 1** Purification of LOX from marine algae (*Ulva fasciata)*

| Purification stage | Total activity (μmol/min) | Total protein (mg) | Specific activity (μmol $min^{-1}$ $mg^{-1}$) | Recovery (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude extract | 44.92 | 187.20 | 0.23 | 100 | 1.0 |
| 35–55% $(NH_4)_2SO_4$ ppt | 38.22 | 115.85 | 0.33 | 87 | 1.4 |
| MacroPrep-Q | 22.88 | 9.96 | 2.30 | 52 | 10.0 |

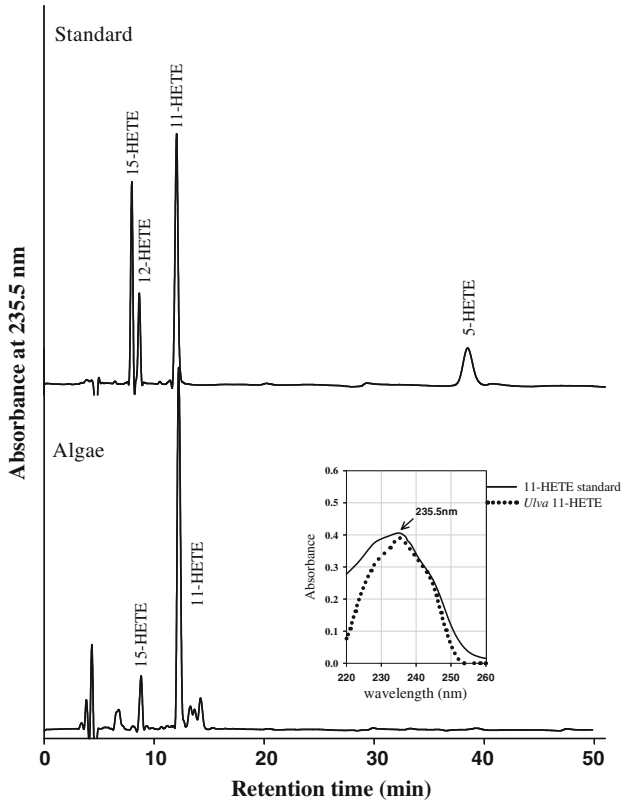


**Fig. 4** Normal phase HPLC detected with absorbance at 235.5 nm and the spectrum of hydroxy derivatives (*HETE*) reduced from hydroperoxy products (*HpETE*) formed from arachidonic acid peroxidation catalyzed by partial-purified algal LOX from *Ulva fasciata*

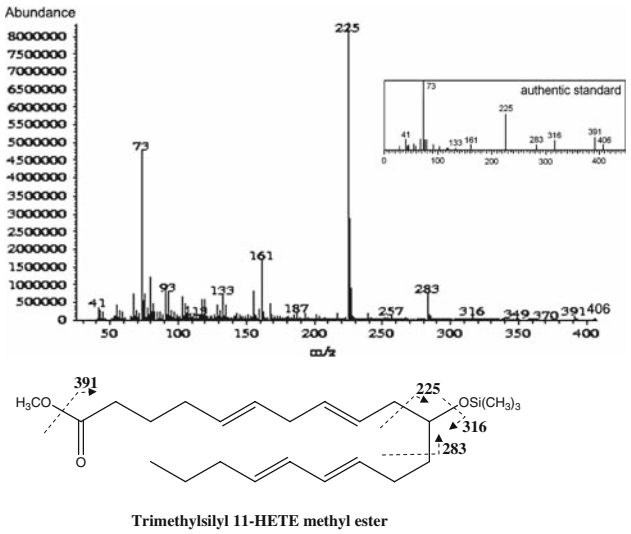


**Fig. 5** Mass spectrum of the product of arachidonic acid treated with algal LOX

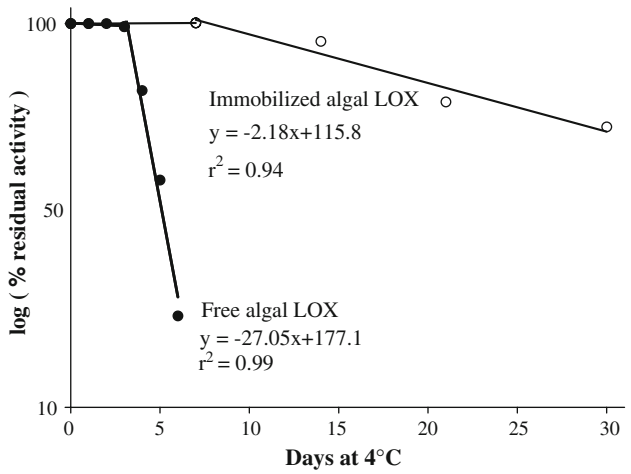


**Fig. 6** A semilogarithmic plot of the residual activity of the free and the alginate-immobilized algal LOX stored in ten volumes of 0.05 M Tris–HCl buffer, pH 7.5 at 4°C

confirmed the insertion of a hydroxyl group at carbon-11 of arachidonic acid (Fig. 5). If the TMS was at carbon 12, the base peak would have been *m*/*z* 295 after hydroperoxidation of arachidonic acid. In this case, the base peak was 225 not 295. Therefore, the TMS was at carbon 11, and the hydroperoxidation of arachidonic acid catalyzed by the algal-LOX occurred at the carbon 11 position. The 11-LOX has been found in marine organisms such as sea urchin [19], mussel [10], and was different from those LOXs existing in land plants.

## Substrate Specificity and Kinetics

Six polyunsaturated fatty acids (PUFAs) and one methyl ester were tested as preferred substrates for the novel 11-LOX. The susceptibility of these PUFAs to algal LOX was examined (Table 2). Octadecatetraenoic acid (C18: 4 $\omega$3) showed the highest affinity to this algal 11-LOX. The activity on C18: 4 was nearly two times of that on linoleic acid (18:2 $\omega$6). The relative reactivity using arachidonic acid (20:4 $\omega$6) as substrate was 133.0%, also higher than that shown on linoleic acid. On the other hand, eicosapentaenoic acid (20:5 $\omega$3, 85.5%), docosahexaenoic (22:6 $\omega$3, 16.7%), linolenic (18:3 $\omega$6, 7.5%) and the methyl ester of octadecatetraenoic acid (4.7%) were substrates of lower affinity than the C18:4 and C20:4 to the 11-LOX. Since among all the PUFAs, linoleic acid has the highest reactivity to land plant LOX, it seems that the algal 11-LOX has a unique affinity for tetraene structure but not the tetraenoate nor other *cis–cis* pentadienes. The binding channel of the algal 11-LOX for PUFA substrate is likely to be different from the conformation of the land plant LOX.

Based on the Lineweaver-Burk plot, arachidonic acid was a preferred substrate than the linoleic acid to the algal LOX giving a $K_m$ 27% and a $V_{max}$ 1.8 fold of the latter

**Table 2** Substrate specificity and relative activity of purified algal LOX

| Fatty acid | Specific activity (μmol $min^{-1}$ $mg^{-1}$ protein) | Relative activity (%) |
|---|---|---|
| C18:2 (ω-6) | 2.01 | 100.0[a] |
| C18:3 (ω-3) | 0.15 | 7.5 |
| C18:4 (ω-3) | 3.97 | 197.5 |
| C20:4 (ω-6) | 2.68 | 133.0 |
| C20:5 (ω-3) | 1.71 | 85.5 |
| C22:6 (ω-3) | 0.34 | 16.7 |
| C18:4 methyl ester | 0.10 | 4.7 |

LOX activity was determined with the spectrophotometric method described under "Materials and Methods"

[a] Linoleic acid (18:2 ω6) is the major PUFA in *Ulva fasciata* (our unpublished data), and in most of the land plants

**Table 3** Michaelis–Menten constant ($K_m$) and maximum velocity ($V_{max}$) of purified algal LOX

| Substrate | $K_m$ (μM) | $V_{max}$ (μmol $min^{-1}$ $mg^{-1}$) |
|---|---|---|
| $C_{18:2}$ | 117.6 | 12.8 |
| $C_{20:4}$ | 31.3 | 23.2 |

LOX activity was determined with spectrophotometric method as described under "Materials and Methods"

(Table 3). The $K_m$ of purified LOX from banana leaves using linoleic acid as substrate was 150 μM with a $V_{max}$ value of 2.4 μM/min mg [14]. The $K_m$ of soybean LOX is 4.8 μM with a $V_{max}$ value of 0.4 μmol/min nmol [20]. Tomato LOX had a $K_m$ value ranging from 1.5 to 4,100 μM and $V_{max}$ value ranging from 186 to 7,400 μmol/min mg protein [21]. The $K_m$ of algal LOX was similar to that from banana leaves, and $V_{max}$ was similar to that of tomato fruit (10 μmol/min mg protein). No previous report on $V_{max}$ and $K_m$ of algal LOX was found in the literature.

## Storage Stability of Free and Immobilized Enzymes

Algal 11-LOX immobilized and stored in 10 volumes of 0.05 M Tris–HCl buffer (pH 7.5) was stable for 7 days, later it lost approximately 25% of its activity in the following 20 days, while free LOX remained 100% active for 4 days then rapidly lost 50% of the activity in 1 day (Fig. 6). The stability of the immobilized LOX was estimated to be sevenfold greater than that of the free LOX stored at 4 °C in 0.05 M Tris–HCl buffer (pH 7.5). The comparison was based on the half life, or the time required for 50% of the original activity of the enzyme to decay in a semilogarithmic plot. Since the majority of reactions occurring in foods obey first-order reaction kinetics, the "logarithmic order of inactivation" was used.

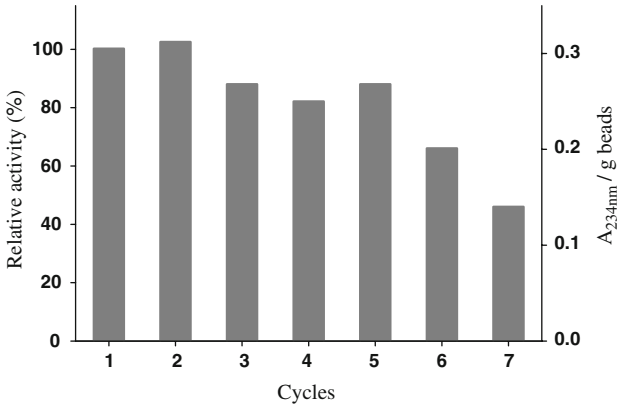


**Fig. 7** Operational stability indicated by the residual activity of immobilized algal LOX from *Ulva fasciata*. Activity was assayed using linoleic acid as substrate. After each reaction cycle, the immobilized LOX beads were washed with Tris–HCl buffer (pH 7.5) followed by adding fresh linoleic acid to conduct a subsequent reaction cycle. The activity after the first cycle was expressed as 100%. Others were expressed as relative activity to the first cycle

A similar study of soybean 15-LOX immobilized in alginate silicate gel demonstrated a complete operational stability at ambient temperature for at least 25 days, whereas the activity of the free enzyme completely decreased in less than 1 day [13]. Other immobilized LOX covalently bonded to carbonyl-imidazole activated support and demonstrated that the stability of the immobilized LOX at 15 °C was approximately ten times greater than that of the unbound soybean LOX [22]. In our study the free algal 11-LOX showed a significant loss of activity during purification and storage. The immobilization of this algal LOX in calcium alginate sol–gel matrix was found to stabilize the enzyme. This is the first report on immobilized LOX from marine algae.

## Reusability

The immobilized algal 11-LOX was repeatedly used for seven reaction cycles (Fig. 7). No substantial loss in enzyme activity was found for up to five cycles. The immobilized LOX activity dropped to 69.5% after six cycles and to approximately 50% after seven cycles. This decrease may be due to the increased diffusion limitation of the substrate into the pores of the beads immobilizing the enzyme [23], or the residual peroxidation products entrapped in the beads caused the suicidal oxidation [24] of the immobilized LOX.

Algal LOXs have been previously identified from *Enteromorpha intestinalis* [4], and *U. conglobata* [7, 9]. This current observation is the first report on the substrate specificity, $K_m$ and immobilization of algal 11-LOX.

## Potential Applicability of Immobilized LOX

During assay of the immobilized LOX with linoleic acid or salad oil as substrate reacted for half an hour, the mixture had slightly algal odor. Our previous studies using algal LOX to modify fish oil abundant in highly unsaturated fatty acids produced desirable volatile compounds [7]. These compounds were identified as *E,Z*-2,6-nonadienal, *E*-2-hexenal, *E,E*-2,4-octadienal, *E,Z*-2,4-decadienals, *E*-2,4-decadienals, *E,E*-3-5-octadien-2-one, and alcohols such as *E*-2-pentanol and 2-butoxyethanol. The 2,4-decadienals which imparted algal odors were produced via 11-hydroperoxyicosatetraenoic acid (HPITE) derived from arachidonic acid exclusively [9]. Monitoring the algal-LOX reactions to generate desirable aroma compounds using the immobilization technique may have practical significance in foods and edible oils.

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