

Fatty Acid Oxidizing Activity in a Red Marine Alga, *Porphyra* sp.

Tadahiko Kajiwar^{a,*}, Kenji Matsui^a, Yoshihiko Akakabe^a, Kaori Okajima^a and Anong Chirapart^b

^a Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, 753–8515 Japan. Fax: +81–839–33–5849.
E-mail: kajiwar@agr.yamaguchi-u.ac.jp

^b Department of Biology, Faculty of Fisheries, Kasetsart University, Chatuchak, Bangkok 10900, Thailand

* Author for correspondence and reprint requests

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A crude enzyme solution prepared from fronds of *Porphyra* sp. showed remarkable oxygen uptake activity when linoleic acid was added as a substrate. Fatty acid oxidizing activity was mainly present in the soluble fraction of the crude homogenate. The activity was purified 769-fold from mature fronds by ammonium sulfate fractionation, ion-exchange and hydrophobic chromatography. SDS-PAGE analysis of the purified proteins indicated that its subunit size was about 13 kDa. Gel filtration chromatography equipped with a photodiode array detector revealed that the activity was associated with a protein having a molecular weight of 12,500–13,000. It eluted with a chromophore having the maximum absorbance at 417 nm, thus, the protein was suggested to be a heme protein. The spectrophotometric property of the protein was highly similar to that of cytochrome *c* suggesting that it has heme *c* as a prosthetic group. The protein showed highest oxygenation activity against linoleic acid, and α -linolenic acid and arachidonic acid followed, but oleic acid could not be oxidized. From linoleic acid the protein formed 9- and 13-hydroperoxides to the same extent, and both were shown to be racemic. These results showed that the oxidizing activity is accountable to a cytochrome, but not to a typical lipoxygenase.

Introduction

Marine alga are a well recognized as a source of unique natural products of diverse structural types (Gerwick, 1994). Recently, the pathway of oxylipin-formation from fatty acids has attracted attention as a novel pathway generating natural products (Gerwick, 1993a; Gerwick, 1990; Jiang and Gerwick, 1991; Gerwick, 1993b). In higher plants, oxylipins are biosynthesized via various pathways, many of which involve initial lipoxygenase-catalyzed formation of an unsaturated fatty acid hydroperoxide (Hamberg, 1993). Lipoxygenases are widely distributed in nature (Gardner, 1991). It is a dioxygenase that catalyzes the conversion of a polyunsaturated fatty acid containing a (1*Z*,4*Z*)-pentadiene system into a conjugated hydroperoxy fatty acid.

Recently, in a red alga, *Gracilariopsis lemaneiformis*, a sodium-dependent 12-lipoxygenase was detected (Hamberg and Gerwick, 1993). A gametophyte-specific cDNA encoding a lipoxygenase from the red alga *Porphyra purpurea* was also

isolated (Liu and Reith, 1994). Nonetheless, there is no report concerning their enzymatic character. Thus, in this study, purification and characterization of the fatty acid oxidizing activity were carried out.

Materials and Methods

Plant material

The fronds of *Porphyra* sp. cultured in Onoda bay in Yamaguchi, Japan, were harvested during December to March in 1995. They were kept frozen at –20 °C until use.

Substrate

Linoleic acid (99% pure), α -linolenic acid (98% pure) and oleic acid (99% pure) were purchased from Sigma Chemical Co., arachidonic acid (99% pure) was purchased from Funakoshi Co (Japan). Each substrate was suspended with 0.2% Tween 20 to be 50 mM. These substrate solutions were

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stored at -20°C until use and were briefly sonicated just prior to use.

Preparation of active solution

Frozen fronds of *Porphyr* sp. (100 g wet weight) were homogenized in two volumes of 0.1 M K-phosphate buffer (pH 7.3) in a mortar on ice. The homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged at $20,000 \times g$ for 20 min. The supernatant was used as a crude enzyme solution. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the enzyme solution for a 30% saturation. After stirring on ice for 60 min, the mixture was centrifuged at $10,000 \times g$ for 10 min. The supernatant was brought to 70% saturation of $(\text{NH}_4)_2\text{SO}_4$, and stirred and centrifuged in the same way. The precipitate was redissolved with a minimum volume of 10 mM Tris [Tris (hydroxymethyl) aminomethane]-HCl buffer (pH 8.0). The ammonium sulfate fraction was dialyzed against 10 mM Tris-HCl (pH 8.0) for 12 h with three changes. The dialyzed enzyme solution was centrifuged at $20,000 \times g$ for 20 min and the supernatant was applied to a DEAE-Cellulofine A500 column (Seikagaku Kohgyo, Japan, 18 mm i.d. \times 385 mm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The column was washed with 500 ml of the same buffer, and elution was done with 1 l of a linear gradient of KCl (0–0.5 M) formed with the same buffer. To the combined active fraction $(\text{NH}_4)_2\text{SO}_4$ was added for a 30% saturation, and the soln. was applied to a Phenyl-Toyopearl 650 M column (9 mm i.d. \times 150 mm) equipped on a HPLC system (L-6200, Hitachi, Japan), that had been equilibrated with 10 mM K-phosphate buffer (pH 8.0) containing $(\text{NH}_4)_2\text{SO}_4$ of 30% saturation. The column was washed with 10 ml of the same buffer, and elution was done with a 30–0% saturation gradient of $(\text{NH}_4)_2\text{SO}_4$ in 10 mM K-phosphate buffer (pH 8.0) with the flow rate of 1.0 ml/min. Elution was monitored by a UV detector (L-4000, Hitachi, Japan) at 280 nm. The eluted active fractions were collected as a purified protein.

Assays

The activity was determined polarographically with a Clark type oxygen electrode (Yellow Spring Instruments.Co) at 25°C . The reaction mixture contained 50 μl of linoleic acid soln (50 mM, in

0.2% Tween 20), 100 μl of the enzyme soln and 1.7 ml of 0.1 M Tris-HCl buffer (pH 8.0). One katal of the activity was defined as the quantity of the protein catalyzing the consumption of 1 mol of oxygen within a second at 25°C . The activity was calculated from the maximum O_2 -uptake rate observed just after addition of the substrate. Typically, the rate progressively decreased. In order to estimate fatty acid oxidizing activities of various heme proteins, hemoglobin from bovine and cytochrome *c* from horse heart (Wako Pure Chemical), or hematin from bovine blood (Sigma Chemical Co.), dissolved in a few drops of 1 N NaOH and diluted with 10 mM Tris-HCl buffer) was used. For estimation of inhibitory effect of chemicals, the purified protein was preincubated with a chemical for 3 min before adding a substrate. Alternatively, spectrometric analysis following formation of the reaction products were performed with a spectrophotometer, UV-160A (Shimadzu). Time course of product formation was monitored by intermittent scanning of the spectrum from 200 to 300 nm, or by following increase in absorbance at 234 nm. For the spectrophotometric analysis, a reaction mixture was used containing 50 μl of Phenyl-Toyopearl active fraction, 850 μl of 0.1 M Tris-HCl buffer (pH 8.0) and 50 μl of 10 mM linoleic acid solution.

Quantification of protein

Protein was quantified by a modified method of Lowry *et al.* (Dulley and Grieve, 1975) with bovine serum albumin as standard.

Estimation of molecular weight

The DEAE active fractions were applied to a TSK-gel G3000 SW column (7.5 mm i.d. \times 600 mm, Tosoh) equipped with a photodiode array detector (SPD-M6A, Shimadzu, Japan) on a HPLC system (LC-10AD, Shimadzu, Kyoto, Japan) that had been equilibrated with 0.1 M Na-phosphate buffer (pH 6.7) containing 0.1 M Na_2SO_4 and 5 mM 3-[(3-cholamidopropyl) dimethylamino]-1-propanesulfonate. The solution was separated with the same buffer with the flow rate of 0.4 ml/min. Elution was continuously monitored at 280 nm and 420 nm.

β -Amylase, bovine serum albumin, ovalbumin and bovine erythrocyte carbonic anhydrase

(Sigma Chemical Co.) were used as molecular weight markers. SDS-polyacrylamide gel electrophoresis was performed on a 15.0% (w/v) polyacrylamide gel by the method of Laemmli (1970). The gel was silver stained (Morrisey, 1981).

Product analyses

To DEAE-Cellulofine active fraction (4 ml) was added 32 ml of 0.1 M Tris-HCl buffer (pH 8.0) and 1 ml of 50 mM linoleic acid. The mixture was incubated at 10 °C for 30 min, and adjusted to pH 3.0 by HCl. Then, the mixture was extracted with diethylether. The fatty acid derivatives were converted to their methyl esters with ethereal diazomethane. The methyl esterified extract was concentrated *in vacuo*, and reduced by triphenylphosphine. The reduced and methyl esterified products were analyzed with HPLC, TLC and LC/MS.

Straight-phase HPLC analyses of the products were performed with a Zorbax-SIL column (4.6 mm i.d. × 250 mm) by using a solvent system of hexane/2-propanol (98.8/1.2) with a flow rate of 1.0 ml/min. The column temperature was 25 °C. Reverse-phase HPLC analyses were performed on a Zorbax-ODS column (4.6 mm i.d. × 250 mm) by using a solvent system of acetonitrile/0.1 M ammonium acetate (90/10, v/v) at 25 °C with the flow rate of 1.0 ml/min. As authentic specimen, 13-hydroperoxyoctadecadienoic acid (13-HPOD) and 9-hydroperoxyoctadecadienoic acid (9-HPOD) were prepared with soybean seed lipoxygenase (Type I, Sigma (Matsui *et al.*, 1989)) and with potato tuber lipoxygenase (Matsui *et al.*, 1989; Mulliez *et al.*, 1987), respectively.

TLC was carried out with precoated plates (Silica gel 60 F254) from E. Merck (Darmstadt, Germany). The developing solvents consisted of a mixture of ether/*n*-hexane (2/1, v/v). After devel-

opment, spots were detected under UV illumination, anisaldehyde-staining or *N,N'*-dimethyl-*p*-phenylenediamine-staining (Dussault and Lee, 1995).

LC/MS analyses were performed with a mass selective detector connected to a LC MS-QP 1000 (Shimadzu, Japan). The temperature of the column was 25 °C and the flow rate was 0.7 ml/min. Elution solvent was acetonitrile/water (90/10, v/v) buffered with 0.1 M ammonium acetate. A Zorbax-ODS column (4.6 mm i.d. × 250 mm) was used, with Tip temp. 250 °C and Block 273 °C in thermospray method.

Absolute configuration and enantiomeric excess were determined by chiral-phase HPLC on a Chiralcel-OB column (cellulose tribenzoate coated on silica, Daicel Japan; 4.6 mm i.d. × 250 mm) with detection at 234 nm. Elution was carried out with hexane/ethanol (99.5/0.5, v/v) at the flow rate of 0.7 ml/min.

Results and Discussion

Purification of fatty acid oxidizing activity

Crude homogenate was prepared from frozen fronds of *Porphyra* sp. Most fatty acid oxidizing activity was recovered in the supernatant fraction after centrifugation of a crude homogenate of *Porphyra* sp. fronds at 20,000 × *g* for 20 min. This is in good accordance with the report showing that the major part (86–94%) of 12-lipoxygenase of the red marine alga *Gracilariopsis lemaneiformis* was present in the soluble fraction (Hamberg and Gerwick, 1993). Fatty acid oxidizing activity in *Porphyra* sp. was then purified as shown in Table I. The activity was purified 769-fold with a specific activity of 261.6 nkat/mg protein. During these purification steps, only one active fraction could be

Table I. Purification of fatty acid oxidizing activity from *Porphyra* sp.

	Total protein (mg)	Total volume (ml)	Total activity (nkat)	Specific activity (nkat/mg)	Yield (%)	Purification (-fold)
Crude enzyme solution (20,000× <i>g</i> sup.)	594.0	200	200.00	0.34	100	1.00
Ammonium sulfate fraction	558.0	30	162.12	0.29	81.06	0.85
Dialyzed solution	457.0	48	145.72	0.32	72.86	0.82
DEAE-Cellulofine A500	42.6	16	88.48	2.08	44.24	6.12
Phenyl-Toyopearl 650M	1.26	33	109.89	261.64	54.90	769.44

found. The activity of the purified protein was stable and could be kept at 4 °C for several days in 0.1 M Tris-HCl buffer (pH 8.0) without any significant loss of the activity. SDS-PAGE analysis showed that the protein having the activity was purified to a homogenous state (Fig. 1). Mr of the main band was estimated to be 12 kDa.

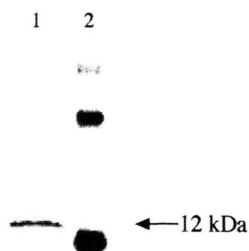


Fig. 1. Silver stained SDS-polyacrylamide gel. Lane 1, active fraction from phenyl-Toyopearl (0.0142 nkat); lane 2, molecular mass marker (bovine cytochrome *c*).

The purified protein was then subjected to analytical gel filtration chromatography using a column of TSK-gel G3000SW. With calibration curve constructed with standard globular proteins, molecular weight of this protein was estimated to be 13 kDa (Fig. 2), which showed good coincidence with the value estimated with SDS-PAGE analysis.

The molecular mass of the active protein estimated by gel filtration and SDS-PAGE was obviously different from those of typical plant lipoxygenases ranging from 90 to 100 kDa. Lipoxygenase in a red marine alga was reported to have a molecular mass of 84–89 kDa (Hamberg and Gerwick,

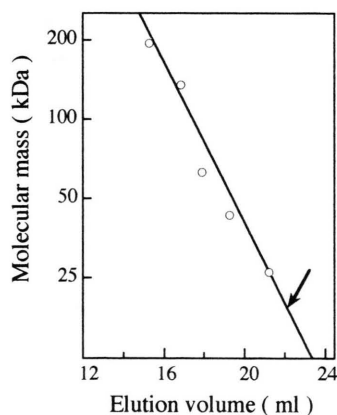


Fig. 2. An estimation of the molecular weight of fatty acid oxidizing activity by gel filtration on TSK-gel H3000SW. Fatty acid oxidizing activity was eluted as the arrow indicates.

1993). Furthermore, this activity was extremely stable at high temperature and essentially no loss of the activity could be seen even after heating at 90 °C for 20 min. This property is also uncommon within plant lipoxygenases previously known.

Substrate specificity

Fatty acid oxidizing activity with various fatty acids was examined. With α -linolenic acid, oleic acid or arachidonic acid, little activity could be detected at pH 8.5 while the highest activity (13.5 nkat/ml) was observed at pH 8.5 with linoleic acid. At pH 6.5, slightly higher activity (2.8 nkat/ml) could be observed with α -linolenic acid than that with linoleic acid (2.5 nkat/ml) observed at same pH. With arachidonic acid, only a little activity was observed. No activity could be detected with oleic acid, which has no (1Z,4Z)-pentadiene moiety. As a result, this activity has a rather strict substrate specificity, and can oxidize only polyunsaturated fatty acid containing a (1Z,4Z)-pentadiene moiety.

Inhibitor studies

Effect of various chemicals on fatty acid oxidizing activity was examined. Partially purified protein was preincubated with the inhibitor for at least three min before starting the reaction by adding linoleic acid. Table II shows that the activity was strongly inhibited by the lipophilic antioxidants, nordihydroguaiaretic acid (NDGA) and phenidone (1-phenyl-3-pyrazolidone). However, with chelating reagents, ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPAC), the activity was little inhibited.

Table II. Effect of various inhibitors on fatty acid oxidizing activity.

Inhibitor	Concentration (mM)	Relative remaining activity (%)
None		100
NDGA	0.1	0
Phenidone	0.1	0
EDTA	1.0	83.1
DETAPAC	1.0	78.2
NaN ₃	1.5	0
KCN	0.1	0
Tiron®	0.1	0
<i>o</i> -Phenanthroline	1.0	118.5

NDGA, nordihydroguaiaretic acid; phenidone, 1-phenyl-3-pyrazolidone; Tiron, 4,5-dihydroxy-1,3-benzene disulfonic acid.

On the contrary, NaN_3 and KCN showed significant inhibitory activity. These are known as typical inhibitors for heme-oxygenase. Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid) and *o*-phenanthroline are used as Fe^{3+} - and Fe^{2+} -chelating agents, respectively. The activity was inhibited by addition of Tiron, but not by *o*-phenanthroline. Thus, it was suggested that a metal ion, especially Fe^{3+} ion is involved in the activity.

These results shown above suggested that the activity is not associated with the general lipxygenase but with oxygenase activity of low molecular weight protein having a metal as a cofactor.

Fatty acid oxidizing activity associated with a heme protein

The purified activity was further examined with a HPLC equipped with a gel filtration column and a photodiode array detector. As shown in Fig. 3, only one symmetrical peak of the activity was detected. The enzymatic activity coeluted with the peak of A420, which suggested that this has a heme chromophore. Absorption spectrum of the purified protein revealed that it was very similar to a spectrum of a reduced form of bovine cytochrome *c* (Nakashima *et al.*, 1996). As shown in Fig. 4, the diagnostic features include the Soret band at 415 nm, α band at 551 nm and β band at 521 nm.

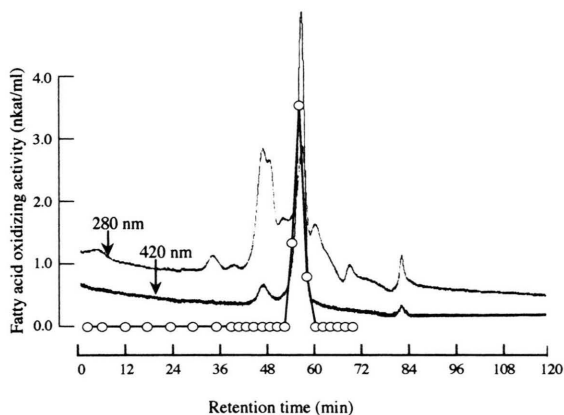


Fig. 3. A representative of elution profile of fatty acid oxidizing activity from TSK-gel G3000SW gel filtration column. Absorptions at 280 nm detecting proteins and at 420 nm detecting a Soret band of a heme were continuously recorded. Fractions eluted in every 2 minutes were collected, and the activity was determined (—○—).

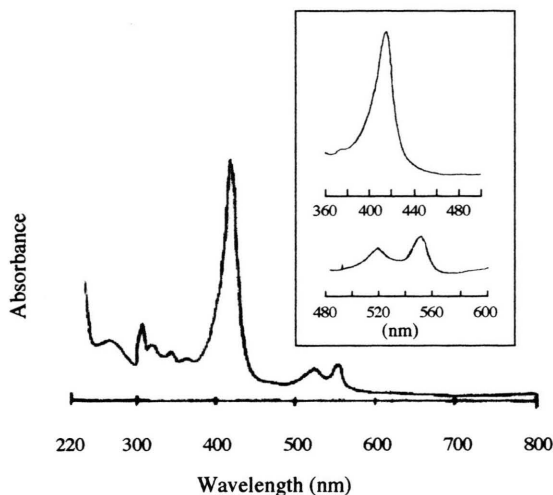


Fig. 4. Absorption spectrum of native-type of Phenyl-Toyopearl active fraction. Spectrum was monitored with UV-160A.

As described above, fatty acid oxidizing activity was suggested to be caused by a heme protein. In order to investigate the difference between the active protein purified here and the other common heme proteins and hematin, the oxidizing activities of them for linoleic acid were estimated under the same condition employed here. Hemoglobin, cytochrome *c* and hematin and ferrous and ferric sulfates, showed little oxidizing activity even if as much as 10 mg/ml of them were used (Chan *et al.*, 1978).

Thus, it is suggested that the active protein in *Porphyra* has different properties from these common hemes with regard to oxygenation capacity.

Product analyses

The reaction products were subjected to TLC analysis. It has been known that triphenylphosphine is converted into the triphenylphosphin oxide by reacting with hydroperoxides. When the reaction products were reacted with triphenylphosphine and subjected to TLC, the spot of triphenylphosphin oxide was detected, and simultaneously, the R_f value of the spot of the product slightly changed, which indicated that the products had hydroperoxide moieties. *N,N'*-Dimethyl-*p*-phenylenediamine-staining was also used to qualify the products. With this indicator, hydroperoxides immediately yield a pink spot, while hydroxides ex-

hibit a pink or green-red color after mild charring. The products exhibited one spot, that immediately yield a pink spot after spraying the indicator (Dussault and Lee, 1995). This spot disappeared by treatment of the products with triphenylphosphine before TLC. On the other hand, one major peak on a reverse-phase HPLC of the methyl esterified products was analyzed by LC/MS. The mass spectrum of the methyl esterified product is shown in Fig. 5: m/z 326 (M^+), 310 ($M-16$), 292 ($M-34$; loss of hydrogen peroxide), 239 ($M-87$; loss of $(CH_2)_4-CH_3$ and O), 178 ($CO(CH_2)_7-CH=CH-CH=CH$), 153 ($CO(CH_2)_7-CH=CH_2$). This finding, coupled with the TLC data which indicated the presence of hydroperoxide, established that the main product was 13-hydroperoxy-9,11-octadecadienoate.

Furthermore, methyl esterified products were reduced with triphenylphosphine, and analyzed

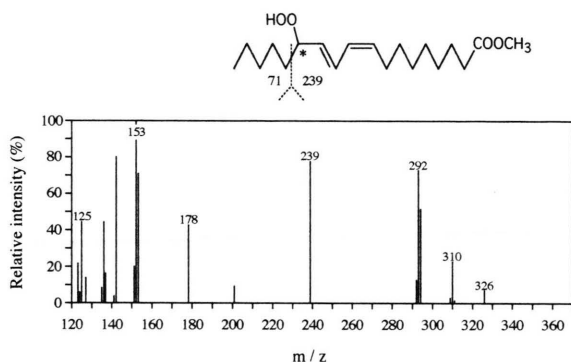


Fig. 5. Mass spectrum of the methyl esterified product.

with a straight phase HPLC. As shown in Fig. 6, two major peaks (compounds A_1 and B_1) and two minor peaks (compounds A_2 and B_2) were detected. By using authentic specimens, the major compounds A_1 and B_1 were identified as methyl 13-hydroxy-9(*Z*),11(*E*)-octadecadienoate and methyl 9-hydroxy-10(*E*),12(*Z*)-octadecadienoate, respectively. Compounds A_2 and B_2 were identified as their geometrical isomers, *i.e.*, methyl 13-hydroxy-9(*E*),11(*E*)-octadecadienoate and methyl

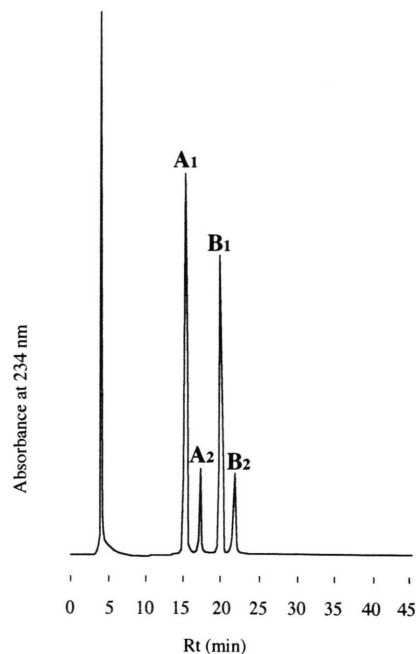


Fig. 6. HPLC analysis of positional and enantiomeric isomers of the reduced methyl-esterified product.

9-hydroxy-10(*E*),12(*E*)-octadecadienoate, respectively (Hatanaka *et al.*, 1984). The separated A_1 and B_1 were subjected to chiral HPLC on Chiralcel OB, respectively, and they were shown to be racemic (Kajiwaru *et al.*, 1980; Marzo *et al.*, 1993).

In conclusion, the fatty acid oxidizing activity detected in the fronds of this marine alga can be mostly ascribed to a heme protein. Lipoyxygenase does not essentially contribute to the activity. Thus, deterioration of the fronds during food-processing, *i.e.*, oxidation of lipids and formation of oily and beany flavor, can be also attributed to this heme protein.

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