C6-Aldehyde Formation by Fatty Acid Hydroperoxide Lyase in the Brown Alga Laminaria angustata

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Some marine algae can form volatile aldehydes such as n-hexanal, hexenals, and nonenals. In higher plants it is well established that these short-chain aldehydes are formed from C18 fatty acids via actions of lipoxygenase and fatty acid hydroperoxide lyase, however, the biosynthetic pathway in marine algae has not been fully established yet. A brown alga, Laminaria angustata, forms relatively higher amounts of C6- and C9-aldehydes. When linoleic acid was added to a homogenate prepared from the fronds of this algae, formation of n-hexanal was observed. When glutathione peroxidase was added to the reaction mixture concomitant with glutathione, the formation of n-hexanal from linoleic acid was inhibited, and oxygenated fatty acids accumulated. By chemical analyses one of the major oxygenated fatty acids was shown to be (S)-13-hydroxy-(Z, E)-9, 11-octadecadienoic acid. Therefore, it is assumed that n-hexanal is formed from linoleic acid via a sequential action of lipoxygenase and fatty acid hydroperoxide lyase (HPL), by an almost similar pathway as the counterpart found in higher plants. HPL partially purified from the fronds has a rather strict substrate specificity, and only 13-hydroperoxide of linoleic acid, and 15-hydroperoxide of arachidonic acid are the essentially suitable substrates for the enzyme. By surveying various species of marine algae including Phaeophyta, Rhodophyta and Chlorophyta it was shown that almost all the marine algae have HPL activity. Thus, a wide distribution of the enzyme is expected.

Key words: Laminaria angustata, n-Hexanal, Fatty Acid Hydroperoxide Lyase

Introduction

In higher plants, short chain aldehydes having six or nine carbon atoms are known to be important flavor compounds, and also known to play a physiological role in response to wounding and pest attack (Howe and Schilmiller, 2002). They are formed from unsaturated fatty acids, such as linoleic and linolenic acids, through an activity of lipoxygenase (LOX) that catalyzes dioxygenation of the fatty acid containing 1Z, 4Z-pentadiene structure to result in hydroperoxy unsaturated fatty acids. Subsequently, fatty acid hydroperoxide lyase (HPL) cleaves the fatty acid hydroperoxides to form aldehydes and oxo-acids (Blée, 1998). Saturated aldehyde (n-hexanal) and unsaturated aldehydes (hexenal, nonenal, and nonadienal) are known to be formed in this enzymatic system (Gardner, 1991; Blée, 1998). Depending on the properties of lipoxygenase, 13- or 9-hydroperoxides (13- or 9-HPO) can be formed, however, in most of higher plants, HPL prefers to act on 13HPO. However, in some plants, such as cucumber or melon, HPL can act on both HPOs, and it prefers the (S)-isomer (Matsui, 1998; Noordermeer et al., 2001). In essential oils obtained from marine algae, short-chain aldehydes can be found as well as long-chain fatty aldehydes, such as (Z, Z, Z)-8, 11,14-heptadecatrienal (Kajiwara et al., 1993, 1996). While the biogeneration of a long-chain aldehyde from fatty acids in marine algae such as linolenic acid has been established to be carried out via α-oxidation activity through formation of 2-hydroperoxy fatty acid as an intermediate (Kajiwara et al., 1993, 1996; Akakabe et al., 1999), little study the on biosynthetic pathway of short chain aldehydes has been done with this diverse group of marine species. In this study, we at-
tempted to characterize an enzymatic system forming a short-chain aldehydes from linoleic acid in a brown alga, *Laminaria angustata* by elucidating chemical structures of the intermediate. Also, HPL was partially purified from the algae, and some properties were studied. In addition, we showed the wide distribution of the HPL activity in marine algae to form *n*-hexanal from (S)-13-hydroperoxy-(Z, E)-9, 11-octadecadienoic acid.

**Materials and Methods**

**Plant material**

*L. angustata* was harvested at Charatsunai beach, Muroran, Hokkaido, Japan. The marine alga listed in Table II were collected at various places as indicated. They were kept at 4 °C during delivery to our laboratory (within 2 days). The fronds were frozen in liquid nitrogen, crushed into small pieces, and stored at −80°C until use.

**Chemical compounds and substrate preparation**

Linoleic acid, linolenic acid and arachidonic acid were purchased from Sigma (99% pure, St. Louis, MO, U.S.A.). Glutathione peroxidase (GPx) and reduced glutathione (GSH) were also purchased from Sigma Chemical Co. (Z)-3-Hexenal, (E)-2-hexenal, *n*-hexanal, (E, Z)-2, 6-nonadienal, (E)-2-nonenal (95% pure) were purchased from (Wako Pure Chemical Industries, Ltd., Japan). (Z, E)-3, 6-Nonadienal and (Z)-3-nonenal were prepared in our laboratory (> 95% pure) according to the protocol described elsewhere (Kajiwara et al., 1975). All the organic solvents were from Kanto Chemical (Japan), and were purified by distillation. (S)-9-hydroperoxy-(E, Z)-10, 12-octadecadienoic acid [9(S)HPOD] and (S)-9-hydroperoxy-(E, Z)-10, 12, 15-octadecatrienoic acid [9(S)HPOT] were prepared by using tomato lipoxygenase (Zamora et al., 1987) and (S)-13-hydroperoxy-(Z, E)-9, 11-octadecadienoic acid [13(S)HPOD], (S)-13-hydroperoxy-(Z, E)-9, 11, 15-octadecatrienoic acid [13(S)HPOT] and (S)-15-hydroperoxy-(Z, Z, E)-5, 8, 11, 13-icosatetraenoic acid [15(S)HPITE] were prepared by using soybean lipoxygenase-1 (Axelrod et al., 1981; Matsui et al., 1989). (S)-12-hydroperoxy-(Z, E, Z)-5, 8, 10, 14-icosatetraenoic acid [12(S)HPITE] was prepared by using purified porcine leukocyte arachidonate 12-lipoxygenase expressed with *E. coli* (Yokoyama et al., 1986; Suzuki et al., 1994). They were purified by SiO2 column chromatography using *n*-hexane/diethyl ether (10−25%) as an elution solvent. 12(S)HPITE was purified by preparative TLC using *n*-hexane/diethyl ether/acetic acid (1/1/0.01, v/v) as the developing solvent. The geometrical and positional purities of 9(S)HPOD, 9(S)HPOT, 13(S)HPOD and 13(S)HPOT were determined with a straight phase HPLC (Hatanaka et al., 1992). From the HPLC analysis, these products were confirmed to be more than 90% pure. The structures of these hydroperoxides were confirmed as described (Boonprab et al., submitted).

**Enzyme reaction**

The fronds were crushed to fine powder with a mortar and pestle, subsequently with a Maxim homogenizer in liquid nitrogen, then transferred into a glass bottle containing three volumes of 0.1 M borate borax buffer, pH 9.0, containing 2% Polyclar VT (Wako Pure Chemical Industries, Ltd., Japan). The suspension was further homogenized by a Polytron mixer to break down polysaccharides, then filtrated through six layers of cheesecloth. The filtrate was centrifuged at 500 × g at 4°C for 15 min to remove cell debris. The supernatant was used as crude enzyme after adjustment of the pH to 6.9. To the chilled mixture of substrate (14 μl of 50 mM LA in ethanol) in 0.1 mM 2-(N-morpholino)ethanesulfonic acid-KOH (MES-KOH) buffer (pH 6.9) prepared with seawater (1 ml), 1 ml of the crude enzyme solution was added. The mixture was incubated in a water bath shaker at 4°C for 30 min, then, 8 μl of 1 mM *n*-heptanal (8 nmol) as the internal standard and 1% 2, 4-dinitrophenylhydrazine (2,4-DNPH) in 2% acetic acid in ethanol (1.4 ml) were added to stop the reaction. The hydrazone derivatives were extracted with hexane, washed with saturated NaCl solution, and then, hexane was removed to yield yellow powder. The powder was dissolved in 50 μl of ether, and fractionated with a preparative TLC (Merck, silica gel 60 F-254, 10 × 20 cm, ethyl acetate/hexane, 2/1). The band at Rf 0.6−1.0 was scraped off and extracted with distilled ether. After filtration with a hydrophobic filter unit (DISMIC-3JP-disposable syringe filter, 0.5 μm), ether was removed, and the resultant residue was dis-
solved in 50 µl CH₃CN for reversed phase HPLC analysis on Zorbax SB C-18 column (4.6 mm i. d. × 250 mm, Hewlett Packard U. S. A.), with a solvent system of CH₃CN/H₂O/THF 66/33/1 (v/v) at a flow rate 1 ml/min by detecting absorbance (A) at 350 nm.

Hydroxy-linoleic acid analysis

Buffer (0.1 M MES-KOH (pH 6.9 in seawater, 5 ml)) containing 3500 nmol of linoleic acid, 6 mM GSH, 2 units/ml GPx was chilled (1 unit of GPx will catalyze the oxidation by H₂O₂ of 1.0 µmol of reduced glutathione to oxidized glutathione per min at pH 7.0 at 25°C), and 1 ml of the crude enzyme solution was added. The reaction was proceeded at 4°C for 30 min. The oxygenated fatty acids were extracted with Sep-Pak C₁₈ cartridge with eluting with methanol. Methanol was removed and the residue was dissolved in diethyl ether, and washed by saturated NaCl. Hydroxyoctadienoic acid(s) (HOD(s)) were purified with preparative TLC (silica gel 60 F-254, 10×20 cm, hexane/diethyl ether/acetic acid 1/1/0.001 v/v). The band at Rf 0.13—0.2 was scraped off, and HOD(s) were extracted with diethyl ether. The solvent was changed into ethanol, then, the concentration was estimated by reading A at 234 nm (23,200 M⁻¹ cm⁻¹ (Graff et al., 1990)). Absolute configuration and enantiomeric excess were determined by Chiralcel OD-H (4.6 mm i. d. × 250 mm, Daicel Chemical IND., LTD.) with photodiode array detector. Elution was carried out with n-hexane/2-propanol (98/2, v/v) at the flow rate of 1 ml/min.

Purification of fatty acid hydroperoxide lyase

L. angustata (25 g. fr. wt.) was washed, cut and homogenized with 2 volumes of ice-cold buffer (10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100) in a mortar. Polyclar VT (2%, w/v) was added just before homogenization. After filtration through four layers of cheesecloth, the filtrate was centrifuged at 500×g for 3 min at 4°C to remove the debris and then centrifuged at 100,000×g for 60 min at 4°C. To remove the sticky polymers of polysaccharides, the supernatant was filtered through 80-µm nylon mesh. The solution was diluted with 3 volumes of distilled water containing 0.1% (w/v) Triton X-100 and applied to a QA-Cellulofine Q-500 column (18 mm i. d. × 88 mm, Seikagaku-Kogyo Co., Japan) equilibrated with 10 mM tris(hydroxymethyl)aminomethane (Tris-HCl) buffer (pH 8.0) containing 0.1% Triton X-100. It was washed with 120 ml of 10 mM Tris-HCl buffer, then the HPL activity was eluted with 80 ml of a linear gradient of NaCl (0–1.0 M) formed with the same buffer. Every 5.0 ml per tube was collected. The protein content was quantified by the modified method of Lowry (Dulley and Grieve, 1975) with bovine serum albumin as a standard.

Enzymatic generation of aldehyde in marine algae

The marine alga (5 g. fr. wt.) was powdered in a mortar under liquid N₂. After evaporation of N₂, the tissue was added to five volumes of 0.1 M sodium phosphate buffer (pH 6.0) and was homogenized with a Polytron mixer. The homogenate was filtrated through four layers of cheesecloth, and the filtrate was used as the crude enzyme solution. The reaction was carried out with 3 ml of the enzyme solution in the presence of 1.0 µmol of 13HPOD at 5°C for 80 min. Aldehydes formed during the incubation were analyzed quantitatively by HPLC as their 2,4-dinitrophenylhydrazone derivatives. HPLC analyses were performed on a Purospher RP-18 column (4.6 mm i. d. × 250 mm, KANTO Chemical co., inc). The products were eluted by using a solvent system of acetonitrile/water/tetrahydrofuran (66/33/1, v/v). The temperature of column was 25°C and the flow rate 1.0 ml/min. Detection was performed at 350 nm.

Results and Discussion

Aldehyde formation from linoleic acid

When linoleic acid was added to the crude enzyme solution increase in the amount of n-hexanal only could be found as shown in Fig. 1. Although a higher amount of (E)-2-hexenal could be found even without addition of the fatty acid, no increase after addition of linoleic acid could be seen. No increase in the amount of the other possible products, namely, nonenals could be seen as well. It is suggested that linoleic acid could be converted to 13-hydroperoxyoctadecadienoic acid in the enzyme solution, then, it was further cleaved to form n-hexanal. C9-aldehydes were hardly formed from linoleic acid although (E)-2-nonenal is one of the
Fig. 1. Short chain aldehyde formation from *L. angustata*. Reaction was performed by incubation of the crude enzyme without (□) and with (■) linoleic acid (C18:2, n-6) at 4°C 30 min. All aldehydes were analyzed in dinitrophenylhydrazone derivative form on reverse HPLC and quantified the amount by internal standard method using n-heptanal as internal standard (see Materials and Methods). The numbering indicates aldehyde compounds, (Z)-3-hexenal (1), (E)-2-hexenal (2), n-hexanal (3), (Z, Z)-3, 6-nonadienal (4), (E, Z)-2, 6-nonadienal (5), (Z)-3-nonenal (6) and (E)-2-nonenal (7).

Hydroxyoctadecadienoic acid as the intermediate of n-hexanal formation

It is well established that addition of GSH with GPx can trap fatty acid hydroperoxides formed as intermediates in an oxylipin pathway (Brodowsky *et al.*, 1992; Hamberg *et al.*, 1986, 1998; Hamberg and Gerwick, 1993; Hombeck *et al.*, 1999). Hydroxyoctadecadienoic acids trapped with this system were partially purified by preparative TLC and subjected to straight phase HPLC analyses. As shown in Fig. 2A, two peaks could be detected by monitoring the absorption at 234 nm. Both the compounds showed a peak at around 234 nm. By comparing their retention times with authentic compounds prepared from soybean or tomato lipoygenase, respectively, they could be identified as 13-hydroxy-(Z, E)-9, 11-octadecadienoic acid (13HOD), and 9-hydroxy-(E, Z)-10, 12-octadecadienoic acid (9HOD), according to their elution order (Hatanaka *et al.*, 1984). This is further confirmed by co-injection of an authentic standard obtained from autoxidation of linoleic acid (Fig. 2B). From this analysis, it was also suggested that the two small peaks eluted at 15.96, and 21.13 min.

![Graph showing aldehyde formation](image-url)

**Fig. 2.** Structural analysis of enzymatic HODE isomers from *L. angustata* 
(A) by co-injection (B) with known isomers from autoxidation of linoleic acid (C18:2(n-6)), (Hatanaka *et al.*, 1984) on straight phase HPLC using Zorbax SIL (0.45 x 25 mm, Du Pont) with solvent, n-hexane:2-propanol; 98:12 (v/v), flow rate 1 ml/min and photodiode array detector 234 nm. The known isomers are indicated as 13(Z,E): 13-hydroxy-(Z)-9, 11-octadecadienoic acid, 13(E,E): 13-hydroxy-(E)-9, 11-octadecadienoic acid, 9(E,Z): 9-hydroxy-(E, Z)-10, 12-octadecadienoic acid and 9(E,E): 9-hydroxy-(E, E)-10, 12-octadecadienoic acid. Traces are indicated as enzymatic-algal-HODEs (a), HODE isomers from C18:2, n-6 autoxidation (b), HODE isomers from C18:2, n-6 autoxidation/ enzymatic-algal-HODEs (c). Enzyme reaction was established using the condition as mentioned in Materials and Methods.
were geometrical isomers of 13-hydroxy-(E, E)-9, 11-octadecadienoic acid and 9-hydroxy-(E, E)-10, 12-octadecadienoic acid, respectively. The ratio of 13-hydroperoxy-(Z, E)-9, 11-octadecadienoic acid (13HPOD) to 9-hydroperoxy-(E, Z)-10, 12-octadecadienoic acid (9HPOD) was estimated to be 89:11. The preferential formation of 13HPOD from linoleic acid has been shown with *L. sinclairii*, *L. setchellii* or *L. saccharina* (Gerwick et al. 1993), or with *Porphyra* sp. (Kajiwara et al. 2000). On the other hand, at least in some green algae formation of 9HPOD is preferred, *e.g.*, *in Ulva lactuca*, the ratio was shown to be 14:86 (Kuo et al. 1997) and in *Enteromorpha intestinalis* it is 34:66 (Kuo et al. 1996).

In order to confirm the stereochemistry of the oxygenation, HOD isolated from the reaction mixture was methylated and subjected to chiral phase HPLC analyses. As shown in Fig. 3B, both the enantiomers of 13HOD prepared by autooxidation of linoleic acid could be completely separated by the HPLC condition employed here. Using 13(S)HOD prepared from soybean lipoxygenase-1, it was confirmed that the peak eluted earlier corresponded to 13(R)-isomer, and the later peak to 13(S)-isomer. Separation of both the enantiomers of 9HOD could be also confirmed, and, by using 9(S)HOD prepared with tomato lipoxygenase, the elution order was confirmed to be 9(R)HOD first, then 9(S)HOD. With the chiral HPLC system, methyl-HOD prepared by the algal enzyme was separated as shown in Fig. 3A. The main peak corresponded to 13(S)HOD, and the peaks before and after the main peak were identified as 9(R)HOD and 9(S)HOD, respectively. No peak for 13(R)HOD could be detected. Thus, the stereo-specificity of 13(S)HOD could be estimated as more than 99% enantiomeric excess (e.e.). This analysis also showed that the ratio of the (R)-isomer and (S)-isomer of 9HOD was 2:1 (30% e.e.). Gerwick et al. (1993) also showed that the predominant configuration of 13HPOD formed from *L. sinclairii*, *L. setchellii* or *L. saccharina* was (S).

In summary, *L. angustata* forms 13(S)HPOD from linoleic acid in a stereo-specific manner, and the hydroperoxide can be further converted to n-hexanal. This enzymatic pathway is almost the same as its counterpart in higher plants (Gardner, 1991; Blée, 1998). Although this alga can form 9-HPOD, both the amounts and the stereo-specificity are low. Thus, these may hardly serve as substrates for HPL to form nonenals.

**Enzymatic properties of HPL**

In order to purify the activity accountable for the formation of n-hexanal from hydroperoxides, a crude enzyme solution was prepared in the presence of 0.1% Triton X-100. The HPL activity to form n-hexanal from 13(S)HPOD could be recovered in a solubilized fraction after centrifugation of the crude enzyme solution at 100,000 × g for 60 min. Further purification was achieved by ion-exchange chromatography on a QA-Cellulofine Q-500 column. HPL activity was eluted between 0.35 and 0.53 M NaCl. By this chromatography, HPL activity was purified 4.8 fold with a specific activity of 213.8 pmol/min×mg. Using partial purified HPL 13(S)HPOD was incubated at 4°C 80 min and the product formed analyzed. As shown in Fig. 4, only n-hexanal could be found as a product. Substrate specificity of the partially purified HPL was...
n-Hexanal forming activity in marine algae

In order to know the distribution of HPL activity in marine algae, the activity to form n-hexanal from 13(S)HPOD was determined with various kinds of algae (Table II). Apparently, all algae investigated here showed this activity. Highest activity could be found with *Alaria crassifolia* collected in January, followed by *Chorda filum*. In some cases, differences of the activities depending on the sampling date, or sampling location could be seen, which suggested that the activity might be developmentally regulated as in higher plants (Blée, 1998). Broadly speaking, higher activity can be found with Phaeophyta (brown algae) than with Rhodophyta (red algae) or Chlorophyta (green algae). This demonstrates that the activity to form n-hexanal from 13(S)HPOD is ubiquitously distributed in marine algae.

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Table II. Distribution of $n$-hexanal-forming activity in marine algae located in Japan and Thailand.

<table>
<thead>
<tr>
<th>Division/Family</th>
<th>Genus/Species*</th>
<th>Location/Collecting time(month/year)</th>
<th>$n$-Hexanal formation (nmol/g. fr. wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phaeophyta</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alariaceae</td>
<td><em>Alaria crassifolia</em></td>
<td>Charatsunai, Muroran, Japan/ Jan. 1999</td>
<td>505.3</td>
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<tr>
<td>Chordaceae</td>
<td><em>Chorda filum</em></td>
<td>Aio, Yamaguchi, Japan/ Mar. 1999</td>
<td>436.8</td>
</tr>
<tr>
<td>Cutleriaceae</td>
<td><em>Cutleria cylindrica</em></td>
<td>Misaki, Iyo, Japan/ Apr. 1999</td>
<td>100.0</td>
</tr>
<tr>
<td>Dictyotaceae</td>
<td><em>Dictyopteris undalata</em></td>
<td>Murotsu, Shimonoseki, Japan/ Apr. 1999</td>
<td>52.6</td>
</tr>
<tr>
<td>Fucaceae</td>
<td><em>Fucus evanescens</em></td>
<td>Charatsunai, Muroran, Japan/ Nov. 1999</td>
<td>52.6</td>
</tr>
<tr>
<td>Ishigeaceae</td>
<td><em>Ishige sinicola</em></td>
<td>Aio, Yamaguchi, Japan/ Dec. 1999</td>
<td>247.4</td>
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<td>Laminariaceae</td>
<td><em>Laminaria angustata</em></td>
<td>Charatsunai, Muroran, Hokkaido, Japan/Mar. 1999</td>
<td>194.0</td>
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<td>Sargassaceae</td>
<td><em>Turbinaria conoides</em></td>
<td>Mannai island, Rayong, Thailand/ Apr. 1999</td>
<td>84.2</td>
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<tr>
<td>Scytosiphonaceae</td>
<td><em>Colpomenia bullosa</em></td>
<td>Murotsu, Shimonoseki, Japan/ Apr. 1999</td>
<td>186.8</td>
</tr>
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<td><strong>Rhodophyta</strong></td>
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<td>Bangiaceae</td>
<td><em>Bangia fuscopurpurea</em></td>
<td>Aio, Yamaguchi, Japan/ Mar. 1999</td>
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<td>173.7</td>
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<td>Endocladiaceae</td>
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<td>Aio, Yamaguchi, Japan/ Apr. 1999</td>
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<td>Gigartinaceae</td>
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<td>Murotsu, Shimonoseki, Japan/ Apr. 1999</td>
<td>36.8</td>
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<td>Gracilariaceae</td>
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<td>Ulvaceae</td>
<td><em>Enteromorpha prolifera</em></td>
<td>Aio, Yamaguchi, Japan/ Apr. 1999</td>
<td>263.2</td>
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</table>

* More algae have been assayed.


