

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 1*Z*, 4*Z*-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 (Kajiware *et al.*, 1993, 1996). While the biogenesis 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 (Kajiware *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 algae 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, 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, Z)-9, 11, 15-octadecatrienoic acid [13(S)HPOT] and (S)-15-hydroperoxy-(Z, 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, Z, E, Z)-5, 8, 10, 14-icosatetraenoic acid [12(S)HPITE] was prepared by using purified porcine leukocyte arachidonate 12-lipoxy-

genase 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 cheese-cloth. 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 M 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 R_f 0.6–1.0 was scraped off and extracted with distilled ether. After filtration with a hydrophobic filter unit (DIS-MIC-3JP-disposable syringe filter, 0.5 µm), ether was removed, and the resultant residue was dis-

solved in 50 μ l CH₃CN for reversed phase HPLC analysis on Zorbax SB C-18 column (4.6 mm *i. d.* $\times$ 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 μ mol 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 $\times$ 20 cm, hexane/diethyl ether/acetic acid 1/1/0.001 v/v). The band at R_f 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.* $\times$ 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 $\times$ g for 3 min at 4 °C to remove the debris and then centrifuged at 100,000 $\times$ g for 60 min at 4 °C. To remove the sticky polymers of polysaccharides, the supernatant was filtrated through 80- μ m 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.* $\times$ 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 μ mol 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.* $\times$ 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

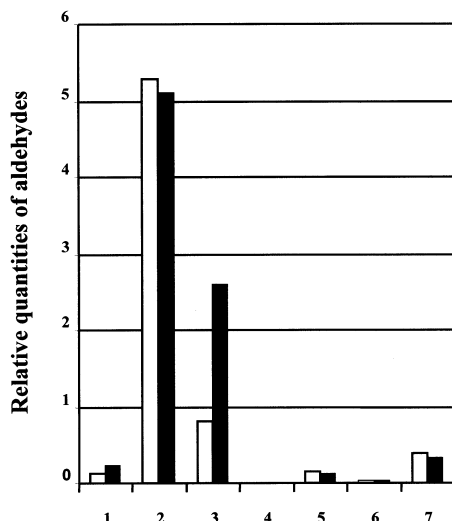


Fig. 1. Short chain aldehyde formation from *L. angustata*. Reaction was performed by incubation of the crude enzyme without (□) and with (■) linoleic acid (C18:2, *n*-6) at 4° C 30 min. All aldehydes were analyzed in dinitrophenylhydrazone derivative form on reverse HPLC and quantified the amount by 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).

major volatile compounds found in essential oil prepared from *L. angustata* (Kajiwara *et al.*, 1993, 1996). The low activity to form C9-aldehydes from linoleic acid may be due to the low activity to form the 9-hydroperoxide of linoleic acid, or to the low activity of the enzyme that can cleave the hydroperoxide, or both. Very recently, we showed that this C9-aldehyde is formed predominantly from arachidonic acid via formation of 12-hydroperoxide of the fatty acid (Boonprab *et al.*, submitted).

Hydroxyoctadecadienoic acid as the intermediate of n-hexenal 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 lipoxygenase, 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 coinjection 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

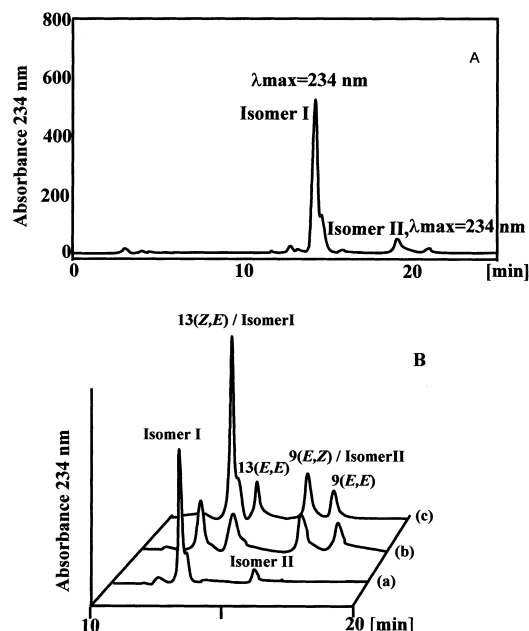


Fig. 2. Structural analysis of enzymatic HODE isomers from *L. angustata* (A) by coinjection (B) with known isomers from autoxidation of linoleic acid (C18:2(*n*-6)), (Hatanaka *et al.*, 1984) on straight phase HPLC using Zorbax SIL (0.45 × 25 mm, Du Pont) with solvent, *n*-hexane:2-propanol; 988:12 (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*, *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. (Kajiwarra *et al.*, 2000). On the other hand, at least in some green algae formation of 9HPOD is preferred, *i. 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 $100,000 \times g$ 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 $\times$ 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

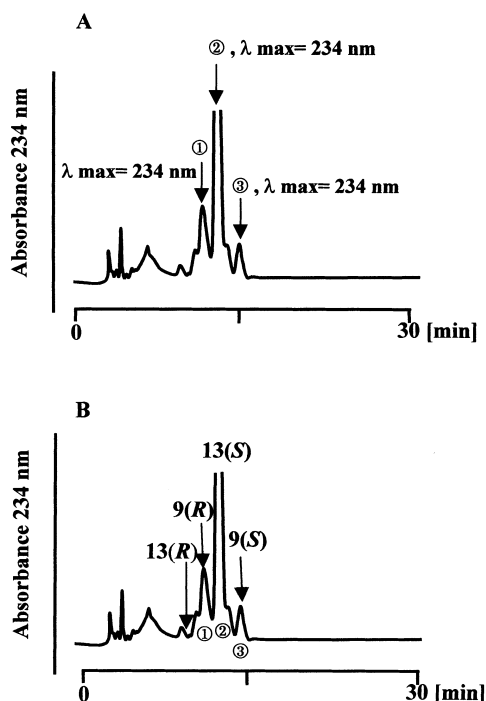


Fig. 3. Stereoisomer analysis (B) of methyl-HODEs from *L. angustata* (A) by chiral phase HPLC (see Materials and Methods).

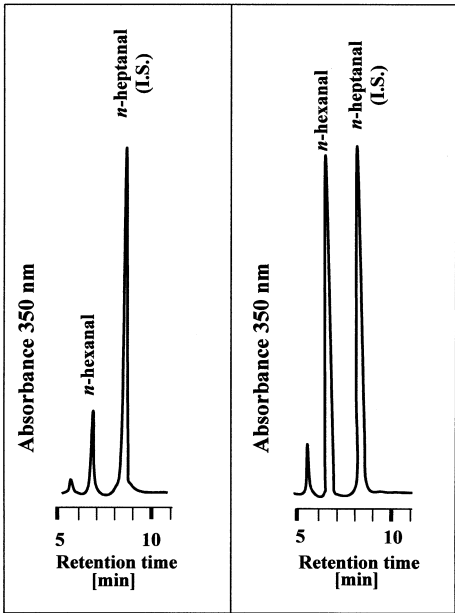


Fig. 4. *n*-Hexanal formation from the partially purified HPL enzyme incubated without (A) and with (B) 13(S)HPOD. The aldehyde formed was derivatized as 2,4-dinitrophenylhydrazone isomer and analyzed by reverse phase HPLC (see Materials and Methods).

determined by using 9(S)HPOD, 9(S)HPOT, 12(S)HPITE, 13(S)HPOD, 13(S)HPOT and 15(S)HPITE (Table I). Among these, 13(S)HPOD was the best substrate followed by 15(S)HPITE that showed half of the activity observed. 13(S)HPOD, 9(S)HPOD, 9(S)HPOT, 12(S)HPITE and 13(S)HPOT were shown to be a poor substrate. Obviously the strict substrate specificity is due to recognition of the hydroperoxy group at the ω-6 position of the fatty acid hydroperoxide. In summary, this study indicated that the C6-aldehyde-forming branch in the brown alga had almost the same properties as that in higher plants. This is in a marked contrast to the C9-aldehyde forming branch, where arachidonic acid instead of linoleic acid are preferentially used as substrate (Boonprab *et al.*, submitted).

Table I. Substrate specificity of partially purified fatty acid hydroperoxide lyase from *L. angustata*.

Substrate*	Product	Relative activity**
9(S)-HPOD	(Z)-3-nonenal	0.0
9(S)-HPOT	(Z, Z)-3, 6-nonadienal	0.0
12(S)-HPITE	(Z)-3-nonenal	0.0
13(S)-HPOD	<i>n</i> -hexanal	1.0
13(S)-HPOT	(Z)-3-hexenal	0.0
15(S)-HPITE	<i>n</i> -hexanal	0.46

* 9(S)-HPOD: 9(S)-hydroperoxy octadecadienoic acid
9(S)-HPOT: 9(S)-hydroperoxy octadecatrienoic acid
12(S)-HPITE: 12(S)-hydroperoxy icosatetraenoic acid
13(S)-HPOD: 13(S)-hydroperoxy octadecadienoic acid
13(S)-HPOT: 13(S)-hydroperoxy octadecatrienoic acid
15(S)-HPITE: 15(S)-hydroperoxy icosatetraenoic acid
** Relative activity expressed relative to the amount of product from each substrate to the amount of *n*-hexanal from 13(S)-HPOD.

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.

Division/ Family	Genus/Species*	Location/Collecting time(month/year)	<i>n</i> -Hexanal formation (nmol/g. fr. wt.)
Phaeophyta			
Alariaceae	<i>Alaria crassifolia</i>	Charatsunai, Muroran, Japan/ Jan. 1999	505.3
Chordaceae	<i>Chorda filum</i>	Aio, Yamaguchi, Japan/ Mar. 1999	436.8
Cutleriaceae	<i>Cutleria cylindrica</i>	Misaki, Iyo, Japan/ Apr. 1999	100.0
Dictyotaceae	<i>Dictyopteris undalata</i>	Murotsu, Shimonoseki, Japan/ Apr. 1999	52.6
Fucaceae	<i>Fucus evanescens</i>	Charatsunai, Muroran, Japan/ Nov. 1999	52.6
Ishigeaceae	<i>Ishige sinicola</i>	Aio, Yamaguchi, Japan/ Dec. 1999	247.4
Laminariaceae	<i>Laminaria angustata</i>	Charatsunai, Muroran, Hokkaido, Japan/Mar. 1999	194.0
Sargassaceae	<i>Turbinaria conoides</i>	Mannai island, Rayong, Thailand/ Apr. 1999	84.2
Scytosiphonaceae	<i>Colpomenia bulbosa</i>	Murotsu, Shimonoseki, Japan/ Apr. 1999	186.8
Rhodophyta			
Bangiaceae	<i>Bangia fuscopurpurea</i>	Aio, Yamaguchi, Japan/ Mar. 1999	26.3
Crytonemiaceae	<i>Grateloupia filicina</i>	Aio, Yamaguchi, Japan/ Mar. 1999	43.2
Corallinaceae	<i>Zostera marina</i>	Aio, Yamaguchi, Japan/ Mar. 1999	173.7
Endocladaceae	<i>Gloiopeltis tenax</i>	Aio, Yamaguchi, Japan/ Apr. 1999	57.9
Gigartinales	<i>Chondrus ocellatus</i>	Murotsu, Shimonoseki, Japan/ Apr. 1999	36.8
Gracilariaceae	<i>Gracilaria verrucosa</i>	Aio, Yamaguchi, Japan/ Apr. 1999	81.6
Rhodomelaceae	<i>Rhodomela gracilis</i>	Charatsunai, Muroran, Japan/ Nov. 1999	20.0
Schizymeniaceae	<i>Schizymenia dubyi</i>	Aio, Yamaguchi, Japan/ Apr. 1999	47.4
Solieriaceae	<i>Solieria robusta</i>	Aio, Yamaguchi, Japan/ Apr. 1999	31.6
Chlorophyta			
Codiaceae	<i>Codium fragile</i>	Aio, Yamaguchi, Japan/ Apr. 1999	147.4
Monostromaceae	<i>Monostroma nitidum</i>	Aio, Yamaguchi, Japan/ Apr. 1999	110.5
Ulvaceae	<i>Enteromorpha prolifera</i>	Aio, Yamaguchi, Japan/ Apr. 1999	263.2

* More algae have been assayed.

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