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2,4-Decadienals are Produced via (*R*)-11-HPITE from Arachidonic Acid in Marine Green Alga *Ulva conglobata*

Yoshihiko Akakabe,* Kenji Matsui and Tadahiko Kajiwara

Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan

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Abstract—Marine green alga *Ulva conglobata* was investigated for the biogeneration of oxygenated products from exogenously added arachidonic acid (ARA). A crude enzyme from the alga afforded the detectable amount of a hydroperoxyicosatetraenoic acid (HPITE), which was identified as (*R*)-11-HPITE by HPLC and GC–MS. Headspace–SPME method indicated that ARA was selectively used to form 2,4-decadienals. These results showed that 2,4-decadienals are produced via (*R*)-11-HPITE from ARA exclusively.

Introduction

The aroma compounds produced by marine algae have been well characterized. Long-chain aldehydes such as pentadecanal, (*8Z*)-8-heptadecenal, (*8Z, 11Z*)-8,11-heptadecadienal, and (*8Z, 11Z, 14Z*)-8,11,14-heptadecatrienal are the most important flavor components among a variety of volatile compounds found in marine green alga *Ulva pertusa*. The biosynthesis of the aldehydes is due to α-oxidation by α-oxygenase of long-chain fatty acids into 2-hydroperoxy acids followed by decarboxylation. We indicated that long-chain saturated and unsaturated fatty acids such as palmitic, oleic, linoleic, and linolenic acid are 2-hydroperoxylated with a crude enzyme of *U. pertusa* to afford the corresponding (*R*)-2-hydroperoxy acids with enantiomeric excess (ee) of >99%.[1–3] In the essential oil of *Ulva conglobata*, short-chain aldehydes such as hexanal, (*2E*)-2-hexenal, (*3Z*)-3-nonenal, (*2E*)-2-nonenal, and (*2E, 6Z*)-2,6-nonadienal were found and identified by GC–MS. The biogeneration was due to oxygenation by lipoxygenases of linoleic or linolenic acid into regio- and stereo-specific hydroperoxides, followed by enzymatic cleavage by hydroperoxide lyases to produce the aldehydes. We reported that linoleic and linolenic acid are oxygenated with a crude enzyme of *Ulva conglobata* to produce (*R*)-9-hydroperoxyoctadecadienoic and (*R*)-9-hydroperoxyoctadecatrienoic acids with a high ee (>99%), respectively.[4] On the other hand, middle-chain aldehydes such as (*2E,4Z*)-2,4-decadienal and (*2E,4E*)-2,4-decadienal were also detected in the oil. Recently, 2,4-decadienals have been proposed to be formed via 11-hydroperoxyicosatetraenoic acid (11-HPITE) from arachidonic acid (ARA) in marine diatom *Thalassiosira rotula*.[5] However, the asymmetric oxygenation of lipoxygenase type in the diatom has not been fully investigated, and the absolute configuration of the hydroperoxides has remained unknown. Here the author describes for the biosynthesis of 2,4-decadienals via hydroxyeicosatetraenoic acids (HETEs) with a crude enzyme of *U. conglobata*.

Results and Discussion

Confirmation and regioseparation of HPITEs

When ARA was incubated with a crude enzyme of *U. conglobata*, one major peak of 9-anthrylmethyl ester was found in RP-HPLC eluted with acetonitrile–water (9/1). Furthermore, a portion of the extract was reduced with triphenylphosphine (PPh_3), the peak with the retention time (t_R) of 11.0 min disappeared whereas the peak with t_R of 11.5 min increased, indicating that the product has a hydroperoxy group (Fig. 1). These compounds were identified by comparison with the 9-anthrylmethyl ester of authentic standards of HITEs (Cayman Chemical Co Ltd.). Thus, incubation of ARA with a crude enzyme *U. conglobata* led to the conversion

*Corresponding author. Tel.: +81-83-933-5851; fax: +81-83-933-5820; e-mail: akakabe@agr.yamaguchi-u.ac.jp

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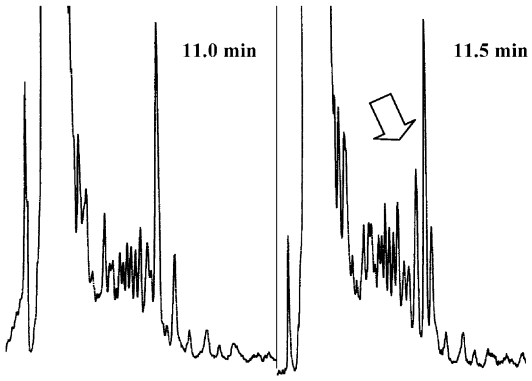

Figure 1. Confirmation of oxygenated products from a crude enzyme of *U. conglobata* by RP-HPLC.

of the exogenous fatty acid into mainly one HPITE. The RP-HPLC analysis eluted with acetonitrile–water (75/25) showed one major peak with t_R of 57.0 min (Fig. 2). The retention property of this peak was identical to that of racemic 11-HITE (Cayman Chemical Co Ltd.). On the other hand, the mass spectrum of the hydrogenated TMS derivative was characterized by a molecular ion (M^+) at m/z 414 and by two intense ions at m/z 229 and 287 (Fig. 3). The characteristic mass fragments of the derivative were consistent with the structure of the derivative of racemic 11-HITE. This HPITE obtained from the alga was characterized as 11-HPITE by means of RP-HPLC and GC–MS techniques. Furthermore, a trace amount of 15-HPITE in the alga was also suggested on the basis of GC–MS data.

Enantioseparation of HITEs

Comparison based on t_Rs and co-chromatography with authentic standards such as racemic 11-HITE with t_R (*R*) of 28.5 and t_R (*S*) of 31.5 min, (*S*)-11-HITE (Cayman Chemical Co Ltd.) revealed that the 11-HITE from the alga was constituted of the former peak (98.9%) and of the latter one (1.1%). Thus, the absolute configuration of the 11-HITE was (*R*)-form and the optical purity was estimated as 97.8% ee.

Detection of 2,4-decadienals by headspace-SPME

Quantification of 2,4-decadienals was performed by reference to dodecanal as an internal standard (IS). The aldehydes were expressed as mean ± SD of three samples. The area of the peaks on the ion chromatograms were calculated and compared to that observed for the IS. When ARA was added to the crude enzyme, (*2E,4Z*)-decadienal and (*2E,4E*)-decadienal were detected at concentration of 1.97 ± 0.15 nmol/g and 0.34 ± 0.07 nmol/g fresh weight (fw), respectively (Fig. 4). Other aldehydes were not confirmed by this GC–MS analysis. On the other hand, when the incubation was performed in the presence of trimethyl phosphite as a reducing agent of hydroperoxy group, the formation of aldehydes is effectively inhibited (0.44 ± 0.02 nmol/g fw), whereas the detectable amount of (*R*)-11-HITE was observed by NP (chiral)-HPLC analysis. Furthermore, with linoleic acid, the amounts of 2,4-decadienals were not significantly increased in this system.

The dioxygenated products of ARA by lipoxygenase isolated from hairy root cultures of *Solanum tuberosum* treated with a fungal elicitor were investigated, and 11-HPITE was the most abundant oxygenated product.[6] In whole homogenates from hydroid species, the homogenates from *Hydra oligactis* and *Halocordyle disticha* produced measurable amounts of a metabolite which was identified as 11-HITE.[7] Particularly, incubation of ARA with homogenates of *Hydra vulgaris* converted into mainly two metabolites. These were characterized as 11-HPITE and 11-HITE. NP (chiral)-HPLC analysis of 11-HITE revealed that this metabolite was composed mainly of the (*R*)-enantiomer.[8] In this paper, the following findings were obtained: (1) the crude enzyme with ARA showed a high preference for

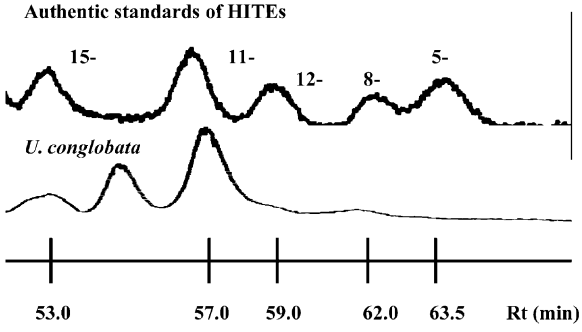

Figure 2. Regioseparation of HODE from *U. conglobata* by NP-HPLC. Rt, retention time.

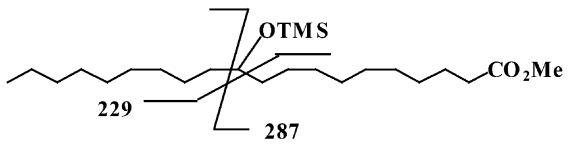

Figure 3.

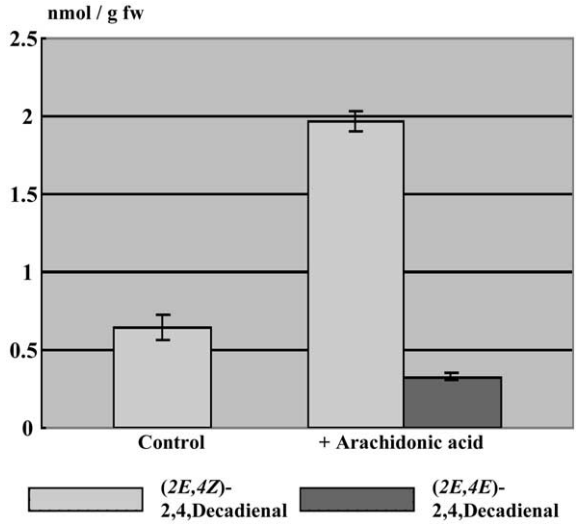

Figure 4. Enantioseparation of HODE from *U. conglobata* NP (chiral)-HPLC.

the formation of 11-HPITE by RP-HPLC and GC–MS; (2) NP (chiral)-HPLC analysis of the 11-HITE obtained from the extract showed the predominance of (*R*)-enantiomer, indicating that 11-HPITE was produced enzymatically; (3) SPME method showed that ARA was selectively used to form 2,4-decadienals and the formation of aldehydes could be inhibited with trimethyl phosphite. These results supported that 2,4-decadienals are produced via (*R*)-11-HPITE from ARA exclusively and strongly suggested the presence of a lipoxygenase and a hydroperoxide lyase in *U. conglobata*.

Experimental

Materials

The alga was collected in the intertidal zone of Hikoshima, Yamaguchi, Japan, in 2002 and was immediately frozen at −20 °C until being used.

Preparation of a crude enzyme of *U. conglobata*

The tissue (25 g, fresh weight) was homogenized with 0.1 M phosphate buffer (125 mL, pH 6.0) containing 0.1% Triton X-100 in a blender. After the homogenate was filtered, the filtrate was used for the enzyme assay.

Recovery of HPITEs

The crude enzyme was incubated with a substrate (0.5 μmol) at 0 °C for 10 min. Then $(NH_4)_2SO_4$, NaCl, and tetrahydrofuran were added and the mixture was centrifuged at 2000*g* for 10 min for separation of the organic layer. The layer was washed with brine and dried over anhydrous $MgSO_4$. The extract was concentrated under reduced pressure and the residue was diluted with *t*-butyl methyl ether. A portion of the solution was treated with 9-anthryldiazomethane at 0 °C for 10 min.

RP-HPLC analyses of HPITEs

For confirmation of HPITEs, the mixture was subjected to RP-HPLC with a Mightysil RP-18 GP Aqua column (5 μm, 4.5×250 mm) eluted with acetonitrile–water (9/1, 1.0 mL/min). The HPITEs were monitored with a fluorescence detector (excitation at 365 nm and emission at 412 nm). The 9-anthrylmethyl esters were also recorded after reduction to the hydroxy forms with PPh_3. Determination of the regioselectivity of HITEs was done with the Mightysil RP-18 GP Aqua column eluted with acetonitrile–water (75/25, 1.5 mL/min).

GC–MS analyses of derivatized HITEs

The extract was esterified with diazomethane (CH_2N_2) at 0 °C for 10 min and the resulting methyl ester was reduced with PPh_3 at 0 °C for 10 min. The hydroxy methyl ester was also analyzed by GC–MS with a DB-WAX (0.25 mm×60 m) after reduction of the double bonds with H_2/PtO_2 and silylation of the hydroxy group with bis(trimethylsilyl)trifluoroacetamide (BSTFA). The injector was set at 240 °C. The oven temperature was programmed from 120 to 210 °C at a rate of 10 °C/min. The carrier gas was helium.

NP (chiral)-HPLC analyses of HPITEs

For identification of the absolute configuration of HITEs, the 9-anthrylmethyl esters were done with a Chiralcel OD-H column (5 μm, 4.6×250 mm) eluted with hexane-2-propanol (96.5/3.5, 1.0 mL/min). The HITEs were monitored with a fluorescence detector (excitation at 365 nm and emission at 412 nm).

Headspace–SPME

An SPME holder (Supelco) was used to perform the experiments. A fused-silica fiber, coated with a 65 μm layer of polydimethylsiloxane-divinylbenzene (PDMS-DVB), was chosen to absorb the volatile components of the flows. For the headspace–SPME process, the crude enzyme (25 mL) was added in the flask. ARA (0.5 μmol) was administered to the flask and the mixture was stirred at rt. After stirred for 90 min, IS (dodecanal, 230 nmol) was added to the mixture. The fiber was exposed in the headspace of the stirred mixture at rt for 30 min and then it was removed from the headspace and introduced into the GC injector where the thermal desorption at 240 °C for 10 min was carried out. The volatile compounds were analyzed by GC–MS with the DB-WAX. The oven temperature was programmed from 50 to 230 °C at a rate of 10 °C/min.

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