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Enantioselective Formation of (*R*)-9-HPODE and (*R*)-9-HPOTrE in Marine Green Alga *Ulva Conglobata*

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Abstract—When linoleic and linolenic acid were incubated with a crude enzyme of marine green alga *Ulva conglobata*, the corresponding (*R*)-9-hydroperoxy-(10*E*, 12*Z*)-10, 12-octadecadienoic acid [(*R*)-9-HPODE] and (*R*)-9-hydroperoxy-(10*E*, 12*Z*, 15*Z*)-10, 12, 15-octadecatrienoic acid [(*R*)-9-HPOTrE] were formed with a high enantiomeric excess (>99%), respectively.

In the intertidal zone of Japan, a variety of seaweeds, green brown, and red algae are widely distributed. Particularly, marine green alga *Ulva* spp. are widely distributed in the temperate coastal waters throughout Japan and grows in spring to summer to form 'green tide'.

An essential oil, which was prepared from marine green alga *Ulva conglobata* by simultaneous distillation extraction, was confirmed the odor components by GC–MS. From this result, not only long-chain aldehydes such as pentadecanal, (8*Z*)-8-heptadecenal, and (8*Z*,11*Z*,14*Z*)-8,11,14-heptadecatrienal but also short-chain aldehydes such as hexanal, (2*E*)-2-hexenal, (3*Z*)-3-nonenal, (2*E*)-2-nonenal, and (2*E*,6*Z*)-2,6-nonadienal were identified by comparison with authentic samples. This result suggested that long-chain unsaturated fatty acids such as linoleic (LA) and linolenic acid (LNA) are oxidized to the corresponding 2-, 9-, and 13-hydroperoxides which subsequently are converted into C17, C9, and C6 aldehydes, respectively.

Recently, we reported that long-chain saturated and unsaturated fatty acids such as palmitic, oleic acid, LA, and LNA are 2-hydroperoxylated with a crude enzyme of marine green alga *Ulva pertusa* to afford the corresponding (*R*)-2-hydroperoxy acids with enantiomeric excess (ee) of >99%.[1,2] Furthermore, we found that not only green algae but also brown and red algae can 2-hydroperoxylate long-chain fatty acids.[3] However, the asymmetric oxygenation of lipoxygenase type in the algae have not been fully investigated, and the absolute configuration of the hydroperoxides has remained unknown.[4,5] In this paper, we describe highly enantioselective oxygenation of LA and LNA with a crude enzyme of *U. conglobata*.

The alga was collected in the intertidal zone of Hikoshima, Yamaguchi, Japan, in 2001 and was immediately frozen at −20 °C until being used. 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. The crude enzyme was incubated with a substrate (0.2 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 and the mixture was subjected to reversed-phase (RP)-HPLC with Mightysil RP-18 GP column (5 μm, 3.0 × 250 mm) eluted with acetonitrile −0.1 M $CH_3CO_2NH_4$ (9:1, 1 mL/min). The oxygenated products 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 alcohol with triphenylphosphine (PPh_3). With LA as a substrate, one major peak of 9-anthrylmethyl ester was

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found in RP-HPLC. Indeed, when a portion of the extract was reduced with PPh_3, the peak with the retention time (R_t) of 6.5 min disappeared whereas the peak with R_t of 7.5 min increased (Fig. 1). These compounds were identified by comparison with the 9-anthrylmethyl ester of autoxidation products of LA.

In the separate experiment, 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. Then the solution was analyzed by normal-phase (NP)-HPLC with a Mightysil Si 60 column (5 μm, 3.0 × 250 mm) eluted with hexane–2-propanol (98:2, 1 mL/min). The oxygenated products were monitored with a UV detector at 234 nm. The NP-HPLC showed one major peak with R_t of 3.5 min (Fig. 2). The retention property of this peak was identical to that of racemic 9-hydroxy-(*10E,12Z*).12-octadecadienoic acid (9-HODE) obtained by autoxidation of LA.

For identification of the absolute configuration of 9-HODE, the hydroxy methyl ester was done with a

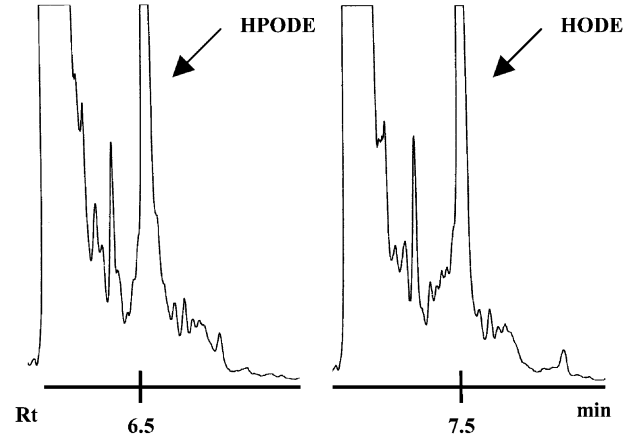

Figure 1. Confirmation of oxygenated products from a crude enzyme of *U. conglobata* by RP-HPLC.

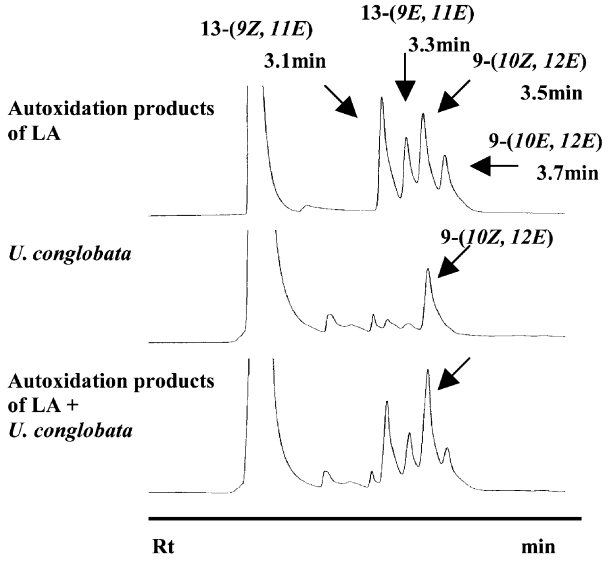

Figure 2. Regioseparation of HODE from *U. conglobata* by NP-HPLC.

Chiralcel OD-H column (5 μm, 4.6 × 250 mm) eluted with hexane–2-propanol (98:2, 1 mL/min). The hydroxy ester was monitored with a UV detector at 234 nm. After comparison with racemic 9-HODE obtained by autoxidation of LA and (*S*)-9-HODE (Cayman Chemical Co Ltd.), the absolute configuration of the product was predominantly (*R*)-9-HODE (>99% ee) as determined by NP(chiral)-HPLC (Fig. 3).

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 80 to 230 °C at a rate of 2 °C/min. The carrier gas was helium. The mass spectrum of the hydrogenated TMS derivative was characterized by a molecular ion (M^+) at *m/z* 386 and by two intense ions at *m/z* 229 and 259 (Fig. 4). The characteristic mass fragments of the derivative were consistent with the structure of the derivative of racemic 9-HODE obtained by autoxidation of LA.

Similarly, oxygenated products of LNA were extracted from the reaction mixture and separated by RP-HPLC with a Mightysil RP-18 GP Aqua column (5 μm, 4.5 × 250 mm) eluted with acetonitrile–water (9:1, 1.5 mL/min). The 9-anthrylmethyl esters of the extract showed one major peak with R_t of 8.1 min. When the ester was reduced with PPh_3, the peak was eluted at 8.6 min. Further determination of the regio and stereoselectivity was done with the Chiralcel OD-H column eluted with hexane–2-propanol (96.5:3.5, 1 mL/min). After comparison based on R_ts and co-chromatography with authentic standards such as racemic 13-hydroxy-(9*Z*,11*E*,15*Z*)-9, 11, 15-octadecatrienoic acid [(±)-13-HOTrE] with R_t (*R*) of 6.6 and R_t (*S*) of 7.5 min, racemic 9-hydroxy-(10*E*,12*Z*,15*Z*)-10, 12,

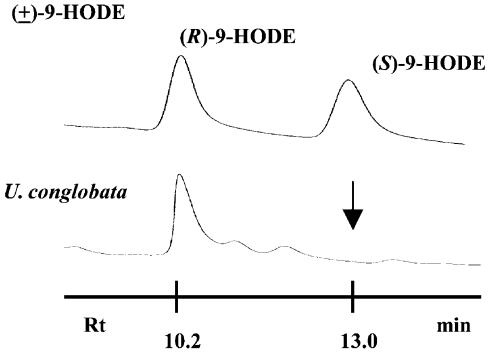

Figure 3. Enantioseparation of HODE from *U. conglobata* NP(chiral)-HPLC.

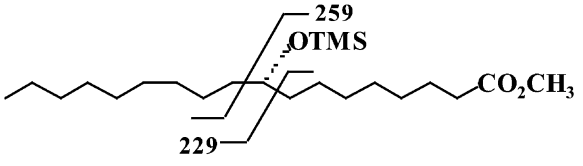

Figure 4.

15-octadecatrienoic acid [(±)-9-HOTrE] with R_t (*R*) of 8.0 and R_t (*S*) of 9.5 min, (*S*)-13-HOTrE constituted of 0.5% (*R*)- and 99.5% (*S*)-form, and (*S*)-9-HOTrE constituted of 3.1% (*R*)- and 96.9% (*S*)-form, the methyl esters of the oxygenated products showed one major peak with R_t of 8.0 min. Thus, based on these and on comparison with the GC–MS analysis of authentic standard, the major peak of the extract was identified as (*R*)-9-hydroperoxy-(10*E*,12*Z*,15*Z*)-10, 12, 15-octadecatrienoic acid [(*R*)-HPOTrE, >99% ee].

Quantification of the identified compounds was performed by reference to 12-hydroxystearic acid as an internal standard (IS). The oxygenated products were expressed as mean ± SD of three samples. Prior to extraction, the IS (100 nmol) was added and the extracts were concentrated under reduced pressure. Following derivatization with CH_2N_2, PPh_3, and BSTFA, the samples were analyzed by GC–MS. The oven temperature was programed from 80 to 200 °C at a rate of 10 °C/min. The area of the peaks on the ion chromatograms were calculated and compared to that observed for the IS. When LA and LNA were added to the crude enzyme, (*R*)-HODE and (*R*)-HOTrE were detected at concentration of 16.98 ± 0.72 and 3.51 ± 0.23 nmol/g (fresh weight), respectively. Other HODE and HOTrE compounds were not detected by this GC–MS analysis. Furthermore, with oleic acid and methyl ester of LA, oxygenated products were not detectable in this system.

Lipoxygenases (EC 1.13.11.12, LOX) catalyze the oxygenation of fatty acids containing a (1*Z*, 4*Z*)-pentadiene moiety in a regio and stereoselective way. These enzymes are widely distributed in plants[6] and mammals[7] and have further been demonstrated to occur in algae,[8] yeast,[9] and bacteria.[10] Among of these, the seeds of barley,[11] wheat,[12] maize,[13] and rice[14] contain one major LOX, which produces only (*S*)-9-hydroperoxy-(10*E*,12*Z*).12-octadecadienoic acid [(*S*)-9-HPODE] from LA. The seeds of legumes such as chickpea, kidney, lentil, and pea bean contain two major LOXs.[15] Although these LOXs produce (*R*)-9-HPODE and (*S*)-13-HPODE from LA, respectively, the enantioselectivity of (*R*)-9-HPODE is relatively low. On the other hand, potato tuber,[16] tomato,[17] and the seed of barley[18] LOXs convert LNA into (*S*)-9-HPOTrE, while *Hydra vulgaris*[19] LOX produces (*R*)-9-HPOTrE.

In this study, when LA and LNA were incubated with a crude enzyme of *U. conglobata*, the corresponding (*R*)-9-HPODE and (*R*)-9-HPOTrE were formed with a high ee (>99%), respectively. This regio and stereoselective way differs from those found in plants. This is the first time to find (*R*)-9-HPODE and (*R*)-9-HPOTrE in marine algae.

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