Oxygen Incorporation in Cleavage of ^{18}O-Labeled 13-Hydroperoxylinoleyl Alcohol into 12-Hydroxy-(3*Z*)-dodecenal in Tea Chloroplasts

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The oxygen atom in the hydroperoxy group of 13-hydroperoxylinoleyl alcohol was primarily incorporated into the carbonyl group of 12-hydroxy-(3*Z*)-dodecenal but scarcely into *n*-hexanal during the incubation with tea chloroplasts.

Introduction

Volatile C_6- and C_9-aldehydes are formed by lipoxygenase-catalyzed hydroperoxydation of C_{18}-unsaturated fatty acids containing a (1*Z*, 4*Z*)-pentadiene system followed by hydroperoxide lyase-catalyzed cleavage of the hydroperoxides formed when plants are macerated or mechanically ruptured under aerobic conditions. Up to date, hydroperoxide lyases which catalyze the conversion of 13- and 9-hydroperoxy-fatty acids to the C_6- and C_9-aldehydes and the C_{12}- and C_9-oxo acids, have been found in the higher plants such as cucumber [1], watermelon [2], tomato [3], alfalfa [4], pear [5], tea [6], and apple [7].

Recently, we have reported that 13-hydroperoxy-(9*Z*, 11*E*)-octadecadienol (13-HPOLAl, **2**) is used for a substrate of hydroperoxide lyase (E_2'') in tea chloroplasts to give hexanal (**3**) as shown in Fig. 1 [6]. However, the C_{12}-counterpart (**4**) of the enzymic cleavage products has not been identified so far.

This paper describes incorporation of the oxygen in the hydroperoxy group of 13-L-HPOLAl (**2**) into the carbonyl group of the C_{12}-oxo-alcohol (**4**) formed during the enzymic cleavage using ^{18}O-labeling.

Materials and Methods

Linoleic acid (99% purity) was gifted from Nippon Oil and Fats Co. Ltd. Linoleyl alcohol (**1**) was prepared by methylation of linoleic acid with diazomethane followed by reduction with $LiAlH_4$ in the usual manner. $^{18}O_2$ (98.75 atom %) was obtained from Commissariat a L'Energie Atomique (CEA), France. Lipoxygenase was purchased from Sigma Chem. Co. (Type V).

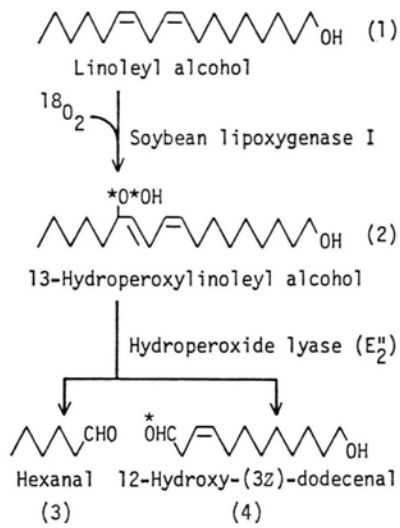

Fig. 1. Enzymic cleavage of 13-HPOLAl (**2**) in tea chloroplasts. *O; ^{18}O.

*Preparation of ^{18}O-labeled **2***

The alcohol **1** (1.0 g; 3.76 mmol) and soybean lipoxygenase I (400 U) were added into M/10-NH_4Cl-NH_4OH buffer (pH 9.0) (300 ml) containing 0.5% Tween 20 in an airtight flask which had been flashed with N_2 for 12 hr at room temperature. The reaction flask (300 ml Elrenmeyer) was sucked by water aspirator, and then $^{18}O_2$ (98.75 atom %) in the gastight syringe was injected into the flask with stirring at 0–5 °C. After the complete mixture was stirred for 12 hr and $(NH_4)_2SO_4$ (50% sat.) was added into the reaction solution, the reaction mixture was extracted with ether and the ether extract was concentrated *in vacuo*. The concentrate was purified by column chromatography on silica gel (for dry column, Woelm Pharma, W. Germany) to give **2** (558 mg; 1.86 mmol) in 50% yield. Positional and geometrical compositions of the prepared **2** were determined by HPLC analysis on a column of Zorbax sil (25 cm × 4.6 mm ∅) using an eluent of hexane containing 2% anhydrous EtOH. The HPLC analyses showed three peaks, which consist of **2** (94% and 92% for the

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labeled and unlabeled peroxides), 13-(9*E*, 11*E*)-HPOLAl (~ 1%), 9-(10*E*, 12*Z*)-HPOLAl (~ 5%) including the 9-(10*E*, 12*E*)-isomer. Enantiomeric compositions (*L*/*D*) of the **2** were determined 84/16 and 78/22 for the labeled and unlabeled peroxides, respectively by GLC analyses of the diastereomeric MTPA derivative as reported previously [9]. Purity of ^{18}O-C (95%) of the ^{18}O-labeled **2** was calculated from ratios of relative intensities of the peak at *m*/*z* 428 (molecular ion containing a ^{18}O-atom) and *m*/*z* 426 on mass spectrum (EI mode) of the TMS derivative obtained after reduction of **2** with $NaBH_4$ and subsequent trimethylsilylation with trimethylchlorosilane/hexamethyldisilazane (TMS-HT) [8]. The ^{18}O-labeled and unlabeled **2** having high purities were used for the enzymic cleavage reaction.

Identification of cleavage products in tea chloroplasts

A mixture of tea chloroplasts (3.0 g) suspended in 50 mM-phosphate buffer (pH 7.0) (50 ml) was shaked for 1 min at room temperature and then for 5 min at 35 °C. A solution of $NaBH_4$ (1 mmol) in MeOH was immediately added into the incubated mixture after 5 min incubation. This procedure was repeated 10 times. The combined reaction mixture was centrifuged at 15,000 × *g* for 10 min. Then $(NH_4)_2SO_4$ (50% sat.) was added to the supernatant and subsequently centrifuged at 15,000 × *g* for 10 min to remove proteins. The cleavage products were extracted with ether from the resulting supernatant. The extracts were purified by silica gel column chromatography (pentane/ether, 9/1–1/9) to give partially purified cleavage products. The cleavage products were subjected to GC-MS analyses after trimethylsilylation and identified as hexanol (**5**) and (3*Z*)-dodecen-1,12-diol (**6**) by comparing GC retention times and MS data with those of authentic specimens; details are given in a separate paper. The GC-MS (EI-mode) analyses were performed on a mass spectrometer (QP-1000 Shimadzu; ion source temperature 250 °C; energy 70 eV) combined with a gas chromatography (GC-9A Shimadzu; 2% OV-17 1.1 m × 2.6 mm ∅; column temperature 50 °C for 10 min and then raised by 5 °C/min to 200 °C). The CI spectra were obtained on a mass spectrometer (GC-MS 6020 Shimadzu; reagent gas isobutane; ion source temperature 250 °C; energy 150 eV using silicone SE-52 1.6 m × 2 mm ∅; column temperature raised from 55 °C to 200 °C by 4 °C/min).

Results and Discussion

Identification of cleavage products in tea chloroplasts

When the ^{18}O-labeled **2** was incubated with tea chloroplasts, $NaBH_4$ was added to the incubation mixture before completion of the cleavage to restrict the fast exchange of the carbonyl oxygen of the oxo-products to water oxygen as far as possible [10]. Thus, an excess of $NaBH_4$ in MeOH was immediately added into the reaction mixture after incubation of ^{18}O-labeled or unlabeled **2** with tea chloroplasts in 50 mM-phosphate buffer (pH 7.0) for 5 min at 35 °C. The cleavage products in the reaction mixture were subjected to GC-MS analyses after trimethylsilylation with TMS-HT. The total ion chromatogram of the enzymic cleavage products from **2** showed that two peaks (A; 4 min and B; 26.7 min) of several peaks had the same retention times as authentic TMS derivatives of **5** and **6** synthesized from suberic acid through unequivocal route. The EI-spectra of A and B from incubation of unlabeled **2** were identical with TMS derivatives of authentic **5** (Fig. 2) and **6** (Fig. 3): *m*/*z* 55 (15), 59 (10), 73 (48), 75 (100), 83 (9), 89 (16), 103 (20), 159 (38); *m*/*z* 55 (18), 73 (100), 75 (34), 103 (86), 147 (10), respectively. The protonated molecular ions of A and B were observed at *m*/*z* 175 as base peak and *m*/*z* 345 with 60% relative intensity to base peak at *m*/*z* 255 ($M^+ + H - 90$) on CI mode respectively. Thus, it was first confirmed that tea chloroplasts catalyzes the cleavage of **2** into the C_6-aldehyde (**3**) and the C_{12}-oxo-alcohol (**4**) as shown in Fig. 1.

With the enzymic cleavage of the ^{18}O-labeled **2**, the EI-spectrum of A showed a base peak at *m*/*z* 75 and a small peak (~ 14%) at *m*/*z* 77, which is probably due to $HO^+ = SiMe_2$ containing an ^{18}O-atom. The protonated molecular ion (*m*/*z* 177) having an ^{18}O-atom was very small (~ 13%) compared with the peak at *m*/*z* 175. However, the relative intensity of the ion at *m*/*z* 177 from the labeled substrate was slightly larger than that from the non-labeled substrate as shown in Fig. 2.

On the other hand the EI mass spectrum of B from the labeled substrate showed the ion at *m*/*z* 73 (base peak) and an ion at *m*/*z* 105 (34% probably due to $CH_2 = O^+SiMe_3$ containing an ^{18}O-atom) (Fig. 3). In the CI-mass spectrum of this peak, the protonated molecular ion containing an ^{18}O-atom appeared at *m*/*z* 347 with 57% relative intensity to that of the ion at *m*/*z* 345 as shown in Fig. 3. When the ^{18}O-labeled

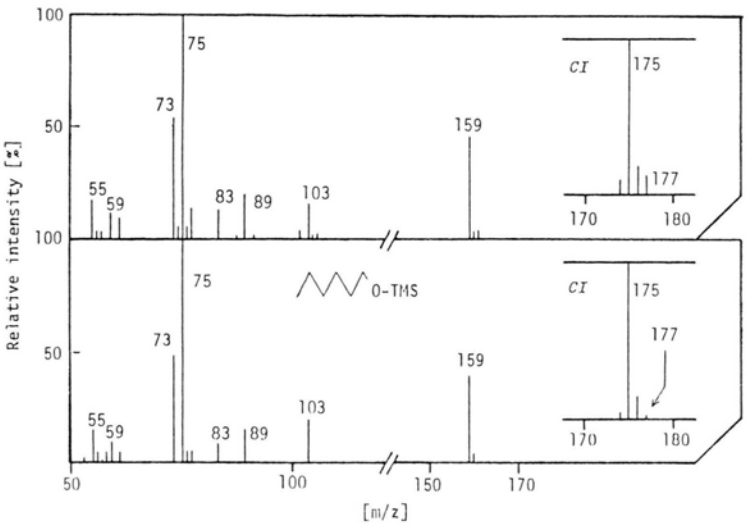

Fig. 2. EI mass spectra including partial CI mass spectra of TMS derivatives of the cleavage product A (top) from ^{18}O-labeled **2** and authentic **5** (bottom).

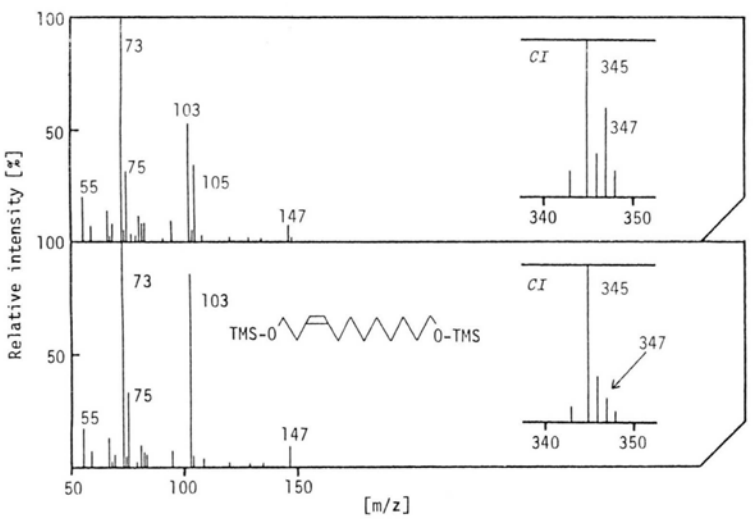

Fig. 3. EI mass spectra including partial CI mass spectra of TMS derivatives of the cleavage product B (top) from ^{18}O-labeled **2** and authentic **6** (bottom).

not the unlabeled **2** was used as a substrate, the relative intensity of the protonated molecular ion *m/z* 347 in the CI spectra of the TMS derivatives of **6** increased 17 to 57% during incubation. Thus comparison of the ratios between the protonated molecular ions and 2 mass unit higher molecular ions on the CI mass spectra of the TMS derivatives from incubation with the ^{18}O-labeled **2** or the unlabeled **2** showed that the oxygen of the hydroperoxy group of the substrate was primarily in the carbonyl group of **4** and scarcely in **3** during the enzymic cleavage as shown in Fig. 1. The origin of the oxygen in the cleavage products during the enzymic reaction presumably is explained by a manner similar to mechanism for acid-catalyzed rearrangement of the 13-hydroperoxide in aprotic solvent [11].

Acknowledgements

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