Oxygen-Isotope Effect in Enzymatic Cleavage Reaction of 13-L-Hydroperoxylinoleic Acid to Hexanal and 11-Formyl-cis-9-undecenoic Acid

Akikazu Hatanaka, Tadahiko Kajiwara, Jiro Sekiya, and Takehiko Fukumoto
Department of Agricultural Chemistry, Faculty of Agriculture, Yamaguchi University Yamaguchi 753 Japan.

Z. Naturforsch. 37 c, 752-757 (1982); received March 31, 1982

Hydroperoxide lyase, 11-Formyl-cis-9-undecenoic Acid, ”O-Labeled 13-L-Hydroperoxylinoleic Acid, Oxygen-Isotope Effect

Hydroperoxide lyase $E''_2$ solubilized with Tween 20 from tea chloroplasts was shown to catalyze cleavage reaction of 13-L-hydroperoxy-cis-9-trans-11-octadecadienoic acid (13-L-hydroperoxylinoleic acid) to hexanal, a C$_6$-compound and 11-formyl-cis-9-undecenoic acid, a C$_{12}$-compound by identification of cleavage products using authentic specimens synthesized through an unequivocal route. An oxygen-isotope effect was first observed in the cleavage reaction of ”O-labeled 13-L-hydroperoxylinoleic acid by solubilized $E''_2$. The ”O-atom of hydroperoxide was not detected in carbonyl group of hexanal formed from ”O-labeled 13-L-hydroperoxylinoleic acid.

Introduction

Leaf alcohol (cis-3-hexenol) and leaf aldehyde (trans-2-hexenal), which are formed from cis-3-hexenal, are widely distributed in fresh leaves, vegetables, and fruits and are responsible for “Green odor” characteristic of leaves [1-6]. We have demonstrated that cis-3-hexenal is biosynthesized by enzymatic splitting ($E''_2$ reaction) of 13-L-hydroperoxylinolenic acid which is produced by stereospecific oxygenation ($E'_2$ reaction) at C-13 of linolenic acid [7-9] in tea chloroplasts and plant tissues as shown in Fig. 1. Also hexanal was shown to be produced from linolenic acid by the same system.

A hydroperoxide lyase which catalyzes cleavage of 13-hydroperoxide into a C$_6$-aldehyde and a C$_{12}$-oxo acid has been found in alfalfa seeds [10], watermelon seedlings [11], tomato fruits [12], bean leaves [13], cucumber fruits [14], and cucumber seedlings [10]. Recently, a hydroperoxide lyase was partially purified from pears [15] by differential centrifugation, gel chromatography and isoelectric focusing.

In a previous paper [16], solubilization and properties of hydroperoxide lyase $E''_2$ from tea chloroplasts have been reported. However, the mechanism of cleavage reaction of 13-L-hydroperoxides into C$_6$-aldehydes and C$_{12}$-oxo acid has remained unknown.

This paper describes identification of cleavage products of 13-L-hydroperoxy-cis-9,trans-11-octadecadienoic acid (13-L-hydroperoxylinoleic acid) by solubilized $E''_2$ and an oxygen-isotope effect in cleavage reaction by solubilized $E''_2$, tea leaves, tea chloroplasts, and watermelon seedlings, using ”O-labeled 13-L-hydroperoxylinoleic acid, whose ”O-atom was introduced into C-13 of linoleic acid.

Reprint requests to Prof. Dr. Akikazu Hatanaka.
0341-0382/82/0900-0752 $ 01.30/0

Fig. 1. Biosynthetic pathway of cis-3-hexenal.
Materials and Methods

Lipoxygenase I was obtained from P. L. Biochemical Inc. (Type I, soybean; activity 50 000 units/mg). Linoleic acid (purity, 99%) was obtained from Wako Pure Chemical Industries Ltd. $^{18}$O (18O; 50.0% atom%) was obtained from Commissariat a L’Energie Atomique (CEA), France.

a) Preparation of solubilized hydroperoxide lyase $E_2'$: Chloroplasts were prepared from fresh leaves of tea (Thea sinensis cv. Yabukita) harvested in August according to the method reported previously [17]. Chloroplasts (2 g wet weight) were suspended in chilled 32 mM citric acid-135 mM Na$_2$HPO$_4$ (McIlvaine’s buffer) (20 ml; pH 7.0) containing 0.5% Tween 20, and homogenized with a teflon-pestle homogenizer for 30 s. The homogenate was centrifuged at 25 000 x g 10 min and the supernatant (20 ml) was used as a solubilized hydroperoxide lyase $E_2'$ [16].

b) Preparation of homogenates containing $E_2'$ activity: Tea leaves (0.5 g) were homogenized in Waring blender for 3 min in McIlvaine’s buffer (10 ml; pH 7.0). The homogenate was filtered through 4 layers of gauze and the filtrate (10 ml) was used as tea homogenate.

The enzyme solution of watermelon seedlings (Citrullus lanatus) was prepared by the method of Vick and Zimmerman [11]. Six-day-old etiolated watermelon seedlings (3 g fresh weight) were ground with McIlvaine’s buffer (10 ml; pH 7.0) at 4 °C. The homogenate was filtered through 2 layers of gauze and the filtrate (10 ml) was used as an enzyme solution.

c) Preparation of $^{18}$O-labeled 13-L-hydroperoxide: A suspension of linoleic acid in a 40 mM NH$_4$Cl-NH$_2$OH buffer (pH 9.0) in the reaction vessel was evacuated by water pump and subsequently by flashing N$_2$ gas to eliminate the dissolved air. After this procedure was repeated three times, soybean lipoxygenase I was injected in the suspension. The complete reaction mixture was incubated in an $^{18}$O$_2$-atmosphere (50 atom%) for 90 min at 0 °C. The reaction mixture was carefully acidified with 2 N HCl and then extracted with ether. The solvent of the extract was evaporated in vacuo to give a crude hydroperoxide, which was purified by silica gel (Woelm Pharma, W. Germany) column chromatography (pet. ether/ether = 1/1) to give pure 13-L-hydroperoxylinoleic acid containing $^{18}$O-labeled 13-L-hydroperoxide in 48% yield. Purities of $^{18}$O-C and $^{18}$O-C of 13-L-hydroperoxide thus obtained were 34% and 66%, respectively. Isotope compositions were calculated from ratios of intensities of the peaks at 225 (+ 2) and 311 (+ 2) on mass spectrum of trimethylsilyl ether derivative of methyl 13-L-hydroxylinolet prepared by reduction of 13-L-hydroperoxide with NaBH$_4$ in methanol and esterification with diazomethane at −20 °C, followed by trimethylsilylation with bis-(trimethylsilyl)-trifluoroacetamide according to the method of Boldingh [18].

The structure of labeled hydroperoxide was fully substantiated by NMR and IR analyses: IR spectrum 3440, 1710, 1450, 980, 730 cm$^{-1}$; NMR spectrum (CHCl$_3$) δ = 7.3 (1 H d), 4.3−6.6 (4 H, m), 4.00 (1 H, m), 3.30 (1 H, s), 2.21 (4 H, m), 1.7 (2 H, m), 1.42 (16 H, s), 0.90 (3 H, t).

d) Identification of cleavage products by solubilized $E_2'$: A solution of solubilized $E_2'$ (4 ml) and McIlvaine’s buffer (6 ml: pH 7.0) were preincubated at 35 °C for 1 min and subsequently incubated with 13-L-hydroperoxylinoleic acid (10 μmol) for 10 min at 35 °C. After 2 N HCl (2 ml) was added in the incubated solution to stop the reaction, the reaction mixture was extracted with ether in a N$_2$ atmosphere. These procedures were repeated 20 times. The combined ether extract was concentrated in vacuo and the concentrate was esterified with diazomethane at −20 °C. The esterified products were converted to methoxime derivatives using methoxiamine hydrochloride/sodium carbonate (pHs 8.0 or 12.0) in the usual manner [19]. The methoximes from cleavage products were identified as methoximes of hexanal and 11-formyl-trans-10-undecenoic acid by comparison of GLC retention times and mass spectra of authentic specimens synthesized through an unequivocal route: [Shimadzu GC-6 A gas chromatograph equipped a glass column (Ø 3 mm x 3 m) with 5% OV-25 on 60−80 mesh Chromosorb W AW and Shimadzu GC-MS 7000].

e) Synthesis of methyl 11-formyl-trans-10-undecenoate: Ozonolysis of methyl 10-undecenoate (2.0 g: 0.01 mol) in dry ethyl acetate at −20 °C for 1.5 h and subsequent hydrogenation over 10% Pd-C (1.0 g) gave methyl 9-formyl-nonanoate, which was purified by silica gel column chromatography in 77% yield.
(1.7 g). The oxo-ester (1.0 g; 0.003 mol) with formylmethylenetriphenylphosphorane [20] (1.0 g; 0.003 mol) was refluxed in benzene for 18 h to afford 11-formyl-trans-10-undecenoate in 81% yield (1.2 g). The structure was substantiated by IR and NMR analyses: IR spectrum 2700, 1730, 1690, 980 cm⁻¹; NMR spectrum (CHCl₃) δ = 9.6 (1H, d), 6.3 (2H, m), 3.55 (3H, s), 2.2 (4H, m), 1.33 (12H, s) [21].

f) Oxygen-isotope effect during incubation of 13-L-hydroperoxylinoleic acid with solubilized E₂′

i) GLC analysis of formed hexanal: Solubilized E₂′ (1 ml) or homogenate (4 ml) were brought to 10 ml with Mcllvaine’s buffer (pH 7.0). The mixture (10 ml) was preincubated at 35 °C for 1 min in a 50 ml-Erlenmeyer flask sealed with a rubber stopper and then [¹⁶O]- or [¹⁸O]-13-L-hydroperoxide (6 µmol) was injected into the mixture. After 10 ml of air was sucked out of the flask by a syringe, the mixture was shaken vigorously for 1 min and subsequently incubated at 35 °C for 10 min with shaking. The head-space vapor (6 ml) in the flask was quantitatively analyzed by the method reported previously [10].

ii) UV analysis of cleavage of 13-L-hydroperoxylinoleic acid: Decrease of absorbance at 234 nm due to the conjugated diene of 13-L-hydroperoxide was measured photometrically (Hitachi model 124 spectrophotometer) at 25 °C. The standard reaction mixture in 1 cm cuvette contained 13-L-hydroperoxide (0.064 µmol), solubilized hydroperoxide lyase E₂′ (0.1 ml) and Mcllvaine’s buffer (pH 7.0) in a final volume of 3 ml. The decrease of absorbance at 234 nm was followed for 10 min after addition of an enzyme solution.

iii) GC-MS analysis of recovered 13-hydroperoxide: A mixture of 13-L-hydroperoxide (10 µmol), hydroperoxide lyase E₂′ (4 ml) and Mcllvaine’s buffer (6 ml) in a 50 ml-Erlenmeyer flask, was incubated for 10 min at 35 °C and then the reaction mixture was acidified to pH 2.0 with 2 N HCl (3 ml) to stop the reaction. After addition of ammonium sulfate (10 g), 13-hydroperoxide was extracted with ether. The ether extract was dried over anhydrous sodium sulfate, concentrated under reduced pressure and reduced with NaBH₄ in methanol: Borate buffer, pH 9.0, 1/1, V/V to give a hydroxy isomer. The hydroxy-acid from the recovered hydroperoxide, was esterified with diazomethane in ether at −20 °C. The resultant methyl 13-L-hydroxylinoleate was converted to the corresponding TMS ether derivatives as described earlier. The TMS ether was subjected to GC-MS analysis: (18.3 min: PEG 20 M (BCL) ⊗ 0.3 mm × 30 m, column temp. 180 °C, injector and detector temp. 200 °C, N₂ flow rate 20 ml/min). Oxygen isotopic compositions were determined by calculations from ratios of relative intensities of the fragment ions containing oxygen atom on mass spectrum [the parent peaks at m/e 382 and 384 (its isotope peak) and the prominent peaks at m/e 225 and 227 (its isotope peak)].

Results and Discussion

a) Identification of cleavage products of 13-L-hydroperoxylinoleic acid by solubilized E₂′: The mixture of products resulting from incubation of 13-L-hydroperoxylinoleic acid with solubilized E₂′ was converted to methoxime derivatives at pH 8.0 according to the usual method. The crude methoximes were subjected to GLC analysis without further purification. From the GLC-tracings of Fig. 2, cleavage products by E₂′ was found to comprise three oxo-compounds (peak A, 5.8 min, peak B, 19.9 min and peak C, 22.0 min) accompanied by endogenous compounds in E₂′ solution. Retention times of peak A and C were the same as those of authentic methoximes of hexanal and methyl 11-formyl-trans-10-undecenoate synthesized through an unequivocal route, respectively. The mass spectra of peak A and C were identical with those of methoximes of hexanal and methyl 11-formyl-trans-10-undecenoate, respectively as shown in Fig. 3. Authentic methoximes of the synthetic C₁₂-oxo ester prepared at both

Fig. 2. GLC analysis of cleavage products of 13-L-hydroperoxide by solubilized E₂′: B: methoxime of methyl 11-formyl-cis-9-undecenoate; C: methoxime of methyl 11-formyl-trans-10-undecenoate.
pH 8.0 and 12.0 showed a single peak on GLC analysis, whereas peak B was shifted to peak C, being prepared the methoxime derivative at pH 12.0, from the cleavage mixture as seen in the upper GLC-tracing of Fig. 2. This reflects the isomerization of the \( \beta,\gamma \)-oxo acid ester (peak B) to the \( \alpha,\beta \)-oxo acid ester (peak C) and was in agreement with that reported on runner bean pods by Zimmerman et al. [22].

Based on these results and findings, peak B was shown to be 11-formyl-cis-9-undecenoate. With denatured \( E' \), which is prepared by heating at 95 °C for 10 min, peak A, B and C were not detected under the condition used for the enzymatic reaction. Thus, hexanal and 11-formyl-cis-9-undecenoic acid, which isomerized to the corresponding trans-10-isomer were enzymatically formed from 13-L-hydroperoxylinoleic acid by solubilized \( E'' \).

b) Isotope effect

Incubation of unlabeled 13-L-hydroperoxylinoleic acid (6 µmol) with solubilized \( E'' \) (1 ml) for 10 min at 35 °C, resulted in 1.2 µmol of hexanal formation, whereas 0.5 µmol of hexanal was formed from the \( ^{18}O \)-labeled 13-L-hydroperoxylinoleic acid. The difference between an amount of hexanal formed from the \( ^{18}O \)-labeled hydroperoxide and that from unlabeled hydroperoxide was also found in the region

Fig. 3. Mass spectra of methoximes of \( C_7 \)-oxo acid and hexanal (A): methoxime of hexanal; (B): methoxime of methyl 11-formyl-trans-10-undecenoate.

Fig. 4. Enzymatic formation of hexanal from \( ^{16}O \)-labeled and unlabeled hydroperoxides by solubilized \( E'' \) (○-○): hexanal formation from \( ^{16}O \)-13-L-hydroperoxide; (●-●): hexanal formation from \( ^{18}O \)-13-L-hydroperoxide.
of substrate concentration as indicated in Fig. 4. This finding is supported by monitoring the course of reaction with decrease at 234 nm due to conjugated diene of 13-hydroperoxide; unlabeled hydroperoxide cleaved faster ca. 2.6 times than the 18O-labeled hydroperoxide did as seen in Fig. 5. Using large excess of E, the labeled hydroperoxide was cleaved to hexanal completely. Whereas, a decrease in absorbance at 234 nm was not detected during incubation of only the substrate 25 °C for 10 min. Thus, these differences in reactivity between 18O-labeled and unlabeled substrates during the cleavage reaction by E could be interpreted in terms of an oxygen-isotope effect.

To demonstrate the isotope effect, use of the difference in purities of 18O-C of 13-hydroperoxide before or after reaction. A significant difference was found between the percentage of 18O-C of TMS ether derivative from recovered 13-L-hydroperoxide after incubation of 18O-labeled 13-L-hydroperoxylinoic acid for 10 min at 35 °C and that of the peroxide for the substrate.

The percentages of 13-hydroperoxides were determined from calculations of relative intensities if fragment ions containing the oxygen atom in mass spectra (the parent peaks at m/e 382 and 384 or the prominent peaks at m/e 225 and 227). Purity of 18O-C of the recovered hydroperoxide increased after incubation of 18O-labeled hydroperoxide which had 34% purity of 18O-C, as seen in Fig. 6 and Table I. With tea chloroplasts and homogenates of tea leaves and watermelon seedlings, hexanal formation from the 18O-labeled hydroperoxide was 44—54% of that from unlabeled 13-L-hydroperoxide as Table II indicates. Based on these results and findings, we have proposed that an oxygen-isotope effect involves in
Table I. Isotopic compositions of the recovered 13-hydroperoxide from relative intensities of mass fragment ions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Relative intensity [%]</th>
<th>Mass ion [m/e] 225</th>
<th>Mass ion [m/e] 227</th>
<th>Mass ion [m/e] 382</th>
<th>Mass ion [m/e] 384</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>66.2</td>
<td>33.8</td>
<td>63.6</td>
<td>36.4</td>
<td></td>
</tr>
<tr>
<td>Recovered hydroperoxide a</td>
<td>58.0</td>
<td>42.0</td>
<td>56.9</td>
<td>43.1</td>
<td></td>
</tr>
</tbody>
</table>

a 13-Hydroperoxide recovered at 23% completion of cleavage reaction by $E''$.

Table II. Comparison of oxygen-isotope effect in $E''$ reaction by plant tissues.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Hexanal [μmol] [16O]$^*$ [18O]</th>
<th>[18O]$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tea leaves$^1$</td>
<td>2.42 (100)$^*$</td>
<td>1.27 (52)</td>
</tr>
<tr>
<td>Tea chloroplasts$^2$</td>
<td>3.68 (100)</td>
<td>1.62 (44)</td>
</tr>
<tr>
<td>Solubilized $E''_3$</td>
<td>2.70 (100)</td>
<td>1.23 (46)</td>
</tr>
<tr>
<td>Watermelon seedlings$^4$</td>
<td>0.83 (100)</td>
<td>0.45 (54)</td>
</tr>
</tbody>
</table>

1 0.5 g fresh weight.  
2 0.1 g [corresponded to 0.5 g leaves (fresh weight)].  
3 1 ml (see Materials and Methods).  
4 10 ml (see Materials and Methods).  
5 hexanal formation from $^{18}$O-13-L-hydroperoxide.  
6 hexanal formation from $^{18}$O-13-L-hydroperoxide.  
7 numbers in parenthesis represent relative values (%).

The enzymatic cleavage reaction of 13-L-hydroperoxylinoleic acid to hexanal and 11-formyl-cis-9-undecenoic acid by $E''$ in tea chloroplasts and plant tissue. On the other hand, the $^{18}$O-atom of hydroperoxy group at C-13 of the substrate was not detected in carbonyl group of formed hexanal under our experimental conditions: reacted at pH 7.0 and stopped the reaction by addition of 2 N HCl to pH 2.0 or of organic solvent at pH 7.0. This suggests an exchange of $^{18}$O-atom originated from the hydroperoxy group to water after and/or during the enzymatic cleavage reaction. However, further experiments on an $^{18}$O-incorporation to 11-formyl-cis-9-, or 11-formyl-trans-10-undecenoic acid and an exchange of oxygen atom of carbonyl groups to water molecule using H$_2^{18}$O are required to elucidate the mechanism of cleavage reaction.

Acknowledgement

The authors thank Mr. H. Miyawaki and C. Yukawa, Taiyo Perfumery Co., Ltd, for GC-MS measurement. This work was supported in part by a Grant-in Aid (No. 56109003) for Special Project Research from the Ministry of Education, Science and Culture of Japan.