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

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Hydroperoxide lyase, 11-Formyl-*cis*-9-undecenoic Acid, ^{18}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 ^{18}O-labeled 13-L-hydroperoxylinoleic acid by solubilized E_2''. The ^{18}O-atom of hydroperoxide was not detected in carbonyl group of hexanal formed from ^{18}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 linoleic 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 ^{18}O-labeled 13-L-hydroperoxylinoleic acid, whose ^{18}O-atom was introduced into C-13 of linoleic acid.

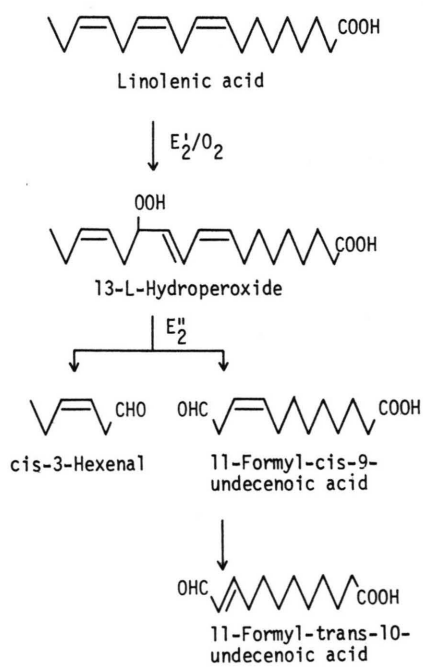

Fig. 1. Biosynthetic pathway of *cis*-3-hexenal.

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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. $^{18}O_2$ (^{18}O; 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_2HPO_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 $25000 \times g$ 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 was centrifuged at $12\,000 \times g$ for 10 min. The supernatant was passed through 2 layers of gauze to remove lipid-like materials floating at the top of tube. The resultant supernatant (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_4Cl-NH_4OH 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 ^{16}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-hydroxylinoleate 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 ($CHCl_3$) $\delta = 7.3$ (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 (∅ 3 mm × 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 ($CHCl_3$) $\delta = 9.6$ (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* E_2''

i) *GLC analysis of formed hexanal:* Solubilized E_2'' (1 ml) or homogenate (4 ml) were brought to 10 ml with McIlvaine'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 [^{18}O]- or [^{16}O]-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 headspace 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 E_2'' (0.1 ml) and McIlvaine's buffer (pH 7.0) in a final volumn 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 E_2'' (4 ml) and McIlvaine'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 $NaBH_4$ 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, N_2 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* E_2'': The mixture of products resulting from incubation of 13-L-hydroperoxylinoleic acid with solubilized E_2'' 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 E_2'' 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 E_2'' 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 C_{12}-oxo ester prepared at both

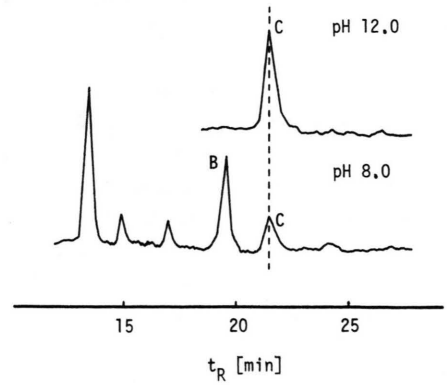

Fig. 2. GLC analysis of cleavage products of 13-L-hydroperoxide by solubilized E_2''. B: methoxime of methyl 11-formyl-*cis*-9-undecenoate; C: methoxime of methyl 11-formyl-*trans*-10-undecenoate.

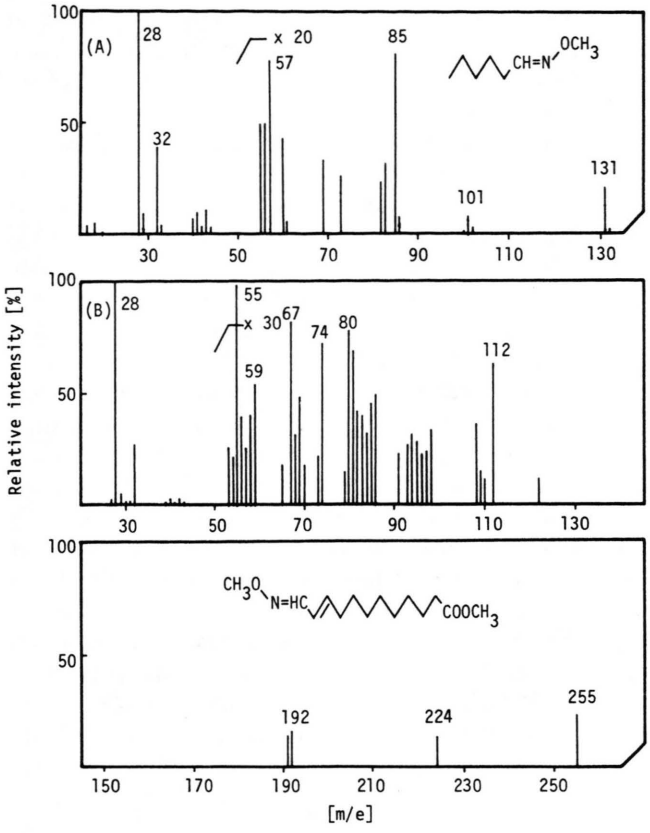

Fig. 3. Mass spectra of methoximes of C_{12}-oxo acid and hexanal (A): methoxime of hexanal; (B): methoxime of methyl 11-formyl-*trans*-10-undecenoate.

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 β,γ-oxo acid ester (peak B) to the α,β-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_2'', 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_2''.

b) *Isotope effect*

Incubation of unlabeled 13-L-hydroperoxylinoleic acid (6 µmol) with solubilized E_2'' (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

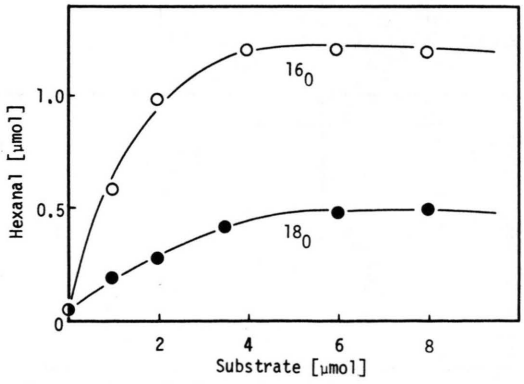

Fig. 4. Enzymatic formation of hexanal from ^{18}O-labeled and unlabeled hydroperoxides by solubilized E_2'' (-●-): hexanal formation from ^{18}O-13-L-hydroperoxide; (-○-): hexanal formation from ^{16}O-13-L-hydroperoxide.

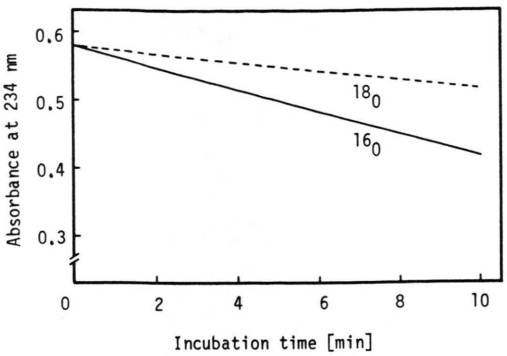

Fig. 5. Decrease in absorbance at 234 nm during E_2'' reaction of ^{18}O-labeled and unlabeled 13-hydroperoxides, (- - -): ^{18}O-13- L-hydroperoxide; (—): ^{16}O-13- L-hydroperoxide.

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 ^{18}O-labeled hydroperoxide did as seen in Fig. 5. Using large excess of E_2'', 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 ^{18}O-labeled and unlabeled substrates during the cleavage reaction by E_2'' could be interpreted in terms of an oxygen-isotope effect.

To demonstrate the isotope effect, use of the difference in purities of ^{18}O-C of 13-hydroperoxide before or after reaction. A significant difference was found between the percentage of ^{18}O-C of TMS ether derivative from recovered 13-L-hydroperoxide after incubation of ^{18}O-labeled 13-L-hydroperoxylinoleic 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 ^{18}O-C of the recovered hydroperoxide increased after incubation of ^{18}O-labeled hydroperoxide which had 34% purity of ^{18}O-C, as seen in Fig. 6 and Table I. With tea chloroplasts and homogenates of tea leaves and watermelon seedlings, hexanal formation from the ^{18}O-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

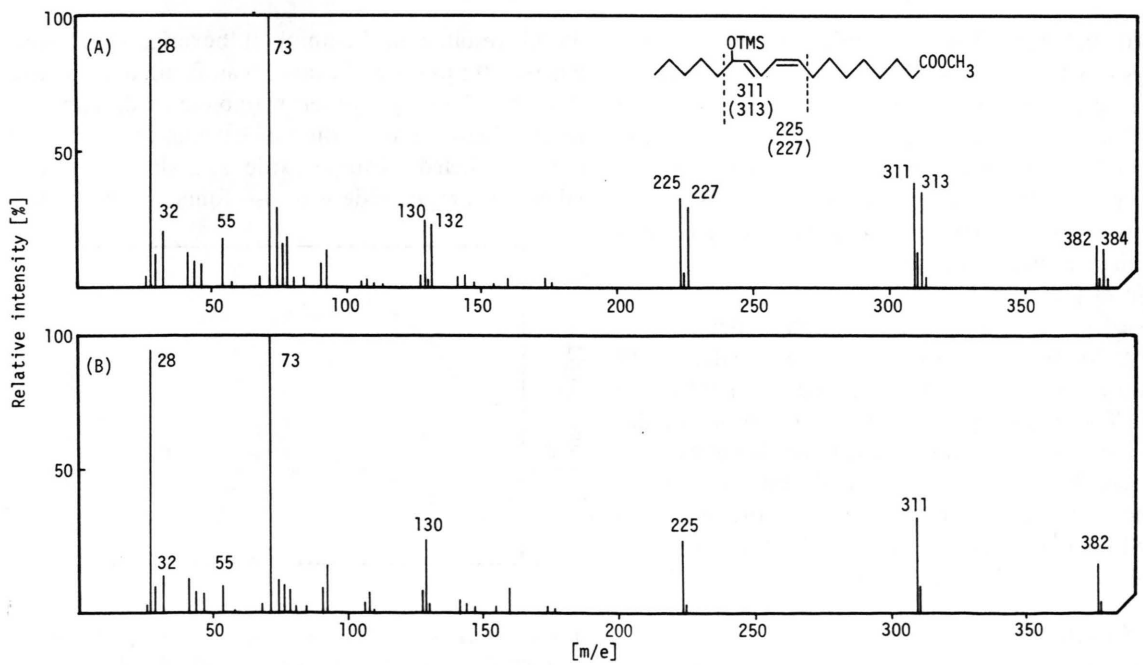

Fig. 6. Mass spectra of TMS derivatives of methyl 13-hydroxylinoleate. (A): ^{18}O-13-hydroxylinoleate recovered after the E_2'' reaction; (B): authentic methyl ^{16}O-13-hydroxylinoleate.

Table I. Isotopic compositions of the recovered 13-hydroperoxide from relative intensities of mass fragment ions.

Condition	Relative intensity [%]			
	Mass ion [*m/e*]		Mass ion [*m/e*]	
	225	227	382	384
Substrate	66.2	33.8	63.6	36.4
Recovered hydroperoxide[a]	58.0	42.0	56.9	43.1

[a] 13-Hydroperoxide recovered at 23% completion of cleavage reaction by E_2''.

Table II. Comparison of oxygen-isotope effect in E_2'' reaction by plant tissues.

Enzyme	Hexanal [μmol]	
	[^{16}O][5]	[^{18}O][6]
Tea leaves[1]	2.42 (100)[7]	1.27 (52)
Tea chloroplasts[2]	3.68 (100)	1.62 (44)
Solubilized E_2'' [3]	2.70 (100)	1.23 (46)
Watermelon seedlings[4]	0.83 (100)	0.45 (54)

[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 ^{16}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_2'' 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.

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