Volatile C₆-Aldehyde Formation via Hydroperoxides from C₁₈-Unsaturated Fatty Acids in Etiolated Alfalfa and Cucumber Seedlings

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1. Etiolated seedlings of alfalfa and cucumber evolved *n*-hexanal from linoleic acid and *cis*-3-hexenal and *trans*-2-hexenal from linolenic acid when they were homogenized.
2. The activities for *n*-hexanal formation from linoleic acid, lipoxygenase and hydroperoxide lyase were maximum in dry seeds and 1~2 day-old etiolated seedlings of alfalfa, and in 6~7 day-old etiolated seedlings of cucumber.
3. *n*-Hexanal was produced from linoleic acid and 13-hydroperoxylinoleic acid by the crude extracts of etiolated alfalfa and cucumber seedlings. *cis*-3-Hexenal and *trans*-2-hexenal were produced from linolenic acid and 13-hydroperoxylinolenic acid by the crude extracts of etiolated alfalfa and cucumber seedlings. But these extracts, particularly cucumber one, showed a high isomerizing activity from *cis*-3-hexenal to *trans*-2-hexenal.
4. When the C₆-aldehydes were produced from linoleic acid and linolenic acid by the crude extracts, formation of hydroperoxides of these C₁₈-fatty acids was observed.
5. When 9-hydroperoxylinoleic acid was used as a substrate, *trans*-2-nonenal was produced by the cucumber homogenate but not by the alfalfa homogenate.
6. As the enzymes concerned with C₆-aldehyde formation, lipoxygenase was partially purified from alfalfa and cucumber seedlings and hydroperoxide lyase, from cucumber seedlings. Lipoxygenase was found in a soluble fraction, but hydroperoxide lyase was in a membrane bound form. Alfalfa lipoxygenase catalyzed formation of 9- and 13-hydroperoxylinoleic acid (35: 65) from linoleic acid and cucumber one, mainly 13-hydroperoxylinoleic acid formation. Alfalfa hydroperoxide lyase catalyzed *n*-hexanal formation from 13-hydroperoxylinoleic acid, but cucumber one catalyzed formation of *n*-hexanal and *trans*-2-nonenal from 13- and 9-hydroperoxylinoleic acid, respectively.
7. From the above results, the biosynthetic pathway for C₆-aldehyde formation in etiolated alfalfa and cucumber seedlings is established that C₆-aldehydes (*n*-hexanal, *cis*-3-hexenal and *trans*-2-hexenal) are produced from linoleic acid and linolenic acid *via* their 13-hydroperoxides by lipoxygenase and hydroperoxide lyase.

C₆-Aldehydes such as *n*-hexanal, *cis*-3-hexenal and *trans*-2-hexenal, which are re-

Abbreviations: DCIP, 2, 6-dichlorophenol indophenol; MB, methylene blue; SKF 525-A, 2-dimethylaminoethyl-2, 2-diphenyl valerate; *p*-CMB, *p*-chloromercuribenzoate; GSH, reduced glutathione; 9-hydroperoxylinoleic acid, 9-hydroperoxy-octadeca-*trans*-10, *cis*-12-dienoic acid; 9-hydroxylinoleic acid, 9-hydroxy-octadeca-*trans*-10, *cis*-12-dienoic acid; 9-hydroperoxylinolenic acid, 9-hydroperoxy-octadeca-*trans*-10, *cis*-12, *cis*-15-trienoic acid; 13-hydroperoxylinoleic acid, 13-hydroperoxy-octadeca-*cis*-9, *trans*-11-dienoic acid; 13-hydroxylinoleic acid, 13-hydroxy-octadeca-*cis*-9, *trans*-11-dienoic acid; 13-hydroperoxylinolenic acid, 13-hydroperoxy-octadeca-*cis*-9, *trans*-11, *cis*-15-trienoic acid.

sponsible for major characteristic odor of green leaves, occur in a variety of plants including vegetables and fruits. These are produced from C₁₈-unsaturated fatty acids containing a *cis*-1, *cis*-4-pentadiene moiety such as linoleic acid and linolenic acid.^{1~6)} Recently, etiolated watermelon seedlings have been reported to produce *n*-hexanal from linoleic acid *via* 13-hydroperoxide by lipoxygenase and hydroperoxide lyase.⁷⁾ Similar pathway for C₆-aldehyde formation are reported in tomato fruits⁸⁾ and bean leaves,⁹⁾ and for C₆- and C₉-aldehydes (*cis*-3-nonenal, *trans*-2-nonenal, *cis*-3, *cis*-6-nonadienal and *trans*-2, *cis*-6-

nonadienal) in cucumber fruits¹⁰⁻¹³⁾ in which C₆-aldehydes are major products from C₁₈-fatty acids.^{10,11,14,15)} However, properties of the enzymes in etiolated seedlings and fruits are not well known.

In the present paper, we describe C₆-aldehyde formation mediated by lipoxygenase and hydroperoxide lyase in etiolated alfalfa and cucumber seedlings: changes in the enzyme activities responsible for C₆-aldehyde formation during germination, properties of C₆-aldehyde formation from C₁₈-unsaturated fatty acids and partial purification and some properties of the concerned enzymes.

MATERIALS AND METHODS

Plant materials and preparation of the crude extracts. Seeds of alfalfa (*Medicago sativa* "Common") and cucumber (*Cucumis sativus* "Suyo") were soaked for 12 hr in tap water, then placed on moistened paper and germinated in the dark at 25°C. Time zero means dry seeds. Etiolated seedlings obtained were homogenized in a chilled mortar for 10 min with 50 mM phosphate buffer, pH 6.0 or 6.5. These homogenates were referred to as "crude homogenates." The crude homogenates were centrifuged at 15,000×*g* for 10 min and the supernatant solutions thus obtained were referred to as "crude extracts."

Enzyme assays. i) *Lipoxygenase activity* was determined from the increase in the absorbance at 234 nm due to conjugated diene of the hydroperoxide with the reaction mixture containing 0.1 ml of linoleic acid solution prepared by the method of Surrey,¹⁶⁾ 0.1 ml of enzyme solution and 2.8 ml of 50 mM phosphate buffer, pH 6.0~6.5. The lipoxygenase activity was also determined by oxygen uptake polarographically with a Clark oxygen electrode (YSI 4004) at 25°C with the reaction mixture containing 0.1 ml of linoleic acid solution (0.28 mg in 0.2% Tween 20 solution), 0.1 ml of enzyme solution and 2.8 ml of 50 mM phosphate buffer, pH 6.5. One enzyme unit was defined as the enzyme amount producing 1 μmol of hydroperoxide or consuming 1 μmol of oxygen per min at 25°C. ii) *Hydroperoxide lyase activity* was determined from the decrease in absorbance at 234 nm at 25°C essentially according to the method described previously.⁷⁾ The standard reaction mixture contained 0.5 ml of 13-hydroperoxylinoleic acid solution,⁷⁾ 0.1 ml of enzyme solution and 2.4 ml of 50 mM phosphate buffer, pH 6.0 or 6.5. One enzyme unit was defined as the enzyme amount consuming 1 μmol of hydroperoxide per min at 25°C. iii) *C₆-Aldehyde formation*

from C₁₈-fatty acids was determined by the headspace vapor analysis. Method A: a standard reaction mixture consisted of a substrate at the concentration indicated and 10 ml of enzyme solution with 50 mM phosphate buffer, pH 6.0 or 6.5, in a 50 ml Erlenmeyer flask with a rubber stopper. Method B: a standard reaction mixture contained 1 ml of linoleic acid (7 μmol) or 13-hydroperoxylinoleic acid solution (6.5 μmol) as a substrate and 1 ml of enzyme solution in 12×105 mm test tube with a rubber stopper. Reaction mixtures for the methods A and B were incubated at 35°C for 10 min after 1 min shaking at room temperature. Then, 6 ml of headspace vapor was taken and analysed by GLC under the condition described elsewhere.¹⁷⁾ Amounts of C₆-aldehydes were calculated from the standard curve obtained with authentic compounds.¹⁸⁾

Preparation of 9- and 13-hydroperoxylinoleic acids and 13-hydroperoxylinolenic acid. 9- and 13-hydroperoxylinoleic acids were prepared from linoleic acid essentially according to the procedure described previously,¹⁹⁾ using potato lipoxygenase^{20,21)} and soybean lipoxygenase (Sigma type I),²²⁾ respectively. 13-Hydroperoxylinolenic acid was prepared from linolenic acid and soybean lipoxygenase.^{18,22)} These hydroperoxides gave a sharp absorbance at 234 nm and a single spot on silica gel TLC.

Centrifugal fractionation of enzymes in cucumber seedlings. Six day-old etiolated seedlings of cucumber (110 g in fr. wt.) were homogenized in a Waring blender for 30 sec with 3 vol of grinding buffer: 50 mM phosphate buffer (pH 7.0) containing 1 mM MgCl₂, 1 mM EDTA, 1 mM GSH and 0.4 M sucrose. The homogenate was filtered through 2 layers of gauze, and the filtrate was centrifuged at 10,000×*g* for 10 min. The supernatant (10,000×*g* supernatant) was again centrifuged at 88,000×*g* (*R*_{max}, Hitachi RP 30A rotor) for 90 min. The fractions thus obtained were referred to as 88,000×*g* supernatant and 88,000×*g* pellet. The 88,000×*g* pellet was suspended gently in a small amount of the grinding buffer and centrifuged at 149,000×*g* (*R*_{max}, Hitachi RP 55 rotor) for 60 min to wash 88,000×*g* pellet. The washed 88,000×*g* pellet was suspended and homogenized with 50 mM phosphate buffer, pH 6.5, containing 1 mM GSH and 0.1% Triton X-100, and stirred gently for 1 hr at 4°C. Then, the 88,000×*g* pellet suspension was centrifuged at 149,000×*g* for 90 min. The supernatant and pellet thus obtained were referred to as Triton X-100 supernatant and Triton X-100 pellet, respectively.

Purification of lipoxygenase. Crude extracts were used as enzyme source. i) *Alfalfa.* (NH₄)₂SO₄ was added to the alfalfa crude extract to bring to 70% saturation. The mixture was centrifuged at 10,000×*g*

for 20 min and separated into pellet, supernatant and surface fluffy layer, among which fluffy layer contained 60% of lipoxxygenase activity. The fluffy layer was collected, dissolved in and dialyzed against 10 mM phosphate buffer, pH 7.5. The dialyzed enzyme solution was applied to CAP-S₁ cellulose (trimethylamino-2-hydroxypropyl cellulose) column equilibrated with 10 mM phosphate buffer, pH 7.5, and eluted with 0, 0.1 and 0.2 M NaCl in 10 mM phosphate buffer, pH 6.5. Lipoxxygenase eluted with 0.1 M NaCl was used as a partially purified enzyme. ii) *Cucumber*. (NH₄)₂SO₄ was added to the cucumber extract to bring to 70% saturation, and the mixture was centrifuged at 10,000 × *g* for 20 min. The pellet which contained lipoxxygenase was dissolved in and dialyzed against 10 mM phosphate buffer, pH 7.5. The dialyzed enzyme was subjected to DEAE cellulose column equilibrated with 10 mM phosphate buffer, pH 7.5. Lipoxxygenase obtained in unadsorbed fraction was precipitated by (NH₄)₂SO₄, collected by centrifugation, dissolved in and dialyzed against 10 mM acetate buffer, pH 5.5. The dialyzed enzyme solution was applied to CM cellulose column equilibrated with 10 mM acetate buffer, pH 5.5, washed with the same buffer and eluted with 0.125 M NaCl in 10 mM acetate buffer, pH 5.5, to yield purified enzyme.

Identification and determination of hydroperoxides of C₁₈-fatty acids. The mixture, after lipoxxygenase reaction was performed, was acidified to pH 3 with 2 N HCl and then extracted with *n*-hexane. The hexane extract was added to excess NaBH₄ in aq. 50% (v/v) MeOH adjusted to pH 9 with 50 mM borate buffer. The mixture under N₂ was stirred for 30 min at room temperature, and diluted with 1 vol of water. After the reaction mixture was acidified to pH 3 with 2 N HCl, the reduced products including hydroxylinoleic acids were extracted with Et₂O. The Et₂O extract was removed under N₂ after esterification with CH₃H₂. The residue thus obtained was subjected to HPLC analysis and methyl 9- and 13-hydroxylinoleates were confirmed by comparison with authentic specimens. HPLC was performed with Shimadzu-Dupont LC-2 using an FD column (7.9 × 250 mm, silicic acid derivatives prepared by Shimadzu) and *n*-hexane-EtOH (95:5) as a carrier solvent (flow rate, 2 ml/min). Detection was performed by the absorbance at 234 nm.

Detection of hydroperoxides produced from linoleic acid or linolenic acid by the crude extracts of etiolated alfalfa and cucumber seedlings was performed as follows. The reaction mixture, after the reaction was performed with the same reaction mixture as that for the headspace method A, was extracted with 5 ml of *n*-hexane. The hexane extract containing hydroperoxylinoleic acid or hydroperoxylinolenic acid was condensed to 2 ml, then 25 μl was subjected to HPLC analysis: an FD column, *n*-hexane-EtOH (80:20) as a carrier solvent (flow rate, 2 ml/min) and detection by

the absorbance at 234 nm. Under this condition, 9- and 13-hydroperoxides could not be separated. Amounts of hydroperoxides were calculated from the standard curve obtained by the same extraction procedures from 13-hydroperoxylinoleic acid dissolved in distilled water.

Protein determination. Protein was determined by the method of Lowry *et al.*,²³⁾ after precipitating protein by 5% trichloroacetic acid, collecting the precipitate by centrifugation and dissolving in 0.2 N NaOH.

RESULTS

Changes in the activities of n-hexanal formation, lipoxxygenase and hydroperoxide lyase during the germination

Figures 1 and 2 show the changes in the activities of *n*-hexanal formation from linoleic acid, lipoxxygenase and hydroperoxide lyase (tentatively termed according to Vick and Zimmerman⁷⁾) of the crude extracts during the germination of seeds in the dark. Fresh weight and protein contents increased with the development of germination.

In alfalfa (Fig. 1), *n*-hexanal formation with or without addition of linoleic acid was high in dry seeds and in the early stage (1~2 day)

FIG. 1. Changes in the Activities of *n*-Hexanal Formation, Lipoxxygenase and Hydroperoxide Lyase of the Crude Extract of Alfalfa during Germination.

(.....) shows fresh weight per gram of initial dry seeds (indicated as g seeds). Crude extract was prepared with 3 vol of 50 mM phosphate buffer, pH 6.5. (•—•) indicates protein content in the crude extract. Activities of lipoxxygenase (○—○) and hydroperoxide lyase (●—●) were determined at pH 6.5 from the increase and the decrease in absorbance at 234 nm, respectively. An amount of *n*-hexanal formed with (▲—▲) or without (△—△) addition of linoleic acid (70 μmol) was determined by the headspace method A at pH 6.5 as in Methods.

of germination, and then the activity lowered. Lipoygenase and hydroperoxide lyase activities were also high till the second day, and thereafter lowered. Seedlings germinated for 5 days or more lost their activities completely.

In cucumber (Fig. 2), the activity for *n*-hexanal formation became maximum in 6 day-old seedlings, and no activity was found in dry seeds and less activity in 10 day-old seedlings. Lipoygenase activity appeared in 2 day-old seedling and then increased rapidly. Hydroperoxide lyase activity began to appear after 5 day-germination and became maxi-

FIG. 2. Changes in the Activities of *n*-Hexanal Formation, Lipoygenase and Hydroperoxide Lyase of the Crude Extract of Cucumber during Germination.

Symbols and procedures are the same as in Fig. 1 except that 50 mM phosphate buffer, pH 6.0, was used.

um in 6~7 day-old seedlings. But no activities of lipoygenase and hydroperoxide lyase could be detected in dry seeds. Thus, the three kinds of activities became maximum in 6~7 day-old cucumber seedlings.

C₆- and C₉-aldehyde formation from C₁₈-unsaturated fatty acids and their hydroperoxides by the crude homogenates of etiolated seedlings

In order to investigate the *C₆*- and *C₉*-products from linoleic acid and its hydroperoxides, the crude homogenates of 12 hr-old etiolated alfalfa and 6 day-old etiolated cucumber seedlings were incubated with linoleic acid or its hydroperoxides, then the essential oil was prepared and analysed with GLC.¹⁴⁾ Optimal pH for *n*-hexanal formation from linoleic acid was 6.0~6.5 both for alfalfa and cucumber. Table I shows that *n*-hexanal was produced from linoleic acid by the homogenate of alfalfa and cucumber but not *trans*-2-hexenal from linoleic acid. A small amount of *trans*-2-nonenal was also produced from linoleic acid with the cucumber homogenate but not with the alfalfa homogenate. When 13-hydroperoxylinoleic acid was used as a substrate, *n*-hexanal was produced in a larger

TABLE I. *C₆*-AND *C₉*-ALDEHYDES PRODUCED FROM LINOLEIC ACID AND ITS HYDROPEROXIDES BY THE CRUDE HOMOGENATES OF ETIOLATED ALFALFA AND CUCUMBER SEEDLINGS

Substrate added	Aldehydes (μmol/g fr. wt.)			
	<i>n</i> -Hexanal	<i>trans</i> -2-Hexenal	<i>trans</i> -2-Nonenal	<i>trans</i> -2, <i>cis</i> -6-Nonadienal
<i>Alfalfa</i>				
None	0.290	0.041	trace	trace
Linoleic acid	0.510	0.051	0	0
13-Hydroperoxylinoleic acid	0.809	0.062	0	0
9-Hydroperoxylinoleic acid	0.412	0.062	0.050	0
<i>Cucumber</i>				
None	0.175	0.021	0.019	trace
Linoleic acid	0.192	0.022	0.050	trace
13-Hydroperoxylinoleic acid	0.331	0	0	0
9-Hydroperoxylinoleic acid	0.138	0.014	0.961	0

The crude homogenates (300 ml) were prepared from 12 hr-old etiolated alfalfa seedlings (50 g) with 50 mM phosphate buffer, pH 6.5, and from 6 day-old etiolated cucumber seedlings (50 g) with 50 mM phosphate buffer, pH 6.0. Each crude homogenate was incubated at 25°C for 3 min with 360 μmol of substrate. Then, the mixture was subjected to steam-distillation to prepare the essential oil, which was analyzed quantitatively by GLC.¹⁴⁾

amount than that from linoleic acid in alfalfa and cucumber and no C₉-aldehydes were produced in both.

In contrast, when 9-hydroperoxylinoleic acid was used, *trans*-2-nonenal was produced but not *trans*-2, *cis*-6-nonadienal. However, though *trans*-2-nonenal was produced extremely from 9-hydroperoxide by the cucumber homogenate, alfalfa homogenate gave only a slight amount of *trans*-2-nonenal. These indicate that *n*-hexanal is produced *via* 13-hydroperoxide from linoleic acid by alfalfa and cucumber homogenates and that *trans*-2-nonenal is produced from 9-hydroperoxylinoleic acid by the cucumber homogenate only.

When linolenic acid was used as a substrate, *trans*-2-hexenal was produced both by the crude homogenates of alfalfa and cucumber seedlings, and a very small amount of *trans*-2, *cis*-6-nonadienal was detected in cucumber but not in alfalfa.

C₆-Aldehyde formation from C₁₈-fatty acids and their 13-hydroperoxides by the crude extracts of etiolated seedlings and detection of hydroperoxides produced from C₁₈-fatty acids

C₆-Aldehyde formation from C₁₈-unsaturated fatty acids and their 13-hydroperoxides and hydroperoxide formation during C₆-aldehyde formation from C₁₈-fatty acids were investigated by the headspace method and HPLC analysis, respectively.

Figure 3 shows that *n*-hexanal formation from linoleic acid increased with the increase in linoleic acid added in the range of the concentration tested (0~20 μ mol) both in alfalfa and cucumber. However, when 13-hydroperoxylinoleic acid was used as a substrate, the highest *n*-hexanal formation was found with addition of 2 μ mol of 13-hydroperoxide and then, *n*-hexanal formation lowered with the increase in 13-hydroperoxide added. This may be due to toxic action of high concentration of 13-hydroperoxylinoleic acid. The formation of hydroperoxylinoleic acid, which could not be identified as 9- or 13-hydroperoxide under this condition performed, was

FIG. 3. Formation of *n*-Hexanal and Hydroperoxylinoleic Acid by the Crude Extracts of Etiolated Alfalfa (A) and Cucumber (B) Seedlings.

The crude extracts were prepared from 12 hr-old alfalfa and 6 day-old cucumber seedlings with 20 vol of 50 mM phosphate buffer, pH 6.5. *n*-Hexanal formation was determined by the headspace method A with addition of the indicated concentration of linoleic acid (○) or 13-hydroperoxylinoleic acid (●). (×) shows *n*-hexanal formation from linoleic acid or 13-hydroperoxylinoleic acid by heat-treated (100°C, 10 min) crude extracts. Hydroperoxide formation from linoleic acid was determined by HPLC analysis of hexane extract of reaction products as in Methods. (Δ) shows hydroperoxide formation from linoleic acid with the crude extract and (▲), that without the crude extracts.

observed, when linoleic acid was used as a substrate (Fig. 3). The amount of detected hydroperoxylinoleic acid was enough to yield *n*-hexanal obtained from linoleic acid. These results both for alfalfa and cucumber are well consistent with the hypothesis of the *n*-hexanal formation *via* 13-hydroperoxide from linoleic acid.

When linolenic acid or 13-hydroperoxylinolenic acid was used as a substrate, *cis*-3-hexenal and *trans*-2-hexenal were produced as C₆-aldehydes (hexenals) by the crude extracts of alfalfa and cucumber, as shown in Table II. In these cases, hexenals were produced with addition of 20 μ mol of 13-hydroperoxylinolenic acid more than with 2 μ mol of that both for alfalfa and cucumber unlike *n*-hexanal formation with 13-hydroperoxylinoleic acid.

Hydroperoxylinolenic acid was also detected from the incubation mixtures of the crude extracts with linolenic acid (Table II).

Whether *cis*-3-hexenal is produced from 13-hydroperoxylinolenic acid at first and then isomerizes to *trans*-2-hexenal is shown in Tables II and III and Fig. 4. *cis*-3-Hexenal and *trans*-2-hexenal were produced from linolenic acid and its 13-hydroperoxide, but a short time incubation (3 min) gave a relatively larger amount of *cis*-3-hexenal than a long-time incubation (10 min), as shown in Table II. These suggest that *cis*-3-hexenal isomerized

to *trans*-2-hexenal during the incubation. The suggestion was confirmed by the fact that *cis*-3-hexenal was rapidly isomerized to *trans*-2-hexenal by the crude extracts of alfalfa and cucumber seedlings, as shown in Fig. 4 and, particularly, cucumber extract showed a high activity for isomerization. Thus, *cis*-3-hexenal was produced *via* 13-hydroperoxide from linolenic acid and then isomerized to *trans*-2-hexenal by the crude extracts of alfalfa and cucumber seedlings. In addition, a large amount of *cis*-3-hexenal was produced from 13-hydroperoxylinolenic acid by using a 88,000

TABLE II. FORMATION OF *cis*-3-HEXENAL AND *trans*-2-HEXENAL FROM LINOLENIC ACID AND ITS 13-HYDROPEROXIDE BY THE CRUDE EXTRACTS OF ETIOLATED ALFALFA AND CUCUMBER SEEDLINGS

Substrate added	Concentration (μ mol)	Incubation time (min)	Hexenals formed (μ mol/flask)		a: b	Hydroperoxides formed (μ mol/flask)
			<i>cis</i> -3-Hexenal (a)	<i>trans</i> -2-Hexenal (b)		
<i>No enzyme (Buffer)</i>						
Linolenic acid	20	10	trace	0	—	0
13-Hydroperoxy-linolenic acid	20	10	trace	trace	—	—
<i>Alfalfa</i>						
None	—	10	trace	0.03	0: 100	0
Linolenic acid	2	3	0.11	0.06	65: 35	0
	2	10	0.11	0.14	44: 56	0.12
	20	3	0.70	0.08	90: 10	0.15
	20	10	0.48	0.19	72: 28	0.17
13-Hydroperoxy-linolenic acid	2	3	0.70	0.07	91: 9	—
	2	10	0.22	0.21	51: 49	—
	20	3	1.05	0.07	94: 6	—
	20	10	0.51	0.22	70: 30	—
<i>Cucumber</i>						
None	—	10	trace	0.20	0: 100	0
Linolenic acid	2	3	trace	0.30	0: 100	0
	2	10	trace	0.42	0: 100	trace
	20	3	trace	0.37	0: 100	0.11
	20	10	trace	0.50	0: 100	0.23
13-Hydroperoxy-linolenic acid	2	3	0.11	0.39	22: 78	—
	2	10	trace	0.52	0: 100	—
	20	3	0.22	0.38	37: 63	—
	20	10	trace	0.50	0: 100	—

The crude extracts were prepared 12 hr-old alfalfa and 6 day-old cucumber seedlings with 20 vol of 50 mM phosphate buffer, pH 6.5. Formation of *cis*-3-hexenal and *trans*-2-hexenal was determined by the headspace method A at 35°C by the indicated periods of incubation with 10 ml of the crude extract containing indicated concentrations of linolenic acid or its 13-hydroperoxide. Hydroperoxide formation from linolenic acid was analysed by HPLC after extraction of reaction mixture with *n*-hexane. Amount of hydroperoxylinolenic acid was calculated from standard curve with 13-hydroperoxylinoleic acid.

TABLE III. FORMATION OF *cis*-3-HEXENAL AND *trans*-2-HEXENAL FROM LINOLENIC ACID AND ITS 13-HYDROPEROXIDE BY 88,000×*g* PELLET OF ETIOLATED CUCUMBER SEEDLINGS

Enzyme	Substrate (10 μmol)	Incubation time (min)	Hexenals formed (μmol/flask)		a: b
			<i>cis</i> -3- Hexenal (a)	<i>trans</i> -2- Hexenal (b)	
None	Linolenic acid	10	trace	trace	—
	13-Hydroperoxy- linolenic acid	10	trace	trace	—
88,000× <i>g</i> pellet	None	10	0	0.08	0: 100
	Linolenic acid	10	0.11	0.19	37: 63
	13-Hydroperoxy- linolenic acid	3	1.43	0.14	91: 9
	13-Hydroperoxy- linolenic acid	10	0.48	0.31	61: 39

A 88,000×*g* pellet was prepared from 6 day-old etiolated cucumber seedlings (35 g) by the procedures as in Methods, then suspended in 60 ml of 50 mM phosphate buffer, pH 6.5, containing 1 mM GSH, and used as an enzyme solution. Formation of *cis*-3-hexenal and *trans*-2-hexenal was determined by the headspace method A.

FIG. 4. Isomerization of *cis*-3-Hexenal to *trans*-2-Hexenal by the Crude Extracts of Etiolated Alfalfa (A) and Cucumber (B) Seedlings.

Relative composition was determined by the headspace method. Ten ml of the crude extract prepared from 12 hr-old etiolated alfalfa seedlings or 6 day-old etiolated cucumber seedlings with 20 vol of 50 mM phosphate buffer, pH 6.5, was incubated with *cis*-3-hexenal (6 μmol) in a 50 ml flask for indicated periods and the products (hexenals) in the headspace vapor were analyzed by GLC. (○) shows *cis*-3-hexenal and (●), *trans*-2-hexenal.

×*g* pellet as a hydroperoxide lyase rich fraction, as shown in Table III. This may be due to the removal of isomerizing factor involved in the crude extract of cucumber seedlings. In cucumber fruits, a similar result was found that *cis*-3-nonenal was detected easily without isomerization as the purification of hydroperoxide lyase proceeded.

The formation of C₆-aldehydes from C₁₈-unsaturated fatty acids has been reported in green leaves in which the enzyme system is

localized in chloroplast lamellae.^{19, 88)} DCIP, MB and SKF 525-A are potent inhibitors for *n*-hexenal formation in green leaves,¹⁷⁾ but these compounds at 1 mM did not inhibit *n*-hexenal formation by the crude extracts of alfalfa and cucumber seedlings. *p*-Chloromercuribenzoate at 0.05 mM did not inhibit the activity for *n*-hexenal formation.

Subcellular localization of lipoxygenase and hydroperoxide lyase in cucumber seedlings

To demonstrate subcellular localization of enzymes responsible for *n*-hexenal formation, we fractionated the subcellular components of cucumber seedlings by differential centrifugation. A 10,000×*g* pellet showed a little activity of hydroperoxide lyase but no activity of lipoxygenase. Most lipoxygenase activity was found in 88,000×*g* supernatant and hydroperoxide lyase with high specific activity was found in 88,000×*g* pellet. Hydroperoxide lyase was solubilized by the treatment with 0.1% Triton X-100 (Table IV). However, Triton X-100 supernatant still exhibit a little activity of lipoxygenase. The result indicates that hydroperoxide lyase is in a membrane bound form. Table V shows *n*-hexenal formation by the coupled reaction of 88,000×*g* supernatant containing lipoxygenase with Triton X-100 supernatant containing hydroperoxide lyase. Hydroperoxide lyase pro-

TABLE IV. SUBCELLULAR LOCALIZATION OF LIPOXYGENASE AND HYDROPEROXIDE LYASE IN ETIOLATED CUCUMBER SEEDLINGS

Fraction	Vol (ml)	Protein (mg)	Lipoxygenase		Hydroperoxide lyase	
			Total activity (U)	Specific activity (U/mg)	Total activity (U)	Specific activity (U/mg)
88,000 $\times$ g supernatant	300	465	270	0.58	1.5	0.03
88,000 $\times$ g pellet						
{ Triton X-100 supernatant	10.5	8.5	4.2	0.49	1.2	0.14
{ Triton X-100 pellet	10.5	14.7	5.3	0.36	0	0

Each fraction was prepared by the procedures as in Methods. Lipoxygenase was determined from oxygen uptake and hydroperoxide lyase was determined at pH 6.5 from the decrease in absorbance at 234 nm as in Methods.

TABLE V. *n*-HEXANAL FORMATION FROM LINOLEIC ACID AND 13-HYDROPEROXYLINOLEIC ACID BY LIPOXYGENASE AND HYDROPEROXIDE LYASE FROM ETIOLATED CUCUMBER SEEDLINGS

Enzyme	<i>n</i> -Hexanal formed (μ mol/assay tube)		
	None	Linoleic acid	13-Hydroperoxylinoleic acid
Lipoxygenase	0.10	0.39	0.36
Hydroperoxide lyase	0.22	0.64	1.69
Lipoxygenase + hydroperoxide lyase	0.25	2.50	1.54
Lipoxygenase + hydroperoxide lyase (heat-denatured)	0.16	0.28	0.38

A 0.5 ml of 88,000 $\times$ g supernatant and 0.5 ml of Triton X-100 supernatant in Table IV were used as an enzyme source of lipoxygenase and hydroperoxide lyase, respectively. A 0.5 ml of 50 mM phosphate buffer, pH 6.5, containing 1 mM GSH with or without 0.1% Triton X-100 (final concentration in reaction mixture, 0.025%) was added to the reaction mixture of lipoxygenase or hydroperoxide lyase alone. Denatured enzymes were prepared by 10 min-heating in boiling water. *n*-Hexanal formed was determined by the headspace method B.

duced *n*-hexanal from 13-hydroperoxylinoleic acid, but lipoxygenase could not catalyze this conversion. Linoleic acid was converted to *n*-hexanal by the combination of lipoxygenase

and hydroperoxide lyase, but not by either lipoxygenase or hydroperoxide lyase. Heat-denatured enzymes could not catalyze *n*-hexanal formation from each substrate. Moreover, cucumber lipoxygenase used in Table V was replaced by soybean lipoxygenase. These results clearly indicate that *n*-hexanal was produced *via* 13-hydroperoxide from linoleic acid by lipoxygenase and hydroperoxide lyase. A 88,000 $\times$ g pellet also catalyzed the formation of *cis*-3-hexenal and *trans*-2-hexenal from 13-hydroperoxylinolenic acid, as shown in Table IV.

Purification and properties of lipoxygenase

i) *Alfalfa*. Lipoxygenase was partially purified 2-fold with the recovery of 15% (Table VI). The activity for *n*-hexanal formation from linoleic acid was still found after (NH₄)₂SO₄ fractionation, but not after CAP-S₁ cellulose chromatography. Thus, lipoxygenase could be separated from hydroperoxide lyase. Optimal pH of the enzyme was 6.5 and the enzyme was relatively stable at pH 5.0~7.5. The ratio of 13- and 9-hydroperoxylinoleic acids produced by this enzyme was determined to be 65:35 based on HPLC analysis (Fig. 5). This ratio was a little different from 50:50 reported by Chang *et al.*²⁴⁾ Alfalfa lipoxygenase was localized in a soluble fraction as well as cucumber one as it was found in 88,000 $\times$ g supernatant.

ii) *Cucumber*. Lipoxygenase was purified 80-fold with the recovery of 40% (Table VI). Lipoxygenase preparation after CM cellulose chromatography did not show the activity for *n*-hexanal formation from linoleic acid. Optimal pH of the enzyme was 5.5~

6.0 and the enzyme was stable at pH 5.5~8.0. Apparent *K_m* value for linoleic acid was 0.11 mM at 25°C. The ratio of 13- and 9-hydroperoxylinoleic acids as products was 85:15 (Fig. 5).

TABLE VI. SUMMARY OF PURIFICATION OF LIPOXYGENASE

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)
<i>Alfalfa</i>			
Crude extract	369	945	2.6
(NH ₄) ₂ SO ₄ fr.	205	500	2.4
CAP-S ₁ cellulose	27.4	136	5.0
<i>Cucumber</i>			
Crude extract	1764	525	0.30
(NH ₄) ₂ SO ₄ fr.	1210	403	0.33
DEAE cellulose	850	490	0.58
CM cellulose	7.8	198	25.4

Purification was started from 12 hr-old alfalfa seedlings (22.5 g) and 6 day-old cucumber seedlings (72 g). Lipoxygenase activity was determined from the absorbance change at 234 nm.

FIG. 5. HPLC of Products by Lipoxygenase Reaction of Alfalfa (A) and Cucumber (B) Seedlings.

Sample preparation and HPLC performance are shown in MATERIALS and METHODS. (I) indicates methyl linoleate; (II), methyl 13-hydroxylinoleate; and (III), methyl 9-hydroxylinoleate.

Properties of hydroperoxide lyase

Hydroperoxide lyase, which catalyzed *n*-hexanal formation from 13-hydroperoxylinoleic acid and *cis*-3-hexenal from 13-hydroperoxylinolenic acid (Tables III and V), was a membrane bound enzyme and it was solubilized by the treatment with Triton X-100 described above in cucumber seedlings (Table. IV). However, the possibility that the hydroperoxide lyase activity determined from decrease in the absorbance at 234 nm (Table IV and Figs. 1 and 2) contains in part hydroperoxide isomerase activity can not be excluded.^{25~30)} Optimal pH of hydroperoxide lyase in the crude extracts was pH 6~7 both for alfalfa and cucumber. Alfalfa hydroperoxide lyase showed a high activity with 13-hydroperoxide as a substrate, but not with 9-hydroperoxide (Table I). In contrast, cucumber enzyme was active with both 9- and 13-hydroperoxides.

DISCUSSION

Although *n*-hexanal formation from linoleic acid in etiolated watermelon seedlings is reported,⁷⁾ the enzymatic properties of this biosynthetic pathway and C₆-aldehyde formation from linolenic acid in etiolated seedlings have not been well established. We have inclusively shown in this paper that *n*-hexanal and *cis*-3-hexenal (which isomerizes easily to *trans*-2-hexenal) are produced *via* 13-hydroperoxides from linoleic acid and linolenic acid, respectively, in etiolated alfalfa and cucumber seedlings, and have characterized some properties of lipoxygenase and hydroperoxide lyase as the enzymes responsible for C₆-aldehyde formation in etiolated seedlings.

The changes in *n*-hexanal formation from linoleic acid, lipoxygenase and hydroperoxide lyase activities during germination paralleled both in alfalfa and cucumber: *n*-hexanal for-

mation occurred when lipoxygenase and hydroperoxide lyase activities appeared (Figs. 1 and 2). Cucumber showed the maximal activities the 6~7 th day of germination but not in dry seeds, being a pattern similar to that of watermelon.⁷⁾ In contrast, alfalfa showed the maximal activities in dry seeds and 1st-2nd day of germination. Thus, the two types of patterns of the activity changes were observed. A similar enzyme system for *n*-hexanal formation from linoleic acid during germination seemed to occur in a wide range of plants, as we observed the *n*-hexanal formation from linoleic acid in some leguminous and other plants (unpublished data).

From the analysis of essential oil as in Table I, *n*-hexanal was found to be a main volatile product both from linoleic acid and its 13-hydroperoxide in alfalfa and cucumber. On the other hand, *trans*-2-nonenal was produced from 9-hydroperoxylinoleic acid in cucumber seedlings, but not in alfalfa seedlings. These suggest that hydroperoxide lyase of cucumber seedlings utilized 13- and 9-hydroperoxides as a substrate to yield *n*-hexanal and *trans*-2-nonenal, respectively, like that of cucumber fruits^{10~13)} and that alfalfa hydroperoxide lyase utilized 13-hydroperoxide, but not 9-hydroperoxide like tomato fruits.⁸⁾ Main products of lipoxygenase reaction were 9- and 13-hydroperoxylinoleic acids for alfalfa seedlings and 13-hydroperoxide for cucumber seedlings, as shown in Fig. 5. Hence, although 9- and 13-hydroperoxylinoleic acids were produced from linoleic acid by lipoxygenase in the crude extract of alfalfa seedlings, hydroperoxide lyase could cleave only 13-hydroperoxide of both to yield *n*-hexanal. In contrast, in the crude extract of cucumber seedlings, hydroperoxide lyase cleaved the 13-hydroperoxide, which was a sole product of lipoxygenase reaction to yield *n*-hexanal though cucumber hydroperoxide lyase utilized 9- and 13-hydroperoxides.

n-Hexanal formation from linoleic acid and its 13-hydroperoxide was also confirmed by the headspace method in alfalfa and cucumber (Fig. 3). When linolenic acid was used as a

substrate, *cis*-3-hexenal was produced from linolenic acid and its 13-hydroperoxide by the crude extracts of alfalfa and cucumber seedlings. From the results of Tables II and IV and Fig. 4, it was established that *cis*-3-hexenal was at first produced as a C₆-aldehyde from linolenic acid. Observation of hydroperoxide formation during C₆-aldehyde formation from C₁₈-fatty acids by the crude extracts of alfalfa and cucumber seedlings (Fig. 3 and Table II) strongly supports the role of 13-hydroperoxides as an intermediate and involvement of lipoxygenase and hydroperoxide lyase in the crude extracts.

Lipoxygenase was partially purified from etiolated alfalfa and cucumber seedlings and hydroperoxide lyase from cucumber seedlings as the enzymes responsible for C₆-aldehyde formation. Lipoxygenase was obtained in a soluble fraction. On the other hand, hydroperoxide lyase was in a membrane bound form in cucumber seedlings as shown in Tables III and IV. The hydroperoxide lyase in this paper seemed to be the same type of enzyme as reported in watermelon seedlings⁷⁾ and cucumber fruits;^{12,13)} watermelon enzyme was reported to be eluted near the void volume on Sephadex G-200, and cucumber fruit one was localized in, at least, the plasma and Golgi membrane. Hydroperoxide lyase of alfalfa seedlings may be also in a membrane bound form.

From the substrate specificity and products of lipoxygenase and hydroperoxide lyase as described in this paper, coupled reaction of these enzymes results in C₆-aldehyde formation from C₁₈-unsaturated fatty acids (linoleic acid and linolenic acid) *via* their 13-hydroperoxides. The proposed biosynthetic pathway for C₆-aldehydes and the related enzymes are summarized in Scheme I. C₉-Aldehyde formation is also summarized in Scheme I, in which compounds in parentheses have not been confirmed in etiolated cucumber seedlings. But *trans*-2-nonenal is produced in cucumber seedlings mainly when 9-hydroperoxylinoleic acid is used as a substrate, but not linoleic acid (Table I). *trans*-2-Nonenal formed from

SCHEME 1. Proposed Biosynthetic Pathway of C₆- and C₉-Aldehydes from C₁₈-Unsaturated Fatty Acid in Etiolated Seedlings.

Alfalfa (—), cucumber (—).

9-hydroperoxylinoleic acid by the crude homogenate of cucumber seedlings may be an isomerized product of *cis*-3-nonenal as well as in cucumber fruits.^{12,14,31} In the same manner the crude homogenate may give *trans*-2, *cis*-6-nonadienal via *cis*-3, *cis*-6-nonadienal from 9-hydroperoxylinolenic acid.^{12,14,31} The fact that C₆-aldehydes are main products from linoleic acid and linolenic acid in cucumber fruits^{10,11,14,15} may be caused by the difference in products of lipoxygenase reaction in fruits and seedlings.

Recently, we reported the C₆-aldehyde formation from C₁₈-unsaturated fatty acids in green leaves such as tea^{6,17~19,32~36} and *Farfugium japonicum*.^{37~39} However, the enzymes responsible for C₆-aldehyde formation in etiolated seedlings are different from those in green leaves in some points: subcellular localization of the enzymes, behavior of 13-hydroperoxides of C₁₈-fatty acids and inhibition by some drugs *etc.*

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