Biosynthesis of Leaf Alcohol

Formation of 3Z-Hexenal from Linolenic Acid in Chloroplasts of *Thea sinensis* Leaves[†]

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The synthetic activity for 3Z-hexenal, an important precursor of 3Z-hexenol (leaf alcohol), was localized in chloroplasts of *Thea sinensis* leaves. 3Z-Hexenal, which is easily isomerized to 2E-hexenal (leaf aldehyde), was formed from linolenic acid in the presence of oxygen. 13-L-Hydroperoxy-linolenic acid also served as a precursor, but the triglyceride and methyl ester of linolenic acid did not. This enzyme system appeared to be tightly bound to the lamellae membranes of chloroplasts.

It has been reported that 3Z-hexenal (II-a) is formed from linolenic acid (I-a) in the presence of oxygen by an enzyme contained in macerated leaves of *Thea sinensis* and that II-a is an important precursor in the biosynthetic pathway of leaf alcohol (3Z-hexenol) and leaf aldehyde (2E-hexenal, III-a)^{1,2)} as shown in Scheme 1. However, the enzyme system responsible for the production of aldehydes has not been isolated.^{3,4)}

SCHEME 1. Biosynthetic Pathway of Leaf Alcohol and Leaf Aldehyde.

* Alcohol dehydrogenase.

In this paper, we describe that the synthetic activities for II-a and III-a are localized in chloroplasts of tea leaves and that I-a and 13-L-hydroperoxy-linolenic acid (IV-a) are splitted into II-a, which is easily isomerized to III-a, by chloroplasts.

[†] Part XXIX in the series "Leaf Alcohol." For part XXVIII, see Ref. 14).

MATERIALS AND METHODS

Materials. Fresh leaves of *Thea sinensis* var. Yabukita of the 2nd crop (May and July, 1975) were used. Linolenic acid (I-a), trilinolenin (V-a), methyl linolenate (VI-a), linoleic acid (I-b), trilinolein (V-b) and methyl linoleate (VI-b) were a high-purity grade of above 99%.

Preparation of chloroplasts and other subcellular fractions. All procedures were carried out at 4°C. Fresh tea leaves were homogenized in a mixer for 3 min under nitrogen with 3 vol. of 4-diluted McIlvaine's buffer, pH 6.3, containing 0.4 M sucrose (MS buffer), and the homogenate was filtered through 3 layers of gauze. The filtrate was centrifuged at 100 g for 3 min. The supernatant was centrifuged at 1000 g for 10 min and the chloroplast fraction was obtained as precipitates (P-I). P-I was washed with MS buffer. The supernatant was centrifuged at 4000 g for 10 min to collect P-II. After the resulted supernatant was centrifuged at 19,000 g for 20 min, P-III and the supernatant (SUP) were separated.

To prepare the chloroplast rich fraction, both P-I and P-II were collected together and washed with MS buffer twice. The chloroplasts* thus obtained were suspended in 2 vol. of 2.1 M sucrose and stored at -20°C.

Assay of the synthetic activity for hexenals and hexanal in chloroplasts. The synthetic activities for hexenals (II-a and III-a) and hexanal (II-b) were determined by the analysis of headspace vapor with GLC. One gram

* Chloroplast rich fraction.

of chloroplasts (wet weight) was suspended in 30 ml of MS buffer. Ten ml of the suspension containing 20 mg of I-a for hexenals or I-b for hexanal was pre-incubated with shaking for 5 min at room temperature in a rubber-stoppered 50 ml Erlenmeyer flask and then incubated at 40°C for 10 min. Six ml of head-space vapor was analyzed with a Shimadzu GC-4B GLC equipped with FID and a 3 mm × 3 m stainless steel column packed with 20% PEG 20M on cellite 545 (60~80 mesh): column temp. 120°C, inject. temp. 150°C, detector temp. 150°C, the flow rate of N₂ 80 ml/min, attenuation range 0.16 V, and sensitivity 10³ MΩ. The products, hexenals (II-a and III-a) and hexanal (II-b), were identified by comparing with respective authentic compounds²⁾ and the synthetic activities for II-a and III-a, and II-b were represented as peak areas (mm²), respectively.

Electronmicroscopy. Chloroplast precipitates (P-I) were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 hr and then postfixed in 1% OsO₄ for 1 hr. Thin sections were prepared by the method of Luft using Epon 812 mixture and examined with a JEM-100B electronmicroscope.

Preparation of 13-L-hydroperoxy-linolenic and -linoleic acid. 13-L-Hydroperoxy-linolenic (IV-a) and -linoleic acid (IV-b) were prepared using soybean lipoxygenase I⁶⁾ as follows. Linolenic (I-a) or linoleic acid (I-b) (200~400 mg) was suspended in 500 ml of 0.1 M NH₄Cl-NH₄OH buffer, pH 9.0, and the whole was incubated with lipoxygenase I in an ice bath for 1 hr under O₂. The reaction mixture was acidified to pH 3.0 with HCl, and hydroperoxide was extracted with 150 ml of ether three times. After removing ether, hydroperoxide was dissolved in a small amount of ether and the ether solution was applied on a silica gel dry-column and developed with 40 ml of petrol: ether (3:1, v/v) and then eluted with 100 ml of petrol: ether (1:1, v/v). The latter eluate was collected and removal of the solvent gave hydroperoxide (IV-a or IV-b; yield, 40~50%). Hydroperoxides (IV-a and IV-b) thus obtained were proved to be pure by TLC and their UV spectra.

Analysis of fatty acid in chloroplasts. The fatty acids in chloroplasts were quantitatively analyzed according to the method reported previously.⁷⁾

RESULTS

Localization of synthetic activities for hexenals and hexanal

From 100 g of fresh tea leaves, four fractions, P-I (7.2 g), P-II (2.0 g), P-III (1.0 g) and SUP (400 ml), were prepared as the chloroplast rich fr., the chloroplast fragment rich fr.,

the mitochondrial rich fr., and the microsomal rich and the soluble fr., respectively. Ninety percent of the synthetic activity for hexenals from I-a and seventy six percent of that for hexanal from I-b were localized in P-I as shown in Table I. Electronmicroscopic ob-

TABLE I. LOCALIZATION OF SYNTHETIC ACTIVITIES FOR HEXENALS AND HEXANAL

Fraction	Synthetic activities (mm ²)			
	Hexenals		Hexanal	
	S.A. ^{a)}	Total activity	S.A. ^{a)}	Total activity
P-I	306	6609 (90) ^{b)}	465	10,044 (76) ^{b)}
P-II	103	618 (8)	267	1602 (12)
P-III	54	162 (2)	159	477 (4)
SUP	2	80 (0)	27	1080 (8)

a) One gram of each precipitate was suspended in 30 ml of MS buffer. Ten ml of each suspension or SUP was used for assay of the synthetic activities (specific activity: S.A.).

b) (): the relative value, %.

servation of P-I fraction shows that this fraction was composed of almost pure chloroplasts and few other cell components (Fig. 1). Although the chloroplasts lacked the outer limiting membrane, the lamellae systems were essentially intact.

The synthetic activities found in P-II and P-III were considered to originate from chloroplast fragments precipitated in these fractions.

In the following experiments, the chloroplast rich fraction, which was composed of P-I and P-II, was used.

Chloroplasts produced hexenals from I-a, and hexanal from I-b in the presence of oxygen. Products from I-a and I-b by chloroplasts are shown in Table II. When hexenals (II-a and III-a) and hexanal (II-b) were produced enzymatically from I-a and I-b, oxygen was required. Under nitrogen, the synthetic activities decreased to 25~40% of those under oxygen.

The synthetic activities for such C₉-aldehydes as 2E,6Z-nonadienal and 2E-nonenal^{7~9)} were not found in the chloroplasts of tea

FIG. 1. Electronmicroscopic Pictures of P-I of Tea Leaves.

TABLE II. EFFECT OF OXYGEN ON THE FORMATION OF HEXENALS AND HEXANAL

Substrate	Products (mm ²)			
	Hexenals			Hexanal
	3 <i>Z</i> -hexenal	2 <i>E</i> -hexenal	Total hexenals	
<i>Under O₂ (Air)</i>				
None	72	25	97	42
Linolenic acid (I-a)	376	174	550	114
Linoleic acid (I-b)	56	35	91	750
<i>Under N₂</i>				
None	64	18	82	24
Linolenic acid (I-a)	156	45	201	48
Linoleic acid (I-b)	56	28	84	297

leaves.

Conditions for synthetic activities for hexenals and hexanal in chloroplasts

i) *Preservation and thermostability.* In chloroplasts suspended in 2.1 M sucrose and stored at -20°C , more than 80% of the synthetic activities remained over 2 months. Chloroplasts suspended in MS buffer retained more than 80% of the synthetic activities for 3 hr at 25°C and for 5 days at 5°C , respectively; but the synthetic activities were almost lost after 20 hr at 25°C and 7 days at 5°C . Over 80% of the synthetic activities were lost after 1 min heating at 60°C . The synthetic activities were considered to be very unstable to heat. Thus, chloroplasts were preserved in 2.1 M sucrose at -20°C in a freezer.

ii) *pH- and temperature-optima.* The synthetic activities had an optimal pH at 6.5~7.0 for both I-a and I-b and an optimal temperature at 40°C for I-a and at 40~45°C for I-b.

iii) *Effects of metal ions on the synthetic activities for hexenals and hexanal in chloroplasts.* As shown in Table III, CuSO₄ in-

TABLE III. EFFECT OF SOME METALS ON THE SYNTHETIC ACTIVITIES FOR HEXENALS AND HEXANAL^{a)}

Addition	Concentration [M]	Relat. synth. act. (%) ^{b)}	
		Hexenals	Hexanal
Control		100	100
CuSO ₄	10 ⁻³	41	67
	10 ⁻⁴	55	74
K ₄ Fe(CN) ₆	10 ⁻³	113	96
	10 ⁻⁴	98	105
K ₃ Fe(CN) ₆	10 ⁻³	98	102
	10 ⁻⁴	109	108

^{a)} One gram of chloroplasts was suspended in 30 ml of MS buffer for assay of hexenal synthetic activity and in 150ml for that of hexanal synthetic activity. Each 10 ml of suspension was used for assay.

^{b)} Relative synthetic activities.

hibited the formation of hexenals and hexanal. Formation of II-a and III-a was more inhibited than that of II-b. K₄Fe(CN)₆ and K₃Fe(CN)₆ which affect some oxygenase activities did not activate nor inhibit the synthetic activities in chloroplasts. Metal ions (10⁻³ to 10⁻⁵ M; Na⁺, K⁺, Ni²⁺, Ca²⁺, Zn²⁺, Sn²⁺, Hg²⁺, Co²⁺, Fe³⁺, Al³⁺) and anions (10⁻³ to 10⁻⁵ M; Cl⁻, NO₃⁻, SO₄²⁻, CH₃COO⁻) were not effective to the synthetic activities in chloroplasts.

Substrate specificity

The chloroplast catalyzed the formation of hexenals and hexanal from I-a and I-b, respectively (Tables II and IV). As shown in Table IV, VI-a and VI-b were poor substrates compared to I-a and I-b. Triglycerides, V-a and V-b, showed no activities.

Over 70% of the activities for I-a and I-b were found when 13-hydroperoxides (IV-a and IV-b) were used as substrates (Table IV).

TABLE IV. SUBSTRATE SPECIFICITY FOR THE SYNTHETIC ACTIVITIES FOR HEXENALS AND HEXANAL

Substrate	Relative synthetic activities (%)			
	Hexenals		Hexanal	
	5 mg	20 mg	5 mg	20 mg
Linolenic acid (I-a)	100	100	0	0
Linoleic acid (I-b)	0	0	100	100
13-OOH Linolenic acid (IV-a)	75	15	0	0
13-OOH Linoleic acid (IV-b)	0	0	73	56
Trilinolenin (V-a)	0	0	0	0
Trilinolein (V-b)	0	0	0	0
Methyl linolenate (VI-a)	0	0	0	0
Methyl linoleate (VI-b)	0	0	5	3
Palmitic acid	0	0	0	0
Stearic acid	0	0	0	0
Oleic acid	10	10	8	10

As shown in Fig. 2-[B], the optimal substrate amount was found to be 7 mg for I-b and IV-b, and the formation of II-b decreased as the substrate concentration increased. On the other hand, the maximum synthetic activity for hexenals was found at 15 mg of I-a (Fig. 2-[A]).

FIG. 2. Effect of Substrate Amount on the Synthetic Activities for Hexenals [A] and Hexanal [B].

[A]: ○—○, linolenic acid; ●—●, 13-OOH linolenic acid. [B]: ○—○, linoleic acid; ●—●, 13-OOH linoleic acid.

Oleic acid showed an appreciable activity (about 10% of that of I-a or I-b), while palmitic and stearic acid gave no hexenals and hexanal. Tea chloroplasts utilized free fatty acids: I-a and I-b being the best substrates.

Chloroplasts contained 2.527 mg of I-a and 1.144 mg of I-b in total (Table V). Ninety eight percent of I-a and 80% of I-b were found

TABLE V. CONTENTS OF LINOLENIC AND LINOLEIC ACID IN CHLOROPLASTS

Fraction	Linolenic acid mg/g. Chl.	Linoleic acid mg/g. Chl.
Free fatty acid	trace (0) ^{a)}	trace (0)
Neutral lipid	2.482 (98)	0.918 (80)
Phospholipid	0.045 (2)	0.226 (20)
Total	2.527 (100)	1.144 (100)

^{a)} (): the relative value (%).

in the neutral lipid fraction, 2% and 20% were in the phospholipid fraction, respectively. Significant amounts of I-a and I-b were not found in the free fatty acid fraction. Tea leaves and chloroplasts contained I-a and I-b which were localized in the neutral lipid and phospholipid fractions, but their total contents were still low compared to their optimum concentrations for the synthetic activities for hexenals and hexanal (Fig. 2).

DISCUSSION

In some leaves^{1,4)} and fruits,^{3,10,11)} crushing or maceration of the cells causes the formation of hexenals (II-a and III-a) as volatile C₆-carbonyl compounds. Addition of I-a enhances the formation of II-a and III-a in tea leaves.¹⁾ Tables I and II indicate that the synthetic activities for hexenals (II-a and III-a) and hexanal (II-b) are localized in the chloroplast fraction and that oxygen is requisite. From the result of substrate-specificity experiment (Table IV and Fig. 2), we speculate that the biosynthesis of C₆-aldehydes is a two-step reaction in which specific oxygenation of I-a and I-b is followed by their cleavage into II-a and II-b, respectively.

It may be concluded that hexenals (II-a and III-a) and hexanal (II-b) are formed from I-a and I-b in chloroplasts in tea leaves as shown by bold arrows in Scheme 1.

In *Ginkgo* leaves,⁴⁾ the hexenal-synthetic activity seems to be located in the chloroplasts. Cell homogenates of some fruits

produce II-a and III-a from I-a,^{3,10,11)} although they contain no chloroplasts. In these cases, the formation of C₆-aldehydes may take place in proplastids which are the subcellular organells similar to chloroplasts. On the other hand, spinach chloroplasts produce no hexenals from I-a.* Consequently, the synthetic activities for hexenals (II-a and III-a) and hexanal (II-b) from I-a and I-b as shown in tea chloroplasts are characteristic to some kinds of plants.

Oleic acid was also utilized slightly as the substrate to generate C₆-aldehydes in chloroplasts. Therefore, it can be presumed that oleic acid is probably desaturated *via* I-b to I-a in chloroplasts.^{12,13)} Desaturation of oleic acid is current by studies in our laboratory.

The amounts of I-a and I-b in chloroplasts (Table V) seem to be sufficient to generate hexenals and hexanal as volatile flavors. The triglycerides (V-a and V-b) and methyl esters (VI-a and VI-b) of I-a and I-b did not act as the substrate for chloroplasts, while I-a and I-b distributed in the neutral lipid and phospholipid fractions. It is considered that neutral lipid and phospholipid are hydrolyzed to I-a and I-b by lipase or phospholipase and then the liberated acids are utilized as precursors.

Attempts to solubilize the enzyme system responsible for the formation of hexenals failed. It is considered that this enzyme system is tightly bound to the lamellae membranes.

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