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FORMATION OF *CIS*-3-HEXENAL, *TRANS*-2-HEXENAL AND *CIS*-3-HEXENOL IN MACERATED *THEA SINENSIS* LEAVES*

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Key Word Index—*Thea sinensis*; Theaceae; tea; *cis*-3-hexenal: leaf aldehyde; leaf alcohol; linolenic acid; biosynthesis of leaf alcohol.

Abstract—Linolenic acid and *cis*-3-hexenal were found in macerated leaves of *Thea sinensis* and this aldehyde may be produced from linolenic acid by an enzyme contained in macerated leaves in the presence of oxygen. This aldehyde was easily isomerized to *trans*-2-hexenal, and was converted to *cis*-3-hexenol by alcohol dehydrogenase. During maceration of freshly picked tea leaves, the amounts of *trans*-2-hexenal quickly increased and were influenced by maceration time, heating, oxygen and the pH. But in unpicked tea leaves the occurrence of *trans*-2-hexenal is extremely doubtful.

INTRODUCTION

Leaf alcohol, *cis*-3-hexenol, and leaf aldehyde, *trans*-2-hexenal, are widely distributed in fresh foliage,[1–8] vegetables[1,9–15] and fruits[16–19] and are responsible for the characteristic leaf odor. In addition, they are both found in some insect excretions as functioning attractants and repellents.[20–28] *trans*-2-Hexenal was obtained by the isomerization of

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* Part XXII in the series "Leaf Alcohol". For Part XXI see Ref. 30.

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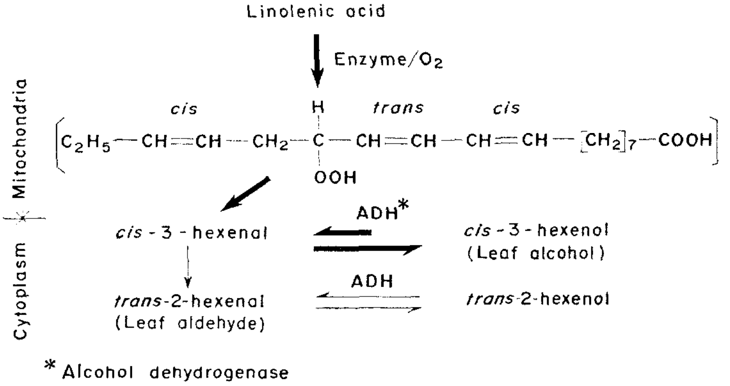
cis-3-hexenal from raspberry[19] and tomato.[14] The investigation of leaf alcohol has been carried out by Takei and Ohno *et al.* since 1938.[1] Recently we reported on the photochemistry[29] and the biosynthesis[30] of leaf alcohol.

In this paper, *cis*-3-hexenal was confirmed in the macerated tea leaves, as being a precursor in the biosynthesis of leaf alcohol, and the process of biosynthesis was clarified (Scheme 1).



SCHEME 1. FORMATION OF LEAF ALCOHOL AND LEAF ALDEHYDE.

RESULTS AND DISCUSSION

Changes of Volatile Components during Maceration of Fresh Tea Leaves

After fresh tea leaves were blended, the crude essential oil was prepared (see Experimental) and each component was analyzed by GLC. During steam distillation, *cis*-3-hexenal was isomerized to *trans*-2-hexenal. *n*-Hexanal, *trans*-2-hexenal and *cis*-3-hexenal notably increased during the maceration of fresh tea leaves as shown in Table 1.

TABLE 1. CHANGES IN VOLATILE COMPONENTS DURING MACERATION

Compounds	Maceration time (min) 0	3	10
n-Hexanal	1·17* (1·0)	3·23 (2·8)	14·29 (3·7)
trans-2-Hexenal	0·96 (1·0)	12·83 (13·4)	15·09 (15·7)
cis-3-Hexenol	14·13 (1·0)	39·08 (2·8)	46·93 (3·3)

* mg/kg fresh tea leaves.
( ) Ratio for maceration time zero.

Effect of Oxygen, Heating and pH on the Formation of Aldehydes

trans-2-Hexenal has been reported to be formed only when the leaves of various plants are macerated in the presence of oxygen.[6] In macerated tea leaves, the formation of *n*-hexanal and *trans*-2-hexenal was impeded by the presence of N_2, which suggests that oxygen is required for the formation of both aldehydes (Table 2). In tea leaves heated at 65° for 30

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[29] HATANAKA, A. and OHGI, T. (1972) *Agr. Biol. Chem.* **36**, 1263.

[30] HATANAKA, A. and HARADA, T. (1972) *Agr. Biol. Chem.* **36**, 2033; HATANAKA, A. (1972) *Bull. Inst. Chem. Res., Kyoto Univ.* **50**, 135.

min, formation of the aldehydes was stopped as shown in Table 2. The relation between the total formation of hexenals; *trans*-2-hexenal and *cis*-3-hexenal, and pH values is shown in Table 3. This table shows that the amount of hexenals is maximum at pH 5·5. However, these vary with the sampling time and/or the variety of tea leaves used.

Table 2. Effect of oxygen and heating on the formation of aldehydes

Condition	*n*-Hexanal	*trans*-2-Hexenal
Control	3·23* (1·0)	12·83 (1·0)
Under nitrogen	2·34 (0·7)	3·60 (0·3)
Heated sample†	1·35 (0·4)	2·11 (0·2)

* mg/kg fresh tea leaves.
† For 30 min at 65°.

Formation of Aldehydes from Unsaturated Fatty Acids in Macerated Tea Leaves

Recently, it has been reported that in a few plants the precursor of *trans*-2-hexenal is linolenic acid.[8,14,31] We therefore prepared linolenic acid, linoleic acid and oleic acid from frozen fresh tea leaves through column chromatography and these were quantitatively analyzed in the ester form by GLC.

Table 3. Effect of pH on the formation of hexenals

Buffer solution	pH	Total hexenals* 10 min†	Peak area (mm^2) 20 min
a	2·5	509·5	626·0
a	3·2	830·4	610·0
a	4·4	999·5	1018·0
a	5·5	1428·5	1778·0
a	7·0	583·5	899·5
b	8·1	70·0	98·4
b	9·0	99·0	94·5

a McIlvain's citrate-phosphate buffer.
b Phosphate buffer (0·1 M).
* *trans*-2-hexenal and *cis*-3-hexenal.
† Standing time.

When fresh tea leaves were macerated with linolenic acid or linoleic acid, increases in *n*-hexanal and *trans*-2-hexenal could be clearly observed (Table 4). When linolenic acid was added to the homogenate of tea leaves heated at 65° for 30 min, *trans*-2-hexenal was not formed. Therefore, the reaction must have been catalyzed by an enzyme which is specifically attached, only to the unsaturated fatty acid containing the *cis,cis*-1,4-pentadien system.

Occurrence of cis-3-hexenal in Macerated Tea Leaves

cis-3-Hexenal was obtained from macerated tea leaves using the head space vapor analysis method. Though this aldehyde was observed immediately after fresh tea leaves were

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[31] Gonzaez, J. G., Coggon, P. and Sanderson, G. W. (1972) *J. Food Sci.* 37, 797.

macerated, after the preparation had stood for 50 min at 40°, it isomerized to *trans*-2-hexenal (Table 5). This isomerization of *cis*-3-hexenal to *trans*-2-hexenal in macerated tea leaves is very fast in comparison with thermal isomerization[32] and is not affected by the introduction of N_2. These results suggested that some unknown factors exist in the isomerization of *cis*-3-hexenal to *trans*-2-hexenal in macerated tea leaves.

TABLE 4. FORMATION OF ALDEHYDES FROM UNSATURATED FATTY ACIDS

Fatty acid added*	*n*-Hexanal	*trans*-2-Hexenal
Control	3·19† (1·0)	21·47 (1·0)
Linolenic acid	5·92 (1·9)	61·87 (2·9)
Linoleic acid	30·79 (9·7)	39·81 (1·9)
Oleic acid	3·33 (1·0)	26·78 (1·2)
Heated sample‡ with linolenic acid	1·15 (0·4)	1·03 (0·1)

* $1{\cdot}8 \times 10^{-3}$ M.
† mg/kg fresh tea leaves.
‡ For 30 min at 65°.

*Formation of Alcohols by Alcohol Dehydrogenase**

We previously reported that alcohol dehydrogenase which catalyzes the conversion of aldehyde to alcohol, is distributed in fresh tea leaves.[30] Although the alcohols increased during the standing of macerated tea leaves, the increase was reduced by the addition of monoiodoacetic acid (Table 6). Therefore, we assume that alcohol dehydrogenase catalyzed the conversion of *cis*-3-hexenal to *cis*-3-hexenol.

TABLE 5. ISOMERIZATION OF *cis*-3-HEXENAL TO *trans*-2-HEXENAL

pH	Standing time	*cis*-3-Hexenal	*trans*-2-Hexenal	pH	Standing time	*cis*-3-Hexenal	*trans*-2-Hexenal
4·4	10	608·0*	391·5	5·5	10	344·0	1084·5
	20	424·0	594·0		20	104·0	1674·0
	30	344·0	711·0		30	68·0	1890·0
	40	292·0	706·5		40	52·0	1710·0
	50	268·0	778·5		50	12·5	1778·5

* Peak areas in mm².

Though no significant amount of *trans*-2-hexenal exists in freshly picked tea leaves, a large amount of *cis*-3-hexenol was detected (Table 1). This seems to be due to the fact that the *cis*-3-hexenal formed from linolenic acid is almost totally converted to *cis*-3-hexenol by alcohol dehydrogenase, which has an equilibrium constant inclined toward the alcohol side in the *cis*-3-hexenol:*cis*-3-hexenal system.[33] And it is possible that the *trans*-2-hexenal which was barely detectable in fresh tea leaves may be formed by isomerization of a small amount of *cis*-3-hexenal during the extraction process. Therefore, in fresh tea leaves the occurrence of *trans*-2-hexenal is very doubtful.

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* Alcohol:NAD oxidoreductase (E.C. 1.1.1.1).

[32] HATANAKA, A. (1972) *Bull. Inst. for Chem. Res. Kyoto Univ.* **50**, 192.

[33] ERIKSSON, C. E. (1968) *J. Food Sci.* **33**, 525; in this report, *cis*-3-hexenal has not been confirmed.

During the standing of macerated tea leaves, the *trans*-2-hexenol, which is not observed in fresh tea leaves, becomes significantly large (Table 6). This supports the fact that *cis*-3-hexenal is isomerized to *trans*-2-hexenal, and then is reduced to *trans*-2-hexenol by alcohol dehydrogenase. These results are summarized in Scheme 1.

TABLE 6. FORMATION OF ALCOHOLS BY ALCOHOL DEHYDROGENASE

Compounds	Control	Standing	Addition of IAA*
n-Hexanal	0·55† (1·0)	0·53 (1·0)	0·67 (1·2)
trans-2-Hexenal	16·60 (1·0)	13·08 (0·8)	14·06 (0·8)
n-Hexanol	2·73 (1·0)	3·65 (1·3)	2·43 (0·9)
cis-3-Hexenol	14·20 (1·0)	20·94 (1·5)	15·28 (1·1)
trans-2-Hexenol	8·02 (1·0)	17·19 (2·1)	9·00 (1·1)

Control: macerated for 2 min.
Standing: kept for an additional 10 min.
* IAA: monoiodoacetic acid, 1×10^{-3} M.
† mg/kg fresh tea leaves.

Hatanaka[2] reported the occurrence of *trans*-2-hexenol and *trans*-3-hexenol in the essential oil prepared from fresh leaves *Thea sinesis* L. (variety; *Yamatomidori*: 1st crop). We assume that the former was the reduction product of *trans*-2-hexenal by alcohol dehydrogenase and that the latter was obtained by isomerization of *cis*-3-hexenol in the hydration of the 3,5-dinitrobenzoate.

EXPERIMENTAL

Materials. Fresh leaves of *Thea sinensis* were the 2nd crops (variety; *Yamatomidori*) and the 3rd crops (variety; *Zairai*) of 1972, in each of the amount of 10 kg. The linolenic acid, linoleic acid and oleic acid were the high-purity grade of above 95%.

Preparation method (a). *Preparation of essential oil*: Fresh tea leaves (150 g) were blended with 500 ml of distilled water in a Waring blender for 3 min and then the mixture was steam distilled until 500 ml of distillate was collected. After the distillate was saturated with NaCl, it was extracted twice with 50 ml of ether. The total extract was washed with 10 ml of distilled water and dried. After evaporation of ether, 20–40 mg of the crude essential oil were obtained. (b) *Preparation of vapor sample*: Fresh tea leaves (5·0 g) were blended with 100 ml of distilled water or buffer solution in a Waring blender for 2 min. Immediately the homogenate was transferred to the vessel which previously had been held at 40° in a water bath. 5·0 ml of headspace volatile were analyzed by GLC. (c) *Preparation of unsaturated fatty acids*: After fresh tea leaves (6·5 g) were soaked in 30 ml H_2O overnight, it was homogenized with 1·0 g of sea sand. After 75 ml MeOH and 37·5 ml $CHCl_3$ were added to the homogenate, the mixture was shaken for 2 min and then kept for about 10 min at room temp. 37·5 ml $CHCl_3$ were added to the mixture and shaken for 30 sec. After addition of 37·5 ml of water, it was shaken again for 30 sec. $CHCl_3$ layer was collected by centrifugation of the mixture at 2500 rpm for 5 min and then $CHCl_3$ was removed under reduced pressure in N_2. The residue was saponified by refluxing with a solution of 0·2 g of KOH, 2 ml EtOH and 0·4 ml H_2O for 1·5 hr under N_2. The mixture was diluted with 20 ml H_2O and it was washed 2× with 20 ml Et_2O. The solution was cooled in ice and acidified with HCl. After the acidified solution was extracted with Et_2O, it was esterified by CH_2N_2 in Et_2O. The methyl ester of fatty acids was applied on silica gel column chromatography (2 × 20 cm) and then eluted with Et_2O–light petrol. (1:9, v/v).

Analytical method. Identification by GLC: GLC equipment used was *Shimazu* GC-4B type. *For samples of preparation method of* (*a*) *and* (*b*): The column made of 3 m by ϕ 3 mm stainless steel was packed with 10% Carbowax 20 M (40–60 mesh). *For sample of preparation method* (*a*). The GLC was operated with the following conditions: column temp., 120°; N_2 flow rate, 60 ml/min; detector, FID; attenuation range, 0·16 V; sensitivity, 10 MΩ; air, 1·1 kg/cm^2 and sample volume, 1·0 μl. *For sample of preparation method* (*b*): column temp., 120°; N_2 flow rate, 60 ml/min; detector, FID; attenuation range, 0·08 V; sensitivity 10^3 MΩ and sample volume, 5·0 ml. *For sample of preparation method* (*c*): The column made in 3 m by ϕ 3 mm stainless steel was packed with PEG-adipate on Chromosorb W (60–80 mesh). The GLC was operated with the following conditions: column temp., 190°, N_2 flow rate, 60 ml/min; detector, FID; attenuation range, 0·04 V; sensitivity, 10^2 MΩ and sample vol., 5·0 μl. For quantitative analysis the calibration curves of the following compounds were obtained; *n*-hexanal (3·16 min*), *n*-hexanol (9·7 min), *trans*-2-hexenal (6·0 min), *trans*-2-hexenol (12·5 min), *cis*-3-hexenal (4·2 min), *cis*-3-hexenol (11·7 min), oleic acid (23 min), linoleic acid (27 min) and linolenic acid (34 min) (97–100% purity).[2,34]

Effect of maceration time, oxygen, heating and pH on the formation of aldehydes. (*a*) *Effect of maceration time*: Volatile components of maceration time 0 min were preparaed without maceration and other samples were prepared after maceration for 3 and 10 min. (*b*) *Effect of oxygen*: Fresh tea leaves were blended with distilled water under N_2 or under the presence of air. (*c*) *Heat treatment*: Fresh tea leaves were added in dist. H_2O at 65° and it was held for 30 min at the same temp. on a H_2O bath. Each of these essential oil of (*c*) from (*a*) was prepared by using the *preparation method* (*a*) and it was analyzed by the GLC under the conditions of analytical method (*a*). (*d*) *Effect of pH*: Vapor samples were obtained by the *preparation method* (*b*), using McIlvain's (pH: 2·5, 3·2, 4·4, 5·5 and 7·0) and phosphate buffer (pH: 8·1 and 9·0) solutions at the various pH values instead of distilled water and it was analyzed by the GLC under the conditions of analytical method (*b*).

Occurrence of linolenic acid, linoleic acid, oleic acid and cis-3-hexenal in macerated tea leaves. The sample containing the unsaturated fatty acids was prepared by the *preparation method* (*c*) and it was analyzed by the GLC under the conditions of analytical method (*c*). The vapor sample containing *cis*-3-hexenal prepared by the *preparation method* (*b*) was analyzed by the GLC under the conditions of analytical method (*b*).

Formation of aldehydes from unsaturated fatty acids. Each of fatty acids at $1{\cdot}8 \times 10^{-3}$ M was added to fresh tea leaves with distilled water. And then essential oil was prepared by the *preparation method* (*a*) and it was analyzed by the GLC under the conditions of method (*a*).

Formation of alcohols by alcohol dehydrogenase. Fresh tea leaves were blended with dist. H_2O for 2 min (control). The mixture was kept for 10 min at room temperature (standing). Effect of inhibition of alcohol dehydrogenase was observed by addition of monoiodoacetic acid at 1×10^{-3} M to macerated tea leaves after blending for 2 min.

* Retention time.

[34] HATANAKA, A. and SAKAMOTO, H., unpublished observations.