<table>
<thead>
<tr>
<th>Title</th>
<th>Biosynthesis of Leaf Alcohol (Commemoration Issue Dedicated to Professor Yuzo Inouye on the Occasion of his Retirement)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Hatanaka, Akikazu</td>
</tr>
<tr>
<td>Citation</td>
<td>Bulletin of the Institute for Chemical Research, Kyoto University (1983), 61(2): 180-192</td>
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</table>

Kyoto University
Biosynthesis of Leaf Alcohol

Akikazu HATANAKA*

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KEY WORDS: Tea Chloroplasts/ Leaf Alcohol/ Biosynthetic Path Way/

INTRODUCTION

Leaf alcohol, cis-3-hexenol, and leaf aldehyde, trans-2-hexenal, are widely distributed in fresh leaves, vegetables, and fruits, and they are responsible for the “Green Odor” characteristics of leaves. They are found in some insect excretions, as functioning attractants and repellents. Investigations on leaf alcohol have been carried out by Takei and Ohno et al. since 1938. From 1957, we have been studying leaf alcohol and leaf aldehyde from different approaches: synthetic chemistry, natural products chemistry, and plant biochemistry.

Scheme 1 Biosynthetic pathway of leaf alcohol.

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Biosynthesis of Leaf Alcohol

We recently have focused our attention on the biosynthesis of leaf alcohol and aldehyde and have demonstrated the biosynthetic pathway with *Thea sinensis* leaves, as shown in Scheme 1. We know now that the leaf alcohol and aldehyde are produced from cis-3-hexenal.39, 42, 49) This C6-compound, cis-3-hexenal, and the C12-compound, 11-formyl-cis-9-undecenoic acid,61,62) biosynthesized from linolenic acid by enzymatic oxygenative splitting through the addition of oxygen to the double bond between C-12 and C-13. In the oxygenative splitting reaction, two enzymes, lipoxygenase (Es) and hydroperoxide lyase (Eh) are involved, and 13-1-hydroperoxylinolenic acid is an intermediate. From this cis-3-hexenal, trans-3-hexenyl6) and trans-2-hexenal are formed by the isomerization and cis-3-hexenol is formed by reduction with alcohol dehydrogenase (E3)*. The C12 fragment isomerizes to give 11-formyl-trans-10-undecenoic acid,61,62) traumatic half aldehyde, which is known as the “wound hormone”.

FORMATION OF “GREEN ODOR”

Changes in fatty acid contents.39,72) First, changes in fatty acid contents in lipids were examined. The Table I shows the result of leaves harvested in June and in November. The summer leaves showed a high activity for C6-aldehyde formation and the winter leaves showed a low activity. Fatty acids were prepared from leaves blended for 3 minutes in a Waring blender or without blending. The homogenate was extracted with chloroform-methanol and separated into phospholipid, neutral fat, and free fatty acid fractions by acetone and ether fractionation. The phospholipids and neutral fats obtained were saponified with potassium hydroxide, then esterified with diazomethane. The obtained fatty acid methyl esters were analyzed and determined by GLC with a 20% PEG-adipate column, 3 mm by 1 m. In this table, the 0 and 3 in parentheses refer to minutes of blending time. With no blending, more than 50% of the fatty acids were found in the neutral fat fraction, and the rest in the

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Blending time (min)</th>
<th>Linoleic acid</th>
<th>Linolenic acid</th>
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<tr>
<td></td>
<td>0</td>
<td>3</td>
<td>0</td>
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<tr>
<td>Free fatty acid</td>
<td>trace</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td></td>
<td>(trace)</td>
<td>(trace)</td>
<td>(trace)</td>
</tr>
<tr>
<td>Neutral fat</td>
<td>55.6 (18.5)</td>
<td>23.5 (13.8)</td>
<td>218.3 (164.0)</td>
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<tr>
<td>Phospholipid</td>
<td>16.8 (13.8)</td>
<td>13.3 (3.5)</td>
<td>11.5 (8.4)</td>
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<tr>
<td>Total lipid</td>
<td>72.4 (32.3)</td>
<td>36.8 (19.3)</td>
<td>229.8 (172.4)</td>
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</table>

mg/100 g fresh tea leaves.
On 6th of June, ( ) on 26th of November.

* trans-2-hexenol,81)
phospholipid fraction. The ratio of linolenic acid to linoleic acid was about 3 in summer leaves and 5 in winter leaves. When the summer leaves were blended for 3 minutes, large quantities of linoleic acid and linolenic acid, about a half of the initial content, disappeared from these two lipid fractions, neutral fat and phospholipids. But no free fatty acids were found. In the winter leaves, blending caused a far lower decrease in fatty acid content in lipid fractions compared with the summer leaves. These results indicate that linolenic acid and linoleic acid hydrolyzed are converted to compounds other than free fatty acids.

**Hexenal and hexanal formation during the blending.** In Fig. 1, the broken line indicates the total linolenic acid content in lipids. During the blending of summer tea leaves, linolenic acid content decreased. Therefore, we expected that C6-compounds, such as trans-2-hexenal and cis-3-hexenol derived from linolenic acid would increase during the blending of leaves with decreasing linolenic acid content. In addition to linolenic acid determination, C6-compound determinations were performed using essential
Biosynthesis of Leaf Alcohol

oil prepared from blending leaves by steam distillation followed by ether extraction. During the blending, trans-2-hexenal, indicated by the open circles and solid line, increased dramatically. cis-3-Hexenol, indicated by the dark circles, increased initially at one minute and then reached a plateau. On the other hand, in the first three minutes, 4 μmoles of linolenic acid were lost from lipids and 1 μmole of trans-2-hexenal and cis-3-hexenal was newly formed, indicating that one-quarter to one-third of the lost linolenic acid was converted to C6-compounds, such as trans-2-hexenal and cis-3-hexenol. In the procedures used here, cis-3-hexenal, which is a labile compound in the homogenate, was not detected because of isomerization to trans-2-hexenal.

**Seasonal changes in the C6-aldehyde forming activity.**52,54) Next we examined the seasonal changes in C6-aldehydes forming activity from linoleic acid and linolenic acid using tea leaf homogenates. Linolenic acid or linoleic acid was incubated at 35°C for 10 minutes with the leaf homogenate in a sealed flask. At the end of incubation time, the headspace vapor gas was analyzed by GLC as having 20% PEG 20M column in 3 mm by 3 m. Hexanal formation is represented by the dark circles and hexenal formation, by open circles. The dotted lines indicate averages of maximum and minimum temperature for 10 days (Fig. 2). The solid line indicates solar radiation. C6-Aldehyde forming activity began to increase in late March to April. In July to August, the activity reached a maximum, then gradually decreased and disappeared completely in December. The activity changes were parallel to the temperature changes.

**Localization of C6-aldehyde formation activity.**47) Since the summer leaves exhibited high activity for C6-aldehyde formation, we fractionated the tea leaves into subcellular fractions. Tea leaves harvested in August were homogenized with McIlvaine's buffer, pH 6.3, containing 0.4 M sucrose for 3 minutes, and the homogenate was filtered through three layers of gauze. Then the filtrate was subjected to successive centrifugation at 1,000 g, 4,000 g and 19,000 g. The activity for C6-aldehyde formation in each fraction was determined by the headspace method. Most of the activity for C6-aldehyde formation was localized in the 1,000 g pellet, which was the chloroplast-rich fraction (Table II). Washing the chloroplast-rich 1,000 g pellet with McIlvaine's buffer, pH 6.3, repeatedly, did not cause a significant decrease in activity. Therefore, the activity for C6-aldehyde formation was concluded to be localized in the chloroplast lamellae membrane. In the following experiments, the

Table II. Localization of C₆-aldehyde formation activity

<table>
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<th>Fraction</th>
<th>C₆-Aldehydes [µmol]</th>
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<tr>
<td></td>
<td>Hexenals</td>
<td>Hexanal</td>
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<tr>
<td>1000 g pellet*</td>
<td>117.5 (90)</td>
<td>22.3 (76)</td>
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<td>4000 g pellet</td>
<td>10.7 ( 8)</td>
<td>3.6 (12)</td>
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<td>19000 g pellet</td>
<td>2.9 ( 2)</td>
<td>1.1 ( 4)</td>
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<tr>
<td>Supernatant</td>
<td>0.4 ( 0)</td>
<td>2.4 ( 8)</td>
<td></td>
</tr>
</tbody>
</table>

* Chloroplasts

(183)
pellet obtained by centrifugation of the 1,000 g supernatant at 4,000 g for 10 minutes was used routinely as the chloroplast-rich fraction.

**Biosynthetic pathway of C₆-aldehydes from ¹⁴C-labeled fatty acids.** When uniformly labeled ¹⁴C-linolenic acid was incubated with isolated tea chloroplasts, two radioactive peaks were found at positions of cis-3-hexenal and trans-2-hexenal in head-space vapor gas with radio gas chromatograph, as shown by the spiked line (Fig. 3). Other radioactive peaks, such as hexanal, alcohols, and short chain aldehydes, were not found. When incubation was prolonged, radioactivity in cis-3-hexenal decreased, and radioactivity in trans-2-hexenal increased. When ¹⁴C-labeled linoleic acid was used, hexanal was labeled by ¹⁴C, but no other volatile compounds were labeled. Thus, the six-carbon aldehydes were produced from linolenic acid or linoleic acid in the presence of isolated tea chloroplasts. When the reaction mixture of ¹⁴C-labeled linolenic acid and isolated tea chloroplasts was incubated, extracted with ether and the extract analyzed by gas liquid chromatograph, five major radioactive peaks were found. One peak was an unreacted substrate peak. Two others were 11-formyl-cis-9-undecenoic acid and 11-formyl-trans-10-undecenoic acid, as presented here. After cleaving linolenic acid and linoleic acid, these two compounds were expected to be moiety twelve-carbon. Other radioactive peaks were azelaic half aldehyde, 9-formyloctanoic acid, and unknown compound. Radioactivity in 11-formyl-cis-9-undecenoic acid decreased with prolonged incubation time, while 11-formyl-trans-10-undecenoic acid increased. These results indicate that cis-3-hexenal and 11-formyl-cis-9-undecenoic acid were first formed from linolenic acid and then cis-3-hexenal was isomerized to trans-2-hexenal and 11-formyl-cis-9-undecenoic acid to 11-formyl-trans-10-undecenoic acid.

![Diagram](image_url)

**Fig. 3.** Radio gas chromatograms of cis-3-hexenal and trans-2-hexenal from linolenic acid [U-¹⁴C] (left) and of 11-formyl-cis-9- and -trans-10-undecenoic acid from linolenic acid [1-¹⁴C] by isolated tea chloroplast.
Distribution of an enzyme system producing cis-3-hexenal and n-hexanal from linolenic and linoleic acids in some plants. The activity of the enzyme system \((E_2 + E_2')\) producing C6-aldehydes from C18-unsaturated fatty acids was investigated using about 40 plants. Green leaves of dicotyledonous plants belonging to the Sphenopsida, Pteropsida, Theaceae and Leguminosae showed a high enzyme activity but edible leafy vegetables and fruits and monocotyledonous plants showed a low activity as shown Table III. Seasonal changes in the enzyme activities were observed. The concentrations of cis-3-hexenol and trans-2-hexenal and the enzyme activities showed a correlation; high concentrations were observed in the summer but they were low in the winter.

Solubilization and properties of the enzyme-cleaving 13-L-hydroperoxylinolenic acid in tea leaves. The membrane bound hydroperoxide lyase \((E_2')\) which catalyzes the cleavage of 13-L-hydroperoxides of linolenic and linoleic acids to C6-volatile aldehydes (hexenals and n-hexanal) was found to be localized in the chloroplast lamellae of tea leaves. It was selectively solubilized from the lamellae with 0.5 % (w/v) Tween 20. The enzymatic cleavage of the hydroperoxides occurred even under anaerobic conditions. The optimal pH of \(E_2'\) was 7-8. The common structural features shown by substrates of \(E_2'\) were the presence of a 13-L-hydroperoxy group at \(\omega-6\) with a conjugated trans, cis-diene at \(\omega-7\) and \(\omega-9\) in a C18-fatty acid. \(E_2'\) had an apparent Km of 2.5 and 1.9 mM for 13-L-hydroperoxylinolenic and 13-L-hydroperoxylinoleic acids, respectively. No significant differences were found between chloroplast and solubilized \((E_2')\).

Participation and properties of lipoxygenase and hydroperoxide lyase in volatile C6-aldehyde formation from C18-unsaturated fatty acids in isolated tea chloroplasts. Isolated tea chloroplasts utilized linoleic acid, linolenic acid and their 13-hydroperoxides as substrates for volatile C6-aldehyde formation. Optimal pH values for oxygen uptake \((E_2'),\) hydroperoxide lyase \((E_2')\) and the overall reaction \(E_2' + E_2'\) from C18-fatty acids to C6-aldehydes were 6.3, 7.0 and 6.3, respectively. Methyl linoleate, linoleyl alcohol and \(\gamma\)-linolenic acid were poor substrates for the overall reaction, but linoleic and linolenic acids were good substrates (Fig. 4, 5). The 13-hydroperoxides of the above fatty acids and alcohol also showed substrate specificity similar to that of fatty acids. Oxygen uptakes (relative \(V_{max}\)) with methyl linoleate, linoleyl alcohol, linolenic acid, \(\gamma\)-linolenic acid and arachidonic acid were comparable to or higher than that with linoleic acid. In winter leaves, the activity for C6-aldehyde formation from C18-fatty acids was reduced to almost zero. This was due to the reduction in oxygenation. The findings presented here provide evidence for the involvement of lipoxygenase and hydroperoxide lyase in C6-aldehyde formation in isolated chloroplasts.

Oxygen-isotope effect in enzymatic cleavage reaction of 13-L-hydroperoxylinoleic acid to hexanal and 11-formyl-cis-9-undecenoic acid. 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, and 11-formyl-cis-9-undecenoic acid by identification of cleavage products using authentic specimens synthesized through an unequivocal route. An oxygen-isotope effect was first observed in the cleavage
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<th>Plant no. and common name</th>
<th>Class</th>
<th>Family</th>
<th>Genus and species</th>
<th>Organ*</th>
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<td>Musci</td>
<td>Polytrichaceae</td>
<td>Pogonatum inflexum</td>
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<td>2. Hordeatil</td>
<td>Sphenopsida</td>
<td>Equisetaceae</td>
<td>Equisetum arvense</td>
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<td>Pteropsida</td>
<td>Osmundaceae</td>
<td>Osmunda japonica</td>
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<td>4. Bracken</td>
<td>Polypodiaceae</td>
<td>Pteridium aquilinum</td>
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<td>5. Ginkgo</td>
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<td>Podocarpaceae</td>
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<td>8. Cucumber</td>
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<td>L (green)</td>
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<td>Brassica oleracea var. botrytis</td>
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<td>Diospyros kaki</td>
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<td>Diospyros kaki</td>
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<td>15. Japanese persimmon</td>
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<td>Pragaria grandiflora</td>
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<td>17. Japanese apricot</td>
<td>Amygdalaceae</td>
<td>Prunus persica</td>
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<td>19. Japanese wisteria</td>
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<td>Robinia pseudoacacia</td>
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<td>20. False acacia</td>
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<td>Trifolium repens</td>
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<td>22. White clover</td>
<td>Soybean</td>
<td>Glycine max</td>
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<td>23. Alfalfa</td>
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<td>Phaseolus vulgaris</td>
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<td>24. Soybean</td>
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<td>Morus bombycis</td>
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<td>29. Egg plant</td>
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* L: leaf. F: fruit.
† Plant materials were harvested in late May–July 1976, except for alfalfa in September.
‡ Total hexenals: cis-3- and trans-2-hexenal.
## Biosynthesis of Leaf Alcohol producing C₆-aldehydes in summer

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<th>cis-3-hexenal</th>
<th>trans-2-hexenal</th>
<th>total hexenals</th>
<th>n-hexenal</th>
<th>cis-3-hexenal</th>
<th>trans-2-hexenal</th>
<th>total hexenals</th>
<th>n-hexenal</th>
<th>Degree of activity</th>
<th>Fresh tissue homogenate (µg/g fr. wt)</th>
<th>4000 g precipitate fraction (µg/g ppt.)</th>
<th>Isomerization rate %</th>
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§ H, L show high, moderate and low enzyme activity, respectively.

| Isomerization rate (%) = \( \frac{\text{trans-2-hexenal}}{\text{total hexenals}} \times 100 \).
Fig. 4. C₆-Aldehyde formation from LA, LNA and their 13-hydroperoxides by isolated tea chloroplasts. Ten milliliters of the chloroplast suspension was incubated at 35°C for 10 min with a single substrate (A or B) or mixed substrates (C) at the concentrations indicated in a sealed 50-ml flask. Numbers given as the substrate concentrations in (C) are the sums of LA and LNA (1:1). At the end of incubation, 6 ml of headspace vapor was analyzed by GLC as described in the text. A, hexanal formation from LA (•) or LAHPO (▲). B, hexenal formation from LNA (◯) or LNAHPO (△). C, hexanal (●) and hexenal (◯) formation from the mixture of LA and LNA as substrate. ♦ shows the total C₆-aldehydes (hexanal and hexenals) produced from the mixed substrates.


Fig. 5. Changes in the amounts of substrate and the products formed during C₆-aldehyde formation. A chloroplast suspension (10 ml) first was incubated for 1 min at 35°C, then transferred to a sealed 50-ml flask which contained LA or LNA (final concentration, 0.6 mM). This mixture was incubated at 35°C. At the times indicated, 6 ml of headspace vapor from the sealed flask was analyzed by GLC. Immediately after headspace analysis, 1 ml of 2 N HCl was added to the reaction mixture, then the contents of the flask were treated with 5 ml of hexane. The hexane extract was concentrated to 2 ml, and 25 μl was used in HPLC analysis to determine the C₁₉-fatty acid hydroperoxides. The rest of the concentrate was esterified with diazomethane, then analyzed by GLC to determine the unreacted C₁₉-fatty acids. A, hexanal formed from LA (●), LAHPO from LA (◯), unreacted LA (△) and the total amount detected as LA, LAHPO and hexanal (□). B, hexenals formed from LNA (●), LNAHPO from LNA (◯), unreacted LNA (△) and the total amount detected as LNA, LNAHPO and hexenals (□).
Biosynthesis of Leaf Alcohol

reaction of $^{18}$O-labeled 13-L-hydroperoxylinoleic acid by solubilized $E_2''$ (Scheme 2, Fig. 6, Table IV). The $^{18}$O-atom of hydroperoxide was not detected in carbonyl group of hexanal formed from $^{18}$O-labeled 13-L-hydroperoxylinoleic acid.

![Scheme 2 Procedure for the preparation of $[^{18}$O]-labeled hydroperoxide and analysis of hexanal during $E_2$ reaction.](image)

![Fig. 6. Enzymatic formation of hexanal from $[^{18}$O]- and $[^{16}$O]-hydroperoxides by $E_2$.](image)
A. HATANAKA

Table IV Comparison of oxygen-isotope effect in E$\delta$ reaction by plant tissues.

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<th>Enzyme</th>
<th>Hexanal [nmol]</th>
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<td>Tea leaves*1</td>
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<td>Tea chloroplasts*2</td>
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<td>Solubilized E$\delta$*3</td>
<td>2.70 (100)</td>
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<td>Watermelon seedlings*4</td>
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</table>

*1; 0.5 g fresh weight.
*2; 0.1 g [corresponded to 0.5 g leaves (fresh weight)].
*3; 1 ml (corresponded to 0.1 g chloroplasts).
*4; 10 ml (corresponded to 3 g fresh seedling).
*5; hexanal formation from $[\text{^{16}O}]$-13-L-hydroperoxide.
*6; hexanal formation from $[\text{^{18}O}]$-13-L-hydroperoxide.
*7; numbers in parentheses represent relative values (%).

REFERENCES


Original references

Biosynthesis of Leaf Alcohol

A. Hatanaka


Original review


