Minireview

The Biogeneration of Green Odour by Green Leaves and It's Physiological Functions – Past, Present and Future

Akikazu Hatanaka, Tadahiko Kajiwara and Kenji Matsui

Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753, Japan

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Green Odour, Lipoxygenase, Fatty Acid Hydroperoxide Lyase

The Green Odour of Plants

(3*Z*)-Hexenol, which is commonly called leaf alcohol, and (2*E*)-hexenal, also called leaf aldehyde, are widely distributed in fresh leaves, vegetables and fruits. They are mainly responsible for the so-called "green odour" characteristic of leaves, along with other C_6-compounds such as (3*E*)-, and (2*E*)-hexenols, their corresponding aldehydes (3*Z*)-, (3*E*)-hexenals, *n*-hexanol and *n*-hexanal. Thus, green odour of green leaves arises from a mixture of the eight volatile C_6-compounds. (2*E*)-Hexenal was first isolated from the green leaves of certain bushes by Curtius at Heidelberg University in 1912 (Curtius, 1912). On the other hand, (3*Z*)-hexenol was found in green tea leaves by A. H's former teacher Sankichi Takei at Kyoto University in 1933 (Takei and Sakato, 1933). His investigations on green odour were carried on until 1942. Since 1957, we have been conducting studies on leaf alcohol, leaf aldehyde and other volatile C_6-compounds found in tea leaves, using multidisciplinary approaches including synthetic chemistry, natural products chemistry and physiological biochemistry (Hatanaka, 1993). In the course of our extensive studies, it has also been revealed that the young and fresh green odour of fruits results from a mixture of not only the eight volatile C_6-compounds but also eight C_9-compounds consisting of (3*Z*, 6*Z*)-, (2*E*, 6*Z*)-nonadienols and (3*Z*)-, (2*E*)-nonenols, and their corresponding aldehydes (Hatanaka *et al.*, 1975). (3*Z*, 6*Z*)-Nonadienol is characteristic of the watermelon, and (2*E*, 6*Z*)-nonadienal of the cucumber. The ratio of C_9 to C_6 is characteristic for each fruit species, e.g., in banana, 90% is C_9- and 10% is C_6-compounds (Hatanaka *et al.*, 1975).

In response to various stimuli, green leaves emit characteristic green odour consisting of the various concentrations of the eight volatile C_6-compounds. The subtle differences in the composition of the green odour are thought to be used by plants to communicate with or attack other species. These are also used to either attract or repel insects. In addition, plants can kill certain bacteria such as *Dermatophytes* and *Staphylococus* by using the green odour mixture at various concentrations thereby providing an example of a phytocide. Certain ants take green odour compounds into their bodies by consuming green leaves and then use them as pheromones for communication, alarm and attack and so on.

The Biogeneration of Green Odour

The biosynthetic pathway to green odour was first demonstrated in tea, *Thea sinensis* leaves, as shown in Scheme 1 (Hatanaka and Harada, 1973). In this scheme, the precursors of green odour are α-linolenic and linoleic acids, as confirmed by ^{14}C-labelling experiments with tea chloropolasts. Later, α-linolenic acid 13-(*S*)-hydroperoxide was isolated as an intermediate in the formation of (3*Z*)-hexenal from α-linolenic acid (Hatanaka *et al.*, 1976). In summary, the enzyme systems of green odour biogeneration involve the following steps: (i) α-linolenic and linoleic acids are liberated from galacto-, phospholipids or triglycerides by a lipolytic enzyme, lipolytic acyl hydrolase

Reprint requests to Prof. A. Hatanaka, present address: Department of Life Science, Graduate School of Integrated Art and Science, University of East Asia, Shimonoseki 751, Japan. Telefax: +81-832-56-9577.

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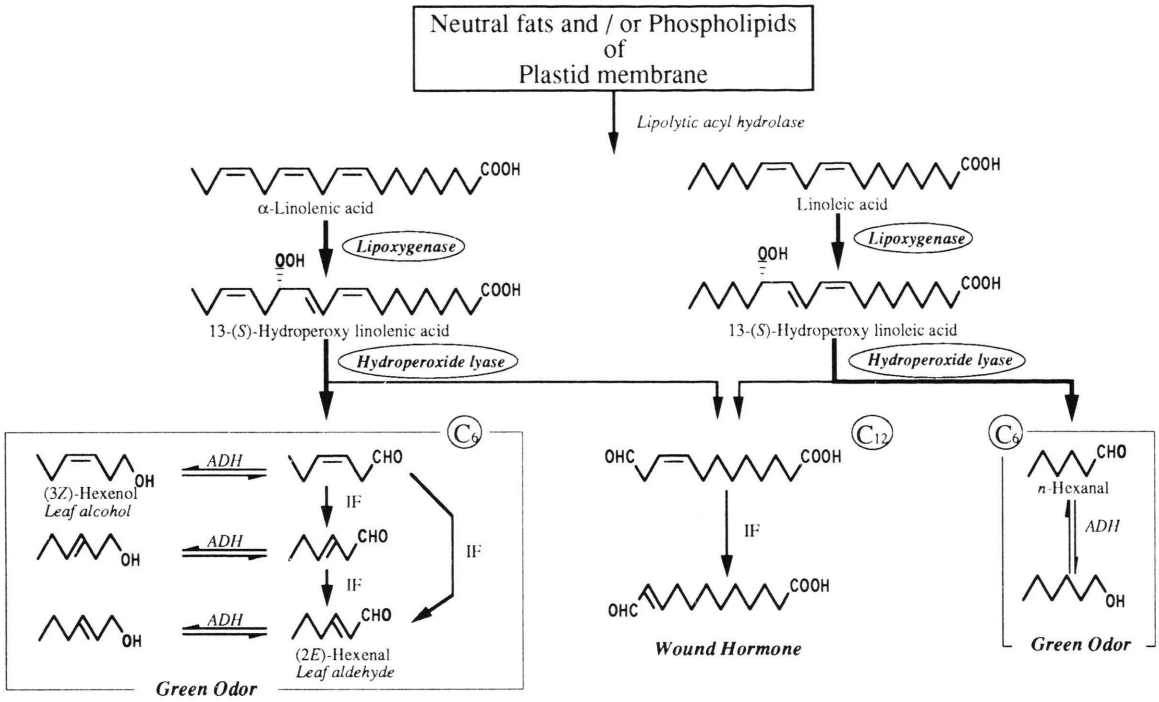

Biosynthesis of Green Odour by Green Leaves

ADH : *Alcohol dehydrogenase.* IF : Isomerization factor.

Scheme 1. Biosynthesis of green odour by green leaves. After liberation of linoleic and linolenic acids from membrane, sequential catyalyses with lipoxygenase and fatty acid hydroperoxide lyase results in the formation of C6- and C12-compounds. Part of C6-compounds are reduced by alcohol dehydrogenase. ADH: alcohol dehydrogenase, IF: isomerization factor.

(LAH) (Galliard, 1978); (ii) these free fatty acids are oxygenated to form their hydroperoxides by a none-heme iron dioxygenase, lipoxygenase (LOX, EC 1.13.11.12); (iii) the fatty acid chains of the hydroperoxides are cleaved between C-12 and C-13 by fatty acid hydroperoxide lyase (HPO) to form C_6-aldehyde and C_{12}-oxo-acid (Scheme I). In some cases, (3*Z*)-hexenal is converted into (3*Z*)- or (2*E*)-hexenol by alcohol dehydrogenase and / or an isomerization factor. *n*-Hexanal and *n*-hexanol are also biosynthesized from linoleic acid through a similar pathway. C_9 compounds are produced analogously via the fatty acid 9-(*S*)-hydroperoxide through a similar pathway (Hatanaka *et al.*, 1975).

Enzyme Systems in Green Odour Biogeneration

In plants, there exist at least two types of lipoxygenases. One, such as in soybean seeds, tea leaves and cucumber cotyledons, oxygenates specifically at C-13 of α-linolenic or linoleic acid (type 1) (Kajiwara *et al.*, 1980, Matsui *et al.*, 1992); the other, such as in potato tuber and wheat seeds, oxygenates at C-9 (type 2) (Galliard, 1978). Fatty acid hydroperoxide lyases can be also grouped into two types that afford cleavage of either 9-(*S*)- or 13-(*S*)-hydroperoxides. These lyases give, respectively, either two C_9 fragments, or a C_6 and a C_{12} fragments. The former type occurs in cucumber fruits and the latter type exists in watermelon seedlings, tea leaves, tomato fruit, alfalfa seedling and soybean seeds etc (for review, see Vick and Zimmerman, 1987). In cucumber cotyledons, there exist at least two types of fatty acid hydroperoxide lyase, one of which specifically acts on fatty acid 9-hydroperoxide while the other acts only on 13-hydroperoxide (Matsui *et al.* 1989). Hydroperoxide lyase was first purified to homogeneity from the membrane fraction of tea leaves. The molecu-

lar weight of the hydroperoxide lyase was found to be 55 000 by SDS-polyacrylamide gel electrophoresis (Matsui *et al.*, 1991). Some natural lipophilic antioxidants, such as nordihydroguaiaretic acid, is a potent irreversible inhibitor.

Substrate specificity

To search for the recognition mechanism of lipoxygenase for the hydrophilic area of a substrate, an entire series of (ω6*Z*,ω9*Z*)-$C_{14\sim24}$ dienoic acids (A group) were synthesized for use as substrates. They have a fixed carbon chain from ω1 to ω10 incorporating a (ω6*Z*,ω9*Z*)-diene structure, together with an elongated carbon chain of varying length from ω11 toward the α-terminal carboxyl group. They are analogous to the natural fatty acid, linoleic acid, and have an essential common structure of (1*Z*,4*Z*)-pentadiene between the ω6 and ω10 carbons (Hatanaka *et al.*, 1989). On the other hand, in order to examine the environment of the hydrophobic area, (9*Z*,12*Z*)-$C_{14\sim24}$ dienoic acids (B group), with a fixed (9*Z*,12*Z*)-C_{13}-diene carboxyl moiety and successively elongated carbon chains from C_{14} to C_{24} were used (Hatanaka *et al.*, 1990). Soybean lipoxygenase-1 was purified to homogeneity from the soluble fraction of soybean seeds using slight modification of an established procedure (Axelrod *et al.*, 1981)). Lipoxygenase-1 showed broad substrate specificities for compounds of the A group with activity increasing from C_{16} to C_{20} and then decreasing from the maximum at C_{20} to C_{24}; no appreciable activity was detected with C_{14} and C_{15}. On the other hand, the B group showed little activity except for C_{18}, linoleic acid. These results indicate that the substrate requirement for the hydrophilic area of the LOX is fairly broad, but in contrast, that for the hydrophobic area is strict. The latter evidence indicates that there is a hydrophobic pocket in LOX to bind the hydrophobic methylene chain of substrates. The tertiary structure recently reported for soybean LOX-1 supports the presence of such a pocket (Boyington *et al.*, 1993). Fig. 1 shows substrate specificities of soybean seed-, cucumber cotyledon- and wheat seed-LOXs for A group (Matsui *et al.*, 1992). These LOXs were extensively purified to almost homogenous state when analyzed with SDS-polyacrylamide gel electrophoresis. Within these LOXs, soybean seed and cucumber cotyledon LOXs are specifically form 13-hydroperoxide, while wheat seed LOX forms 9-isomer. Furthermore, soybean seed LOX shows an optimum activity at pH 9.0 to 10.0 where the carboxylic acid group ionized to form corresponding carboxylate anion, while the others are most active at pH 6.0 to 7.0 where the carboxylic acid form is highly exclusive. Relative activities of the substrates of group A for the three LOXs showed different specificity profiles. Soybean LOX-1 showed broad specificity having an optimum activity with the C_{20}-dienoic acid. Cucumber LOX was most active with C_{19}-dienoic acid, and with the longer substrate than C_{19} the activity decreased gradually like that observed with soybean LOX-1. Wheat LOX was most active with the natural substrate,

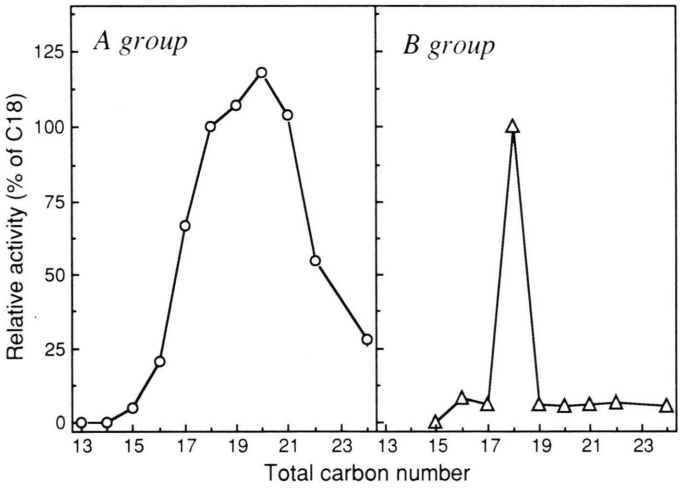

Fig. 1. Substrate specificities of soybean lipoxygenase-1 for entire series of (ω6*Z*,ω9*Z*)-$C_{14\sim24}$ dienoic acids (A group) and (9*Z*,12*Z*)-$C_{14\sim24}$ dienoic acids (B group). Relative activities when the activity of C18-fatty acid (linoleic acid) is designated as 100% are plotted against total carbon number of each substrate.

linoleic acid, of total carbon number 18, and either addition or deletion of even one methylene unit decreased the activity drastically. The other dienoic acids were not oxygenated by wheat LOX. This relatively narrow specificity observed with wheat LOX suggests that recognition of the terminal carboxyl function of a substrate has a crucial role.

In Fig. 2, the relative conversion rates of 13-hydroperoxides prepared from fatty acids of (ω6*Z*,ω9*Z*)-$C_{14\sim24}$ dienoic acids and (ω6*Z*,ω9*Z*,ω12*Z*)-$C_{14\sim24}$ trienoic acids into aldehyde products by tea leaf fatty acid hydroperoxide lyase are shown (Hatanaka *et al.*, 1992). For both the dienoic and trienoic acid hydroperoxides, product specificity is broad. Elongation between the terminal carboxyl group and the hydroperoxy group over all chain lengths from C-18 to C-22 caused enhancement of the activity towards the lyase. However, elongation beyond C-22 decreased the activity. It should be noticed that reactivities of the trienoic acid hydroperoxides were always four to seven times higher than those of the dienoic acid having the same carbon number. This indicates that introduction of a (*Z*)-double bond between ω3 and ω4 carbon positions is very effective in increasing the activity, and its is assumed that the compact turning of the side arm at the ω-terminal end caused by a (*Z*)-double bond, facilitates recognition by the lyase. Decomposition of γ-linolenic acid 13-hydroperoxide was catalyzed at a rate of only about 2% of that of α-linolenic acid 13-hydroperoxide. Thus, introduction of a (*Z*)-double bond into the carbonyl side arm, between ω12 and ω13 carbon positions, decreases the activity strikingly. In summary, recognition of the chain length between the ω10-carbon and the terminal carboxyl group is not so strict for tea leaf fatty acid hydroperoxide lyase, particularly when the length is longer than 18.

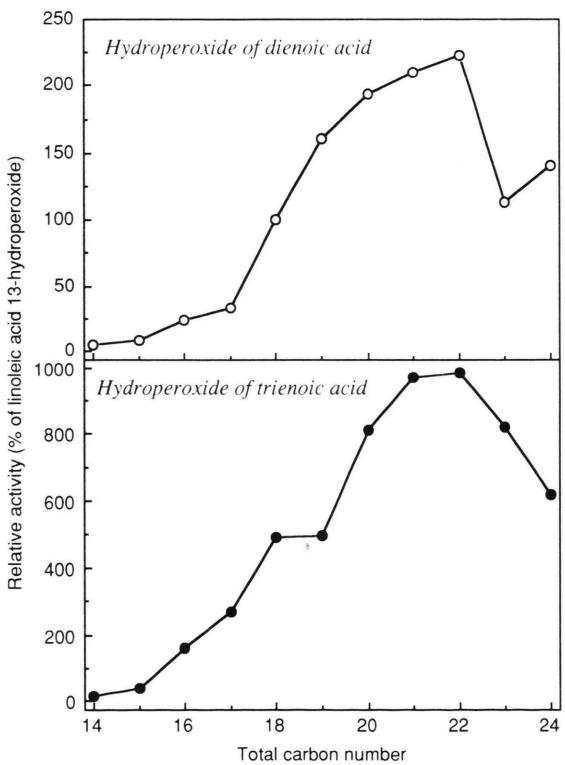

Fig. 2. Substrate specificities of tea leaf hydroperoxide lyase for entire series of 13-hydroperoxides prepared from (ω6*Z*,ω9*Z*)-$C_{14\sim24}$ dienoic acids (upper panel) and (ω6*Z*,ω9*Z*,*w*12*Z*)-$C_{14\sim24}$ trienoic acids (lower panel) by tea leaf fatty acid hydroperoxide lyase. Relative activities when the activity of 13-hydroperoxide of C18-dienoic acid (linoleic acid 13-hydroperoxide) is designated as 100% are plotted against total carbon number of each substrate.

Reaction mechanism

Using either a tea chloroplast preparation or a soybean seeds LOX, peroxidation of linoleic acid results in the same product, 13-(*S*)-hydroperoxy-(9*Z*, 11*E*)-linoleic acid. On the basis of the ESR signals from the spectrum of the spin adduct of 2-methyl-nitrosopropane (Aoshima *et al.*, 1977), we concluded that a free radical is formed during the incubation of linoleic acid with tea chloroplasts. Hyperfine constants of 15.25G and 2.00G indicate the presence of *b*-hydrogen. We can deduce that the enzymic oxygenation involves the formation of a free radical at the C-13 position of linoleic acid, and that in the initial step, the pro-(*S*) hydrogen is abstracted stereospecifically from the two hydrogens of the methylene group at the C-11 position. The double bond (*Z*-form) at C-12 is delocalized non-enzymically to C-11 (*E*-form) to produce a free radical at the C-13 position, and it is assumed that this radical would be stabilized by formation of a fatty acid-lipoxygense complex. Oxygen attacks the radical center at the C-13 position specifically from the *Si*-face of the molecule.

Fatty acid 13-hydroperoxide specific lyase in tea leaves is shown to cleave only the (*S*)-enantiomer

to form the C_6-aldehyde and C_{12}-oxo acid (Kajiwara *et al.*, 1982). To clarify the mechanism of the cleavage reaction, both oxygen atoms of the hydroperoxide group were labeled with ^{18}O, and this hydroperoxide was incubated with tea chloroplasts. As a result, one of the ^{18}O of the hydroperoxide was not transferred to C_6 but to C_{12} (Hatanaka *et al.*, 1986). From these findings concomitant with the chemical knowledge (Gardner and Plattner, 1984), a representation for the cleavage reaction mechanism may be put forward. In the first step, hydroperoxide lyase protonates the hydroperoxide which loses a molecule of water to form an allylic ether cation with a positive charge localized at carbon-13. Spontaneous rearrangement of the intermediate would result in formation of C12 oxo acid with ^{18}O and C6 aldehyde without ^{18}O.

The Relationship of Enzyme Activities to Various Stimuli

In order to examine the distribution in plants of enzyme activities which produce green odour, about 40 plant species have been investigated. It was found that almost all the plants have the activities of lipoxygenase and fatty acid hydroperoxide lyase although the levels are highly different (Hatanaka *et al.*, 1978). At the seasonal changes considering hexenal formation in tea leaves, the activities began to increase in April, and in August reached a maximum, then gradually decreased and disappeared completely in December, below 10°C (Sekiya *et al.*, 1977). The changes were parallel to temperature and solar radiation. Lipoxygenase activities showed maximal activity from 3 to 4 units per gram fresh weight, in summer leaves. On the other hand, fatty acid hydroperoxide lyase activity in summer leaves quite substantial, but was higher still in winter. This high lyase activity was found throughout the year, and the activity does not disappear as does LOX activity in winter. The overall C_6-aldehyde-forming activity, which signifies a sequential reaction of LOX and HPO, therefore shows seasonal changes similar to that of LOX. The step determining the seasonal changes is in LOX rather than the lyase (Sekiya *et al.*, 1977).

Forthcoming work

This research on the biosynthesis of volatile compounds in terrestrial plants is being extended to a study of the biogeneration of sex pheromones in marine brown algae. These are acyclic or cyclic hydrocarbons ($C_{11}H_{14}$, $C_{11}H_{16}$ and $C_{11}H_{18}$) (Kajiwara *et al.*, 1993). We presume that the pheromones are biosynthesized in female gametes from polyunsaturated C_{20}-fatty acids (eicosapentaenoic and arachidonic acids) by a sequence of oxygenation and cleavage reactions which are catalyzed by LOX and fatty acid hydroperoxide lyase (Kajiwara *et al.*, in preparation). Very recently, we found oxygenation activity in gametes although the cleavage enzyme is as yet unknown. Gametes secrete not only the species-specific pheromone but a complex mixture of the related compounds (Kajiwara *et al.*,). The composition of the pheromone bouquet depends on the specificity of the enzymes involved in the biosynthesis. We believe that future studies are envisaged on the site of pheromone biosynthesis, the cellular pathways up to secretion, precise structure-activity relations and the nature of chemoreceptors for pheromones.

In the plant enzyme system forming C_6-aldehydes and / or alcohols, the first step, i.e., liberation of free fatty acid, catalyzed by lipolytic acyl hydrolase, should be a key step which regulate the whole sequence. This is because (a) the substrates of acyl hydrolase, lipids, are abundant in plant cells, (b) contents of free fatty acids and their hydroperoxides in plant cells are very low, and (c) C_6-aldehydes and -alcohols are formed very rapidly upon homogenizing plant tissues (Matsui *et al.*, 1993). Almost the same regulatory system is found with an arachidonic cascade in mammalian cells, although calcium ion has no significance in the regulatory system in plants. There must be a novel and plant-specific regulation mechanism in the aldehyde / alcohol formation system of plants. Probably external stimuli, such as wounding and pest invasion are amplified by special signal transduction pathway to respond to them, and a regulation mechanism for free fatty acid liberation must be included in such a signal transduction pathway (Matsui *et al.*, 1993).

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