Linolenic Acid and Its 13-Hydroperoxide Inhibit Hexanal Formation from Linoleic Acid in Plant Tissues

Hexanal and hexenals (*cis*-3-hexenal and *trans*-2-hexenal) are flavor components produced by lipoxygenase and hydroperoxide lyase in plant materials from linoleic (LA) and linolenic (LNA) acids, respectively. C_6 aldehyde formation from LA was reduced by addition of LNA or its 13-L-hydroperoxide (LNAHPO), but the reverse did not occur. Inhibition was observed with isolated tea chloroplasts and crude enzyme preparations from kidney bean leaves or alfalfa seeds. LNA did not substantially reduce hexanal formation from 13-L-hydroperoxide of LA, but LNAHPO did. This suggests that the site of LNA inhibition was the lipoxygenase reaction, and LNAHPO inhibited hydroperoxide lyase and possibly also lipoxygenase reaction.

Hexanal and hexenals (*cis*-3-hexenal and *trans*-2-hexenal) are important flavor components of fruits, vegetables, and green leaf products, along with the corresponding alcohols, especially when plant tissues are processed. Precursors of the saturated and the unsaturated C_6 compounds are linoleic acid (LA) and linolenic acid (LNA), respectively. Lipoxygenase (LPO) and hydroperoxide lyase (HPO lyase) are responsible for C_6 aldehyde formation from C_{18} fatty acids (Vick and Zimmerman, 1976). The ratios of *trans*-2-hexenal to hexanal are more than 6 in apple, about 3 in grape, 30 in grape leaves, and 2 in banana when these tissues are macerated (Drawert et al., 1966). In blueberry, the ratio of a total amount of unsaturated C_6 aldehydes and alcohols to a total one of saturated C_6 compounds is 12 (Parliment and Kolor, 1975). Macerated tea leaves give more than 10 times as much unsaturated volatile C_6 compounds as saturated C_6 compounds without addition of exogenous substrate (Hatanaka and Harada, 1973). However, the ratio of LNA to LA as a constituent of lipids is about 3 in tea leaves (Saijyo and Takeo, 1972). A similar relation of C_9 aldehydes and alcohols with C_{18} fatty acids is found in cucumber fruits (Forss et al., 1962; Hatanaka et al., 1975; Galliard et al., 1976). These works suggest that the formation of C_6 and C_9 aldehydes is regulated in one or both of the steps: hydrolysis of lipids into free fatty acids and oxidative cleavage of free fatty acids into C_6 or C_9 aldehydes. Our previous paper suggested that hexanal formation was reduced when both LA and LNA were incubated with isolated tea chloroplasts (Hatanaka et al., 1982a). In the present paper, we describe the inhibitory effects of LNA and its 13-L-hydroperoxide on hexanal formation by isolated tea chloroplasts and homogenates of other plant tissues.

EXPERIMENTAL SECTION

Palmitic acid (99%), LA (99%), and LNA (99%) were purchased from P-L Biochemicals, Inc. The 13-hydroperoxide of LA (13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid, LAHPO) and 13-hydroperoxide of LNA (13-L-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid, LNAHPO) were prepared according to the method described previously (Hatanaka et al., 1982b).

Fresh tea leaves (*Thea sinensis* cv. Yabukita) were obtained from a local commercial tea garden in August, and chloroplsts were prepared and stored as a 4000-g pellet (Hatanaka et al., 1982a). Chloroplasts (0.1 g wet weight) were washed with 10 mL of 50 mM phosphate buffer, pH 6.3, by centrifugation, and suspended in 10 mL of the same buffer. Primary leaves (5 g fresh weight) of kidney bean (*Phaseolus vulgaris* L. cv. Masterpiece) grown for 2 weeks in a greenhouse (Sekiya et al., 1982b) were homogenized with 100 mL of 50 mM phosphate buffer, pH 6.3. Alfalfa seeds (1 g fresh weight) (*Medicago sativa*) immersed in tap water for 2 h were homogenized with 200 mL of 50 mM phosphate buffer, pH 6.3. Ten milliliters of each homogenate filtered through four layers of gauze were used as an enzyme solution.

Hexanal formation from LA or LAHPO and hexenal (*cis*-3-hexenal and *trans*-2-hexenal) formation from LNA or LNAHPO were determined by the headspace method (Hatanaka et al., 1982a). The substrate (6 μmol) dissolved in diethyl ether was pipetted into a 50-mL flask and the solvent was evaporated in vacuo. Then, 10 mL of the enzyme solution was added. The mixture was sealed in

Table I. Hexanal and Hexenal Formation from Mixtures of C_{18} Fatty Acids or Their 13-Hydroperoxides[a]

substrate	C_6 aldehyde formation, μmol/flask: hexanal	hexenals	total C_6 aldehydes
none	0.03	0.27	0.30
LA	2.38	0.27	2.65
LA + LNA	0.91	2.92	3.83
LA + LNAHPO	0.78	2.73	3.51
LAHPO	3.52	0.28	3.80
LAHPO + LNA	2.84	2.60	5.44
LAHPO + LNAHPO	0.95	2.53	3.48
LNA	0.04	3.22	3.26
LNAHPO	0.04	3.28	3.32

[a] Each substrate (0.6 mM) or a mixture of substrates (each 0.6 mM) was incubated with isolated tea chloroplasts, and the C_6 aldehydes formed were determined by the headspace method with GLC.

Table II. Hexanal and Hexenal Formation from LA and LNA by Homogenate of Kidney Bean Leaves or Alfalfa Seeds[a]

substrate	C_6 aldehyde formation, μmol/flask: hexanal	hexenals
Kidney Bean Leaf		
none	0.10	1.45
LA	2.60	1.45
LNA	0.10	2.39
LA + LNA	0.89	2.29
Alfalfa Seed		
none	0.18	0.03
LA	0.90	0.03
LNA	0.18	0.25
LA + LNA	0.56	0.22

[a] Each substrate (0.6 mM) or a mixture of LA and LNA (each 0.6 mM) was incubated with the homogenate of kidney bean leaves or alfalfa seeds. The C_6 aldehydes formed were determined by the headspace method with GLC.

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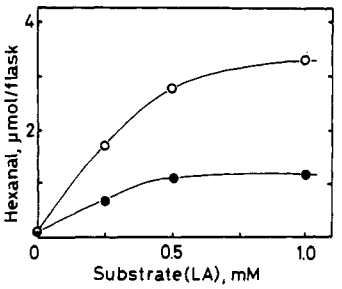

Figure 1. Inhibition of hexanal formation from LA by LNA. Isolated tea chloroplasts were incubated with LA as a substrate in the absence (○) and the presence (●) of LNA (0.6 mM). Hexanal formed was determined by the headspace method.

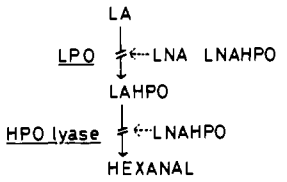

Figure 2. Biosynthesis of hexanal from LA and possible inhibition sites.

the flask with a rubber stopper. After vigorous shaking of the flask for 1 min, the mixture was incubated at 35 °C for 10 min. C_6 aldehydes formed were determined by analysis of the headspace vapor with GLC (Hatanaka et al., 1979).

RESULTS AND DISCUSSION

Hexanal formation by isolated tea chloroplasts from LA was reduced in the presence of LNA (Figure 1). Hexanal formed from LA was reduced to about 40% of hexanal formed with LA alone when both LA (0.6 mM) and LNA (0.6 mM) were incubated with tea chloroplasts, but the presence of LA caused only 10% reduction of hexenal formation from LNA (Table I). LNAHPO also inhibited hexanal formation from LA (Table I). The extent of inhibition by LNA and LNAHPO was similar. Hexanal formation from LAHPO was strongly inhibited by LNAHPO but not by LNA. LNA and LNAHPO were the precursors of hexenals, but the reduction of hexenal formation from LNA or LNAHPO by the addition of LA was less than 25%. Thus, C_6 aldehyde formation from LA was inhibited by LNA and LNAHPO but the reverse did not occur. Since LNA did not inhibit hexanal formation from LAHPO, the inhibition site of LNA is LPO reaction (Figure 2). On the other hand, LNAHPO inhibited hexanal formation from both LA and LAHPO; thus LNAHPO inhibited HPO lyase and possibly also LPO reaction (Figure 2). This possible inhibition of LPO by the addition of LNAHPO is suggested by the fact that LPO is easily inactivated or inhibited by the treatments of tea chloroplasts with detergents (Hatanaka et al., 1982b), lipolytic acyl hydrolase (Sekiya et al., 1982a), and low temperature (Hatanaka et al., 1982a) while HPO lyase is not inhibited by these treatments. Increasing the amount of LA as a substrate did not overcome the inhibition of hexanal formation by LNA (Figure 1).

Lineweaver–Burk plots indicated that the inhibition of hexanal formation caused by LNA (0.6 mM) was noncompetitive when LA as a substrate ranged from 0.25 to 1 mM.

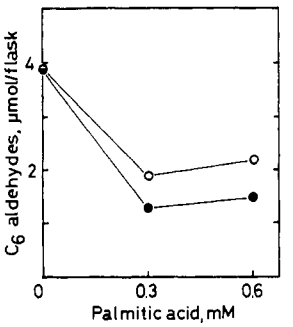

Figure 3. Inhibition of C_6 aldehyde formation from LA and LNA by addition of palmitic acid. Isolated tea chloroplasts were incubated with LA (0.6 mM) or LNA (0.6 mM) in the presence of palmitic acid at the concentrations indicated. Hexanal (○) and hexenal (●) formation was determined by the headspace method.

Dixon plots also indicated noncompetitive inhibition of hexanal formation from LA (0.2 and 0.8 mM) by the addition of LNA (0–0.6 mM).

Isolated tea chloroplasts contain LPO and HPO lyase which are tightly bound to the lamellar membrane of chloroplasts (Hatanaka et al., 1982a). On the other hand, kidney bean LPO is not always chloroplast lamellae bound in young green leaves (Sekiya et al., 1982c), although HPO lyase is membrane bound (Matthew and Galliard, 1978). Alfalfa seeds also have soluble LPO and membrane-bound HPO lyase (Sekiya et al., 1979). Therefore, we examined an inhibitory effects of LA and LNA on the enzymes in kidney bean leaves and alfalfa seeds. Hexanal formation from LA was inhibited by LNA in the kidney bean leaves and alfalfa seeds but LA did not inhibit hexenal formation from LNA (Table II). Therefore, the inhibition of the hexanal formation by LNA seems to be common in a variety of plants.

The fact that the ratio of unsaturated C_6 compounds to saturated C_6 compounds is over 1 in most plant tissues has been explained by the ratio of LNA to LA in lipids. However, the findings presented here provide a second explanation: hexanal formation may be inhibited by LNA.

Presence of palmitic acid (0.3 and 0.6 mM) caused reduction of hexanal and hexenal formation by isolated tea chloroplasts from LA and LNA, respectively (Figure 3), although palmitoleic, stearic, and oleic acids at 0.6 mM inhibited neither hexanal nor hexenal formation. This finding suggests that the presence of palmitic acid also influences formation of hexanal and hexenals.

ACKNOWLEDGMENT

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Registry No. Hexanal, 66-25-1; *cis*-3-hexenal, 6789-80-6; *trans*-3-hexenal, 6728-26-3; LPO, 9029-60-1; HPO lyase, 71833-11-9; palmitic acid, 57-10-3; LA, 60-33-3; LNA, 463-40-1; LNAHPO, 67597-26-6; LAHPO, 33964-75-9.

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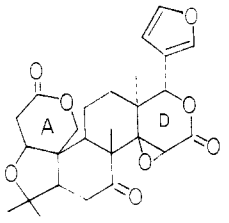
Reduction of Nomilin Bitterness in Citrus Juices and Juice Serum with *Arthrobacter globiformis* Cells Immobilized in Acrylamide Gel

Nomilin debittering of citrus juices and juice serum was successfully demonstrated with *Arthrobacter globiformis* cells immobilized in acrylamide gel. When juice serum was passed through a column packed with immobilized cells, nomilin was converted to nonbitter 17-dehydronomilinoate A-ring lactone by two enzymes, limonin D-ring lactone hydrolase and limonoate dehydrogenase. The enzyme system in the immobilized cells was as effective toward nomilin as it was toward limonin.

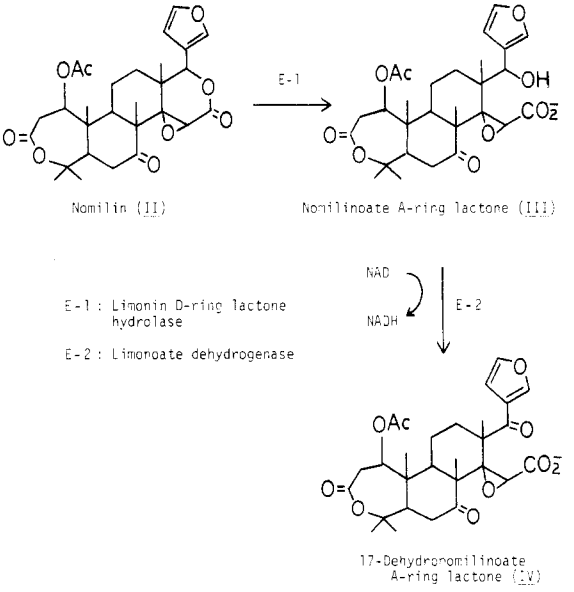
Limonoids are a group of chemically related triterpene derivatives found in Rutaceae and Maliaceae families. Limonin (I) is an intensely bitter limonoid that occurs



widely in *Citrus*. The development of bitterness in certain citrus juices due to I, sometimes referred to as delayed bitterness, is one of the primary determinants for juice acceptability and has significant ecomomic impact on the industry. We have recently developed a I debittering process that employs limonoid-metabolizing bacterial cells immobilized in acrylamide gel (Hasegawa et al., 1982). The limonoid-metabolizing enzyme system in the immobilized *Arthrobacter globiformis* cells very effectively converted I to a nonbitter metabolite, 17-dehydrolimonoate A-ring lactone, in navel orange juice and juice serum. The system could be used many times without losing its effectiveness.

Twenty-nine limonoids, 18 neutral and 11 acidic, have been isolated from *Citrus* and *Citrus* hybrids. Four of them, I, nomilin (II), ichangin, and nomilinic acid, are bitter (see Scheme I). Compound I is the major limonoid present in certain citrus juices and is the primary cause of delayed bitterness. Recently, the II content of citrus juices was reported (Rouseff et al., 1981; Hashinaga and Itoo, 1981). We have also observed the presence of II in commercial orange juices (Hasegawa and Patel, 1980), and

Scheme I. The Nomilin-Metabolizing Enzyme System in *A. globiformis* Immobilized in Acrylamide Gel



it, also, appears to play a role in the development of delayed bitterness. Organoleptic tests of Hashinaga et al. (1977) estimated that II is twice as bitter as I and II bitterness threshold is about 3 ppm. Therefore, we have tested our I debittering process to determine whether it also reduces the II content of citrus juice and juice serum.

EXPERIMENTAL SECTION

Orange juices and juice sera were prepared, *A. globiformis* cells were grown, and bacterial cells were immo-