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Lipoxygenase from Banana Leaf: Purification and Characterization of an Enzyme That Catalyzes Linoleic Acid Oxygenation at the 9-Position

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The objective of the present study was to purify and characterize the lipoxygenase (LOX) from banana leaf (*Giant Cavendishii*, AAA), an unutilized bioresource. LOX was extracted, isolated, and purified 327-fold using 25−50% saturation of ammonium sulfate fractionation, hydroxyapatite column separation, and gel filtration on Superdex 200. The molecular mass of the purified LOX was 85 kDa, K_m was 0.15 mM, and V_{max} was 2.4 μM/min·mg using linoleic acid as substrate. Triton X-100 was required in the extraction medium; otherwise, no LOX activity was detected. LOX activity increased with the concentration of Triton X-100 with an optimum at 0.1%. The optimal pH of the purified LOX from banana leaf was 6.2, and optimal temperature was 40 °C. The LOX showed the highest reactivity toward 18:2 followed by 18:3 and 20:4. A very low reaction rate was observed toward 20:5 and 22:6. On the basis of retention time in normal phase HPLC, the products of 18:2 or 18:3 catalyzed by purified LOX were hydroperoxyoctadecadienoic acid or hydroperoxyoctadecatrienoic acid. It seems that 9-LOX is the predominant enzyme in banana leaf. Banada leaf dried at 110 °C for 2 h developed algal aroma. Banana leaf extract stored at 10 °C for 12 h formed an oolong tea-like flavor. Banana leaf extract reacted with 18:2 or soybean oil pretreated with bacterial lipase produced green and melon-like aroma, whereas the same reaction with 18:3 produced a sweet, fruity, cucumber-like flavor note.

KEYWORDS: Lipoxygenase; banana leaf; purification; V_{max}; K_m; flavor formation

INTRODUCTION

Aldehydes and alcohols of six and nine carbons are widely used in flavors to provide a green odor note and an aroma of fruity freshness. The characteristic aroma compounds responsible for these flavor note may include (2*E*)-hexenol, (2*Z*)-hexenol, (3*E*)-hexenol, (3*Z*)-hexenol, hexanol, hexanal, (2*E*)-hexenal (*1*, *2*), (3Z)-nonenal, and (3*Z*,6*Z*)-nonadienal (*3*, *4*). The market of these flavor compounds is estimated at about U.S. $20−40 million per year (*4*). These compounds are formed naturally via the lipoxygenase (LOX) pathway including four major enzymes, that is, lipase, LOX, hydroperoxide lyase (HPLS), and alcohol dehydrogenase (ADH). Among them, LOX and HPLS seem to play the most important role in flavor formation (*1*).

Plant LOXs are generally classified according to their positional specificity for linoleic acid (LA) oxygenation, mainly at carbon atom 9 (9-LOX) and at carbon atom 13 (13-LOX). Among the various plant sources, soybean (*5*), pea (*6*), and cucumber (*7*) contain 13-LOX activity. Potato (*8*), tomato (*9*, *10*), and almond (*11*), contain 9-LOX acitivity. Green macroalga contains both 9- and 13-LOX (*12*). The C-6 and C-9 volatile flavor compounds were derived from the action of 13- and 9-LOX, respectively. Biogeneration of flavor compounds had been developed using different sources of LOX pathway enzymes (*1*, *3*, *12*−*14*). In our preliminary studies, banana leaf extract was able to generate volatile compounds contributing to melon, cucumber, fruity, or oolong tea-like flavor from different treatments, indicating LOX activity may exist in the banana leaf (*15*). However, isolation and characterization of LOX from banana leaf and its effect on aroma have not yet been reported in the literature.

Moreover, banana is an important subtropical annual crop not only in Taiwan but also in the areas of Southeast Asia and South America. The production of banana in Taiwan is

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>198 000 tons per year. The farmer cuts off the tree after the harvest of banana in order to grow new plants. Thousands of banana trees and leaves are wasted and burnt without further utilization and result in air pollution. Thus, the objective of the present study is to identify the LOX activities in banana leaf and to study LOX's role in flavor formation to find possible applications for banana leaf as a bioresource.

MATERIALS AND METHODS

Materials. Banana (*Giant Cavendishii*, AAA) leaves were harvested in July 2003 from a farm in southern Taiwan. The leaves were treated with liquid nitrogen and stored at −75 °C until use. Fresh tomato was obtained from a local supermarket. Soybean LOX1 prepared by affinity chromatography was purchased from Sigma Chemical Co. (St. Louis, MO).

Enzyme Purification. All steps were conducted at 4 °C. All of the buffers used consisted of 0.1% Triton X-100 (Sigma). The banana leaves were homogenized with phosphate buffer (25 mM, pH 6.3) in a ratio of 1:10, w/v. The homogenate was centrifuged at 20000*g* for 20 min to obtain a crude enzyme extract. One liter of crude extract was fractionated by ammonium sulfate precipitation at 25−50% saturation and centrifuged at 20000*g* for 20 min. The pellet was dissolved in a minimal volume of phosphate buffer (25 mM, pH 6.3) and dialyzed against the same buffer.

The dialysate was applied to a hydroxyapatite (ceramic grade, Bio-Rad, Hercules, CA) column (2.6 × 15 cm) equilibrated with phosphate buffer (25 mM, pH 6.3) and eluted stepwise with 0.12 and 0.4 M potassium phosphate buffer (pH 6.3) at a flow rate of 1.0 mL/min. The active fraction from the elution of 0.12 M phosphate buffer was collected and concentrated using ultrafiltration (molecular mas cutoff of 10 kDa).

The active fraction from hydroxyapatite separation was then pooled onto a Superdex pg 200 (Amersham Pharmacia, Uppsala, Sweden) column (1.6 × 60 cm) using a Fast Protein Liquid Chromatrophy system (FPLC, Amersham Pharmacia). The enzyme was eluted with 0.05 M potassium phosphate (pH 6.3) at a flow rate of 1.0 mL/min. The active fraction was collected, concentrated, and pooled again onto the same column for separation. The purified LOX was obtained from the elution at a flow rate of 0.3 mL/min, and the purity of the enzyme was determined with native polyacrylamide gel electrophoresis (PAGE). The purified LOX was performed on 7.5% polyacrylamide gel using a Bio-Rad mini-PAGE instrument and silver stained for protein detection (*16*).

Assay of LOX Activity. The enzyme extract was incubated with linoleic acid (200 μM in ethanol) at 28 °C for 5 min. The LOX activity was determined by measuring the increase of absorbance at 234 nm (*17*−*19*) with a spectrophotometer (Amersham Pharmacia U-2000). Ten microliters of enzyme extract was diluted with 0.98 mL of 0.05 M phosphate buffer (pH 6.3) containing 0.06% Tween-20. The reaction was initiated by adding 10 μL of linoleic acid (20 mM). LOX activity was determined by the increase in fatty acid hydroperoxide using a molar absorptivity of 25000 L/mol·cm at 234 nm for estimatation (*20*). Triplicate analyses were used in the assay of LOX activity.

Hydroperoxide Analysis. The products from LOX-catalyzed peroxidation of 18:2 or 18:3 were extracted with ethyl acetate and then reduced with $NaBH_4$ and methylated with diazomethane. The resulting compounds were separated with a solid-phase extraction column (Si form, J&W Scientific, Folsom, CA) and then subjected to normal phase high-pressure liquid chromatographic (NP-HPLC) analysis (*19*). NP-HPLC analyses were performed on a Bondclone silica column (30 cm × 3.9 mm, 10 μm, Phenomenex, Torrance, CA) equipped with a UV diode array detector (UV-DAD) (Hitachi, L7000, Tokyo, Japan). The hydroperoxy derivatives measured at 234 nm were eluted isocratically with a solvent system of hexane/2-propanol/acetic acid (98:1.9:0.1, v/v/v) at a flow rate of 1.2 mL/min. The LOX-catalyzed products, 18:2-9OOH (9-HpODE, hydroperoxyoctadecadienoic acid) and 18:3-9OOH (9-HpOTE, hydroperoxyoctadecatrienoic acid), were confirmed in comparison to authentic standards (Caman, Ann Arbor, MI). 9-HpODE and 9-HpOTE were also prepared from 18:2 or 18:3 treated with tomato LOX; 13-HpODE and 13-HpOTE were from those treated with soybean LOX1 (*18*, *19*).

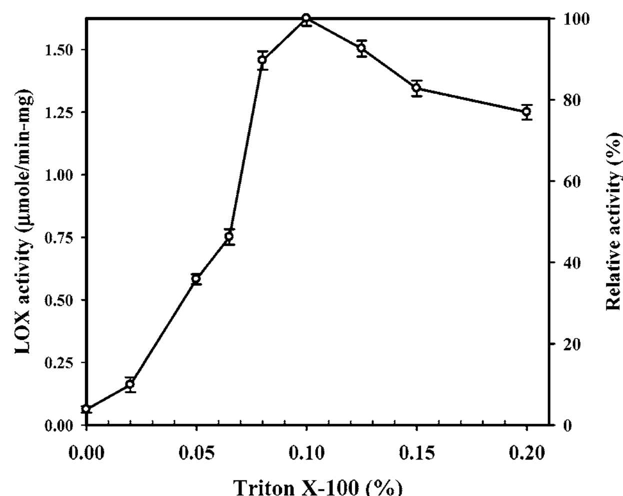

Figure 1. Effect of Triton X-100 on LOX activity of banana leaf using linoleic acid as substrate.

Table 1. Purification of Lipoxygenase from Banana Leaf (*Giant Cavendishii*, AAA)

stage	total activity (μmol/min)	total protein (mg)	specific activity (μmol/mg·min)	recovery (%)	purification (fold)
crude extract	498.4	330.20	1.51	100	1.0
25–50% $(NH_4)SO_4$	199.9	63.06	3.17	40.1	2.1
hydroxyapatite	114.1	3.62	31.52	22.9	20.9
Superdex pg 200[a]	58.3	0.46	126.74	11.7	83.9
Superdex pg 200[b]	49.3	0.10	493.01	9.9	326.5

[a] Flow rate of 1 mL/min. [b] Flow rate of 0.3 mL/min.

RESULTS AND DISCUSSION

Purification of LOX. The use of Triton X-100 was necessary for extraction of the enzyme from banana leaf. When Triton X-100 was not added in the extraction media, LOX activity was almost undetectable. LOX activity increased with the concentration of Triton X-100 added. The concentration of 0.1% Triton X-100 was optimized to compromise between solubilization and enzyme inhibition (**Figure 1**). It was also suggested that the banana leaf LOX was probably membrane-bound (*9*, *21*). Therefore, all buffer used in the following purification was added with 0.1% Triton X-100. The crude enzyme from banana leaf treated with 0.1% Triton X-100 showed high specific activity similar to crude LOX extracted from soybean (unpublished data). The crude extract of banana leaf LOX was further purified by ammonium sulfate fractionation, hydroxyapatite column chromatography, and gel filtration on Superdex pg 200. The fraction precipitated with ammonium sulfate between 25 and 50% resulted in a 2.1-fold purification with 40.1% recovery (**Table 1**). The fraction of 0−25% saturation of ammonium sulfate still contained ∼9% of enzyme activity and 80% of protein (data not shown) including chlorophyll. Most of the protein was removed after $(NH_4)_2SO_4$ fractionation; however, 60% of enzyme activity was lost in this step.

The inorganic gel of hydroxyapatite (HPT) was used as a powerful separation medium in the purification of LOX (*22*). Fractions eluted with 0.025 or 0.4 M phosphate buffer resulted in higher amounts of protein with no enzyme activity (**Figure 2**). The LOX activity was eluted with 0.12 M phosphate buffer,

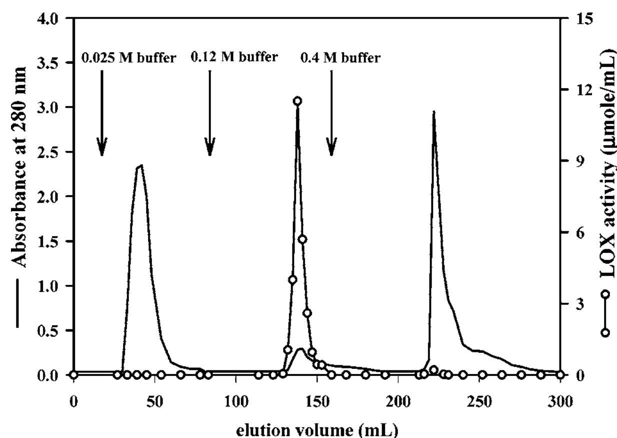

Figure 2. Elution profile of banana leaf LOX on hydroxyapatite column (2.6 × 15 cm). Column was equilibrated with 25 mM phosphate buffer (pH 6.3) containing 0.1% Triton X-100 and eluted stepwise with 0.12 and 0.4 M potassium phosphate buffer at a flow rate of 1 mL/min. Fractions of 3 mL were collected and assayed for protein as absorbance at 280 nm and for LOX activity.

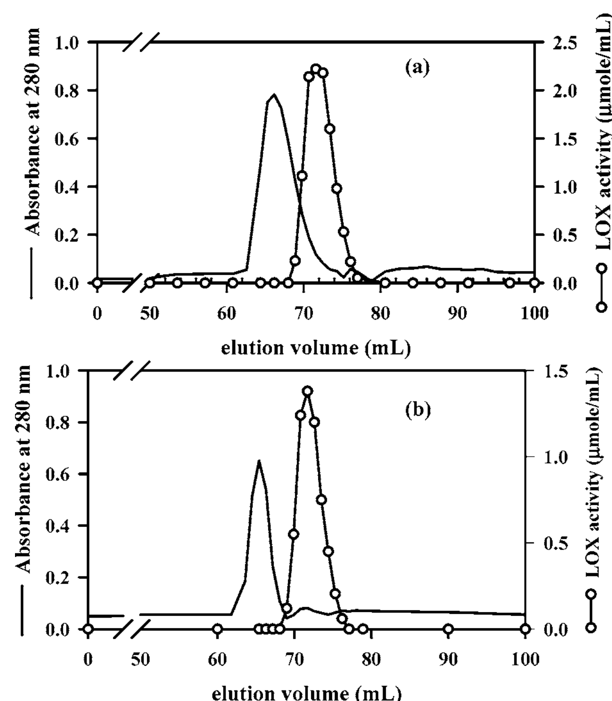

Figure 3. Elution profile of banana leaf LOX on Superdex pg 200 column (1.6 × 60 cm). Column was equilibrated with 25 mM potassium phosphate buffer (pH 6.3) containing 0.1% Triton X-100 and eluted with the same buffer at a flow rate of (**a**) 1 mL/min. Fractions of 1 mL were collected and assayed for protein as absorbance at 280 nm and for LOX activity. The active fraction from (**a**) was repooled onto the same column and eluted with the same buffer at a flow rate of (**b**) 0.3 mL/min.

resulting in a 20.9-fold purification with 22.9% recovery. The LOX fraction from hydroxyapatite column separation was pooled onto a Superdex pg 200 column and eluted between 70 and 76 mL in the first gel filtration (**Figure 3a**); the recovery of LOX activity in this fraction was 11.7% with 83.9-fold purification. In this condition, the enzyme and the impurity were not separated. Thus, the active fraction from the first gel filtration was collected, concentrated, and pooled again onto the Superdex pg 200 column for a low flow rate separation. Two protein peaks were well separated, and LOX activity was found in the minor protein peak (**Figure 3b**). When the gel

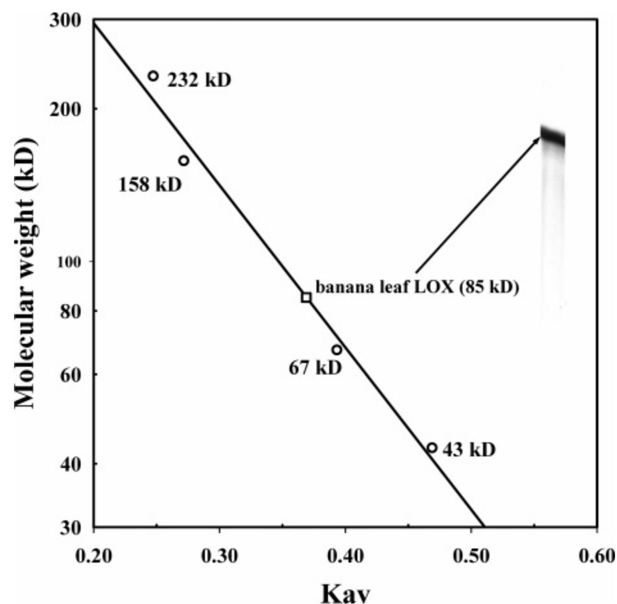

Figure 4. Estimation of the molecular weight of banana leaf LOX on Superdex pg 200 gel filtration.

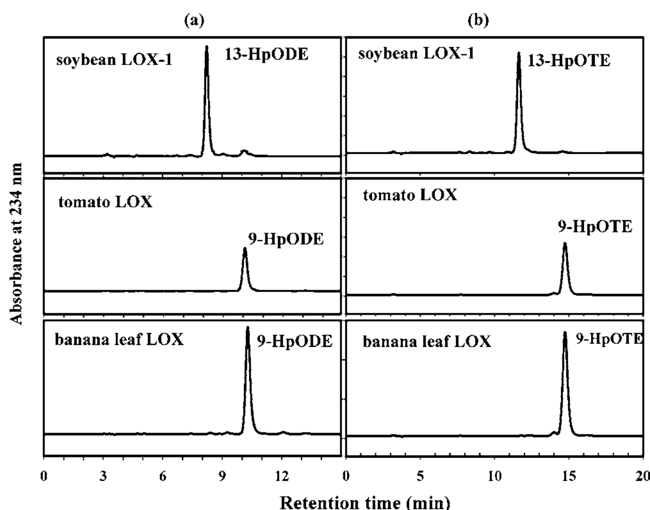

Figure 5. NP-HPLC chromatogram (Bondclone silica column, 30 cm × 3.9 mm, 10 μ) of (**a**) linoleic acid or (**b**) linolenic acid treated with purified LOX of banana leaf.

filtration was performed at low flow rate, a single run done in a working day is sufficient in contrast to five runs done in these circumstances. The recovery of LOX activity after the second gel filtration was 9.9% with 326.5-fold purification. The purity of banana leaf LOX was identified by a 7.5% mini-PAGE and showed only one band with a molecular mass of 85 kDa in the electropherogram (**Figure 4**). The molecular mass of LOX from a land plant of *Phaseolus vulgaris* L. (*23*) was 86.7 kDa; that of almond seed, 97.7 kDa (*11*); that of the three isozymes from soybean, 98 kDa; that of the two isozymes from pea, both 95 kDa; those of the two isozymes from potato tuber, 35 and 85 kDa (*24*); that of freshwater algae, 124 kDa (*25*); and that of microalgae, 182 kDa (*26*). The molecular mass of LOX from banana leaf was similar to those from land plants, but smaller than those found in marine plants.

Identification of 9-LOX. The chromatograms of NP-HPLC elution profiles of 18:2 (**Figure 5a**) or 18:3 hydroperoxide (**Figure 5b**) were obtained from the LOX activity of banana leaf in comparison to those from soybean and tomato. Soybean LOX-1 forms primarily 13-HpODE or 13-HpOTE with a small

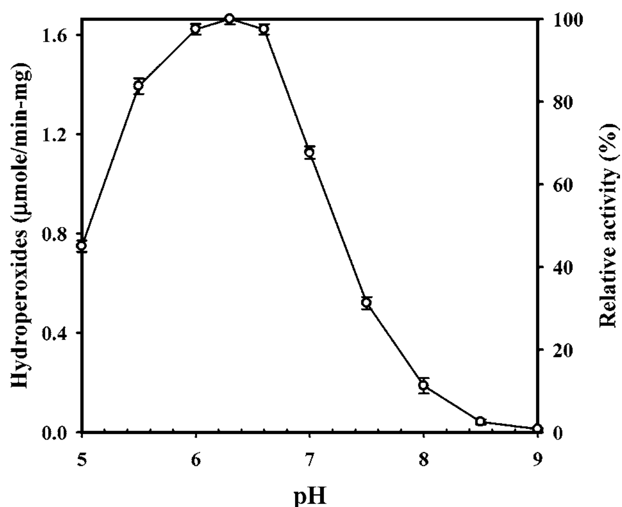

Figure 6. pH profile of LOX activity from banana leaf. Linoleic acid was used as substrate and reacted at 28 °C for 5 min. The buffer systems included acetate buffer ranging from pH 5.0 to 6, phosphate buffer ranging from pH 6.5 to 7.5, Tris buffer at pH 8 and 8.5, and borate buffer at pH 9.

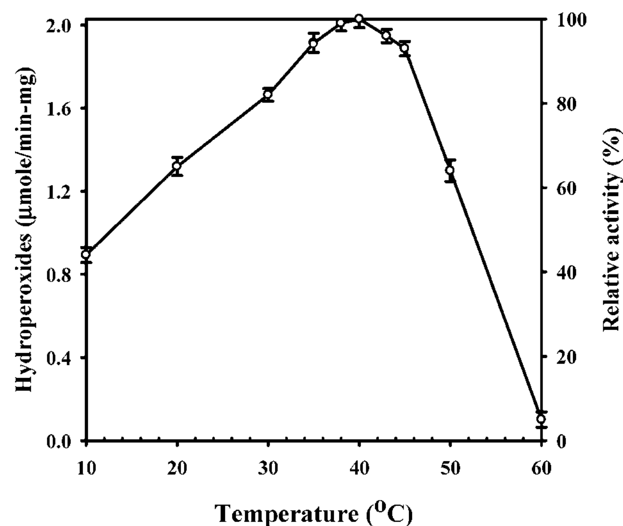

Figure 7. Temperature profile of LOX activity from banana leaf in 0.05 M potassium phosphate buffer (pH 6.3) containing 0.1% Triton X-100 using linoleic acid as substrate.

amount of 9-isomer (*27*). Tomato LOX produces 9-HpODE or 9-HpOTE (*28*). The products derived from banana leaf LOX reacted with 18:2 or 18:3 were 9-HpODE and 9-HpOTE, respectively. The identification of these hydroperoxides was based on the retention time and absorbances at 234 nm in comparison to those of the authentic standards as well as in reference to those reported in the literature.

LOX from corn germ (*29*), potato (*8*), tomato (*28*), or almond (*11*) produced 9-HpODE or 9-HpOTE from 18:2 or 18:3. Those from soybean LOX-1 (*27*), pea (*6*), cucumber (*7*), and a land plant of *P. vulgaris* L. (*23*) formed 13-HpODE or 13-HpOTE. Sea algae of *Ulva lactuca* and *Enteromorpha intestinalis* yield 9- and 13-HpODE at ratios of 86:14 (*19*) and 66.3:33.7 (*18*). It seems that 9-LOX is the only or predominant form of LOX in banana leaf.

Properties of LOX. The optimal pH of banana leaf LOX was 6.2 (**Figure 6**). At pH 5.5, 84% activity was observed, and at pH 8.0 only 11% activity. The optimal pH of soybean LOX-2 was 6.6 (*30*); that of freshwater algal LOX, 8.8 (*25*); and that of sea algal (*Enteromorpha intestinalis*) LOX, 7.8 (*18*).The optimal temperature of purified LOX from banana leaf was 40 °C (**Figure 7**). An abrupt decline in activity occurred above 45 °C, whereas 80% of the maximal activity remained at 26 °C. The optimal temperature of sea algal (*E. intestinalis*) LOX was 35 °C (*18*). That for both mullet gill (*22*) and trout gill (*31*) was 20 °C.

Fatty acids with chain lengths of 18−22 carbons possessing a *cis*,*cis*-1,4-pentadiene unit, that is, 18:2, 18:3, 20:4, 20:5, and 22:6, are substrates for LOX. The susceptibility of these fatty acids to LOX of banana leaf was assayed at 27 °C (**Table 2**). The enzyme showed the highest reactivity toward 18:2, followed by 18:3 and 20:4. The 20:5 and 22:6 fatty acids showed very low reaction rate to the LOX of banana leaf. Soybean LOX-catalyzed oxidation showed reaction rates on 18:2, 22:6, 20:5, 18:3, and 20:4 in a decreasing order (*27*). Tomato LOX showed a substrate preference for 18:2, followed by 18:3 and 20:4 (*28*), similar to that of banana leaf LOX. The LOX from *A. niger* showed the highest substrate specificity toward 18:2 and 18:3 followed by 20:4, its activity being 85% relative to the former two substrates (*32*). The algal LOX showed the highest reactivity and hydroperoxide production toward 18:2, followed by 20:4,

Table 2. Formation of Hydroperoxide from Polyunsaturated Fatty Acid Reacted with Purified Lipoxygenase from Banana Leaf (*Giant Cavendishii*, AAA)

fatty acid	hydroperoxide (μmol/mg of protein·min)	relative activity (%)
18:2 (ω−6)	1.51	100
18:3 (ω−3)	1.39	92.3
20:4 (ω−6)	0.26	17.3
20:5 (ω−3)	0.03	2.1
22:6 (ω−3)	0.07	4.3

Table 3. Kinetic Parameters of Banana Leaf LOX Using Linoleic Acid as Substrate

pH	6.3
ionic strength	50 mM
temperature	28 °C
K_m	0.15 mM
V_{max}	2.4 μM/min·mg

20:5, 22:6, and 18:3. Reactivity of shrimp hemolymph LOX on fatty acids increased with degree of unsaturation of polyunsaturated fatty scid (*33*). Grey mullet gill LOX showed the highest reactivity toward 20:4 followed by 22:6, 18:2, 18:3, and 20:5 (*22*). It seems that LOX from banana leaf and other land plants showed higher reactivity toward 18:2 than other PUFA, whereas animal LOX prefers C20:4.

Kinetics of 9-LOX. On the basis of a Lineweaver−Burk plot, the K_m of the purified LOX from banana leaf was 0.15 mM and the V_{max} was 2.4 μM diene/min·mg using linoleic acid as substrate (**Table 3**). For soybean LOX-1 K_m was 0.02 mM (*34*), for canola seed it was 0.2 mM (*35*), for tomato it was 0.52 mM (*36*), for a land plant of *P. vulgaris* L. it was 1.4 mM (*23*), and it was 2.8 mM for broad bean (*37*). For *Aspergillus niger* V_{max} was 0.095 μM/mg of protein/min (*32*), for English pea it was 2 μM/mg of protein/min (*6*), for tomato fruit it was 10 μmol/mg of protein/min (*36*), and for soybean LOX-1 it was 16 μmol/mg of protein/min (*6*). The K_m of banana leaf LOX is similar to that of canola seed, and V_{max} is similar to that of English pea.

Effects of Banana Leaf LOX on Aroma Formation and Its Possible Application. The banana leaf extract stored at 10 °C for 12 h formed am oolong tea-like flavor. When the crude

Table 4. Odor Developed[a] from Different Treatments of Banana Leaf Extract

treatment	odor
pickling[b]	oolong tea-like
+ soybean oil + lipase[c]	green, melon-like
linoleic acid[d]	green, melon-like
linolenic acid[e]	sweet, fruity, cucumber-like

[a] Odor developed was analyzed by sensory evaluation with 10 panelists. [b] Banana leaf extract was stored in 0.05 M phosphate buffer (pH 6.3) at 10 °C for 12 h. [c] Banana leaf extract was reacted with soybean oil pretreated with bacterial lipase (according to ref *13*). [d] Banana leaf extract was reacted with 400 μM linoleic acid at 26 °C for 1 h. [e] Banana leaf extract was reacted with 400 μM linolenic acid at 26 °C for 1 h.

LOX extract reacted either with 18:2 or with soybean oil pretreated with bacterial lipase, a green and melon-like aroma was produced. When it reacted with 18:3, a sweet, fruity, cucumber-like flavor note was produced (**Table 4**). The flavor compounds of tea were possibly derived from the LOX−HPLS system (*38*, *39*). The aroma compounds of cucumber were formed from hydroperoxide of 18:2 catalyzed by LOX followed by HPLS (*1*). The HPLS activity was detected in the banana leaf extract at pH 6.2 when the hydroperoxide of 18:2 was used as substrate (unpublished data). Both LOX and HPLS from banana leaf exhibited the same optimal pH between 6.3 and 6.5. This phenomenon is different from those of soybean LOX-1, of which the optimal pH was 9, and the optimal pH of HPLS was between 6.3 and 6.5. Thus, compounds contributing to melon, fruity, cucumber, or oolong tea-like flavor notes developed from the banana leaf extracts were likely catalyzed by LOX−HPLS actions. These volatile components are now being identified with GC-MS.

[^{14}C]Linoleic acid and [^{14}C]linolenic acid were converted to hexanal, *trans*-2-hexenal, and 12-oxododecenoic acid by ripening banana fruit extract. Unripe banana formed 2-nonenal, 2,6-nonadienal, and 9-oxododecenoic acid from the same fatty acids (*40*). Data suggested that 9-LOX probably existed in the unripe banada, whereas 13-LOX occurred in the ripening banana fruit (*1*). However, attempts to isolate these enzymes were not reported (*40*). In this study, we purified 9-LOX from banana leaf regardless of whether it was ripened or not.

The C-6 compounds having green and fruity characteristics were biosynthetically derived from the action of 13-LOX (*1*). For example, the organoleptic impression of hexanol has a green flavor note; 2-hexenol gives green and fruity notes; and 3-hexenol (leaf alcohol) gives green and fresh notes (*2*). The C-9 volatile compounds biogenerated from 9-LOX action (*1*) possessed melon, cucumber, and fruity flavor notes; others such as 3-nonenal had a green melon note, 6-nonenal possessed a a fresh melon note, 2,6-nonadienal had a green-cucumber note, and 3,6-nonadienal had a melon−cucumber note (*41*). Both C-6 or C-9 compounds are widely used in flavors to provide different impressions. These compounds could be obtained by extraction from plants or from enzyme-catalyzed reactions. According to the *U.S. Code of Federal Regulations*, flavors produced by enzymes could be designated "natural". Thus, the production of these natural green, fruity compounds via LOX pathway enzymes has been widely studied in many applications. A hollow-fiber reactor immobilized with LOX and HPLS was used to produce tomato flavor volatiles (*14*). Biocatalytic production of natural green flavor compounds was established from hydrolyzed linseed oil treated with LOX and HPLS (*4*). Many patents for the production of green-note compounds were derived from the reaction of fatty acids or vegetable oil with different sources of LOX and HPLS (*2*, *3*). Further studies will be conducted in line with these approaches.

Conclusion. A membrane-bound enzyme of 9-LOX is present in banana leaf (*Giant Cavendishii*, AAA). This enzyme shows high reactivity to linoleic acid. The banana leaf has the same optimal pH for both LOX and HPLS, similar to those from other plant sources such as guava fruit (*42*). This makes using banana leaf extract convenient for the continuous generation of flavor compounds by operating at the same pH for both enzymes. Guava fruit also has the same optimal pH for both LOX and HPLS except its LOX is 13-LOX. The aroma compounds formed from the guava fruit system will be different from those of the banana leaf systems. In the meantime, the characterization of HPLS of banana leaf is being studied.

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