

STUDIES ON LIPOXYGENASE AND FATTY ACID HYDROPEROXIDE LYASE
IN PLANTS

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Dedicated to my father who died on April 3, 1991.

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ABBREVIATIONS

c.p.	: cotyledon pair
GLC	: gas-liquid chromatography
EDTA	: ethylenediaminetetraacetic acid
EGTA	: ethyleneglycol bis(2-aminoethylether) tetraacetic acid
fr. wt	: fresh weight
HPLC	: high performance liquid chromatography
HPO lyase	: fatty acid hydroperoxide lyase
LA 13-HPO	: 13-hydroperoxy-(9Z,11E)-octadecadienoic acid
α LNA 13-HPO	: 13-hydroperoxy-(9Z,11E,15Z)-octadecatrienoic acid
γ LNA 13-HPO	: 13-hydroperoxy-(6Z,9Z,11E)-octadecatrienoic acid
MES	: 2-(N-morpholino)-ethanesulfonic acid
PCMB	: <i>p</i> -chloromercuribenzoate
PEG	: polyethyleneglycol
PMSF	: phenylmethanesulfonylfluoride
PVP	: polyvinylpyrrolidone
SDS-PAGE	: sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris	: tris(hydroxymethyl)aminomethane

INTRODUCTION

Flavors described as grassy, beany and leafy have been attributed to *n*-hexanal, (2E)- and (3Z)-hexenals which evolve from plant tissues upon wounding or homogenization. Although these volatile aldehydes have been familiar to mankind, the biosynthetic pathway of these aldehydes had not been elucidated until recently. Early workers reported that these aldehydes originated from linoleic and/or linolenic acids and that their formation required oxygen and enzyme activity [1, 2]. In 1973, Tressl and Drawert [3] firstly reported that these volatile aldehydes formed directly from fatty acid hydroperoxide by an enzyme which is later termed hydroperoxide lyase by Vick and Zimmerman [4]. On the other hand, it has been long known that lipoxygenase which catalyzes the incorporation of molecular oxygen into polyunsaturated fatty acid to form a fatty acid hydroperoxide is present in a wide variety of higher plants [5]. These observations and subsequent intense researches have shown that the formation of the aldehydes is carried out by sequential reactions of lipids as depicted in Fig. 1 [6]. That is, (1) linoleic and linolenic acids are liberated from galactolipid, phospholipid and triglyceride by lipolytic enzymes [7], and (2) these free fatty acids are oxidized to form their hydroperoxides by oxygenation catalyzed by lipoxygenase, then (3) the fatty acid chains of the hydroperoxides are cleaved between the hydroperoxide group and the α -olefinic carbon by fatty acid hydroperoxide lyase to form an aldehyde and an oxoacid. In some plants the formed aldehydes are further converted into the other forms either by isomerization [8] or reduction to form respective alcohols [6]. Although the biosynthetic pathway and the participating enzymes have been fully elucidated as above, physiological role(s) of this system

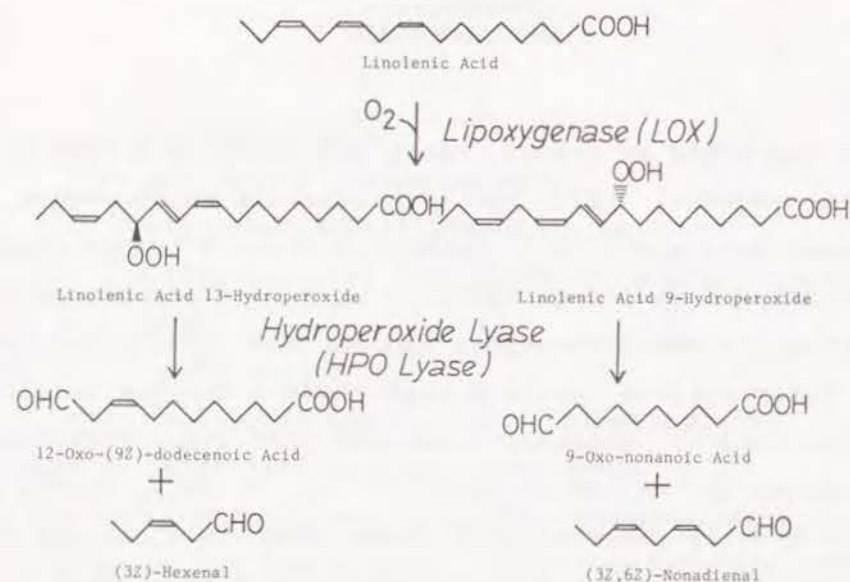


Fig. 1. Short chain aldehyde-forming system in plants.

is still in enigma. It was thought to be needed to characterize the enzymes involved in the system by biochemical and physiological manners to elucidate still-unknown physiological role(s) of the system. In this thesis the author attempted to characterize fatty acid hydroperoxide lyase and lipoxygenase in several kinds of plant species to attain the final end.

One of the enzyme involved in the system, fatty acid hydroperoxide lyase, affords cleavage of either 9-hydroperoxides or 13-hydroperoxides into either two C_9 fragments or C_6 and C_{12} fragments, respectively (Fig. 1). This enzyme has been attracted much attention because its products, volatile aldehydes, have been well known to cause pleasant and in some cases unpleasant flavors of processed foods originated from plants. The cleavage reaction performed by the lyase resembles a known acid-catalyzed cleavage of the hydroperoxide. Although labeling experiment by using oxygen-18 supported that the acid-catalyzed, heterolytic cleavage

is performed by this enzyme [9], the precise mechanism of the reaction has not been fully elucidated. This enzyme mostly associates with membrane and its content in plant tissues is always extremely low [10], which is presumably a reason for little progress with its isolation. The author purified fatty acid hydroperoxide lyase from tea leaves and revealed its properties, which will appear in Chapters 1 and 2. In Chapter 3, partial purification to separate two isozymes of fatty acid hydroperoxide lyase in cucumber cotyledons will be also described.

The other enzyme involving in the system, lipoxygenase, has been well known to be a key enzyme in arachidonate cascade in mammalian cells to form varieties of compounds including leukotrienes which show various effects on the cells such as anti-inflammation [11]. In plants, the enzyme in legume seeds or cereal grains have been mostly investigated, but recent researches indicate that it exists in wide variety of plant species including lower plant such as green algae and cyanobacteria [12, 13]. Furthermore, it has been reported that it exists in most plant tissues including cotyledons, leaves, roots, stems, fruits and so on, in some cases as a soluble-form and in the other cases as a membrane associated-form [10]. Above all, lipoxygenase in cucumber cotyledons is a suitable specimen to elucidate physiological role of this enzyme because cotyledons have specialized roles in early growth of plants. In Chapter 4 and 5, properties of lipoxygenase from cucumber cotyledons and its developmental change during early growth will be described. On the other hand, the author firstly detected lipoxygenase activity in a bryophyte, *Marchantia polymorpha* cultured cells. Cultured cells also have advantages to elucidate physiological role of lipoxygenase because the growth and state of the cells would be easily regulated. In Chapter 6, purification and properties of lipoxygenase in *M. polymorpha* will be described.

Reaction mechanism of lipoxygenase has been intensely investigated with soybean lipoxygenase-1 which has been used as a model. The enzyme catalyzes oxygenation of the (Z),(Z)-pentadiene moiety of polyunsaturated fatty acids into enantiomeric hydroperoxide with (Z),(E)-diene conjugation. This enzyme contains a non-heme iron essential to catalysis. The prosthetic group exists as Fe(III)-state in an active enzyme. The iron is reduced to ferrous state while removing pro-(S) hydrogen from the bis allylic methylene in a substrate. Molecular oxygen is introduced to the resultant alkyl radical in an antarafacial manner to form a peroxy radical which in turn is nonenzymatically reduced to fatty acid hydroperoxide [12]. In the case of soybean lipoxygenase-1, C₁₃-position of linoleic and linolenic acids is oxygenized specifically to form their 13-(S)-hydroperoxide derivatives (Fig. 2).

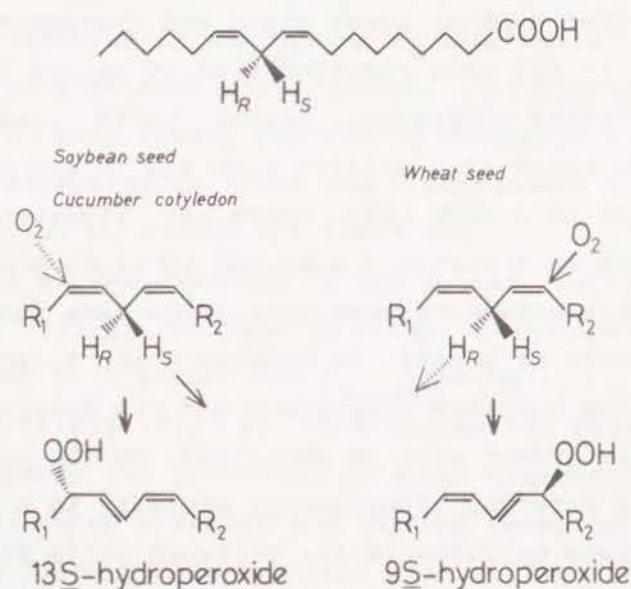


Fig. 2. Mechanism of the dioxygenase reaction catalyzed by lipoxygenases. Linoleic acid as substrate.

Other types of lipoxygenase such as in potato tuber or wheat seed removes pro-(R) hydrogen from the C₁₁-methylene in linoleic and linolenic acids specifically to form their 9-(S)-hydroperoxides (Fig. 2). Because lipoxygenase locates in a division point of the volatile aldehyde forming system, substrate and product specificities of lipoxygenase must play an important role in the system. In Chapter 7, substrate and product specificity of plant lipoxygenases having different properties will be described.

INTRODUCTION

Volatile aldehydes of chain lengths C_6 and C_9 are widely distributed in fresh leaves, vegetables and fruits [6, 13]. These aldehydes are rapidly formed from endogenous lipids when plant cells are wounded in the presence of oxygen. Lipolytic acyl hydrolase liberates linoleic and linolenic acids which in turn are converted to 13- or 9-hydroperoxides by lipoxygenase. Hydroperoxide cleaving enzyme named as fatty acid hydroperoxide lyase (HPO lyase) by Vick and Zimmerman [4] cleaves 13-hydroperoxides to form C_6 -aldehydes and C_{12} -oxoacid or 9-hydroperoxides to form C_9 -aldehydes and C_9 -oxoacid [6, 13]. It was reported that partially purified HPO lyase from cucumber fruits could cleave both 9- and 13-hydroperoxides [14], and the author separated 9-hydroperoxide specific and 13-hydroperoxide specific lyases from cucumber seedlings (see Chapter 3).

Although HPO lyase is thought to be an important enzyme for food processing, little information was given about the properties of this enzyme [10, 15], mainly because this enzyme has not been purified homogeneously yet. Membrane-binding and a low amount of HPO lyase in plant cells make the purification of this enzyme difficult. In this chapter, the author purified HPO lyase from tea leaves to a homogenous state and investigated its properties.

MATERIALS AND METHODS

Enzyme assay. 13-(*S*)-Hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid (LA 13-HPO) was prepared by oxygenation of linoleic acid with soybean lipoxygenase-1 (Sigma, type I) (see Chapter 7). Stock solution of LA 13-HPO (0.5 M in ethanol) was kept at -20°C and used within a month. For the assay of HPO lyase activity, 10 μl of the stock LA 13-HPO solution was added into 10 ml of 0.1 M K-phosphate, pH 7.5, containing enzyme solution in 50 ml flask and capped with a rubber stopper. After incubation for 9 min at 35°C with vigorous shaking, head space gas (6 ml) was directly introduced to GLC system equipped with a PEG 20M (3.0 mm x 3.0 m) column. Column temperature was 100°C and a flow rate of N_2 gas was 60 ml/min. Detection was carried out with a flame-ionization detector held at 150°C . The amount of *n*-hexanal was determined with the peak area by a calibration curve constructed with the authentic *n*-hexanal. The activity was expressed as the amount of *n*-hexanal formed (μmol) from LA 13-HPO during 9 min. For determination of the substrate specificity of HPO lyase, spectrophotometric assay by following decrease of the absorbance at 234 nm was used. To 0.1 M K-phosphate, pH 7.5, containing appropriate amount of the purified HPO lyase I, 10 μl of the substrate solution (5 mM in ethanol) was added and incubated for 10 min at 25°C . Products were identified by the retention time of HPLC of the respective 2,4-dinitrophenylhydrazone derivatives with a Zorbax ODS column (4.6 x 150 mm) eluted with acetonitrile/distilled water/acetic acid = 6/4/0.01 (v/v) as detected by the absorbance at 350 nm. As authentic specimens, *n*-hexanal (Kanto Chemical, Japan), and (3*Z*)- and (2*E*)-hexenal (kindly provided from Nippon Zeon, Japan) were used. Water insoluble inhibitors were dissolved in ethanol. Final concentration of ethanol in the assay mixture was always below 0.5% (v/v). Presence of 0.5%

ethanol did not affect the HPO lyase activity.

Fractionation centrifugation. To fresh tea leaves cut into small pieces by a razor blade (50 g fr. wt) 150 ml of 0.1 M K-phosphate, pH 7.0, containing 3 mM glutathione, 5 mM Na-ascorbate, 3 mM EDTA and various amounts of solid PVP K-30 (for cosmetics, Wako Pure Chemicals, Japan) were added and homogenized with a blender (Tohshiba, Japan) for 2 min at 4°C. After filtration through four-layers of cheesecloth, the homogenate was successively centrifuged at 1000 *g* for 10 min, 4000 *g* for 10 min, 20,000 *g* for 20 min and 100,000 *g* for 60 min. Each precipitate was resuspended in 0.1 M K-phosphate, pH 7.0.

Purification of HPO lyase. Fresh tea leaves (300 g fr. wt) were homogenized with 0.1 M K-phosphate, pH 7.0, containing 4% (w/v) PVP K-30, 3 mM glutathione, 5 mM Na-ascorbate and 3 mM EDTA using a blender. After filtration through eight layers of cheesecloth, the homogenate was centrifuged at 1000 *g* for 10 min. The resultant supernatant was centrifuged at 100,000 *g* for 60 min. The membrane fraction was resuspended in 50 mM K-phosphate, pH 7.0, and chlorophyll content was adjusted to 50 µg/ml by dilution with the same buffer. To the membrane fraction, Triton X-100 was added to 0.15% (w/v). The mixture was stirred for 60 min on ice and centrifuged at 100,000 *g* for 60 min. The supernatant (solubilized enzyme fraction) was stirred for 60 min on ice after the addition of solid PEG 6000 to 13% (w/v), and centrifuged at 12,000 *g* for 10 min at 7°C. After further addition of solid PEG 6000 to 30% (w/v), the supernatant was stirred for 180 min on ice. The precipitate obtained by centrifugation at 12,000 *g* for 10 min at 7°C was dissolved in 50 ml of 20 mM Tris-Cl, pH 8.5, containing 0.1% Triton X-114 (TT buffer) and incubated for additional 30 min with shaking on ice. After centrifugation at 20,000

g for 20 min to remove insoluble materials, the supernatant was applied to a DEAE-Cellulofine A-500 column (Seikagaku Kohgyo, Japan; 21 x 305 mm) equilibrated with the TT buffer. HPO lyase activity was eluted with 0-0.5 M KCl gradient formed in TT buffer. Active fractions were collected and dialyzed against TT buffer for 6 h with three changes of the buffer. The dialyzed enzyme was applied to a DEAE-Toyopearl 650M column (Tosoh, Japan; 19 x 170 mm) equilibrated with TT buffer, and HPO lyase activity was eluted with 0-0.5 M KCl gradient formed in TT buffer. Active fractions were collected and dialyzed against 10 mM K-phosphate, pH 6.8, containing 0.1% Triton X-114 (KT buffer) for 6 h with three changes. The enzyme was applied to a hydroxyl apatite Bio-Gel HT column (Bio-Rad; 10 x 95 mm) equilibrated with KT buffer, and HPO lyase was eluted with 10-400 mM K-phosphate gradient in KT buffer.

Protein and chlorophyll content. Protein content was estimated by the modified method of Lowry [16] with bovine serum albumin as a standard. Chlorophyll content was determined by the method of Arnon [17].

RESULTS AND DISCUSSION

Effect of homogenizing conditions on the activity and is membrane-binding state of HPO lyase. Because tea leaves were rich in polyphenols which would aggregate proteins during maceration of the tissue [18], effect of the addition of the polyphenol-adsorbing, polyvinylpyrrolidone (PVP) on the HPO lyase activity and its membrane-binding state were investigated. After homogenization of fresh tea leaves with three volumes of the homogenizing medium with and without addition of soluble PVP (PVP K-30), the homogenate was fractionated into 1000 *g*, 4000 *g*,

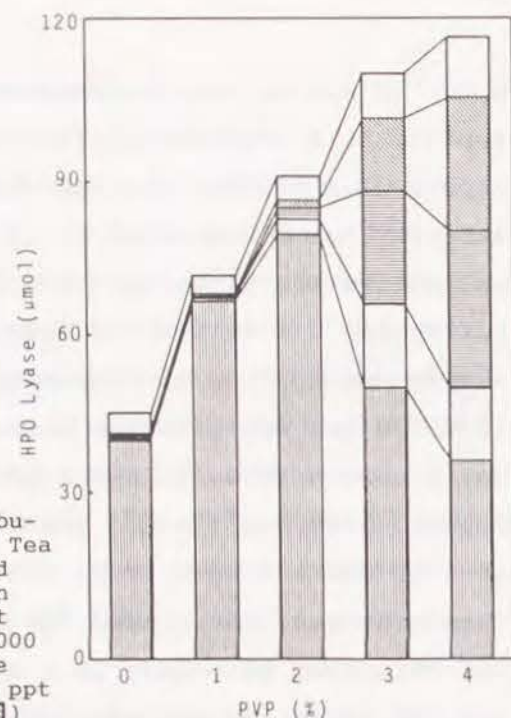


Fig. 1-1. Effect of PVP-K30 on the distribution of HPO lyase activity in tea leaves. Tea leaves were homogenized with the indicated concentration of PVP-K30 and 30 ml of each homogenate was successively centrifuged at 1000 *g* for 10 min, 4000 *g* for 10 min, 20,000 *g* for 20 min and 100,000 *g* for 60 min. The total activity in 1000 *g* ppt (□), 4000 *g* ppt (▤), 20,000 *g* ppt (▥), 100,000 *g* ppt (▧) and 100,000 *g* supernatant (□) were determined by the head space assay method. HPO lyase activity was expressed as the total amount of hexanal formed from each fraction originating from 30 ml of the crude homogenate.

20,000 *g*, and 100,000 *g* precipitates and 100,000 *g* supernatant by successive centrifugations, and the activity in each fraction was determined (Fig. 1-1). As increasing the PVP concentration to 4%, total HPO lyase activity in the homogenate gradually increased and finally reached about 2.5-fold of the activity obtained without PVP. Up to 2% of PVP, increase of the total activity was mostly attributed to the increase of the activity in the 1000 *g* precipitate fraction, but the addition of more PVP decreased the activity in 1000 *g* precipitate while those in 4000 *g*, 20,000 *g* and 100,000 *g* precipitate fractions were much enhanced. Addition of more PVP than 4% up to 10% resulted in no further change on the total activity and on the distribution profile of the activity. It was likely that PVP changed the binding nature of HPO lyase with tea leaf membrane because solubilization of HPO lyase

from whole membrane fraction prepared without PVP needed as much as 0.7% Triton X-100 although 0.15% Triton X-100 was enough to solubilize the activity from the membrane fraction prepared with 4% PVP. Previously it was reported that HPO lyase in tea leaves tightly bound to chloroplast membranes [19]. The results shown above suggests that polyphenols oxidized by polyphenol oxidase during maceration non-specifically bind to HPO lyase and cause the aggregation of the enzyme with other proteins or membrane constituents, which would be easily sedimented with centrifugation at low gravity [20].

Purification of HPO lyase. Tea leaves were homogenized with the buffer containing 4% PVP K-30, glutathione and ascorbate. In order to obtain the starting material of a high specific activity, 1000 *g* precipitate was discarded and the resultant supernatant was centrifuged at 100,000 *g* for 60 min to get the membrane fraction. At this step, only 50% of the initial activity could be obtained, but the specific activity was increased by 3.61-fold (Table 1-1). Treatment of the membrane fraction with 0.15% Triton

Table 1-1. Purification of hydroperoxide lyase from tea leaves

	Total protein (mg)	Total activity (μmol)	Specific activity (μmol mg ⁻¹)	Yield (%)	Purification (fold)
Crude homogenate	9515	1444	0.15	100	1
Membrane	1332	720	0.54	49.9	3.61
Solubilized	925	1383	1.5	95.8	9.97
PEG 6000	73.5	799	10.9	55.4	72.5
DEAE-Cellulofine	2.49	366	147	25.4	984
DEAE-Toyopearl	nd*	160	—	11.1	—
Hydroxyl Apatite					
Fraction I	0.055	44.7	872	3.10	5816
Fraction II	0.010	9.84	879	0.68	5860

*nd; not determined.

X-100 and following separation of the solubilized enzyme by centrifugation doubled HPO lyase activity. Because the activity was enhanced only by the addition of Triton X-100 to the membrane fraction, this detergent by itself may activate HPO lyase. Other detergents, Tween 20 and Tween 80, also enhanced the activity, although these detergents could solubilize much less activity (data not shown). Fractionation with PEG 6000 (13-30%, w/v) effectively concentrated and purified the enzyme. This fraction was most stable in 20 mM Tris-Cl, pH 8.5 containing 0.1% Triton X-114, and essentially no activity was lost during keeping at 4°C for 96 h. With deoxycholic acid or Tween 20, 75 and 35% of the initial activity was lost during 96 h, respectively. About 45% of the initial activity was lost during 96 h even with Triton X-100 which has almost homologous structure with Triton X-114. Relatively low HLB (hydrophilic-lipophilic balance) value of Triton X-114 (12.4) might be effective to stabilize the activity. Then, the PEG 6000 fraction was dissolved in 20 mM Tris-Cl, pH 8.5, containing 0.1% Triton X-114.

Although HPO lyase activity was eluted as a single activity peak in DEAE-Cellulofine and DEAE-Toyopearl chromatographies, further purification by hydroxylapatite chromatography separated the HPO lyase activity into two fractions (Fig. 1-2). The fraction eluted first was designated as HPO lyase I and that eluted secondly was as HPO lyase II. HPO lyases I and II were revealed to be almost homogeneous on SDS-polyacrylamide gel electrophoresis and M_r were estimated to be 55,000 and 53,000, respectively (Fig. 1-3). With estimation by gel filtration method, the M_r of the partially purified HPO lyase from tomato fruit [21] and watermelon seedlings [4] were reported to be over 200,000. Olias et al. reported M_r of HPO lyase from germinating soybean seedlings to be 240,000-260,000 by gel filtration method but 62,000 by SDS-polyacrylamide gel electrophoresis [22]. Although M_r of

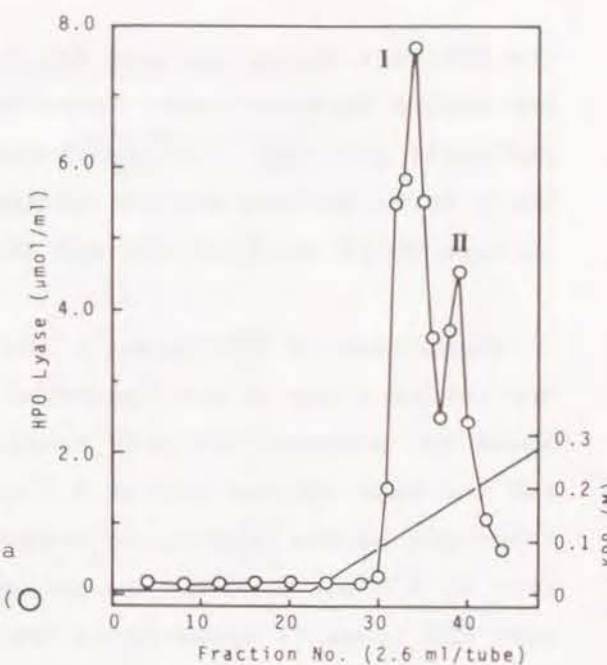


Fig. 1-2. Elution profiles of HPO lyase in tea leaves on a hydroxylapatite column. The active fraction of the DEAE-Toyopearl 650M chromatography was collected and applied to a Bio-Gel HT column, and then eluted with a K-phosphate gradient (—). HPO lyase activity (○) was determined by the head space assay method.

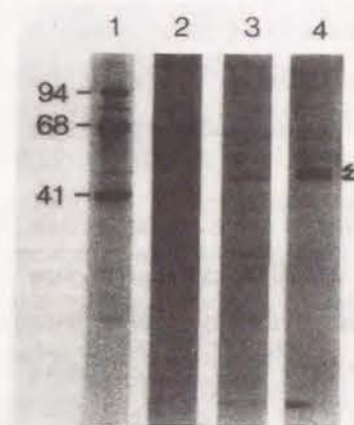


Fig. 1-3. SDS-polyacrylamide gel electrophoresis of the purified HPO lyase. Purified HPO lyase I (lane 2) and II (lane 3) and HPO lyase I kept at 4°C for one week (lane 4) were analyzed with SDS-polyacrylamide gel electrophoresis (12.5% gel). The M_r standards used (lane 1) were: soybean lipoxygenase-1 (94,000), bovine serum albumin (68,000) and yeast alcohol dehydrogenase (41,000).

the native form of tea leaf HPO lyase has not been estimated, it may exist as a tetramer. Recently, other type of HPO lyase partially purified from cyanobacteria [23] and green algae [24], which forms pentane but not hexanal from LA 13-HPO, was reported to have M_r of about 56,000 and 48,000, respectively.

Comparison of HPO lyases I and II. In order to reveal whether HPO lyases I and II are isoenzyme or not, pH-activity profiles of these two preparations were compared. Almost the same profiles and the same optimum pHs of 7.5 were obtained with both the fractions by the head-space method. When purified HPO lyase I was kept at 4°C for a week, new protein band having the same mobility with HPO lyase II appeared on SDS-polyacrylamide gel electrophoresis (Fig. 1-3). The appearance of HPO lyase II was not observed when HPO lyase I was kept at -20°C. This result suggests that HPO lyase II is a partially digested form of HPO lyase I. However, the addition of neither 10 mM diisopropyl fluorophosphate, 1 mM *p*-chloromercuribenzoate, 5 mM EDTA nor 10 µg/ml pepstatin A to the purified HPO lyase I prevented the appearance of the new protein band. It must be noted that HPO lyases I and II had almost the same specific activity (Table 1-1).

Inhibitors. In order to gain information about the active site of HPO lyase in tea leaves, inhibitors for the activity were systematically investigated with purified HPO lyase I. As reported before for HPO lyases of several plant tissues [15], metal chelating reagents such as EDTA, Tiron, diethylenetriaminepentaacetic acid, *o*-phenanthroline and sodium azide or metal cations such as Ca^{2+} , Cu^{2+} , Fe^{2+} , Fe^{3+} and Mn^{2+} (all tested at 1 mM) did not affect the activity. However, 1 mM Hg^{2+} effectively inhibited (Table 1-2). Other sulfhydryl reagents, *p*-chloromercuribenzoate and monoiodoacetate, moderately inhibited the activity as report-

Table 1-2. Effect of reagents on hydroperoxide lyase activity

Reagents	Remaining activity (%)	
	0.2 mM	1 mM
Cumene hydroperoxide		71.4
<i>tert</i> -Butyl hydroperoxide		94.1
Methylene blue	19.4	
Butylated hydroxyanisole	69.6	14.4
Butylated hydroxytoluene	102.4	89.4
<i>nor</i> -Dihydroguaiaretic acid	13.4	0
Esculetin	88.8	66.3
<i>p</i> -Chloromercuribenzoate	74.7	44.0
HgCl_2	3.6	0
Monoiodoacetate	87.9	60.7
Diethyldithiocarbamate	84.3	70.2

ed with HPO lyases from watermelon seedlings [4], cucumber fruits [14] and tomato fruits [21]. These results suggest the participation of sulfhydryl group in the reaction center of HPO lyase or in the conformation of the enzyme. The electron transfer mediator, methylene blue (0.2 mM) inhibited over 80% of the activity, but dichlorophenolindophenol (0.2 mM) did not. Although it was expected that organic hydroperoxide competitively inhibited the activity, cumene hydroperoxide inhibited only slightly and *tert*-butyl hydroperoxide did not (Table 1-2). Recently Andrianarison et al. reported that the inhibitors for lipoxygenase which have phenolic hydroxyl groups are also good inhibitors for HPO lyase of cyanobacteria [23]. This was also the case with HPO lyase purified from tea leaves. Nordihydroguaiaretic acid and butylated hydroxyanisole were potent inhibitors, and esculetin was a moderate inhibitor (Table 1-2). Butylated hydroxytoluene which has almost the same structure with butylated hydroxyanisole did not cause substantial effect on the activity. This suggests that the inhibitor of HPO lyase has a high structural requirement.

Substrate and product specificity. LA 13-HPO, γ LNA 13-HPO and α LNA 13-HPO were prepared by soybean lipoxygenase-1. Because these hydroperoxides contained less than 3% of geometrical and positional heterogeneity, they were used as substrates of HPO lyase I without further purification. Relative activities were determined by following a decrease of the absorbance at 234 nm due to conjugated diene of the substrates. The initial velocities of the purified HPO lyase for LA 13-HPO, α LNA 13-HPO and γ LNA 13-HPO were determined to be 100, 920 and 7%, respectively. This indicates that HPO lyase from tea leaves has a strict substrate specificity. The products, C₆-aldehydes and C₁₂-oxoacid, were converted to respective 2,4-dinitrophenylhydrazone derivatives and analyzed with reversed phase HPLC with detection at 350 nm. Comparison of the retention time with those of authentic specimens revealed that (3Z)-hexenal but not (2E)-hexenal was formed from α LNA 13-HPO, and that 12-oxo-(9Z)-dodecenoic acid but not the 10E-isomer was formed from both LA 13-HPO and γ LNA 13-HPO. These results suggest that HPO lyase retains Z-configuration in substrates, and the isomerization to the E-isomer occurs after the cleavage reaction of HPO lyase.

SUMMARY

Hydroperoxide cleaving enzyme, hydroperoxide lyase (HPO lyase), was first purified to homogeneous state from membrane fraction of tea leaves. The activity was separated by hydroxylapatite gel chromatography into two fractions, HPO lyases I and II, and their molecular weights were 55,000 and 53,000, respectively, as estimated by SDS-polyacrylamide gel electrophoresis. Several lines of evidence are presented to show that HPO lyase I is a native form. Almost the same pH-activity profiles were ob-

served with these two forms of HPO lyase. Antioxidants which are potent inhibitors for lipoxygenase such as nordihydroguaiaretic acid and butylated hydroxyanisole inhibited the activity of HPO lyase I. Activity for 13-hydroperoxide of α -linolenic acid was about 10 times higher than that for 13-hydroperoxide of linoleic acid, but positional isomer, 13-hydroperoxide of γ -linolenic acid was a poor substrate.

Chapter 2. Inactivation of Tea Leaf Hydroperoxide Lyase by Fatty Acid Hydroperoxide

INTRODUCTION

Fatty acid hydroperoxide lyase (HPO lyase) is the enzyme which cleaves fatty acid hydroperoxide to form short-chain aldehydes and is ubiquitous in the plant kingdom. The hydroperoxides (HPOs) of either linoleic or linolenic acids are the natural substrates of the enzyme. This enzyme plays a key role in the formation of the flavor of certain leaves, fruits, and vegetables. Flavors described as grassy, beany, and leafy have been attributed to the products of this enzyme, and in some cases they cause pleasant and in other cases unpleasant flavors [15]. Although the importance of this enzyme in food processing has been well recognized, investigation on the properties and reaction mechanisms of the enzyme have been scarcely reported. In tea leaves, 13-(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid (LA 13-HPO) is formed from linoleic acid by lipoxygenase, and this HPO is cleaved into *n*-hexanal and 12-oxo-(9*Z*)-dodecenoic acid by HPO lyase. *n*-Hexanal and (3*E*)-hexenal (formed from linolenic acid) have been shown to be important components in tea flavor. The author first purified HPO lyase from tea leaves and elucidated some properties (see Chapter 1). Tea leaf HPO lyase preferentially cleaves fatty acid 13-HPO, while the 9-isomer is a poor substrate. Because Hg^{2+} and *p*-chloromercuribenzoic acid strongly inhibited the activity, participation of an SH-group in the reaction was proposed. During further investigation to reveal the reaction mechanism, the author found that the enzyme was rapidly inactivated by its natural substrates, fatty acid HPOs. HPOs are potentially reactive to cause deterioration of food proteins or amino acids [25].

However, the effect of HPOs on enzyme activity has not been reported. In this chapter, kinetics and mechanism of inactivation of tea leaf HPO lyase by fatty acid HPOs are described.

MATERIALS AND METHODS

Materials. Linoleic acid (LA) 13-hydroperoxide (HPO) and LA 9-HPO were prepared from LA (Wako Pure Chemicals, Osaka, Japan) using soybean lipoxygenase (Sigma, type I) and potato tuber lipoxygenase, respectively. Both the HPOs contained less than 10% of geometrical and positional isomers. LA 13-hydroperoxide was prepared by the reduction of LA 13-HPO by sodium borohydride. Other chemicals were of reagent grade.

Methods. Enzyme Preparation. HPO lyase was purified from tea leaves grown in the University's tea garden essentially as described in previous chapter, but the first DEAE-Cellulofine chromatography was omitted. The specific activity of HPO lyase was 18 U/mg protein under the standard assay condition described below. Although HPO lyase activity was separated by hydroxylapatite chromatography into HPO lyases I and II, only HPO lyase I was used because this isoform is a native form, and, in this chapter it is simply denoted HPO lyase. Alcohol dehydrogenase (120 U/mg protein, from yeast) was purchased from Oriental Yeast Co. (Osaka, Japan).

Determination of Enzyme Activities. HPO lyase activity was measured by the decrease of absorbance at 236 nm caused by conjugated diene of LA 13-HPO at 25°C. A typical reaction mixture contained 50 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES)-KOH, pH 5.5, 40 μM LA 13-HPO and enzyme in a total volume of 1 ml. Alcohol dehydrogenase was measured by the decrease of absorbance at 340 nm caused by NADH at 25°C [26]. A typical reaction mixture

contained 100 mM Na-phosphate buffer, pH 7.0, 10 mM β -mercapto-ethanol, 100 mM acetaldehyde, 0.1 mM NADH and enzyme in a total volume of 1 ml. One unit of the HPO lyase and alcohol dehydrogenase activities were defined as the amount of enzyme consuming 1 μ mol LA 13-HPO and NADH for 1 min using 25,000 and 6220 $M^{-1}cm^{-1}$ as the extinction coefficients, respectively. Inactivation kinetics of HPO lyase by LA 13-HPO was followed by the determination of reaction velocities at 15 or 20 second intervals for 10 min using a Shimadzu (Kyoto, Japan) MPS-2000 multi-purpose spectrophotometer at 25°C. Otherwise, HPO lyase and alcohol dehydrogenase were incubated with HPO for 10 min, then, the residual activities were measured after 10- or 100-fold dilution with the assay mixture containing respective substrates. Details are described in the legends to Figures and Tables.

Protein and HPO Determinations. The amount of HPO group was determined by the ferrous thiocyanate method as described in [27]. Protein content was determined by the modified Lowry's method [16] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

For determination of HPO lyase activity, the author previously quantified the amount of a volatile product, *n*-hexanal, formed from LA 13-HPO in the head space of a reaction vessel by GLC reference. In this chapter, however, the decrease of the absorption at 236 nm caused by LA 13-HPO was followed in order to observed kinetics of HPO lyase reaction. As shown by the traces in Fig. 2-1, the addition of the enzyme to LA 13-HPO resulted in a decrease of the absorption with no apparent lag phase. No other change in absorbance in a range between 190 and 350 nm was observed. Reaction conditions were optimized to obtain initial

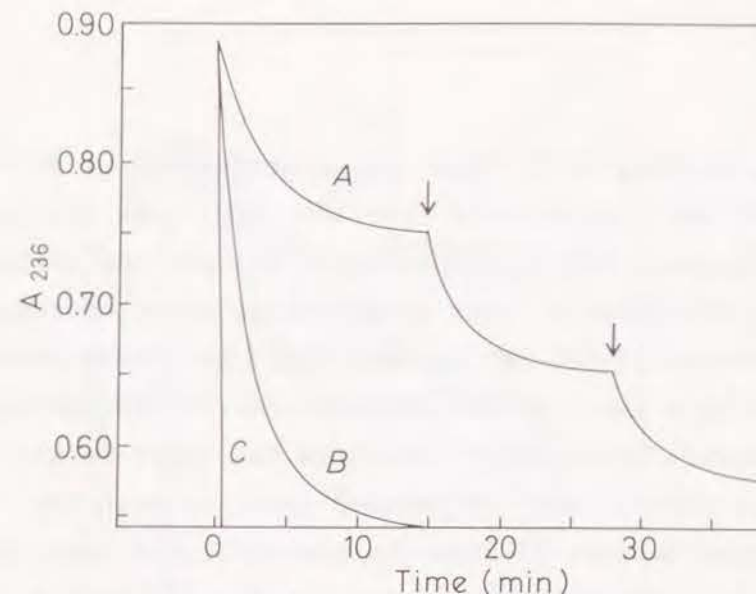


Fig. 2-1. Breakdown of LA 13-HPO by tea leaf HPO lyase. The reaction mixture contained 35.6 μ M LA 13-HPO and 1.74 (trace A), 4.64 (trace B) or 17.4 (trace C) nM HPO lyase in 50 mM MES-KOH, pH 5.5 (total volume, 1 ml). Reaction were initiated by the addition of HPO lyase. Where arrow indicates, additional 1.74 nM HPO lyase was added to the reaction mixture.

slopes of traces for the determination of an initial reaction rate. Based on this assay procedure, HPO lyase activity obeys the Michaelis-Menten kinetics and the K_m for LA 13-HPO of HPO lyase was 1.46×10^{-5} M at the optimum pH of 5.5. When a low concentration of the enzyme was used, the reaction velocity was gradually decreased and finally reduced to nil within 15 min prior to cleavage of all the added substrate (traces A and B in Fig. 2-1). Increasing the LA 13-HPO concentration did not cause further reaction; but the successive addition of the same amounts of fresh enzyme induced again the reaction (trace A). The primary and secondary initial rates of reaction were almost the same.

Inactivation of HPO lyase was also evident when the decrease of the HPO group was monitored by using the ferrous thiocyanate method. Extensive dialysis of the inactivated enzyme against 50 mM MES-KOH, pH 5.5, did not restore the activity. Dialysis against the same buffer containing 1 mM dithiothreitol also

failed to restore the activity. Thus, the inactivation of HPO lyase by LA 13-HPO was irreversible. Tea leaf HPO lyase utilizes only 13-HPOs of linoleic and linolenic acids but not the respective 9-HPOs [28]. Furthermore, this enzyme shows enantioselectivity and only catalyzes 13-(*S*)-HPO isomers [30]. LA 13-HPO used in this study contained a small amount of enantiomeric, geometrical and positional isomers which would remain in the reaction mixture. However, the commencement of renewed reaction with the addition of the same amounts of fresh enzyme indicated that the inactivation of HPO lyase was not responsible for increasing the ratio of non-catalyzable HPO isomers. The activity did not decrease even after a 30 min-incubation under the same conditions without HPO, and furthermore, incubation of HPO lyase with either or both of the products, *n*-hexanal or/and 12-oxo-(9*Z*)-dodecenoic acid, did not affect the activity. These results indicate that HPO lyase is progressively and irreversibly inactivated by its substrate, LA 13-HPO or its reaction intermediate.

To obtain inactivation kinetics, the reaction rates were determined at 15 or 20-second intervals by following the reaction progress curves. Because lowering the substrate concentration during catalysis would much affect the reaction velocity of HPO lyase and thus inactivation kinetics, a low concentration of the enzyme and a high concentration of the substrate ($>2 \times K_m$) were used. Inactivation of HPO lyase by LA 13-HPO proceeded as time- and concentration-dependent manners. Semilogarithmic plots of the residual activities were linear and obeyed the first-order kinetics law (Fig. 2-2). Plotting the apparent first-order rate constant (k_{app}) vs initial concentration of LA 13-HPO gave a reasonably good straight line. This indicates that the kinetics of inactivation are consistent with the simple model shown in Equation 1 [30].

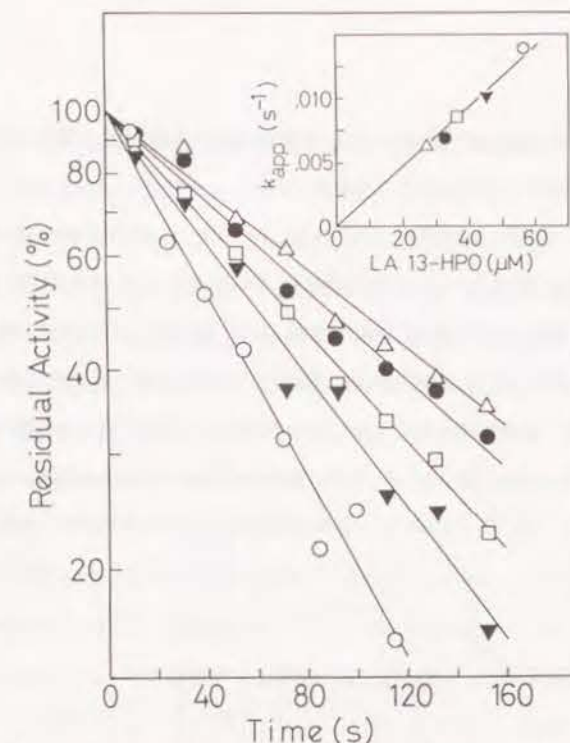
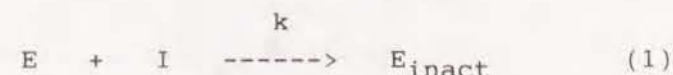


Fig. 2-2. Inactivation of tea leaf HPO lyase as a function of the concentration of LA 13-HPO. Tea leaf HPO lyase, at 1.16 nM in 50 mM MES-KOH, pH 5.5, and 25°C was exposed to LA 13-HPO in a total volume of 1 ml. Inactivation was followed by the determination of reaction rates at 15 or 20-second intervals. The initial concentrations of LA 13-HPO were: 26.8 (Δ), 32.3 ($\bullet$), 36.8 ($\square$), 45.1 ($\blacktriangledown$) and 56.9 ($\circ$) μ M. Plotted on the ordinate is the logarithm of the concentration of active enzyme remaining at the indicated times after addition of LA 13-HPO. inset; Apparent rate constant of inactivation (k_{app}) was plotted against the initial concentration of LA 13-HPO.



The second-order rate constant for inactivation was determined to be $265 \text{ M}^{-1}\text{s}^{-1}$. Little and O'Brien [31] reported that the second-order rate constants for the oxidation of thiols in bovine serum albumin by autooxidized hydroperoxide of linoleic acid were 140 and $500 \text{ M}^{-1}\text{s}^{-1}$ (for native and denatured albumins, respectively) in the reaction mixture involving cytochrome *c* as a catalyst. Heme compounds convert HPO to a highly reactive form such as radical species [32, 33]. This means that fatty acid HPO *per se* is not so reactive as to destroy amino acid residues in

proteins, and it would be essential to convert LA 13-HPO to a reactive form for inactivating HPO lyase.

As shown in Fig. 2-3, HPO lyase showed a pH-activity profile having pH optimum at 5.5. While, a higher degree of inactivation was observed in more acidic pH and showed no peak. Experiment below pH 4 was not carried out because the insolubility of LA 13-HPO interfered with an accurate estimation. The marked difference between pH-dependencies of the two parameters suggests that a mechanism to inactivate HPO lyase, probably involving activa-

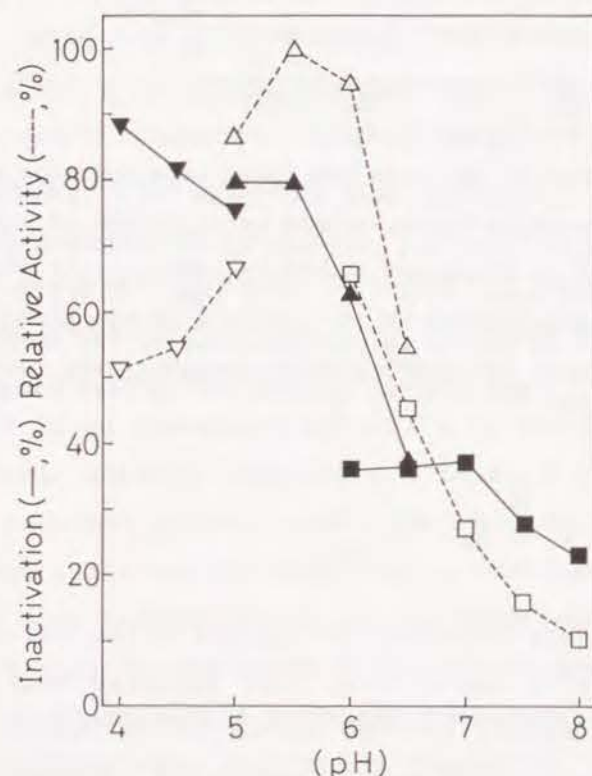


Fig. 2-3. The degree of inactivation of HPO lyase by LA 13-HPO (straight line) and relative activity of HPO lyase (dotted line) as a function of pH. Tea leaf HPO lyase (46.4 nM) was incubated at 25°C for 10 min with 40 μ M LA 13-HPO at indicated pH. Residual activity was determined by 10-fold dilution of the mixture with 33 μ M LA 13-HPO in 50 mM MES-KOH, pH 5.5. Control experiment without LA 13-HPO was also carried out at each pH and percent of inactivation of respective control activity was plotted. Relative activity of HPO lyase was determined with 30 μ M LA 13-HPO. Buffers used were; 20 mM sodium acetate buffer (pH 4.0-5.0; ∇ , ∇), 20 mM MES-KOH buffer (5.0-6.5; $\blacktriangle$, $\triangle$) and 20 mM sodium phosphate buffer (6.0-8.0; $\blacksquare$, $\square$).

tion of fatty acid HPO, was not completely the same as the catalytic mechanism of the reaction usually forming the products from fatty acid HPO. The pH-inactivation curve revealed one pK value of about 6.5. This indicates that a functional group in the enzyme having a pK at around 6.5 participates in the inactivation. Matsushita et al. [34] reported almost the same pH dependent profile with inactivation of pancreas ribonuclease by autooxidized LA, although inactivation mechanism of the ribonuclease has not been specified.

Inactivation of HPO lyase was investigated with other HPOs and related compounds (Table 2-1). In order to assess the sensitivity and specificity of HPO lyase against various HPOs, inactivation

Table 2-1. Effect of HPOs and related compounds on the activity of HPO lyase and alcohol dehydrogenase^a.

Reagent added	Residual activity (%)	
	HPO lyase	Alcohol dehydrogenase
None	100	100
Linoleic acid 13-hydroperoxide	24.5	20.9
Linoleic acid 9-hydroperoxide	15.0	nd ^b
Linoleic acid 13-hydroxide	90.7	nd
<i>tert</i> -Butyl hydroperoxide	98.2	9.4
(96 μ M)	106.9	nd
Cumene hydroperoxide	103.3	8.3
(96 μ M)	98.6	nd
Hydrogen peroxide	104.6	60.7

^a HPO lyase (46.4 nM) or yeast alcohol dehydrogenase (46.5 nM) was incubated with 40 μ M (or 96 μ M) of HPOs or hydroxide in 50 mM MES-KOH, pH 5.5, for 10 min at 25°C. Residual activity was determined after 10-fold dilution of the mixture with 50 mM MES-KOH, pH 5.5, containing 29.5 μ M LA 13-HPO or 100-fold dilution with 100 mM phosphate buffer, pH 7.0, containing 0.1 M acetaldehyde, 0.1 mM NADH and 10 mM 2-mercaptoethanol, for HPO lyase or alcohol dehydrogenase, respectively. Values are means of three repeats. ^b nd; not determined.

of yeast alcohol dehydrogenase caused by these compounds was also investigated. Yeast alcohol dehydrogenase was selected because this enzyme has an SH-group essential to catalytic activity as HPO lyase does (see Chapter 1) and is readily inactivated irreversibly by exposure to active oxygen formed by mixed-function oxidation reaction [35]. Under the conditions employed here, HPO lyase lost 75% of the initial activity during a 10-min incubation with LA 13-HPO. A positional isomer of LA 13-HPO, LA 9-HPO, also inactivated HPO lyase. LA 13-hydroxide, however, little inactivated the enzyme, which indicates that the HPO group is required for the inactivation of HPO lyase. Matsushita et al. [34] proposed that binding of fatty acid HPO to hydrophobic regions of ribonuclease, rather than a chemical reaction, is responsible for modification of the activity. Because HPO lyase was little inactivated by LA 13-hydroxide, which has almost the same hydrophobic moiety as the corresponding HPO, binding of fatty acid HPO to the hydrophobic domain of HPO lyase is not responsible for the inactivation. Organic HPOs, such as *tert*-butyl and cumene HPOs had substantially no effect on the activity of HPO lyase even if these HPOs were used at high concentrations. Furthermore, H₂O₂ also showed no effect.

Yeast alcohol dehydrogenase was inactivated by LA 13-HPO 75% of the initial activity during a 10-min incubation, but over 90% of the initial activity by either *tert*-butyl or cumene HPOs. Hydrogen peroxide also appreciably inactivated the dehydrogenase. The specificity of inactivation of HPO lyase indicates that HPO lyase specifically introduces fatty acid HPO into highly oxidizable domain near the reaction center and converts the bound HPO to a reactive species at the restricted site. Because fatty acid HPOs are natural substrates for HPO lyase, it is reasonable to expect an existence of specific recognition mechanism for fatty HPOs.

Reagents listed in Table 2-2 were co-incubated with HPO lyase and LA 13-HPO. The reduced form of glutathione had no effect on the inactivation although this reagent was effective to extract HPO lyase activity efficiently from tea leaves (Chapter 1). Dithiothreitol substantially protected HPO lyase from the inactivation. As previously reported with HPO lyases from various plant tissues [4, 14, 21], the participation of an SH-group in the

Table 2-2. Protection against inactivation of HPO lyase^a.

Compound added	HPO	Residual Activity (U/mg)	Relative Residual Activity (% without HPO)
None	-	18.9 (100.0) ^b	
	+	4.8	25.2
5 mM Glutathione	-	18.0 (95.2)	
	+	4.9	27.1
5 mM Dithiothreitol	-	19.2 (101.6)	
	+	9.5	50.0
0.2 mM α -Tocopherol	-	14.8 (78.3)	
	+	10.7	72.6
0.2 mM Nordihydro-guaiaretic acid	-	11.9 (63.0)	
	+	6.6	55.4
0.5 mM Butylated hydroxyanisole	-	14.6 (77.2)	
	+	10.1	68.6
0.5 mM Butylated hydroxytoluene	-	17.5 (92.6)	
	+	11.5	65.4
1 mM DETAPAC ^c	-	18.0 (95.2)	
	+	4.7	26.3
50 mM Mannitol	-	18.9 (100.0)	
	+	4.7	25.0
2.5% Ethanol	-	18.4 (97.4)	
	+	6.5	35.3
5% Ethanol	-	17.0 (89.9)	
	+	9.8	57.9
0.1 mM LA 13-hydroxide	-	10.6 (56.1)	
	+	5.1	48.0

^a HPO lyase (46.4 nM) was preincubated with or without 40 μ M LA 13-HPO in 50 mM MES-KOH, pH 5.5, containing indicated reagent for 10 min at 25°C. Residual activity was measured by diluting the preincubated mixture by 10-fold with 32 μ M LA 13-HPO in 50 mM MES-KOH, pH 5.5. Values are means of three repeats. ^b HPO lyase activities in the presence of indicated reagent is shown as a relative activity (%). ^c DETAPAC; diethylenepentaacetic acid.

reaction of this enzyme has been suggested. Protection by dithiothreitol suggests that the oxidation of an essential SH-group of HPO lyase at least partly contributes to the inactivation. Anti-oxidants such as α -tocopherol, nordihydroguaiaretic acid, butylated hydroxyanisole and butylated hydroxytoluene also protected. These anti-oxidants have inhibitory effects on HPO lyase activity (Table 2-2) and also believed to scavenge radical species originated from fatty acid or other organic HPOs [36]. Ethanol also showed the protection although a relatively high concentration was needed. Ethanol scavenge radicals and also a little inhibited HPO lyase activity (Table 2-2). A hydrophilic radical scavenger, mannitol, affected neither the activity nor the degree of inactivation. Furthermore, diethylenetriaminepentaacetic acid, which suppresses metal-catalyzed activation of HPOs, did not show any effect. As a whole, a hydrophobic radical scavenger which inhibited HPO lyase activity always protected it from the inactivation by LA 13-HPO.

From these results, the inactivation mechanism is deduced as follows; that is, fatty acid HPO specifically introduced into the reaction center of HPO lyase is converted to a hydrophobic radical species which in turn oxidizes an SH-group essential to HPO lyase activity. The finding that fatty acid HPO potentially inactivates HPO lyase in tea leaves indicates that HPO lyase activity is finely regulated *in vivo*. This finding may also offer a suggestion when one wishes to regulate flavor of processed tea.

SUMMARY

Tea leaf hydroperoxide lyase (HPO lyase) was rapidly and irreversibly inactivated by linoleic acid 13-hydroperoxide (13-(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid (LA 13-HPO)). The rate

of the inactivation was directly dependent upon the concentration of the hydroperoxide, and its second-order rate constant at pH 5.5 and 25°C was $265 \text{ M}^{-1}\text{s}^{-1}$. Linoleic acid 9-hydroperoxide (9-(*R*)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid) which is little catalyzed by tea leaf HPO lyase also inactivated the enzyme, but other organic hydroperoxides such as cumene and *tert*-butyl hydroperoxide and H_2O_2 did not. Inactivation by LA 13-HPO was protected either by dithiothreitol or LA 13-hydroxide, which suggests that the inactivation is caused by oxidation of essential SH-groups of the enzyme. Hydrophobic radical scavengers such as α -tocopherol and nordihydroguaiaretic acid effectively protected HPO lyase from the inactivation, but mannitol and diethylenetriaminepentaacetic acid showed no effect.

Chapter 3. Separation of 13- and 9-Hydroperoxide Lyase Activities in Cotyledons of Cucumber Seedlings

INTRODUCTION

It has been reported that cucumber seedlings have both the activities of 13- and 9-HPO lyases [37]. Matthew and Galliard also reported the existence of both the activities in *Phaseolus vulgaris* leaves [38]. However, it has not been elucidated yet whether these activities are attributable to one enzyme which can cleave both 13- and 9-HPO or to two or more enzymes each of which specifically cleaves 13- or 9-HPO. The work presented here provides an evidence for the existence of at least two HPO lyases differing in substrate specificity in cucumber seedlings.

MATERIALS AND METHODS

Cucumber (*Cucumis sativus* L. cv. Suvo) seeds were soaked in tap water for 12 hr and germinated under fluorescent light (7,000 lux) with a 14 hr-photoperiod at 25°C. 9-HPO and 13-HPO were prepared from linoleic acid, using potato lipoxygenase [39] and soybean lipoxygenase (Sigma, type I), respectively [19]. Both the HPOs contained less than 10% of geometrical and positional isomers. 13-HPO lyase activity was determined by the head space vapor method (Chapter 1), and 9-HPO lyase activity was determined from the amount of the 2,4-dinitrophenylhydrazones derivatives of the products by HPLC analysis (Chapter 1). For quantification, calibration curves were constructed with authentic *n*-hexanal and (*E*)-2-nonenal (Wako Pure Chemicals, Osaka). For determination of 13-HPO lyase activity, 5 μ mol of 13-HPO was incubated with the

enzyme for 3 min at 25°C, but for 9-HPO lyase activity, 9-HPO (5 μ mol) was incubated for 15 min at 25°C. The reaction was terminated while the rate of aldehyde formation was still linear. Protein was determined by the method of Bradford [40] standardized with bovine serum albumin.

Cucumber cotyledons (6-day-old) were homogenized with 3 volumes of 50 mM Na-phosphate buffer, pH 8.0, containing 4% Triton X-100 and 2 mM ascorbate in a chilled mortar, and the homogenate was filtered through four layers of cheesecloth. The filtrate was stirred for 1 hr at 0°C and centrifuged at 20,000 *g* for 10 min. The supernatant (20 ml) was made to 100 ml with 50 mM Na-phosphate buffer, pH 7.0, and solid PEG 6000 was added to 8% (w/v). After incubation for 1 hr on ice, the enzyme solution was centrifuged at 20,000 *g* for 30 min. The resultant supernatant was made to 22% (w/v) solution of PEG 6000 and stirred for 1 hr on ice. The precipitate collected by centrifugation at 20,000 *g* for 30 min was dissolved in a small volume of the elution buffer (5 mM Na-phosphate buffer, pH 7.5, containing 0.1% Triton X-100, 10% glycerol, 0.1 mM EDTA, 2 mM ascorbate and 5 mM dithiothreitol) and applied to a DEAE Cellulofine A-500 column (17 x 170 mm, Seikagaku Kohgyo, Tokyo) equilibrated with the elution buffer. After the column was washed with 100 ml of the elution buffer HPO lyases were eluted with 400 ml of a linear gradient of NaCl (0-0.3 M) in the elution buffer.

RESULTS AND DISCUSSION

Although 13- and 9-HPO lyase activities could be detected in cotyledons, hypocotyl and roots of 6-day-old cucumber seedlings, cotyledons were used as an enzyme source because this organ had the highest activity of both the lyases. Preliminary experiments

revealed that 80 and 50% of 13- and 9-HPO lyase activities, respectively, were bound to a fraction precipitable with a centrifugation at 100,000 *g* for 60 min and that 4% of Triton X-100 was necessary to extract both the activities. The extract was fractionated with PEG 6000 and applied to a DEAE Cellulofine A-500 column. Both the activities were adsorbed to this resin and could be eluted with a NaCl gradient. As shown in Fig. 3-1, 13-HPO lyase was eluted first, and 9-HPO lyase was eluted thereafter. Although further attempts to separate these activities failed because of an instability of 9-HPO lyase, the elution profile clearly showed that at least two different enzymes were existed in cucumber cotyledons, each of which was specific to 13- or 9-HPO. By this purification step 13-HPO lyase was purified about 9-fold with a yield of 61%, and 9-HPO lyase was purified 18-fold with a yield of 82%.

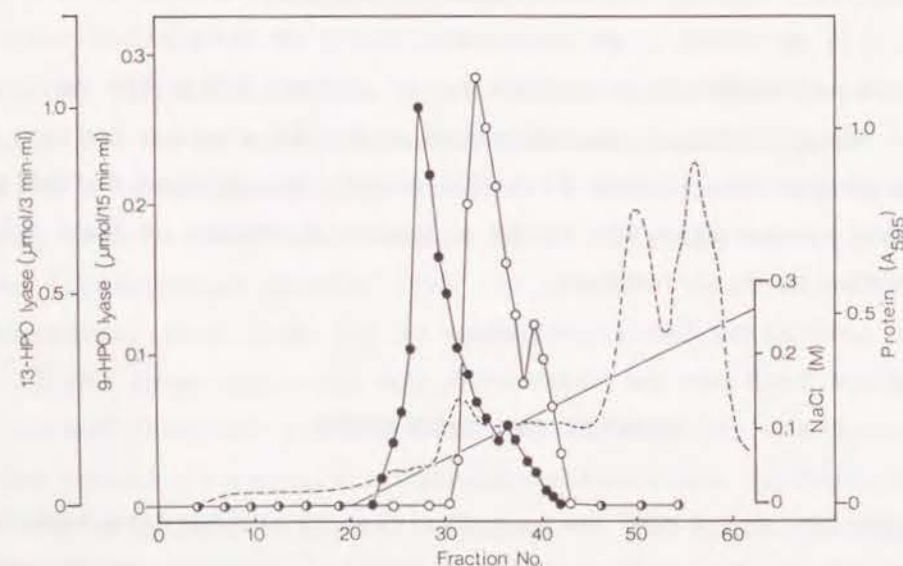


Fig. 3-1. Separation of 13-HPO lyase (●) and 9-HPO lyase (○) activities from cucumber cotyledons on DEAE-Cellulofine A-500 (1.7 x 17 cm). (---); protein content.

Properties of these partially purified lyases were examined. As shown in Fig. 3-2, 13-HPO lyase had a pH optimum at 8.0 while 9-HPO lyase at pH 6.5. At higher pH, the activities was rapidly decreased in both the cases. As shown in Table 3-1, both the activities were much affected by *p*-chloromercuribenzoate (PCMB) which has been shown to be an inhibitor for HPO lyase in several plants [10]. Monoiodoacetate decreased 9-HPO lyase activities whereas 13-HPO lyase activity was hardly affected. A serine

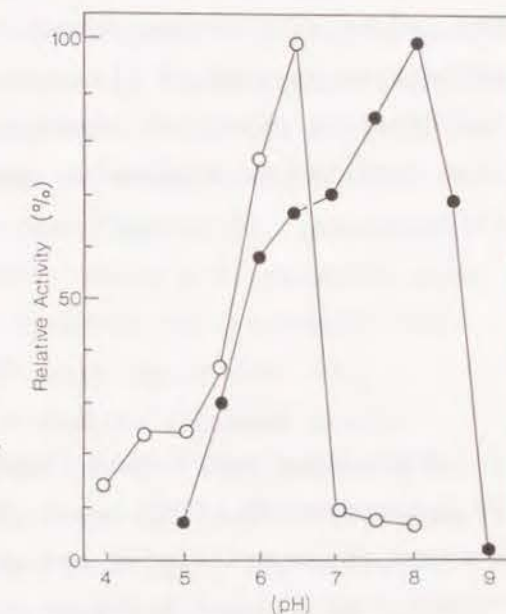


Fig. 3-2. pH-Dependence curves of the partially purified HPO lyases. (●), 13-HPO lyase activity; (○), 9-HPO lyase activity. McIlvaine's buffer (pH 4.0-8.0) and 50 mM Na-pyrophosphate buffer (pH 8.0-9.0) were used.

Table 3-1. Effect of reagents on 9- and 13-hydroperoxide lyase.

Reagents	Relative activity [%]			
	9-HPO lyase 0.1 mM	9-HPO lyase 1 mM	13-HPO lyase 0.1 mM	13-HPO lyase 1 mM
No added	100	100	100	100
EDTA	85	80	98	98
Monoiodoacetate	76	30	98	92
Mercaptoethanol	96	93	104	77
PCMB	53	13	87	1
PMSF	80	40	95	85

protease inhibitor, phenylmethanesulfonylfluoride (PMSF), also decreased 9-HPO lyase activity, whereas 13-HPO lyase activity was little affected.

These results show that in cucumber cotyledons C₆- and C₉-aldehydes were formed by different enzymes. It is well known that the substrate of HPO lyase is provided by lipoxygenase. Lipoxygenase which formed 13- and 9-HPO at a ratio of 85/15 from linoleic acid increased rapidly after germination and reached a maximum by the fifth day of germination of cucumber (see Chapter 4). Both HPO lyases also showed almost the same developmental course of activities as that of lipoxygenase (results not shown). This coordinative change of lipoxygenase and HPO lyase activities was also reported in watermelon seedlings [4] and cotton seedlings [41].

SUMMARY

In cucumber cotyledons, both C₆- and C₉-aldehydes were formed by hydroperoxide (HPO) lyase. Because it has not been elucidated whether these activities are attributed to one enzyme which can cleave both 13- and 9-HPO or to two or more enzymes each of which specifically cleaves 13- or 9-HPO, an attempt to separate HPO lyase activity was done. Ion exchange chromatography separated this activity into two fractions, one of which specifically cleaved 13-hydroperoxylinoleic acid and the other specifically cleaved the 9-isomer. 13-HPO-specific activity was most active at pH 8.0 and 9-HPO-specific one was at pH 6.5. SH-reagents inhibited both the lyases but to different extents.

Chapter 4. Tissue specific heterogeneity of lipoxygenase in cucumber seedlings

INTRODUCTION

Lipoxygenase exists in a wide variety of plants including lower plants such as bryophytes (see Chapter 6), green algae [42] or cyanobacteria [43]. The physiological role of lipoxygenase in plants, however, still remains ambiguous although the enzyme is suggested to be involved either in growth and development, pest resistance, senescence or wounding responses [13]. The product of lipoxygenase, fatty acid hydroperoxide, is known to be further converted into short-chain aldehydes (see Chapter 1), jasmonic acid [44] or fatty acid hydroxides [45]. Above all, jasmonic acid was recently reported to be an inter-plant or an inter-cellular signal to regulate gene expression in plant cells [46, 47].

Lipoxygenase activity has been reported to increase during several days after germination of seeds [4, 48, 49]. Recently Morita and his coworkers [50, 51] have reported that rice plants contain three forms of lipoxygenase and that there are differences in the changes in the activity during development of the seedlings among the three forms. In addition, lipoxygenase activity was detected not only in the cotyledons but also in the root of watermelon seedlings [4]. In this chapter, it is shown that cucumber root lipoxygenase is different from the cotyledon one.

MATERIALS AND METHODS

Seeds of cucumber (*Cucumis sativus* L. Suyo) purchased from Sanyo Seeds Co. (Yamaguchi, Japan) were soaked in running tap water for 12 hr, and germinated in plastic planting pots in ver-

miculite under a 14 hr light (7,000 lux) and 10 hr dark regime at 25°C. Lipoxygenase activity was assayed polarographically using a Clark type oxygen electrode (Yellow Spring Instruments. Co) with 50 mM Na-phosphate buffer, pH 6.3, (for enzyme solutions prepared from cotyledons and hypocotyls) or 50 mM Tris-Cl, pH 8.0, (for that from roots). Reaction was started with the addition of 50 μ l of 50 mM linoleic acid dispersed with 0.2% Tween 20 to a final volume of 1.75 ml. One unit of enzyme is defined as the amount of the enzyme consuming 1 μ mol of O₂ per min at 25°C. Protein was measured by the modified Lowry's method [16] standardized with bovine serum albumin.

The cotyledons (6 days old, 16 g fr. wt) were homogenized with 2 volumes of 10 mM Na-phosphate buffer, pH 8.0, and filtered through four layers of cheesecloth. The filtrate was centrifuged at 22,000 *g* for 20 min, and the resulting supernatant was put on a DEAE-Sephadex A-50 column (2.8 x 22 cm) equilibrated with 10 mM Na-phosphate buffer, pH 8.0. The column was washed with 1 L of the equilibration buffer and eluted with 1.5 L of a linear gradient of NaCl concentration (0-0.5 M) in the same buffer. The roots (6 days old, 72 g fr. wt) were homogenized with an equal volume of 20 mM Tris-Cl, pH 8.0, containing 0.1 mM EDTA, 2 mM ascorbate, and 0.25 M sucrose (buffer A) and filtered through four layers of cheesecloth, then centrifuged at 20,000 *g* for 20 min. The supernatant was precipitated by 30-50% saturation of ammonium sulfate and dissolved in buffer A. After dialysis against buffer A, Triton X-100 was added to 0.1% and put on a DEAE-Toyopearl 650M column (2.2 x 23 cm) equilibrated with buffer A containing 0.1% Triton X-100. The column was washed with 200 ml of the equilibration buffer and eluted with a linear gradient of NaCl concentration (0-0.5 M) in 500 ml of buffer A containing 0.1% Triton X-100.

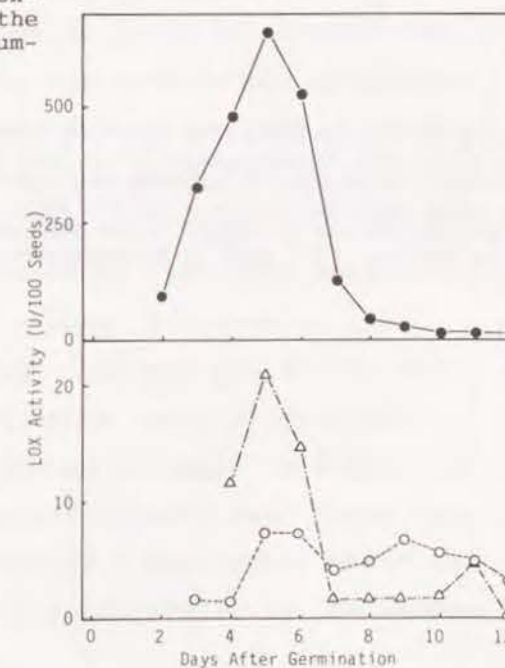
The product specificities by the purified lipoxygenases were

identified by HPLC analysis after the reduction with sodium borohydride and trans-esterification with ethereal-diazomethane. HPLC analysis were performed with a Zorbax-SIL column (0.46 x 25 cm) eluted with *n*-hexane/ethanol (99.5/0.5, v/v) at a flow rate of 2.0 ml/min. Each derivative was detected at 234 nm and identified with retention times of the authentic specimens.

RESULTS

Fig. 4-1 shows the developmental course of lipoxygenase activities in the cotyledons, hypocotyl, and root of cucumber germinated on vermiculite. The activity could not be detected in ungerminated seeds. After the second day of germination, a rapid rise of the activity in the cotyledons occurred and by the fifth day, the activity reached a maximum and rapidly declined thereafter.

Fig. 4-1. The developmental course of lipoxygenase activity in the cotyledons (●), the hypocotyl (Δ), and the roots (○) of cucumber seedlings.

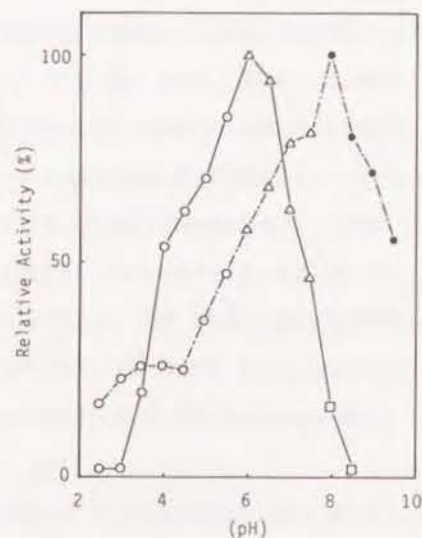


ter. Although almost the same course was observed for the activity in the hypocotyl, the activity in the root was persisted for a longer time.

The cotyledons and root of 6-day-old cucumber seedlings were homogenized with isotonic solution and fractionated by differential centrifugation. Of all the activity, 31.4% for the cotyledons and 48.2% for the root were recovered in the cytosol fractions. The soluble enzymes in the cotyledons and the root were respectively purified about 20-fold to 14.1 U/mg protein with a yeild of 57% and about 10-fold to 0.679 U/mg protein with a yeild of 28%, respectively. In both the cases, activities were eluted as a single peak from a DEAE-Cellulofine column.

The pH-activity profiles of the two purified lipoxygenases are shown in Fig. 4-2. Cotyledon lipoxygenase gave the highest activity at pH 6.0, but root lipoxygenase at pH 8.0. The pH-activity profiles observed with the crude extract of each tissue were almost identical with those observed with purified lipoxygenases and changed little during the development of the seedlings (data

Fig. 4-2. The pH-activity profiles of the purified root lipoxygenase (---) and the purified cotyledon lipoxygenase (—). Buffers used were MacIlvain's buffer (○), 50 mM Na-phosphate buffer (△), 50 mM Na-pyrophosphate buffer (□), and 50 mM ammonium buffer (●).



not shown). These observations indicate that the purified enzymes represent the major ones in the respective tissues.

Product specificities of the two lipoxygenases were identified with HPLC analysis of the products derived from linoleic acid (Table 4-1). Both the two enzymes produced 13-hydroperoxy-(9Z,11E)-octadecadienoic acid predominantly from linoleic acid.

Table 4-1. POSITIONAL SPECIFICITIES OF LOXs

Enzyme source	13-Hydroperoxide		9-Hydroperoxide	
	(Z,E)	(E,E)	(E,Z)	(E,E)
Roots	95.6	1.28	1.51	1.62
Cotyledons	86.4	1.85	8.94	2.77

DISCUSSION

The rapid increase of lipoxygenase activity in cucumber seedlings is accounted for by mostly the activity in the cotyledons, because the activity in this tissue occupied over 90% of that in the whole seedlings at an early period of germination. The intensive enhancement of the activity in the cotyledons occurred while this tissue rapidly expanded and increased their own mass, which suggests that lipoxygenase in this tissue is needed to its growth. The activity in the root kept constant six to ten days after germination when that in cotyledons rapidly decreased. Furthermore, the two purified lipoxygenases could be distinguished from each other by the pH-activity profiles. These results suggest that at least two forms of lipoxygenase exist in cucumber seedlings and are expressed specifically to the respective tissues.

SUMMARY

Most of the lipoxygenase activity in cucumber seedlings was found in the cotyledons. Other tissues, such as the hypocotyl and the roots, showed low but appreciable activities. During seed germination, lipoxygenase activity increased once, then declined in both the cotyledons and roots. The activity was predominantly recovered in the cytosol fractions after differential centrifugation of both the tissues. The soluble enzymes were partially purified from both the tissues. Root lipoxygenase was most active at pH 8.0, but the cotyledon one was at pH 6.0. Thus, lipoxygenase in the cotyledons may differ from that in the roots.

Chapter 5. Developmental change of lipoxygenase activity in cotyledons of cucumber seedlings

INTRODUCTION

With number of plants, biosynthesis of lipoxygenases is induced temporarily during the early stages of seedling growth [4, 41, 52-55]. In Chapter 4, it has been shown that lipoxygenase activity in cucumber cotyledons increased once during the post-germinative stage. Kato et al. reported appearance of new lipoxygenase isozymes in soybean cotyledons after germination [55]. Because soybean seeds are rich in lipoxygenases which remain after germination, they had to use mutant soybeans which lost a part of lipoxygenase isozymes in the seeds. On the other hand, cucumber cotyledons are thought to be convenient materials to examine developmental pattern of the activity during germination because ungerminated cucumber seeds have no lipoxygenase activity (Chapter 4).

Because cotyledons play an essential role in an early growth of plants, lipoxygenase in the organ must participate in the developmental processes. In this chapter, further characterization of the developmental pattern of lipoxygenase in cucumber cotyledons during an early growth of the seedlings will be presented.

MATERIALS AND METHODS

Plant materials. Cucumber seeds (*Cucumis sativus* L. cv. Suvo) were soaked and germinated as described in Chapter 4. Otherwise, the seeds soaked for 12 hr were sterilized with 2% NaClO for 10

min and cut transversely to remove one-third portion of the caruncular end and germinated on moist paper towels. The excised seeds were grown under the continuous dark or light.

Purification of cucumber cotyledon lipoxygenase. The cotyledons grown under the light/dark cycle for 6 days, or the excised cotyledons grown under the continuous light for 10 days were used for enzyme sources. With both the cotyledons the same purification procedure was used. In a typical experiment whose results are shown in Table 5-1, the excised cotyledons (163 g fr. wt.) were homogenized thoroughly with 400 ml of cold acetone (-20°C) with a Polytron homogenizer, and the homogenate was vacuum-filtered through a sheet of filter paper. The residue was again homogenized with 200 ml of cold acetone and the residue obtained by vacuum-filtration was dried *in vacuo*. To the acetone powder (27.3 g) 546 ml of buffer A (20 mM Na-phosphate, pH 8.0, containing 0.5 mM phenylmethanesulfonylfluoride, 1 mM ascorbate and 1 mM EDTA) were added and stirred for 1 hr on ice. The suspension was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 20,000 *g* for 20 min. To the resultant supernatant solid $(\text{NH}_4)_2\text{SO}_4$ was added to 20% saturation adjusting the pH to 8.0, and the mixture was stirred for 1 hr on ice. After centrifugation at 13,000 *g* for 10 min, to the resultant supernatant was added $(\text{NH}_4)_2\text{SO}_4$ to 40% saturation adjusting the pH to 8.0, and the mixture was stirred and centrifuged as above. The resultant precipitate was dissolved in a small volume of buffer B (10 mM Na-phosphate, pH 8.0). The enzyme (33 ml) was dialyzed against 1 L of buffer B for 12 hr with three changes, and the dialyzed enzyme was applied to a DEAE-Cellulofine A-500 column (Seikagaku Kohgyo, Tokyo, Japan, 2.2 x 20.0 cm) equilibrated with buffer B, then the column was washed with 200 ml of buffer B. The enzyme was eluted with 500 ml of a linear gradient of NaCl, 0 to 0.5 M, in buffer B. To the active fractions $(\text{NH}_4)_2\text{SO}_4$ was added to 20%

saturation adjusting the pH to 8.0, and the enzyme was applied to a Butyl-Toyopearl 650M column (Toyo Soda, Tokyo, Japan, 1.75 x 15.5 cm) equilibrated with 20% saturation of $(\text{NH}_4)_2\text{SO}_4$ in buffer B. After washed with 100 ml of the equilibrating buffer, the enzyme was eluted with 300 ml of a linear gradient of $(\text{NH}_4)_2\text{SO}_4$, 20 to 0% saturation, in buffer B. Purified enzyme was stored at -20°C until use.

Enzyme assays. The cotyledons were homogenized with 10 volume of buffer A with an ice-cold mortar. After filtration through four layers of cheesecloth, the homogenate was centrifuged at 20,000 *g* for 20 min and the resultant supernatant was carefully collected to exclude fatty layer and used immediately for the assay. Lipoxygenase activity was determined as described in Chapter 4 with an oxygen electrode. The activity (1 U) is defined as the quantity of enzyme catalyzing the consumption of 1 μmol of O_2 per minute at 25°C .

Protein and chlorophyll content. Protein content was determined by the modified method of Lowry [16] or of Bradford (for the solution containing $(\text{NH}_4)_2\text{SO}_4$) [40] with bovine serum albumin as a standard.

RESULTS

Purification of lipoxygenase from intact cotyledons. Lipoxygenase was purified from cucumber cotyledons grown under 16 hr-light/8 hr-dark cycle. Although chromatography of the $(\text{NH}_4)_2\text{SO}_4$ fraction with a DEAE-Cellulofine column showed only one active peak, two activity peaks were found in hydrophobic chromatography using a Butyl-Toyopearl column (Fig. 5-1B). The author tentatively designated the activity eluted first as fraction I and the one eluted subsequently as fraction II. On SDS-PAGE fraction I showed

one major band with an apparent molecular mass of 90 kD and two minor bands of 96 and 95 kD (Fig. 5-2). Fraction II showed one major band of about 96 kD and two minor bands of about 91 and 90

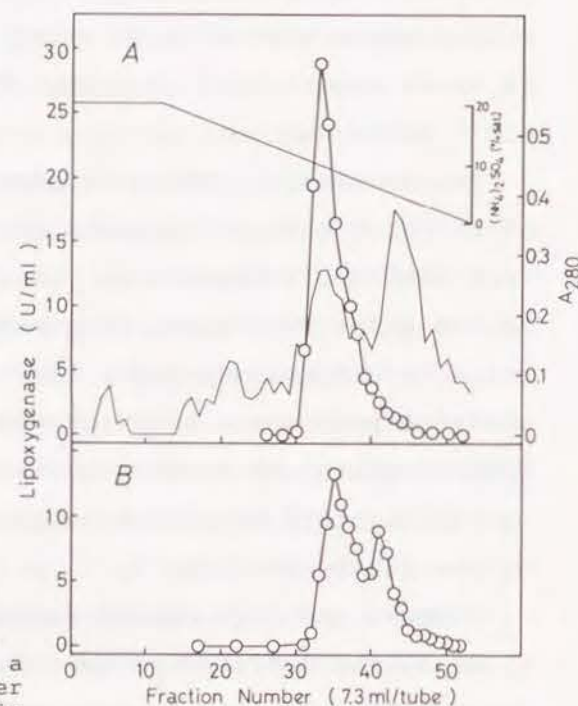


Fig. 5-1. Butyl-Toyopearl 650M column chromatography of cotyledon lipoxygenase. Lipoxygenase was extracted from the intact light-grown cotyledons (B) or the excised light-grown ones (A). The DEAE-Cellulofine fraction of each homogenate was applied on a Butyl-Toyopearl 650M column and eluted under the same conditions. (○) lipoxygenase activity, (—) absorbance at 280 nm.

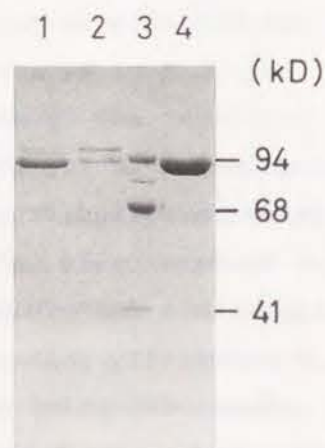


Fig. 5-2. SDS-PAGE analyses of the purified cotyledon lipoxygenases. The lipoxygenases purified from the intact light-grown cotyledons, fraction I (lane 1), fraction II (lane 2) and that purified from the excised dark-grown cotyledons (lane 4) were electrophoresed with molecular weight marker (lane 3) consisting of soybean lipoxygenase-1 (94 kD), bovine serum albumin (68 kD) and yeast alcohol dehydrogenase (41 kD).

kD. From SDS-PAGE analyses of the every fractions eluted from a Butyl-Toyopearl column, the band intensities of 90 and 96 kD were revealed to well coincide with the activities of fractions I and II, respectively. Further attempt to purify 90- and 96-kD proteins to a homogeneous state by either another hydrophobic, metal chelating affinity, or gel filtration chromatography, or chromatofocusing was failed.

Purification from the excised cotyledons. Because cotyledons grown without the embryonic axis showed much higher activity than the intact cotyledons (see later), the cotyledons grown under continuous light for 10 days without the axis were used as an enzyme source. In Butyl-Toyopearl chromatography, lipoxygenase was eluted as a single peak at the same concentration of $(\text{NH}_4)_2\text{SO}_4$ where fraction I was eluted (Fig. 5-1A). With these purification steps, lipoxygenase was purified from the excised cotyledons 66.4-fold with a specific activity of 317 U/mg protein (Table 5-1). On SDS-PAGE this preparation showed only one distinct band with a molecular mass of 90 kD (Fig. 5-2).

Table 5-1. Purification of lipoxygenase from the light-grown excised cotyledons.

step	total protein (mg)	total activity (U)	specific activity (U·mg ⁻¹ protein)	yield (%)	purification (fold)
acetone powder	988.2	4723.3	4.8	100.0	1.0
ammonium sulfate	177.5	4421.2	24.9	93.6	5.2
DEAE-Cellulofine	8.9	2107.9	236.7	44.6	49.5
Butyl-Toyopearl	1.8	578.4	317.2	12.2	66.4

Comparison of the lipoxygenases. All of the three lipoxygenases (fractions I and II from intact cotyledons, and that purified from the excised ones) showed highest activities around pH 6.0 and showed completely the same pH vs activity curves. Furthermore, these lipoxygenases showed the highest activity with α -linolenic acid, followed by linoleic acid. γ -Linolenic acid was a poor substrate for all the three lipoxygenases. As a whole, these lipoxygenases could not be distinguished each other by their enzymatic properties. Then, peptide maps of 96- and 90-kD proteins which were thought to associate with lipoxygenase activity were constructed and compared by the method of Cleveland et al. [56]. Fraction II was separated with SDS-PAGE and 96-kD protein was cut off from the polyacrylamide gels. The 90-kD protein purified from the excised cotyledons was also electrophoresed and cut off. Both the proteins showed the same peptide

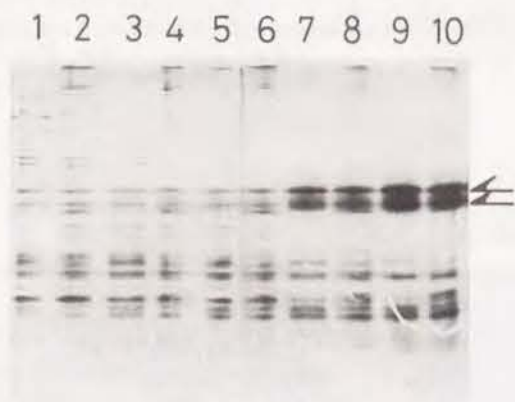


Fig. 5-3. Peptide maps of 96- and 90-kD forms of cucumber cotyledon lipoxygenases. Lipoxygenase-associated 96 kD protein was carefully cut off from polyacrylamide gel after SDS-PAGE of fraction II. Another form of lipoxygenase, 90-kD form purified from the excised cotyledons, was also electrophoresed and cut off. The gel pieces of 90 kD (lane 1, 3, 5, 7, 9) and 96 kD (lane 2, 4, 6, 8, 10) were partially digested with 6 (lane 1, 2), 20 (lane 3, 4), 60 (lane 5, 6), 200 (lane 7, 8) and 600 (lane 9, 10) pg of *S. aureus* V8-protease in polyacrylamide gel buffered with 62.5 mM Tris-Cl, pH 6.8, containing 2% (w/v) SDS and 5% β -mercaptoethanol for 15 min at 25°C. Resultant peptides were separated with 15% acrylamide gel and silver-stained. Arrows indicate the bands of added V8-protease.

fragments after the digestion by V8-protease at various extents (Fig. 5-3). These results indicate that the 90-kD protein was a protein which lost peptide fragment of 6 kD from the 96-kD protein.

When cucumber cotyledons grown under the light/dark for 6 days were homogenized with a buffer containing 2% (w/v) SDS and 8 M urea to prevent proteolysis, only one protein band of 96 kD was detected with immunoblotting using an antibody prepared by fraction I as an antigen, and the band pattern did not change even after 24-hr incubation of the homogenate at 25°C (Fig. 5-4). When the cotyledons were homogenized with 0.1 M Na-phosphate, pH 8.0,



Fig. 5-4. Western immunoblotting analysis of cucumber cotyledon lipoxygenase. Lane 7-9; cucumber lipoxygenase, purified from the intact light-grown cotyledons, fraction I (lane 8) and fraction II (lane 9), and that purified from the excised light-grown cotyledons (lane 7) were electrophoresed and blotted on nitrocellulose membrane. Lane 1-6; crude homogenates of the intact light-grown cotyledons were prepared with either 62.5 mM Tris-Cl, pH 6.8, containing 2% (w/v) SDS, 5% β -mercaptoethanol and 8 M urea (SDS-urea sample buffer, lane 1, 2), 0.1 M Na-phosphate, pH 8.0 (lane 3, 4) or 0.1 M Na-phosphate, pH 6.0 (lane 5, 6). The homogenates were mixed with SDS sample buffer (same as SDS-urea sample buffer but without urea) and heated at immediately 60°C for 15 min (lane 1, 3, 5) or after incubation at 25°C for 24 hr (lane 2, 4, 6). These samples were electrophoresed and blotted on nitrocellulose membrane. Cucumber lipoxygenase was detected with an antibody prepared with fraction I as an antigen.

only one band of 96 kD was detected just after homogenization, but a protein band of 90 kD appeared after 24-hr incubation (Fig. 5-4). An attempt to protect this proteolysis by adding either SDS (2%, w/v), phenylmethanesulfonylfluoride (3 mM), moniodoacetate (5 mM), leupeptin (0.5 mM), pepstatin (0.5 mM) or metal-chelating agents such as EDTA (5 mM) and EGTA (5 mM) to the crude homogenate of intact cotyledons was all failed. These results indicate that lipoxygenase exists as a single form of 96 kD in the cotyledons and after homogenization 6-kD fragment is readily removed by an endogenous peptidase. When the cotyledons were homogenized with 0.1 M Na-phosphate, pH 6.0, the 96-kD band observed just after homogenization was evidently weakened after 24 hr-incubation without appearance of any new bands. This suggests that at pH 6.0 lipoxygenase is extensively digested by another peptidase(s) differing from the one active at pH 8.0 to form much shorter peptides which cannot be detected by immunoblotting.

In summary, there exists only one form of lipoxygenase with a molecular mass of 96 kD in the intact cucumber cotyledons. Homogenization of the cotyledons would cause the contact the lipoxygenase with a peptidase, and a peptide corresponding molecular mass of 6 kD is excised from the intact 96-kD lipoxygenase to form the 90-kD lipoxygenase which still has the activity. There must exist a site susceptible to attack by a peptidase in the intact 96-kD lipoxygenase.

Developmental changes of lipoxygenase. Lipoxygenase activity in cucumber cotyledons increased once, then declined after germination as described in the previous chapter. Because the morphological feature of the cotyledons after germination is much affected by illumination during growth, lipoxygenase activity in this organ was also expected to be affected by illumination. Then, lipoxygenase activities of cucumber cotyledons grown under

the light/dark (16 hr/8 hr) cycle and the continuous darkness were compared.

Early developments were the same whether illuminated or not, i.e., from day 1 to 3 the fresh weight little changed and at about day 4 cotyledons emerged above ground level. After day 4, difference between the light- and the dark-grown seedlings became evident. The light-grown cotyledons started to increase its weight (Fig. 5-5) and accumulates chlorophyll from day 4. The hook straightened and pressed cotyledons started to open at day 5 and fully opened at day 6. Hypocotyl elongated from day 3 but the elongation stopped by day 6 when the cotyledons fully opened and greened. While, the dark-grown cotyledon showed little increase

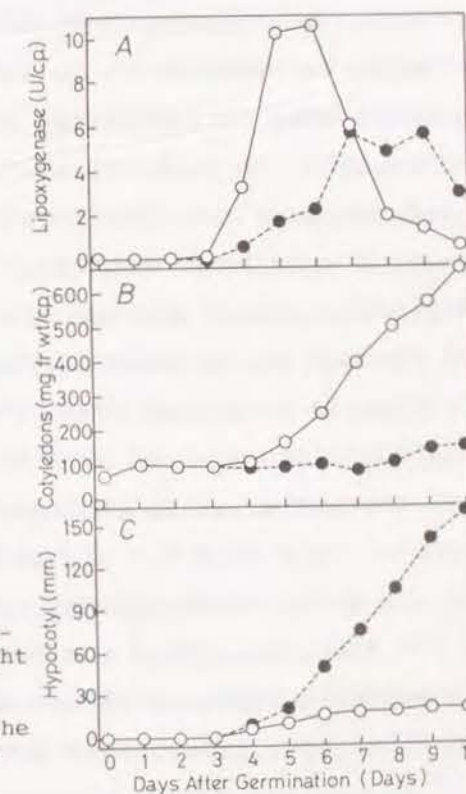


Fig. 5-5. Developmental changes of lipoxygenase activity in cotyledons (A), fresh weight of cotyledons (B), and length of hypocotyl (C). Cucumber seeds were imbibed for 12 hr and germinated on moist vermiculite under the 16 hr-light/8 hr-dark photo-regime (○) or the continuous dark (●). c.p.: cotyledon pair.

in its weight and did not open until day 10, and no accumulation of chlorophyll. Hypocotyl remained hooking but kept elongating until day 10 (Fig. 5-5).

Apart from most plants such as soybean [53], rice [50] and lupin [54], no lipoxygenase activity was detected in dry cucumber seeds (Chapter 4). Lipoxygenase activity in the light-grown cotyledons started to increase from day 3 and reached a peak at day 5 to 6 and declined to extensively low level by day 10 (Fig. 5-5). The activity in the dark-grown cotyledons also started to increase from day 3, but the rate was slower than that in the light-grown ones. It reached a broad peak at day 7 to 9 and slightly declined. Even at day 10 the activity was appreciably high.

Because it has been reported that free chlorophyll is a potent inhibitor of lipoxygenase [57], rapid decline of lipoxygenase activity observed with the light-grown, green cotyledons might be caused merely by inhibition of the activity by accumulating chlorophyll. In order to check this possibility, crude homogenates prepared from the light- and the dark-grown cotyledons were analyzed with immunoblotting. In the case of the light-grown cotyledons a band stained with the antibody appeared from day 3 and reached the highest intensity at day 5 to 6, then absolutely disappeared by day 10 (Fig. 5-6). In the case of the dark-grown cotyledons the stained band also appeared from day 3 and intensified thereafter as in the case of the light-grown ones, but it weakened only slightly and remained appreciable intensity even at day 10. These developmental patterns well corresponded to those of the activity, which indicates that the increase and decline of lipoxygenase activity is accounted for by the changes in the amount of the lipoxygenase protein.

Effect of illumination on lipoxygenase activity. When the

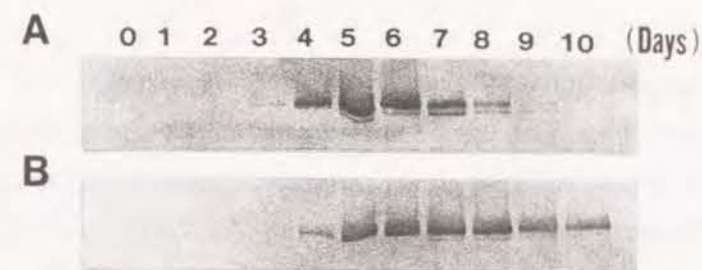


Fig. 5-6. Western immunoblotting analyses of crude homogenates prepared daily from the intact light-grown (A) and the dark-grown (B) cotyledons. Crude homogenates prepared as legend of Fig. 5 were electrophoresed and immunoblotted.

dark-grown seedlings were transferred to the continuous light at day 4, the development was the same as that of the ones grown under the light. Developmental changes of lipoxygenase activity was also same each other (Fig. 5-7A and C). This indicates that illumination during 1-4 days after germination does not affect the development of lipoxygenase activity as well as the morphology (Fig. 5-7B and D). While, when the dark-grown seedlings were transferred to the continuous light at day 6, the cotyledons rapidly greened and opened, and elongation of the hypocotyl stopped within 24 hr. Lipoxygenase activity in the cotyledons once increased during the 24 hr, then rapidly declined thereafter to the level of the activity of the cotyledons grown under the continuous light from day 1 (Fig. 5-7A and C).

When the light-grown seedlings were transferred to the continuous darkness at day 4, the morphology of the seedlings was almost same as the ones grown under the continuous darkness from day 1, but the cotyledons were slightly colored to pale green. Developmental change of lipoxygenase activity in the cotyledons transferred to the dark at day 4 was almost same as that in the

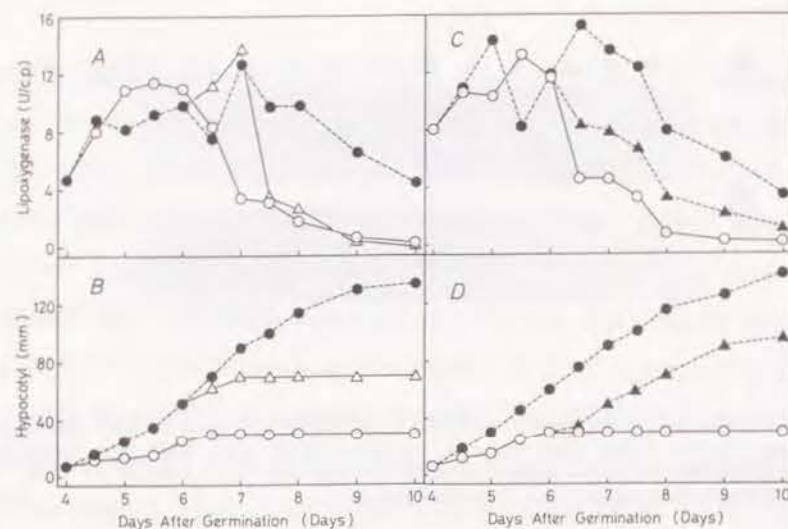


Fig. 5-7. Effect of illumination on lipoxygenase activity in the cotyledons (A, C) and on hypocotyl length (B, D). A, B; cucumber seedlings grown under the continuous dark were transferred to the continuous light from 4 days (○) or 6 days (Δ) after germination, or held under the continuous dark (●). C, D; cucumber seedlings grown under the continuous light were transferred to the continuous dark from 4 days (●) or 6 days (▲) after germination, or held under the continuous light (○). c.p.: cotyledon pair.

ones grown under the continuous dark (Fig. 5-7C and A). When the light-grown seedlings were transferred to the darkness at day 6, the enlargement of cotyledons and accumulation of chlorophyll were stopped while hypocotyl elongation resumed (Fig. 5-7D). Although lipoxygenase activity in these cotyledons also declined as that in the light-grown ones, higher activities than that in the light-grown cotyledons were always observed by day 10 (Fig. 5-7C). Immunoblotting analyses showed that these changes of the activity were elucidated to be due to the changes of the amount of lipoxygenase protein. These observations suggest that increase and decline of lipoxygenase activity are not controlled directly by the light. Thus, lipoxygenase increases whenever cucumber seedlings imbibe and grow, and it rapidly declines after cotyledons sufficiently greened for the start of photosynthesis.

Effect of the embryonic axis on lipoxygenase activity. It is well known that an early development of germinated seeds is highly regulated by plant hormones, and an embryonic axis and a testa are important sources of them. Davies and Chapman reported that the embryonic axis and/or the testa affected both the composition of the free fatty acid pool and the rate of lipid utilization in cucumber cotyledons [58]. In order to elucidate whether the developmental pattern of lipoxygenase activity in cucumber cotyledons is under a control of the embryonic axis and/or the testa or not, cucumber seeds were cut transversely to remove one-third portion of the caruncular end and peeled off the testa, then the rested cotyledons were grown on wetted paper towels.

The excised and peeled cotyledons swelled and enlarged under either the light or the dark (Fig. 5-8B), but, only those under the light greened and their cotyledons curled to open. Lipoxygenase activity in the excised cotyledons under the light started to increase from day 2, faster by a day than that in the intact light-grown cotyledons (Fig. 5-8A). By day 4 the activity exceeded the highest value of the intact ones and continued to increase thereafter till day 10 to as high as 30 U/cotyledon pair (c.p.). The activity did not substantially decreased even after the cotyledons greened. The activity in the excised cotyledons grown under the dark started to increase from day 3, again faster by a day than that in the intact dark-grown ones. The rate of the increase was much higher than the intact ones. The activity continuously increased without any decline as observed with the light-grown excised cotyledons. These developmental patterns of lipoxygenase activity are accounted for by the changes in the amounts of lipoxygenase protein as determined by immunoblotting. Such a rapid and continuous increase of lipoxygenase in cucumber cotyledons could be also observed with axis-less but testa-at-

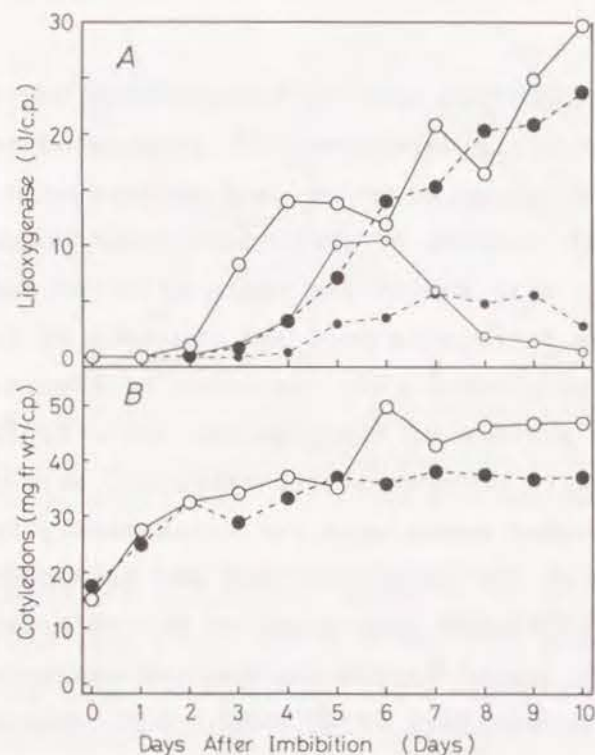


Fig. 5-8. Developmental changes of lipoxxygenase activity (A) and fresh weight of cotyledons (B) of the excised cucumber cotyledons grown under the continuous light (○) or the continuous darkness (●). After 12 hr-imbibition, cucumber seeds were cut transversely to remove one-third portion of the caruncular end and peeled off the testa, then the rested cotyledons were grown on wetted paper towels. For comparison, developmental changes of lipoxxygenase activity in the intact cotyledons were also plotted on panel A with the smaller symbols as those of Fig. 5.

tached cotyledons (result not shown), which indicates that the axis but not the testa affect the development of lipoxxygenase. In order to examine wounding effect which has been reported to induce lipoxxygenase activity [61], cucumber seeds were cut transversely to remove one-third portion of the apical end and grown under the same conditions. Although a little induction of the activity was evident, the developmental changes of the activity

did not substantially differ from those of the intact cotyledons (results not shown). Thus, embryonic axis would emit a signal to repress the induction of lipoxxygenase, and when the enzyme becomes unnecessary.

DISCUSSION

Although isozymes of lipoxxygenase have been found in many plant species [13], the results obtained here indicate that in intact cucumber cotyledons there exists only one form of lipoxxygenase with a molecular mass of 96 kD. Noticeable feature of this lipoxxygenase is that it is rapidly digested by an endogenous peptidase to form the active 90-kD enzyme when cotyledons are homogenized. Recently, van Aarle et al. [60] reported that barley lipoxxygenase purified 450-fold showed four bands of approximately equal intensity on SDS-PAGE, two of about 90 kD and two of about 63 kD, and they indicated that 63-kD proteins were degradation products of the 90-kD enzyme. Furthermore, Peterman and Siedow [53] reported that in soybean cotyledons there exist smaller peptides which must be associated with a catalytically active, but somewhat degraded, form of lipoxxygenase. Such a smaller lipoxxygenase-related peptide was also observed in a *de novo* synthesized lipoxxygenase purified from germinated soybean seeds (L-4) [55]. Their observations and the presented results suggest that most plant lipoxxygenases are susceptible to proteolysis to form a smaller form of the active enzyme. A physiological meaning of this feature is unclear at present. One possibility is that lipoxxygenase should be destined to be degraded rapidly if the activity become unnecessary, because the product, fatty acid hydroperoxide, is highly toxic to plant cells. The degradation to form a smaller but active lipoxxygenase may be the first and

'marking' step of entire degradation to form inactive and much shorter peptides as observed when cucumber cotyledons are homogenized at pH 6.0. To clarify the physiological meaning of this degradation, the 96-kD form of cucumber cotyledon lipoxygenase must be purified homogeneously and the peptidase which is responsible for this degradation should be characterized.

With number of plants, lipoxygenase has been reported to be induced temporarily during an early stages of seedling growth. The author first examined and compared developmental patterns of lipoxygenase activity in the greened and the etiolated cotyledons. The patterns resemble with those of glyoxysomal enzymes such as isocitrate lyase or malate synthetase, which are involved in gluconeogenesis [61]. A preliminary experiment to reveal subcellular localization of lipoxygenase in cucumber cotyledons showed that the enzyme exists in cytosol but not in glyoxysomes. Activities of the glyoxysomal enzymes in light-grown cucumber cotyledons have been reported to reach a peak at day 3 to 4, and to decline to very low level at day 5 [61]. While, lipoxygenase activity reaches its peak at day 5. These indicate that lipoxygenase in cucumber cotyledons is irrespective of the lipid mobilization performed in glyoxysomes, although lipoxygenase catabolizes fatty acids which are substrates for gluconeogenesis. Transition of the dark-grown cucumber seedlings to the light and *vice versa* changed the developmental patterns of lipoxygenase activity in the cotyledons. The results suggest that the activity in cucumber cotyledons starts to accumulate a few days after imbibition of the seeds irrespective of illumination until the cotyledons developed an ability to transit to photo-autotrophy upon illumination. The activity is kept at high level during the transition proceeding, but, after the cotyledons have fully equipped an autotrophic machinery, the activity rapidly decreases. Cucumber cotyledon lipoxygenase may participate in

reconstitution of intracellular structures concomitant with transition from heterotrophy to autotrophy.

Davies and Chapman reported that the removal of the embryonic axis resulted in reduced rates of lipid degradation [62] and that the induction rates of lipase and glyoxysomal enzymes also reduced in the excised cucumber cotyledons [58]. In the case of lipoxygenase, however, removal of the embryonic axis enhanced the rate of its induction and took away its rapid decline which was observed with the light-grown intact cotyledons. The axis might negatively regulate the accumulation of lipoxygenase protein and positively regulate its degradation. Such an effect of embryonic axis on an enzyme has been reported with one of a hydrolytic enzymes important for an early growth of seedlings, amylases. α - and β -amylase activities in the cotyledons of germinating peas which have been removed embryonic axis continue to increase without decline but the intact cotyledons show a decline [63]. It is well known that gibberellin promotes the formation of amylases but, abscisic acid inhibits the promotion [64]. Lipoxygenase in cucumber cotyledons may be regulated by the same manner as amylases. Interestingly, participation of lipoxygenase activity in the formation of abscisic acid has been reported [65, 66].

SUMMARY

Lipoxygenase in intact cucumber cotyledons germinated under the light/dark cycle was highly purified. With hydrophobic chromatography, the activity separated into two fractions. Lipoxygenase activity in each fraction revealed to a protein with a molecular mass of 96 and 90 kD, respectively. If the cotyledons grown without the embryonic axis were used as an enzyme source, only one form of lipoxygenase with a molecular mass of 90 kD

could be obtained homogeneously. These three lipoxygenases showed the same enzymatic properties and peptide maps. Because only 96-kD protein could be detected by Western immunoblotting of crude homogenate of the cotyledons, it is suggested that lipoxygenase exists as only one form of 96 kD and that upon homogenization it rapidly degrades to form active 90 kD form.

Although dry cucumber seeds have no lipoxygenase activity, the activity in the light-grown cotyledons increased a few days after germination to reach a peak at day 5 to 6. Thereafter it declined to a very low level. The activity in the dark-grown cotyledons increased in the same manner, but the rate of the increase was lower and the enhanced activity persisted for a longer time. Considering the results as a whole, lipoxygenase activity is high while cucumber cotyledons have developed an ability to transit to photo-autotrophy upon illumination, but after the transition the activity rapidly reduced. On the other hand, the activity in the cotyledons from which the embryonic axis and the testa had been removed was continuously increased during development of the cotyledons without any apparent decrease even if the cotyledons were grown under the light. These organs are thought to negatively regulate the induction of lipoxygenase in the cotyledons.

Chapter 6. Purification and properties of lipoxygenase in *Marchantia polymorpha* cultured cells

INTRODUCTION

In the course of a comprehensive survey for the better enzyme source suitable to elucidate the role of lipoxygenase in plant cells, the author detected appreciable activity of lipoxygenase in cultured cells of *Marchantia polymorpha* (liverwort). It has been reported that not only higher plants but lower plants such as cyanobacteria [43] and green algae [42] also have lipoxygenase activity. It has not been known whether the properties of the enzymes in lower plants are the same as those in higher plants. Thus, the author purified lipoxygenase in *M. polymorpha* cultured cells and characterized.

MATERIALS AND METHODS

Materials. *Marchantia polymorpha* cells [67] were kindly provided by Dr. K. Ohyama, Kyoto University. The cells were grown under continuous fluorescent lamp at 25°C in 100 ml of 1-M51C medium [67] in 300 ml flask with shaking at 120 rpm. The stock cultures were subcultured every 14 days. Fresh wt of the cells was determined after filtration through a sheet of filter paper under reduced pressure for 5 min.

Linoleic acid (99% pure, from Sigma) was purified with an SiO_2 column before use. Linoleyl alcohol was prepared from linoleic acid by the reduction with LiAlH_4 . Other fatty acids (99% pure) were kindly provided by Nippon Oil and Fats, Co. *L*- α -Phosphatidylcholine (Sigma, type IV-S) was further purified with repetitive acetone precipitation to remove free fatty acids.

Purification. Cultured cell suspension (197 g fr. wt) was harvested during a later stationary phase (14 days) and homogenized with 1/4 McIlvaine's buffer, pH 8.0, containing 0.5 mM EDTA, 1 mM phenylmethanesulfonylfluoride and 4% (w/v) Polyclar AT. The homogenate was centrifuged at 80,000 *g* for 80 min. To the resultant supernatant solid ammonium sulfate was added, and the fraction obtained at 40-70% saturation was collected. The ammonium sulfate fraction was redissolved in a minimum volume of 50 mM Tris-Cl, pH 8.5, containing 25% saturation of ammonium sulfate and applied to a Butyl-Toyopearl 650M column (9 x 1.9 cm, Tosoh, Tokyo) equilibrated with the same buffer. Lipoxygenase was eluted with the same buffer in which the concentration of ammonium sulfate was decreased to zero. Active fraction was pooled and dialyzed against 100 volumes of 50 mM Tris-Cl, pH 8.5, for 4.5 h with three changes. The following steps were carried out with HPLC (Hitachi L-6200). The dialyzed enzyme was applied to a QA-824 column (75 x 8 mm, Showa Denko, Tokyo) equilibrated with the dialyzing buffer and the lipoxygenase was eluted with 0-0.5 M KCl gradient in the same buffer. The active fractions were collected and concentrated with Centricon-30 (Amicon). The enzyme was applied to a TSKgel G3000SW column (600 x 7.5 mm, Tosoh, Tokyo) equilibrated with 0.1 M Na-phosphate, pH 7.0, containing 0.1 M Na₂SO₄ and eluted with the same buffer.

Lipoxygenase assay. The standard assay mixture (3 ml) consisted of 50 mM borate buffer, pH 9.0, and 25 μ l of substrate solution (10 mM linoleic acid dissolved in 0.2% Tween 20) and appropriate volume of the enzyme solution. The reaction was initiated by the addition of the substrate solution and formation of linoleic acid hydroperoxide was followed by the absorbance increase at 234 nm ($\epsilon = 2.5 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) at 25°C. One unit of the enzyme activity was defined as the enzyme forming 1 μ mol of the product per min.

Analysis of reaction products. Linoleic acid (5 mg) was incubated with 0.5 U of the purified lipoxygenase in 50 mM borate buffer, pH 9.0, at 25°C for 15 min. After the formed hydroperoxide was extracted with *n*-hexane/diethyl ether (1/1, v/v), reduced with sodium borohydride and esterified with ethereal diazomethane, the positional and the geometrical isomers were analyzed in a form of methyl hydroxy-linoleate with HPLC (Shimadzu LC-5A) equipped with a Zorbax-SIL column (250 x 4.6 mm, Shimadzu, Kyoto; *n*-hexane/ethanol 99.5/0.5, flow rate 2.0 ml/min, detection at 234 nm). Methyl hydroxy-ester derivative of the 13-(*Z,E*)-isomer was collected and the optical isomer ratio was analyzed with HPLC equipped with a Chiralcel OB column (250 x 4.6 mm, Daicel, Tokyo; *n*-hexane/ethanol 99/1, flow rate 1.0 ml/min).

Other methods. Protein content was determined with BCA protein assay reagents (Pierce) with bovine serum albumin as a standard [68]. Isoelectric point was determined by chromatofocusing on a PBE 94 (Pharmacia) column as described in manufacture's instruction manual.

RESULTS AND DISCUSSION

Subcellular localization. Lipoxygenase activity was first detected in a bryophyte, *M. polymorpha* cultured cells. The activity was depleted by boiling the crude extract for 10 min. The cells cultured for 13 days were harvested and homogenized using a Polytron mixer with three volumes of 67 mM K-phosphate, pH 7.2, containing 0.4 M sucrose, 10 mM KCl, 1 mM MgCl₂, 1% (w/v) bovine serum albumin, 1 mM EDTA and 10 mM ascorbic acid. After filtration through a cheesecloth, the differential centrifugation of the filtrate revealed over 90% of the total lipoxygenase activity to exist in the supernatant of centrifugation at 80,000 *g* for 80

min. Thus, the enzyme was thought to be cytosolic. Next to the cytosolic fraction, chloroplast fraction (1200 g ppt) had the largest amount of the activity (about 8%). The activity in chloroplast fraction was not decreased by repeated washing with hypotonic medium, but brief sonication (10 sec x 4) solubilized all the activity. This suggests that a part of lipoxxygenase activity loosely bound to chloroplast membrane. Occurrence of lipoxxygenase activity in chloroplast membrane was reported for wheat leaves [49]. In this chapter, cytosolic lipoxxygenase in *M. polymorpha* cells was purified and characterized.

Time course. In order to reveal the time courses of growth and lipoxxygenase activity of the cells, 6 g (fr. wt) of the cells were inoculated to grow in the fresh culture medium (100 ml) and harvested every 2 days. As shown in Fig. 6-1A, *M. polymorpha* cells grew logarithmically by 10 days after about 4 days of a lag phase, thereafter, fr. wt of the cells turned to decrease. Al-

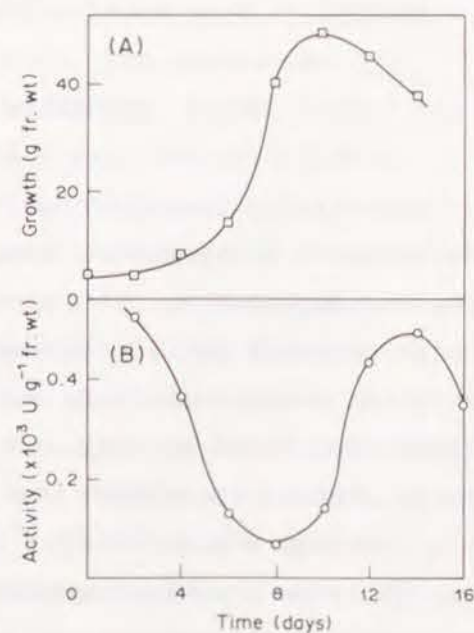


Fig. 6-1. Time course of growth (A) and lipoxxygenase activity (B) of *M. polymorpha* cultured cells. After transplanting the cells to fresh culture medium, the cells were harvested every two days and fr. wt per 100 ml culture medium (□) and lipoxxygenase activity (○) were determined.

though high lipoxxygenase activity was observed at the start of the culture, the activity rapidly decreased, and the cells in a logarithmic phase showed the lowest activity (Fig. 6-1B). Lipoxxygenase activity turned to increase to the original level when growth of the cells almost ceased. Almost the same time course of the activity was observed when the activity was expressed on the basis of a protein content. This result suggests an essential role of the lipoxxygenase in a later stationary phase, but not in other phases of the cell growth.

Purification. The cells in a later stationary phase was homogenized with 1/4 McIlvaine's buffer, pH 8.0, containing 0.5 mM EDTA, 1 mM phenylmethanesulfonylfluoride and 4% (w/v) Polyclar AT and centrifuged to obtain the 80,000 g sup fraction (Table 6-1). A single elution peak of the activity was obtained with hydrophobic chromatography of the ammonium sulfate fraction and succeeding ion exchange chromatography on a QA 824 column. Gel filtration chromatography on a TSKgel G3000SW column efficiently purified the lipoxxygenase to a homogeneous state (Fig. 6-2). With this purification steps, the lipoxxygenase was purified 531-fold with a yield of 5.9% (Table 1). Specific activity of the purified lipoxxygenase was 15.3 U/mg protein with linoleic acid as a substrate. This value was almost equivalent to that reported for

Table 6-1. Purification of lipoxxygenase from *M. polymorpha* cells

	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Yield (%)	Enrichment (fold)
Crude extract	1470	42.2	2.88×10^{-2}	100.0	1
80 000 g supernatant	705.0	57.9	8.21×10^{-2}	137.0	3
(NH ₄) ₂ SO ₄ fraction	212.0	52.1	0.246	123.0	9
Butyl Toyopearl	18.0	23.7	1.32	56.2	46
QA-824	1.24	6.6	5.35	15.6	186
G3000SW	0.164	2.5	15.3	5.9	531

Fig. 6-2. Purification of *M. polymorpha* lipoxygenase with gel filtration chromatography on a TSKgel G3000SW column. The active fraction of ion exchange chromatography were collected and concentrated, then applied to the column. The lipoxygenase was eluted with 0.1 M Na-phosphate, pH 7.0 containing 0.1 M Na_2SO_4 at a flow rate of $0.5 \text{ ml} \cdot \text{min}^{-1}$ with detection at 280 nm. The bar represents lipoxygenase activity.

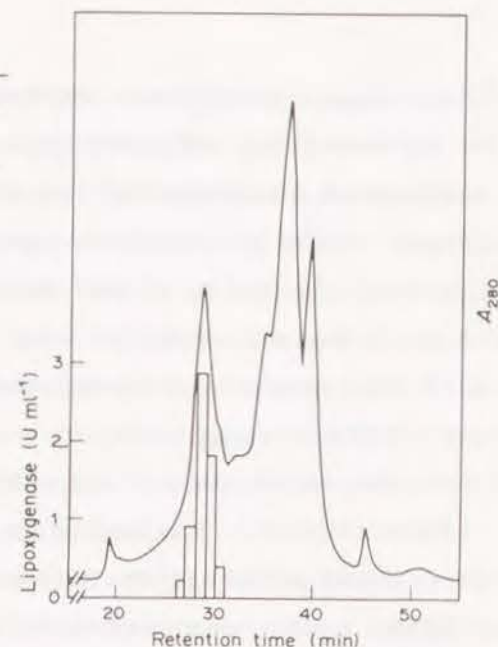


Fig. 6-3. SDS-polyacrylamide gel electrophoresis of the purified lipoxygenase.



avocado lipoxygenase [69], but almost one order of magnitude smaller than those of potato tuber [70], rice embryo [50] or soybean seed lipoxygenases [5]. The lipoxygenase showed only one distinct protein band on SDS-polyacrylamide gel electrophoresis and a subunit M_r of 109,600 was calculated from the mobility of the lipoxygenase in relation to those of marker proteins (Fig.

6-3). Although, The M_r of the native enzyme was estimated to be 261,000 by the gel filtration chromatography using a G3000SW column. Thus, *M. polymorpha* lipoxygenase seems to be a homodimer.

General properties. Purified lipoxygenase had an optimum pH at around 9.0, and its half-maximum activity was found at pH 6.5 and 10.0. This optimum pH was an anomalous value, because most of the optimum pHs of plant lipoxygenases were reported to be 5.5 to 7.0. Soybean lipoxygenase-1 (pH optimum is 9.0-10.0) was a well-known exception [5]. Chromatofocusing with a PBE 94 column revealed the pI value of *M. polymorpha* lipoxygenase as 4.8. This value was relatively low when compared with those of isozymes of soybean lipoxygenase (5.68, 6.25 and 6.15 for isozyme-1, -2 and -3, respectively [13]) or avocado lipoxygenase (5.9) [69]. The K_m value of *M. polymorpha* lipoxygenase for linoleic acid under the standard conditions was estimated to be $18.4 \mu\text{M}$, which was almost equivalent to that reported for soybean lipoxygenase-1 [5]. The presence of 1 mM nordihydroguaiaretic acid or 1 mM mercuric chloride inhibited 100 and 80% of the activity of *M. polymorpha* lipoxygenase, respectively. Both phenidone ($16.7 \mu\text{M}$) and α -tocopherol ($16.7 \mu\text{M}$) inhibited 55% of the activity. Neither 1 mM KCN, NaN_3 nor EDTA inhibited. These results were the same as those obtained with lipoxygenases of higher plants [13].

Substrate and product specificity. All the fatty acids used here were dissolved in 0.2% Tween 20 solution and sonicated for 30 sec just before use. As shown in Table 6-2, *M. polymorpha* lipoxygenase was the most active for γ -linolenic acid (18:3 (n-6)) although the positional isomer, α -linolenic acid (18:3 (n-3)) was a poor substrate. Such a high reactivity to γ -linolenic acid has not known for other plant lipoxygenases. For example, with γ -linolenic acid the activities of soybean lipoxygenase-1, Bengal gram lipoxygenase-1 and -2 were 39, 49 and 17% of those

Table 6-2. Substrate specificity of *M. polymorpha* lipoxygenase

Fatty acids	Relative activity (%)
18:2 (<i>n</i> -6)	100.0
18:3 (<i>n</i> -6)	200.0
18:3 (<i>n</i> -3)	33.7
20:4 (<i>n</i> -6)	58.3
20:4 (<i>n</i> -3)	75.0
20:5 (<i>n</i> -3)	31.6
22:6 (<i>n</i> -3)	37.5
Linoleyl alcohol	10.9
Phosphatidyl choline	5.4

obtained with linoleic acid, respectively [73, 74]. Polyenoic fatty acids having chain length of C-20 and C-22 were also oxygenated by the enzyme but to the lesser extent.

The product specificity of the purified lipoxygenase was analyzed with straight- and chiral-phase HPLC. By using linoleic acid as a substrate, the lipoxygenase produced 13-hydroperoxy-(9*Z*,11*E*)-, 13-hydroperoxy-(9*E*,11*E*)-, 9-hydroperoxy-(10*E*,12*Z*)- and 9-hydroperoxy-(10*E*,12*E*)-octadecadienoic acids at the ratio of 86.5, 4.5, 8.5 and 0.4%, respectively. Of these, optical isomer ratio (*R/S*) of the 13-(*Z,E*)-isomer was estimated as 2/98. These results were almost coincident with those of soybean lipoxygenase-1 [5].

SUMMARY

Lipoxygenase activity was first detected in a bryophyte, *Marchantia polymorpha* (liverwort) cultured cells. The activity was enhanced at a later stationary phase of the culture. Most of the activity occurred in the cytosolic fraction. The lipoxygenase

was purified to homogeneous state by ammonium sulfate fractionation and hydrophobic, ion-exchange, and gel filtration chromatographies. From the SDS-polyacrylamide gel electrophoresis, the M_r of the lipoxygenase was estimated to be 109,600. The pH optimum was about 9.0 and the pI value was 4.8. This enzyme formed mainly 13-(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid from linoleic acid. It showed twice higher the activity for γ -linolenic acid than for linoleic acid.

Chapter 7. Comparison of substrate specificities of lipoxygenases purified from soybean seed, wheat seed and cucumber cotyledons

INTRODUCTION

Lipoxygenase (EC 1.13.11.12) catalyzes the stereospecific dioxygenation of unsaturated fatty acids containing at least one methylene-interrupted (*Z,Z*)-pentadiene system. Lipoxygenases in plants can be classified into two types which are distinguishable primarily by differences in their pH-activity profiles. Type 1 enzymes show optimum activity around pH 9 while type 2 enzymes are most active between pH 6.5 and 7 [13]. Soybean lipoxygenase-1 is a well characterized type 1-lipoxygenase. Plant lipoxygenases can be also classified by their product specificities. Soybean lipoxygenase-1 and cucumber cotyledon lipoxygenase oxygenate linoleic acid to form (13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid while removing a pro-(*S*) hydrogen from the bis allylic methylene. Whereas linoleic acid 9-lipoxygenases such as those in wheat seed and potato tuber catalyze the formation of (9*S*)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid while removing a pro-(*R*) hydrogen from the bis-allylic methylene (Fig. 7-1) [12]. Because pentadiene system of a substrate is symmetrical, hydrogen removal and O₂ insertion are carried out by both the types of lipoxygenase in spatially identical manner. Thus, strict positional specificities of dioxygenation observed with most lipoxygenases indicate that there exists a mechanism to recognize the orientation of a fatty acid. Gardner [73] assumed that soybean lipoxygenase-1 recognizes a carboxyl anion to arrange a fatty acid only in one orientation.

Hatanaka et al. [74] have examined substrate and product specificity of soybean lipoxygenase-1 using an entire series of (*ω*6*Z*, *ω*9*Z*)-C₁₃~C₂₀-dienoic acids, which have different distances

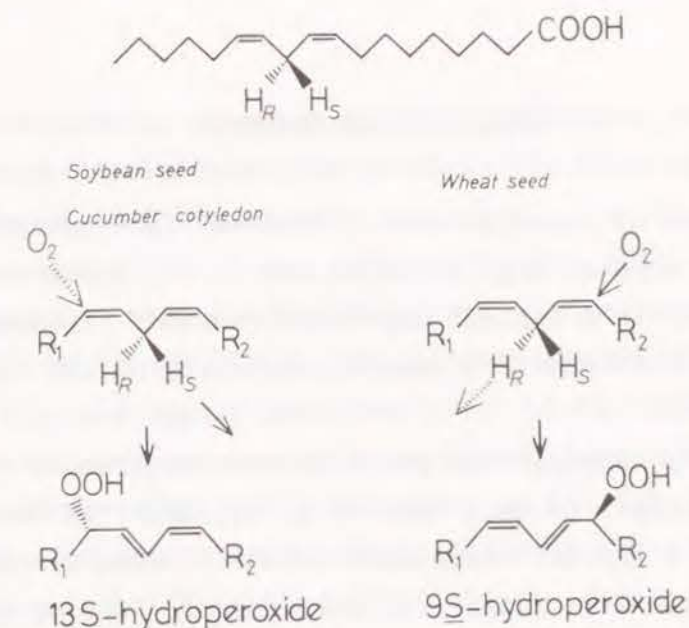


Fig. 7-1. Mechanism of the dioxygenase reaction catalyzed by lipoxygenases. Linoleic acid as substrate.

from the terminal carboxyl group to the pentadiene moiety, and suggested that the interaction of a carboxyl anion of a substrate with the reaction center of soybean lipoxygenase-1 is important to oxygenize a specific position of a substrate but hydrophobic interaction and recognition of the pentadiene system are also important to secure the activity.

It seemed of interest to try to reveal whether the manner of substrate recognition postulated for soybean lipoxygenase-1 can be generalized to those of other plant lipoxygenases. To this end, the author purified lipoxygenases not only from soybean seed, but also from wheat seed and cucumber cotyledons which differ in optimum pHs and/or product specificities. Substrate and product specificities of these three lipoxygenases were compared by using an entire series of (*ω*6*Z*, *ω*9*Z*)-C₁₃~C₂₄-dienoic acid.

MATERIALS AND METHODS

Purification of lipoxygenases. Soybean lipoxygenase-1 was purified from soybean seed (*Glycine max* L. cv. Tamahomare) as described [75]. In a typical experiment soybean lipoxygenase-1 was purified 20-fold with a specific activity of 135 U/mg protein.

Cucumber lipoxygenase was purified from cotyledons of intact cucumber seedlings (*Cucumis sativus* L. cv. Suvo) as described in Chapter 5. In a typical experiment cucumber lipoxygenase was purified 19-fold with a specific activity of 103 U/mg protein.

Wheat lipoxygenase was purified from wheat seed (*Triticum vulgare* Desf. cv. Nourin 61 gou). Wheat seed was finely ground and the flour was stirred with three volumes of 0.12 M Na-phosphate buffer, pH 6.9, at 4°C for 1 hr. After filtration through four layers of cheesecloth, the filtrate was centrifuged at 23,000 g for 20 min, and the supernatant was fractionated with ammonium sulfate (35-55% saturation). After dialysis against 10 mM Na-phosphate buffer, pH 6.9, the solution was applied to a DEAE-Cellulofine A-500 column (20 x 305 mm) equilibrated with the same buffer. At this step three peaks of lipoxygenase activities appeared, and the most active fraction which was eluted first was collected. The active fraction was concentrated by ultrafiltration with Ultra Filter UP-20 (Advantec Toyo, Tokyo) and was dialyzed against 10 mM Tris-Cl buffer, pH 8.0. The dialyzed enzyme was applied to a QA-824 column (Showa Denko, Tokyo, 8 x 75 mm) equilibrated with the same buffer, and eluted with a linear gradient of NaCl concentration (0-0.1 M) in the same buffer. The active fraction was concentrated with Centricon-30 (Amicon) and was applied to a TSKgel G3000SW column (Tosoh, Tokyo, 7.5 x 600 mm) equilibrated with 0.1 M Na-phosphate buffer, pH 7.2, containing 0.1 M sodium sulfate. The active fraction was eluted with the

same buffer and used as purified wheat lipoxygenase. In a typical experiment wheat lipoxygenase was purified 450-fold with a specific activity of 155 U/mg protein.

Enzyme assay. Linoleic acid (99% pure) was purchased from Sigma Chemical Co. (ω 6Z, ω 9Z)-C₁₃~C₂₀-dienoic acids (Fig. 7-2) were prepared as previously described [74]. (ω 6Z, ω 9Z)-C₂₁~C₂₄-dienoic acids were synthesized via the Wittig coupling between a C₉-phosphorane salt and C₁₁~C₁₄-aldehydes as in [74]. Geometrical purity of the synthesized fatty acids were determined with HPLC analyses as previously described [76]. All the dienoic fatty acids were used as substrates after purified by silica gel column chromatography to 99% purity.

Fig. 7-2. Structures of the substrate used.

Total carbon number (n)	Structure
13	
14	
15	
16	
17	
18	
19	
20	
21	
22	
23	
24	

Lipoxygenase activities were determined spectrophotometrically at 25°C by following the formation of hydroperoxides at 234 nm ($\epsilon=25,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$). The buffers used were, for soybean lipoxygenase-1; 50 mM Na-borate buffer, pH 9.0, for cucumber lipoxygenase; 50 mM Na-phosphate buffer, pH 6.3, and for wheat lipoxygenase; 50 mM Na-phosphate buffer, pH 6.9. To 0.98 ml of the respective buffer, 10 μl of substrate solution (10 mM dienoic acid dispersed with 0.2% Tween 20) was added, and the reaction was started by the addition of 10 μl of enzyme solution (7.0×10^{-3} U). One unit of enzyme activity was expressed as the amount of enzyme forming 1 μmol of hydroperoxides at 25°C.

Product specificity. Each lipoxygenase (1 U) was added to a reaction mixture consisting of 50 ml of the respective buffers containing 1 ml of the substrate solution, and the mixture was incubated for 10 hr at 4°C under O_2 atmosphere. Then the reaction mixture was acidified with 2N HCl and extracted with 100 ml of ether. The ether layer was washed twice with 100 ml of saturated NaCl solution, and was evaporated *in vacuo*. Then 3 ml of ether was added to the residue and the ether solution was treated with ethereal diazomethane and subsequent triphenylphosphine to give the corresponding hydroxy-esters. Compositions of positional isomers of the products were analyzed by straight phase HPLC (Shimadzu LC-5A) equipped with a Zorbax-SIL column (Dupont-Shimadzu, 4.6 x 250 mm) with detection at 234 nm. Elution was carried out with *n*-hexane/*iso*-propanol (99/1, v/v) at a flow rate of 1 ml/min at 25°C.

RESULTS AND DISCUSSION

Lipoxygenases purified from soybean seed, wheat seed and

cucumber cotyledons were almost homogenous when analyzed with SDS-polyacrylamide gel electrophoresis. Reactivities of the substrates, ($\omega 6Z, \omega 9Z$)- $\text{C}_{13}\sim\text{C}_{24}$ -dienoic acids (Fig. 7-2), were examined at a concentration of 100 μM . The reactions were monitored at the respective optimum pHs, *i.e.*, pH 9.0 for soybean lipoxygenase-1 [75], pH 6.9 for wheat lipoxygenase [77] and pH 6.3 for cucumber lipoxygenase (see Chapter 4). As shown in Fig. 7-3, the three lipoxygenases showed different profiles of substrate specificities.

Soybean lipoxygenase-1 showed broad specificity having an optimum activity with C_{20} -dienoic acid. Cucumber lipoxygenase 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 lipoxygenase-1. Contrary to soybean lipoxygenase-1, cucumber lipoxygenase catalyzed little the oxygenation of C_{17} -dienoic acid and not that of C_{16} - and the shorter dienoic acids.

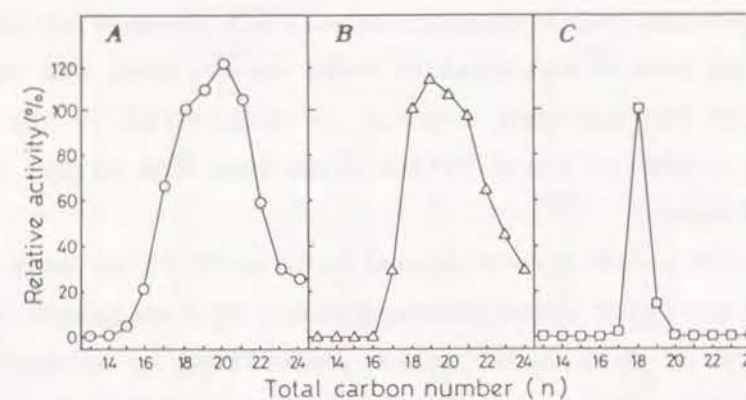


Fig. 7-3. Substrate specificities of soybean lipoxygenase-1 (A), cucumber lipoxygenase (B) and wheat lipoxygenase (C) for the synthetic substrates. The reactivity of a substrate relative to that of C_{18} -dienoic acid (linoleic acid) was plotted against total carbon number (n).

With C₁₇-dienoic acid, cucumber lipoxygenase initiated the oxidation only after a lag period of about 10 min although the other lipoxygenases did not show such a long lag period even if a substrate of low reactivity was used. Furthermore, cucumber lipoxygenase did not show such a long lag period with C₂₄-dienoic acid which showed almost the same reactivity as C₁₇-dienoic acid. Lag period was believed to be the time to activate the inactive form of lipoxygenase by its own product, hydroperoxide [12, 13]. The product formed from C₁₇-dienoic acid was thought not to be an efficient activator for cucumber lipoxygenase under the reaction condition employed here.

Wheat lipoxygenase showed a relatively narrow specificity when compared with other lipoxygenases. Wheat lipoxygenase was most active with a natural substrate, linoleic acid, and either addition or deletion of only one methylene unit decreased the activity drastically. Other dienoic acids were not oxygenated by wheat lipoxygenase. Relatively narrow specificity observed with wheat lipoxygenase suggests that recognition of the terminal carboxyl group of a substrate has a crucial role. This suggestion is supported by the result obtained by Kühn et al. [78] who reported that the site of the hydrogen removal is determined by the distance from the carboxyl group rather than from the methyl end of the fatty acid chain.

The substrates which showed appreciable activities were oxygenated with the purified lipoxygenases under O₂ atmosphere at 4°C and the position of dioxygenation was determined by straight-phase HPLC analyses. All the substrates used here had two possible oxygenation sites, *i.e.*, ω 6- and ω 10-positions. As reported previously, by either soybean lipoxygenase-1 [13] or cucumber lipoxygenase (see Chapter 4), linoleic acid was mainly oxygenated at ω 6-position to form (13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid as a main product but wheat lipoxygenase oxygenated at

ω 10-position to form (9*S*)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid as a main product [78]. Soybean lipoxygenase-1 oxygenated ω 6-position of a substrate shorter than C₂₂ with high specificity (over 95%). However, low specificities were observed with C₂₃- and, more pronouncedly, with C₂₄-dienoic acids (Table 7-1). It must be noticed that C₁₅-dienoic acid which showed only little reactivity was oxygenated more specifically than the two longest acids although they had higher reactivities. This indicates that a fatty acid suited for peroxidation is not always peroxidized specifically. In other words, a factor needed to be oxygenated and that needed to be specifically oxygenated are not same. Gardner [73] suggested that the carboxyl anion form of a substrate arranges itself at the active site of soybean lipoxygenase-1 only in one orientation although its protonated form of the substrate can arrange itself in either orientation in head to

Table 7-1. Positional and geometrical specificity of plant lipoxygenases.

Total carbon number (<i>n</i>)	ω 6		ω 10	
	(<i>Z,E</i>)	(<i>E,E</i>)	(<i>E,Z</i>)	(<i>E,E</i>)
Soybean				
15	94.4	1.5	3.1	1.0
16	99.3	trace	0.7	trace
17	95.3	1.7	1.0	2.0
18	96.3	0.3	3.0	0.4
19	95.0	1.9	1.5	1.6
20	93.6	3.2	3.2	trace
21	90.2	5.3	2.1	2.4
22	92.6	3.4	4.0	trace
23	86.2	3.4	9.9	trace
24	75.6	2.3	22.1	trace
Cucumber				
17	68.7	4.0	19.4	7.9
18	79.2	2.9	17.4	4.1
19	72.5	4.9	16.3	6.3
20	90.0	3.4	4.4	2.2
21	92.4	4.0	2.8	0.8
22	93.1	5.2	1.1	0.6
23	90.4	7.3	1.8	0.5
24	69.2	2.5	27.6	0.7
Wheat				
17	10.8	2.8	84.1	2.4
18	7.1	2.8	87.7	2.4
19	16.1	5.3	74.7	3.9

tail alignment. At the pH used for the assay (9.0), the carboxyl anion forms of the substrates are predominant if the result obtained with linoleic acid (apparent pKa of 7.9 was reported [79]) was applicable to the other fatty acids used here. High specificity of oxygenation to the substrates shorter than C₂₂ indicates that the recognition of the anion fully functions to arrange these substrates only in one orientation even if the reaction center of the enzyme is apart from the pro-(S) hydrogen attached to the bis-allylic methylene. On the contrary, the recognition may be weakened with the two longest dienoic acids by much elongating the distance between the anion and the site of hydrogen removal of a substrate. As a result, these substrates get more chance to arrange themselves in reversal orientation to be removed the pro-(R) hydrogen and oxygenated ω 10-position (Fig. 7-4).

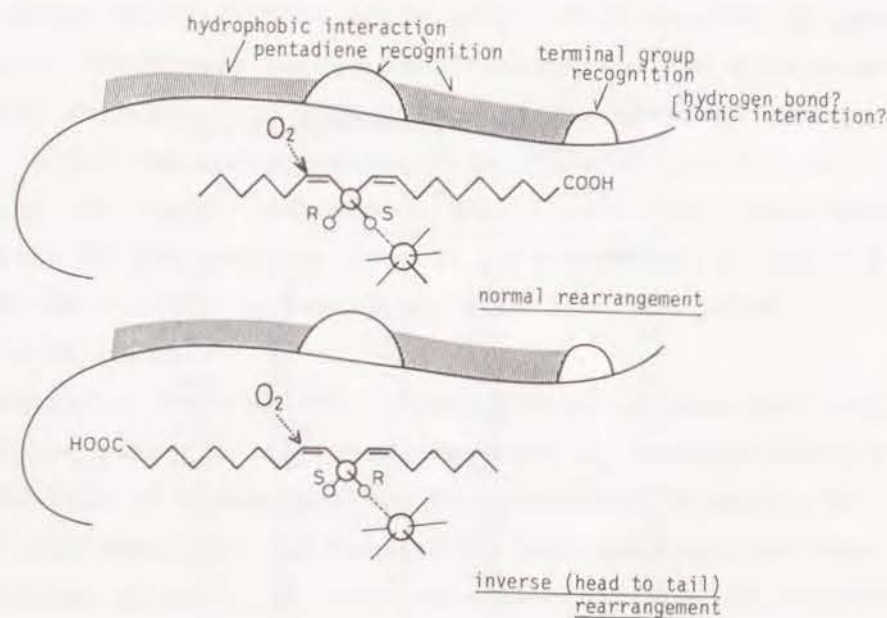


Fig. 7-4. The normal and inverse rearrangement of a substrate (linoleic acid) in the reaction center of lipoxygenases.

Cucumber lipoxygenase introduced oxygen mainly to ω 6-position of any substrates, which indicates that cucumber lipoxygenase recognizes the undissociated form of the terminal group to arrange a substrate only in one orientation because the carboxylic acid form of a substrate is abundant at pH 6.3. However, the distance between the terminal functional group and the site of hydrogen removal affected the positional specificity of dioxygenation of cucumber lipoxygenase to a higher extent than that of soybean lipoxygenase-1. The substrates shorter than C₁₉ and the longest substrate, C₂₄, were oxygenated by cucumber lipoxygenase with low positional specificities (Table 7-1). Unexpectedly, dioxygenation to C₁₉-dienoic acid which showed the highest reactivity (see Fig. 7-3) was not so specific. The highest specificity was observed with C₂₁- and C₂₂-dienoic acids which showed only about 50% of the reactivity of C₁₉-dienoic acid. This result indicates that a fatty acid suited for peroxidation is not always dioxygenized specifically. The recognition of the terminal group of a substrate may not be so strong as that observed with soybean lipoxygenase-1 and the substrate recognition site of cucumber lipoxygenase can arrange a substrate shorter than C₂₀ in both the orientations (Fig. 7-4). One should be noticed that highest specificity was observed with fatty acids which were not detected in cucumber cotyledons [58]. Because cucumber cotyledons have at least two kind of hydroperoxide lyases differing in substrate specificity as described in Chapter 3, the low specificity observed with linoleic acid may have significance to explain the still-unknown physiological role of this enzyme *in vivo*.

Only little information was obtained with analyses of the products obtained with wheat lipoxygenase because only three products could be formed (Fig. 7-3). However, it was suggested that recognition of the carboxyl group but not carboxyl anion had an important role to arrange a substrate only in one orientation.

SUMMARY

Lipoxygenases were highly purified from soybean seed, wheat seed and cucumber cotyledons. Substrate specificities of these lipoxygenases were studied by using an entire series of (ω 6Z, ω 9Z)-C₁₃~C₂₄-dienoic acids as synthetic substrate analogues. Soybean lipoxygenase-1 and cucumber lipoxygenase showed broad specificities for these substrates while wheat lipoxygenase showed narrow specificities. Position of dioxygenation to each substrate was analyzed by high performance liquid chromatography. With soybean lipoxygenase-1 long distance between the terminal carboxyl group and the site of hydrogen removal in a substrate decreased the positional specificity of dioxygenation, while, with cucumber lipoxygenase, shortening the distance decreased the specificity. It was suggested that cucumber lipoxygenase and soybean lipoxygenase-1 recognized the terminal carboxyl group of a substrate to arrange it only in one orientation at the reaction center. In case of wheat lipoxygenase, recognition of the carboxyl group was thought to have crucial and essential role to secure the activity.

CONCLUSION

An ability to form short chain volatile aldehydes is distributed in plant kingdom including bryophytes and algae. However, the physiological role(s) of the aldehyde-forming activity has not been elucidated yet. In this study, the author attempted to elucidate physiological role(s) of the volatile aldehyde forming system in plants. To this end, properties of the enzymes involved in the system should be clarified at first. The author firstly purified fatty acid hydroperoxide lyase from tea leaves and elucidated properties of the enzyme. The enzyme has an SH-group essential to the catalytic activity. Antioxidants having catechol moieties such as nordihydroguaiaretic acid are potent inhibitors against the lyase. Because these catechols are rich in tea leaves, the lyase activity is thought to be at least partly regulated in the leaves by these compounds. Furthermore, the lyase is rapidly inactivated by its own substrate, fatty acid hydroperoxide. Investigation on the inactivation kinetics indicates that the lyase is inactivated in a mechanism-based manner. These unique properties of the lyase indicate that the activity is under a subtle regulation *in vivo*. As the substrate of the enzyme, fatty acid hydroperoxide, is toxic to plant cells, the product, short chain aldehyde, is also toxic. This may be a reason why the activity of hydroperoxide lyase is complexly regulated. On the other hand, these properties could be applicable to modify flavors of foods originated from plant tissues.

There exist two isozymes of fatty acid hydroperoxide lyase in cucumber cotyledons, which are different in substrate and product specificities, i.e., one cleaves 13-hydroperoxide of fatty acid to form C₆-aldehydes and the other cleaves 9-hydroperoxide to form C₉-aldehydes. Both the activities should be implicated in an early growth of seeds because they increased temporary until the

seedlings acquires photosynthetic activity.

With most plants whichever it is monocotyledonous or dicotyledonous, lipoxygenase activity temporary increases during an early growth of the germinating seeds. This indicates that lipoxygenase is also implicated in the germination process. Study on lipoxygenase activity in cucumber seedlings suggests that it is not involved in gluconeogenesis which has an important role in mobilization of carbon source stored in seeds as lipid. Developmental changes of the activity under various light conditions suggest that high activity is needed when cucumber cotyledons are acquiring an ability to transit to photo-autotrophic growth from heterotrophic one. Thus, lipoxygenase in cucumber cotyledons may function to promote the transition in aspects of either reconstitution of intracellular organelle or sustainment of the growth after gluconeogenesis has terminated.

Lipoxygenase activity could be also detected in a bryophyte, *Marchantia polymorpha* cultured cells. Properties of the lipoxygenase purified from the moss cells are same as those of the enzyme purified from higher plants, but substrate specificity is somewhat differed. It showed the highest activity for γ -linolenic acid which is rare in higher plants. The lipoxygenase activity is enhanced in late-stationary phase of the cell growth. Because products of lipoxygenase, fatty acid hydroperoxides, would disorder the integrity of membranes, the activity in *M. polymorpha* cells is thought to be associated with senescence of the cells. Although lipoxygenases in cucumber cotyledons and *M. polymorpha* cells are elucidated to have almost the same properties, physiological roles of these two lipoxygenases are very different each other.

Because lipoxygenase locates at a division point of the aldehyde forming system, substrate and product specificities of the enzyme are thought to play an essential role to determine the

structure of final products, short chain aldehydes and oxoacids. Lipoxygenases in soybean seed, wheat seed and cucumber cotyledons are distinguishable each other in terms of product specificity and pH-dependency. Comparison of the specificities of these three lipoxygenases by using sequentially synthesized substrate analogs having different length from the terminal carboxyl group to the site of hydrogen removal indicates that mechanisms of the enzyme for recognizing the substrate differ each other. Thus, lipoxygenase should have diverged to several forms to meet physiological requirement in plants during the molecular evolution.

Although physiological role(s) of the short chain aldehyde-forming system in plants could not be fully elucidated, the present study is thought to offer much information to promote the progress to the final end.

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